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**PhD Program in Advanced Chemistry**

# *IN VITRO* ANTITUMOR ACTIVITY OF ARACHIDONIC AND DOCOSAHEXAENOIC ACIDS AS BOTH MONOACYLGLYCEROLS AND FREE FATTY ACIDS ON COLORECTAL CANCER CELLS

(Actividad antitumoral *in vitro* de los ácidos araquidónico y docosahexaenoico como monoacilgliceroles y ácidos grasos libres en células de cáncer colorrectal)

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**CERTIFICO:** que el presente trabajo titulado "*In vitro* antitumor activity of arachidonic and docosahexaenoic acids as both monoacylglycerols and free fatty acids on colorectal cancer cells" ha sido realizado en los laboratorios de este Departamento en el edificio de la Escuela Superior de Ingeniería bajo mi dirección por la Ingeniera Agrónoma María José González Fernández para aspirar al grado de Doctora.

El doctorando

El director de la Memoria

Fdo.: María José González Fernández

Fdo.: José Luis Guil Guerrero

*A mis padres*

*"Ahora lo entiendo. Los protagonistas de esas historias se rendirían si quisieran, pero no lo hacen, siguen adelante porque todos luchan por algo"*

*J.R.R. Tolkien*

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# CONTENTS

# ABSTRACT

## 1. ABSTRACT

Colorectal cancer (CRC) is one of the most common and mortal types of cancer. There is an increasing evidence that some polyunsaturated fatty acids (PUFA) are very effective as antitumor compounds on cancer cells through different mechanisms. PUFA occurs in the human body at different forms, from which monoacylglycerols (MAG) and free FA (FFA) promote important health benefits; for instance, they prevent the onset of CRC.

This document is structured in three chapters:

**Chapter I** is related to two articles corresponding to two different methodologies to obtain highly purified PUFA. **In the first article**, a process involving six edible oils, namely: olive, linseed, sunflower, evening primrose, DHASCO® and ARASCO® to obtain different monounsaturated FA (MUFA)- and PUFA- based MAG is described. The purification process starts with an enzymatic hydrolysis using porcine pancreatic lipase, then the reaction products were fractionated by using two liquid chromatography columns. The first chromatography column process with silica gel as stationary phase allowed to separate MAG form the rest of hydrolysis products. In the second step an open column chromatography containing silver nitrate coated silica gel as stationary phase allowed to purify the MAG-enriched fractions containing the different MUFA- and PUFA-based MAG from the corresponding oils. Overall, MAG based on oleic (OA, 18:1*n*-9), linoleic (LA, 18:2*n*-6), α-linolenic (ALA, 18:3*n*-3), γ-linolenic (GLA, 18:3*n*-6), arachidonic (ARA, 20:4*n*-6) and docosahexaenoic (DHA, 22:6*n*-3) acids were isolated at high yields and purities (92.6, 97.4, 95.3, 90.9, 100.0 and 95.3% purity, respectively). **The second article** describes the process to obtain highly concentrated eicosapentaenoic acid (EPA, 20:5*n*-3), DHA and ARA using the urea complexation methodology. For this study, the urea:FA ratio was optimized. Five oil sources were used: commercially available tuna oil, DHASCO®, ARASCO®, and MARINOL®, and the oil from the microalga *Nannochloropsis*. Concentrates were obtained at 98.0, 43.0 and 82.8% of total FA for DHA, EPA, and ARA, respectively.

In **Chapter II,** the *in vitro* antitumor activities of DHA- and ARA-based FFA molecules on HT-29 CRC cells are described. For stablishing activities, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT) along with lactate

dehydrogenase (LDH) and caspase-3 assays were used. In addition, proteome changes were assessed by "sequential windowed acquisition of all theoretical mass spectra" quantitative proteomics (SWATH-MS), followed by pathway analysis, to determine the affected molecular mechanisms. In all assays, DHA inhibited cell proliferation of HT-29 CRC cells to a higher extent than ARA and acted primarily by downregulating proteasome particles, while ARA presented a dramatic effect on all six DNA replication helicase particles.

In **Chapter III**, similar design and technology were used to investigate the actions of both DHA and ARA as MAG molecules, and a comparison of results with those obtained using the corresponding FFA was effected. Cell assays revealed that ARA- and DHA-MAG exercised dose-dependent antiproliferative actions, with DHA-MAG acting on cancer cells more efficiently than ARA-MAG. SWATH-MS, validated by parallel reaction monitoring and followed by pathway analysis, revealed that DHA-MAG had a massive effect in the proteasome complex, while the ARA-MAG main effect was related to DNA replication. Prostaglandin synthesis also resulted inhibited by DHA-MAG. Results clearly demonstrated the ability of both ARA- and DHA-based MAG to induce cell death in CRC cells, which suggests a direct relationship between chemical structure and antitumoral actions.

In short, the present doctoral dissertation demonstrates the antitumor activity of ARA and DHA as both MAG and FFA against HT-29 CRC cells. This activity was stablished by MTT, LDH and Caspase-3 test, and by analyzing the changes observed in the cell proteome after ARA and DHA treatment. The results of this doctoral dissertation indicate that both DHA and ARA constitute potential chemopreventive agent candidates.

**Resumen**

El cáncer colorrectal (CRC) es uno de los tipos de cáncer más comunes y mortales. Cada vez hay más evidencias de que algunos ácidos grasos poliinsaturados (PUFA) son muy eficaces como compuestos antitumorales en células de cáncer a través de diferentes mecanismos de acción. Los PUFA están presentes en el ser humano en diferentes formas, siendo los monoacilgliceroles (MAG) y los ácidos grasos libres (FFA) los que promueven importantes beneficios para la salud, por ejemplo, previenen la aparición del CRC. Por ello, la tesis está estructurada en tres capítulos:

El capítulo I contiene dos artículos que engloban dos metodologías para obtener PUFA altamente purificados. En el primer artículo se describe un proceso que implica seis aceites comestibles (oliva, lino, girasol, onagra, DHASCO® y ARASCO®) para obtener diferentes FA monoinsaturados (MUFA) y PUFA en forma de MAG altamente purificados. El método de purificación consiste en una hidrólisis enzimática con lipasa pancreática porcina y un posterior fraccionamiento de los productos de hidrólisis mediante dos columnas de cromatografía líquida. La primera cromatografía con gel de sílice como fase estacionaria permitió separar la fracción de MAG del resto de los productos de hidrólisis. La segunda, que contenía gel de sílice recubierto de nitrato de plata como fase estacionaria, permitía purificar las fracciones enriquecidas de MAG para obtener MAG a base de MUFA y PUFA de los aceites correspondientes. En general, se aislaron el ácido oleico (OA, 18:1*n*9), linoleico (LA, 18:2*n*-6), α-linolénico (ALA, 18:3*n*-3), γ-linolénico (GLA, 18:3*n*-6), araquidónico (ARA, 20:4*n*-6) y docosahexaenoico (DHA, 22:6*n*-3) con altos rendimientos y purezas (92,6, 97,4, 95,3, 90,9, 100,0 y 95,3% de pureza, respectivamente). En el segundo artículo se describe la obtención de los ácidos eicosapentaenoico (EPA, 20:5*n*-3), DHA y ARA altamente concentrados utilizando el método de la urea, en el cual se optimizó la relación urea:FA. Para preparar este artículo se utilizaron cinco fuentes de aceite: el aceite de atún disponible en el mercado, los aceites DHASCO®, ARASCO® y MARINOL®, y el aceite de la microalga *Nannochloropsis*. Finalmente, se obtuvieron concentrados de DHA, EPA y ARA con purezas de 98,0, 43,0 y 82,8% del total de FA.

En el capítulo II se determinaron las actividades antitumorales *in vitro* de las moléculas de FFA basadas en DHA y ARA en las células HT-29 de CRC. Para estudiar las actividades antitumorales se utilizó el ensayo de bromuro de 3-(4,5-dimetiltiazol-2-

il)-2,5-difeniltetrazolio (MTT), junto con el ensayo de lactato deshidrogenasa (LDH) y la caspasa-3. Además, los cambios de los proteomas se evaluaron mediante proteómica cuantitativa (SWATH-MS), seguida de un análisis de las rutas o pathways, para determinar los mecanismos moleculares afectados. En todos los ensayos, DHA inhibió la proliferación celular de las células HT-29 en mayor medida que ARA y actuó principalmente reduciendo la regulación de las partículas de proteasoma, mientras que ARA presentó un efecto significativo en las seis partículas de la helicasa de replicación del ADN.

En el capítulo III se utilizó un diseño y una tecnología similares al capítulo II para investigar las acciones del DHA y ARA como moléculas de MAG, y se efectuó una comparación de los resultados con los obtenidos utilizando los FFA. Los ensayos celulares revelaron que ARA- y DHA-MAG ejercían acciones antiproliferativas dependientes de la dosis y el tiempo, y que el DHA-MAG actuaba sobre las células cancerosas de forma más eficiente que el ARA-MAG. SWATH-MS, validado por la monitorización de reacciones paralelas y seguido por el análisis de pathways, reveló que DHA-MAG tenía un efecto masivo en el complejo proteasoma, mientras que el efecto principal de ARA-MAG estaba relacionado con la replicación del ADN. La síntesis de prostaglandinas también resultó inhibida por DHA-MAG. Los resultados demostraron claramente la capacidad del ARA y del DHA-MAG para inducir la muerte celular en las células de CRC, lo que sugiere una relación directa entre la estructura química y las acciones antitumorales.

En resumen, la presente tesis doctoral demuestra la actividad antitumoral de ARA y DHA como MAG y FFA contra las células HT-29 del CRC. Esta actividad fue establecida por las pruebas MTT, LDH y Caspasa-3, y por el análisis de los cambios observados en el proteoma de la célula después del tratamiento de ARA y DHA. Los resultados de esta tesis doctoral indican que tanto DHA como ARA constituyen potenciales candidatos a agentes quimiopreventivos.

# INTRODUCTION

# 2. INTRODUCTION

## 2.1. Lipids

Lipids comprise a wide variety of organic compounds which are insoluble in water due to their hydrocarbon chains but soluble in organic solvents such as hexane, methanol, acetone, or ether. Digestion, absorption, and transport in the blood as well as metabolism at the cellular level are affected by this property. Lipids can be classified into two major groups: acyl-lipids (saponifiable) and simple lipids (not saponifiable). Among the former, the most important are triacylglycerols (TAG) and phospholipids. The second group is represented, among others, by sterols and carotenoids (Ramírez et al., 2001; Aranceta and Gil, 2010). Lipids are necessary macronutrients in human nutrition because they regulate many physiological processes in the body, and they provide energy. Fatty acids (FA) are the basic components of TAG. FA are carboxylic acids with long aliphatic chains which may be straight or branched, saturated or unsaturated. Saturated FA (SFA) are those FA without double bonds while those with double bonds are unsaturated. Within unsaturated FA, they are known as polyunsaturated FA (PUFA), the FA with more than one double bond. Other functions of lipids are: i) they are essential in the formation of cellular structures such as membranes, ii) they are the vehicle for fat-soluble vitamins and iii) they provide the palatability and taste of foods (Valenzuela et al., 2002). Glycerol is the other compound necessary for the formation of lipids. It is formed by three polar groups -OH and is soluble in water. The position of FA in the glycerol structure determines physical properties (emulsifiers), chemical properties (stability against hydrolysis and oxidation) and nutritional properties (FA located in *sn*-1,3 positions will have a different metabolic fate from that of *sn*-2 position). Thus, lipids are defined as mixtures of different TAG (Castro González, 2002).

### 2.1.1. *n*-3, *n*-6, and *n*-9 series

According to IUPAC (International Union of Pure and Applied Chemistry) standards, the FA chain is numbered from the first carboxyl carbon. The position of the double bond can be indicated by the Greek letter ω or the letter *n*, hence the names *n* (ω)-9, *n* (ω)-3 and *n* (ω)-6. The *n*-3 family is characterized by the presence of a double bond attached to the third carbon from the end of the methyl. The enzyme Δ-9 desaturase is responsible for inserting double bonds from the end of the methyl to the ninth carbon atom, however, because mammals do not have such an enzyme, *n*-3 FA cannot be

synthesized by the organism and must therefore be present in the diet, so they are named essential FA (EFA) (Barcelo-Cobljin and Murphy, 2009) (Figure 1). The *n*-3 FA are linked to an increasing number of health benefits related to the prevention of certain types of cancer and beneficial effects on bone metabolism and brain function (Dillon et al., 2013). The main sources of *n*-3 FA are vegetable and fish oils, being the latter, the main source of eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) while in vegetable oils, α-linolenic acid (ALA, 18:3*n*-3) stands out (Castro González, 2002). The *n*-6 family is defined by the presence of a double bond in the sixth carbon of the methyl end and is mainly represented by linoleic acid (LA, 18:2*n*-6). One of the main functions of LA is its biotransformation into arachidonic acid (ARA, 20:4*n*-6), which originates eicosanoids (prostaglandin PGE2, thromboxanes TX, and related metabolites) involved in numerous physiological processes such as blood coagulation or inflammatory and immunological responses (Aranceta and Gil, 2010). Oleic acid (OA, 18:1*n*-9) is the representative FA of the *n*-9 family and is found in vegetable oils such as olive oil and in animal tissues. Since the body can synthesize OA by unsaturation of stearic acid (SA, 18:0) or by elongation and subsequent unsaturation of palmitic acid (PA, 16:0), it lacks essentiality (Aranceta and Gil, 2010).

The *n*-6/*n*-3 ratio is commonly used as an index to evaluate the nutritional quality of dietary FA, and it has relevance on human health. *n*-3 FA help to reduce inflammation, while most *n*-6 FA tend to increase it (Rodríguez-Cruz et al., 2005). Dietary LA and ALA play an important role in inflammation and anti-inflammation processes. These effects are due to the bioactive signaling lipids called eicosanoids which are derived from ARA, EPA, and DHA by cyclooxygenase (COX-1 and COX-2), lipoxygenase (5-LOX and 15-LOX), and epoxygenase (cytochrome P450 or CYP). Since *n*-6 and *n*-3 PUFA compete for corresponding desaturase and elongase enzymes, bioconversion of LA and ALA to their respective PUFA depends on the *n*-6/*n*-3 ratio (Saini and Keum, 2018). It was stablished that the recommended dietary *n*-6/*n*-3 ratio for health benefits ranged between 1:1 and 2:1 (Simopoulos, 2016) whereas an imbalanced *n*-6/*n*-3 ratio contributes to the development of diseases (Rodríguez-Cruz et al., 2005). Western diet contains 14 to 25 times more *n*-6 FA than *n*-3 FA while Mediterranean diet consist of a healthy balance between *n*-6/*n*-3, which means less tendency to suffer heart disease, stroke or diabetes (Rodríguez Cruz et al., 2005). However, in recent years, epidemiological studies have suggested that *n*-6 PUFA may be linked to reduced inflammation, so they have a positive

effect in cardiovascular diseases (CVD), on contrary to what has been stipulated so far. Results of Marklund et al., (2019) supported that high *in vivo* circulating and tissue levels of LA was associated with low risk of CVD. Even in a Japanese trial with an ARA treatment (Kakutani et al., 2011) cardiovascular, inflammatory, and allergic diseases were not stimulating. In that sense, Harris (2018) concluded that high consumption of LA and ARA is not the problem in Western cultures with high rates of chronic disease, the problem is the consumption of too little EPA and DHA. So, the problem is not the presence of the *n*-6 but the absence of the *n*-3. Thus, *n*-6/*n*-3 ratio in the context of inflammation is complex and still not properly understood (Innes and Calder, 2018).

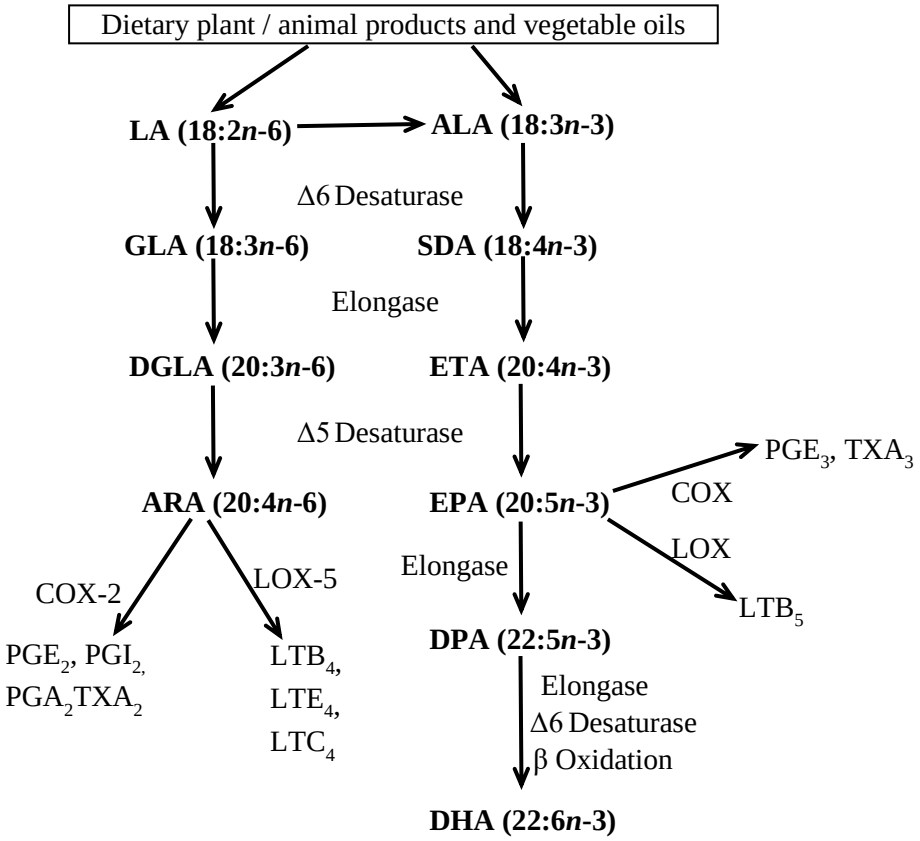


*Figure 1*. Biosynthetic pathway of *n*-3 and *n*-6 PUFA from their common precursors (Saini and Keum, 2018)

### 2.1.2. Lipid metabolism

Fats are the main source of energy in the diet as they provide 9 kcal/g, and the contribution of protein 4 kcal/g. Then, a poor regulation of the lipid metabolism can affect both the cardiovascular, immune, and nervous systems (Mataix and Gil, 2004). As shown in Figure 2, once fats enter the body through the mouth, two physiological processes are initiated, digestion and absorption and the resulting TAG-rich particles called chylomicrons can be assimilated by the cells of our body (Mataix and Gil, 2004). Lipid

digestion takes place in the mouth and in the gastric and intestinal cavity where the hydrolysis of the TAG with lipases and bile salts is taken place (Valenzuela et al., 2002). The enzyme that first act is the lingual lipase. This acil-ester-hydrolase has a great specificity to the *sn*-3 position of the TAG being less effective in the *sn*-1 position and does not act in the *sn*-2 position. So, the main digestion products of this first gastric phase are diacylglycerols (DAG) and free FA (FFA), which are absorbed by the enterocytes of the intestinal wall (Ramírez et al., 2001; FAO, 2010).

Depending on the size of the hydrocarbon chain FA released by the lingual lipase, the metabolic fate will be different. Short chains FA (C4-C10) are transported through the blood as albumin complexes or as lipids esterified in lipoproteins to the liver and they will be used for energy purposes (oxidized by mitochondrial β-oxidation). The lipoproteins found in the liver are called very low-density lipoproteins or VLDL, which are released into the bloodstream and transformed into intermediate density lipoproteins or IDL to low density lipoproteins or LDL (Valenzuela et al., 2002). The rest of the hydrolysis products that do not pass to the liver continue their transit to the small intestine, where the pancreatic and carboxyl-ester hydrolase enzymes act together bile salts form micelles (Arishima et al., 2009). Pancreatic lipase specifically hydrolyzes the *sn*-1,3 positions of the TAG so, *sn*-2 MAG and FFA are obtained (Valenzuela et al., 2002). According to Benito-Gallo et al., (2015), this specificity may be due to a combination of physicochemical properties of TAG, *sn*-2 MAG and FFA, i.e. the drop size of TAG, the solubility of *sn*-2 MAG within micelles and the stability of FFA. In the human metabolism, when TAG are ingested, the FA in the *sn*-1,3 positions of the glycerol skeleton are released by the pancreatic lipase, while the esterified FA in the *sn*-2 position remains unreacted, being adsorbed by the intestinal mucosa as *sn*-2 MAG. Therefore, PUFA that are esterified in the *sn-2* position of the TAG are more easily absorbed than those in the *sn*-1,3 positions, and are more bioavailable for metabolic needs (Iwasaki and Yamane, 2000). Finally, the FFA are assimilated by enterocytes by passive diffusion and are quickly re-esterified in form of chylomicrons. Once in the bloodstream, the chylomicron distributes its lipid load through different tissues (FAO, 2010). On the other hand, bile salts remain in the intestinal light and are reabsorbed by the ileum while some FFA are incorporated into the phospholipids that are within the cell membranes or act as precursors of eicosanoids (Valenzuela et al., 2002).

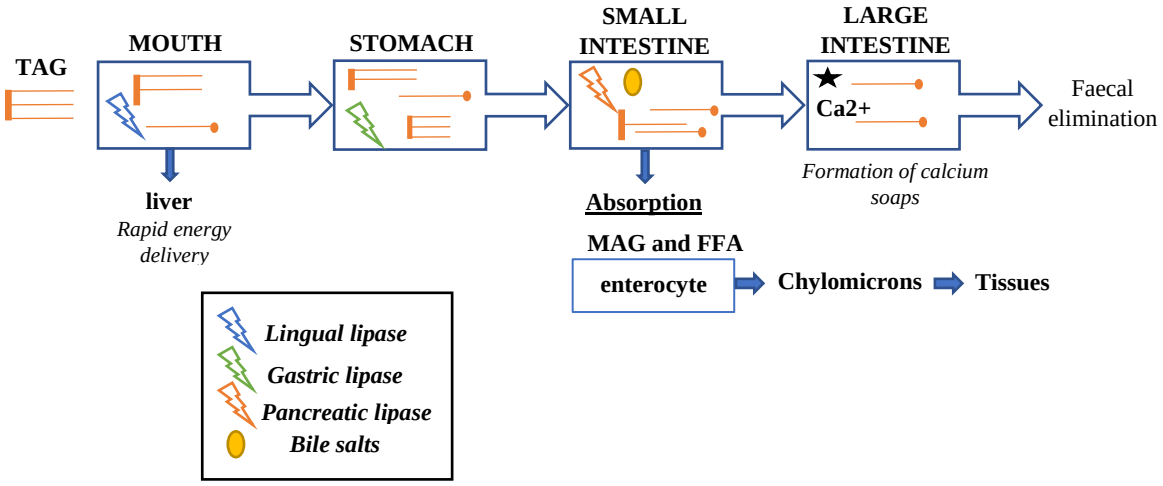


*Figure 2*. Diagram of the digestive process of TAG (Valenzuela et al., 2002)

## 2.2. Bioactive FA

Vegetable oils have always been considered cheap and the healthiest oils due to their suitable lipid profile. However, interest in marine oils has increased considerably, due to the health benefits provides by their PUFA contents, especially those due to DHA and EPA (Sijtsma and Swaaf, 2004). According to Fennema (2000), in seed oils unsaturated FA are preferably stored in the *sn*-2 position and SFA are almost always in the *sn*-1,3 position. Vegetable oils mostly stand out as a good source of *n*-6 (mainly LA) and have low amounts of *n*-3 PUFA (Saini and Keum, 2018). The richest source of *n*-3 PUFA are fish oils, which are obtained from the lean liver of fish such as herring, mackerel, sardine, or anchovy. Fish oil contains EPA from 5 to 26% and DHA from 6 to 26% of total FA (Alkio et al., 2000) which are mainly placed in the *sn*-2 position of the glycerin molecule. Despite their health effects, the PUFA in these oils oxidize easily at room temperature, reducing the nutritional value of fish oil. For that reason, oxidative processes are critical parameters for the shelf life of fish oils and the food products that contain them (Camino Feltes et al., 2013).

The targeted bioactive FA in this doctoral dissertation are the following:

i) ARA: it is a long chain PUFA belonging to *n*-6 family (Figure 3). Human can biosynthesize by desaturation, elongation and partial oxidation reactions from LA or can be obtained from diet. It acts as a precursor for the biosynthetic production of PGE2, TX and related metabolites, which influence various metabolic activities such as platelet aggregation, inflammation, and immune function (Aranceta and Gil, 2010). Both ARA and DHA are incorporated into fetal tissue membranes when the brain and vascular

system are growing at a very fast rate through breast milk (Arterburn et al., 2000). The sources of ARA are mainly microorganisms such as the microalgae *Porphyridium cruentum* and *Parietochloris incisa*, and the fungus *Mortierella alpina* (Yamauchi et al., 2005). This fungus is the most widely used microorganism for the commercial production of ARA, for instance ARASCO® (ARA-rich Single Cell Oil). The oil extracted from *Mortierella alpina* is then diluted to a standard concentration of 40% ARA by the addition of high oleic sunflower oil and mixed with antioxidants such as tocopherols (0.025%) and ascorbyl palmitate (0.025%) (Arterburn et al., 2000).

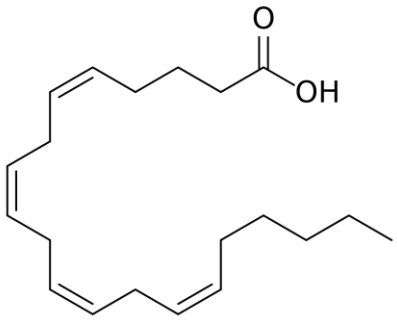


*Figure 3.* ARA estructure

ii) DHA: it is a *n*-3 PUFA composed of 22 carbon atoms and six double bonds (Figure 4). This PUFA is the predominant FA in the structural phospholipids of the human brain and retina (more than 60% of the total FA) and accumulate rapidly in fetal and infant neural tissue during the childhood. For this reason, it is very important that DHA is incorporated into children diet, and it has been recognized as an important component of breast milk (Sijtsma and Swaaf, 2004). At present, fish oil is the main source of DHA, however, the application of PUFA from fish oil in infant formulas, or for pharmaceutical applications, may have some disadvantages due to fish odor or unpleasant taste. In addition, as marine fish oil is a complex mixture of FA with different lengths and degrees of unsaturation, a costly DHA purification process may be necessary prior to application (Sijtsma and Swaaf, 2004). Alternatively, DHA can be produced using microorganisms, mainly marine, which sometimes contain large amounts of DHA. Single Cell Oil (SCO) is a relatively new product. One of the advantages of the use of microorganisms is that preferably they contain a specific PUFA, rather than a mixture of several PUFA, which gives to microbial oils an additional value compared to fish oils, which contain mixtures of PUFA. Commercial DHASCO® (DHA-rich Single Cell Oil) is obtained from *Crypthecodinium cohnii*, a non-photosynthetic marine microalga, which contains about

40% DHA. The oil is extracted from the biomass by mixing the biomass with *n*-hexane in a continuous extraction process. The extracted oil is then diluted to a standard concentration of 40% DHA by the addition of high oleic sunflower oil and mixed with antioxidants such as tocopherols (0.025%) and ascorbyl palmitate (0.025%) (Sijtsma and Swaaf, 2004).

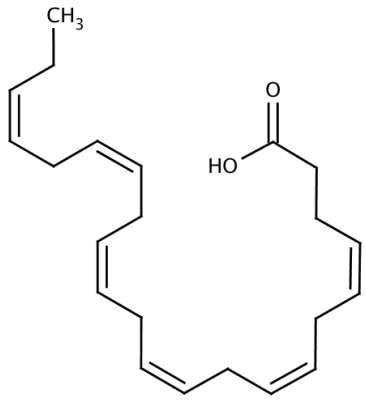


*Figure 4.* DHA structure

### 2.3. PUFA purification

Highly concentrated PUFA from oil or fat are needed for medical or dietary administration, as well as for chemical experimentation. Processes for PUFA concentration are based on the differences in polarity and/or spatial configuration of the PUFA present in the extract, i.e. the number of double bonds in the carbon chain (Guil-Guerrero et al., 2003). For this purpose, there is a wide range of methodologies to isolate PUFA. These methods are low temperature crystallization, the formation of urea complexes (UC), molecular distillation, enzymatic methods, supercritical fluid extraction and various chromatographic methods (Latyshev et al., 2014). Physical methods, such as crystallization or UC, are simple and conventional methods that allow the rapid separation of unsaturated from SFA, however yields are much lower. On the contrary, the use of chromatography and supercritical methods guarantee high purity and yields, but they are considered expensive on an industrial scale. On the other hand, esterification with lipases are used for the enrichment of PUFA according to the specificity of lipase (Baeza Jiménez et al., 2014). PUFA such as GLA or ARA were purified using winterization, chromatography, UC, supercritical extraction and, most recently, lipase catalyzed esterification (Baeza Jiménez et al., 2014). UC methodology is based on the degree of unsaturation, so, SFA and MUFA which have a linear structure, can be easily trapped by

the cavity of the urea complex while the non-urea complex fraction is enriched with PUFA (Magallanes et al., 2016). Winterization (or crystallization) and UC methods have been successfully applied to obtain PUFA-rich concentrates, but in most cases, no upper purity limit is obtained, so these techniques are usually linked to liquid chromatography in order to reach a purity higher than 90% (Guil-Guerrero et al., 2000; Guil-Guerrero and Belarbi, 2001; Guil-Guerrero et al., 2003). One of the advantages of that methods is that they are scalable processes and environmentally friendly due to the use of inexpensive renewable materials (urea and ethanol or methanol as solvent) (Hayes, 2006). Column chromatography using silica gel ($SiO_2$) impregnated with silver nitrate ($AgNO_3$) as stationary phase has been shown to be effective to purify any PUFA (Guil-Guerrero et al., 2001). According to Bhandari et al., (2013) and Dillon et al., (2013), this chromatographic method using $AgNO_3$ has not been accepted industrially due to the presence of residual silver and its high cost. However, some authors argued that the silver ion can be removed successfully by adding NaCl solution, so harmful effects are negligible (e.g. Rincón-Cervera et al., 2009).

### 2.4. MAG purification

Structured lipids formed by a PUFA of high beneficial value in the *sn*-2 position flanked by medium chain FA in the *sn*-1,3 positions are highly bioavailable, so several methodologies were developed to obtain them (Rahman et al., 2015). MAG can be produced through hydrolysis or glycerolysis of TAG and esterification of FA. They can also be obtained by oil alcoholisis, such as methanolysis or ethanolysis (Zhong et al., 2013). However, alcoholisis process may present some problems such as the deactivation of lipase and acyl-migration, which is caused by lipase immobilization, solvents, or high temperatures (Esteban et al., 2009). Enzymatic processes have become an attractive alternative approach to produce MAG since the reactions can be carried out under mild conditions (García Solaesa et al., 2016). Once MAG are obtained, *sn*-2 MAG purification can be carried out by chromatography and solvent extraction, and by combining both procedures high purities and yields are obtained (Nieto et al., 1999; Compton et al., 2013; Rahman et al., 2015). Other method such as flash chromatography, short run distillation process and $CO_2$ have proven to be a practical option for large-scale purification of MAG (Compton et al., 2012; Compton et al., 2013; García-Solaesa et al., 2016). It is necessary to take into account that when *sn*-2 MAG purification is attempted, several variables

influence the acyl-migration, e.g. temperature, polarity of the solvent, length of the FA chain and storage time, among others (Compton et al., 2013).

## 2.5. Colorectal cancer

### 2.5.1. Epidemiology

CRC is a heterogeneous disease that occurs in the colon and the rectum, which are parts of the gastrointestinal system. The majority of CRC develop slowly from adenomatous polyps or adenomas (Aran et al., 2016). Over 1.8 million new CRC cases and 881,000 deaths are estimated to occur in 2018, accounting for about 1 in 10 cancer cases and deaths (Bray et al., 2018). Overall, CRC is the third most common cancer worldwide and the fourth leading cause of cancer-related deaths with mortality approaching 50%, second only to lung cancer as the leading cause of death in Western countries (Vasaikar et al., 2019). According to Bray et al. (2018) the highest CRC incidence rates are found in Europe (e.g. Hungary, Slovenia, the Netherlands, and Norway), Australia, Northern America, and Eastern Asia. These regional differences suggest a close relationship between genetic and environmental factors. Environmental factors include diet and lifestyle. Different epidemiological studies have suggested that there is a close relationship between the quantity and quality of fat and the risk of CRC. For this reason, it could be said that approximately 45% of CRC cases can be avoided by changing diet and lifestyle (Bishop and Ferguson, 2015; Sala-Vila et al., 2010). It is estimated that 30-50% of CRC in males and 20% in females can be prevented by the adoption of a balanced diet and change in lifestyle (Tsoukas et al., 2015). As mentioned above, Mediterranean countries have lower rates of CRC, compared with other western countries, and it has been suggested that it may be due to diet (Nkondjock et al., 2003). This beneficial effect of Mediterranean diet could be attributable to some foods with anti-inflammatory properties and antioxidant effects such as fruits, vegetables, fish, and olive oil (Notarnicola et al., 2018). Excessive caloric intake can increase uncontrolled cell proliferation, so we must incorporate into the diet some components that contribute to inhibit the loss of cell cycle control (Bishop and Ferguson, 2015).

### 2.5.2. CRC cell lines

The cell lines derived from colon cancer have provided a very useful tool for the study of genes regulating intestinal differentiation and its involvement in cancer, largely due to the difficulty of obtaining cultures from normal intestinal epithelial cells. The HT-

29 cell line was established in 1975, derived from human colon adenocarcinoma cells (Fogh, 1975). At the morphological level, the cultures of this cell line are heterogeneous and contain subpopulations of cells with diverse differentiation capacity. Thus, under standard culture conditions, the population contains more than 95% of morphologically undifferentiated cells. During growth towards post-confluence, cells are stacked and do not polarize, forming multilayers, and do not express differentiation markers characteristic of adult intestinal epithelial cells. Some CRC cell lines have preserved parts of the intestinal epithelial differentiation program, expressing many of the characteristic genes of epithelial cells. Altering culture conditions or treating the cells with various inducers, the morphology of HT-29 CRC cells can be modulated to express distinct pathways of enterocyte differentiation, results in a differentiated and polarized morphology, characterized by the redistribution of membrane antigens and development of an apical brush-border membrane (Cohen et al., 1999). Figure 5 shows HT-29 CRC cell line used in this doctoral thesis.

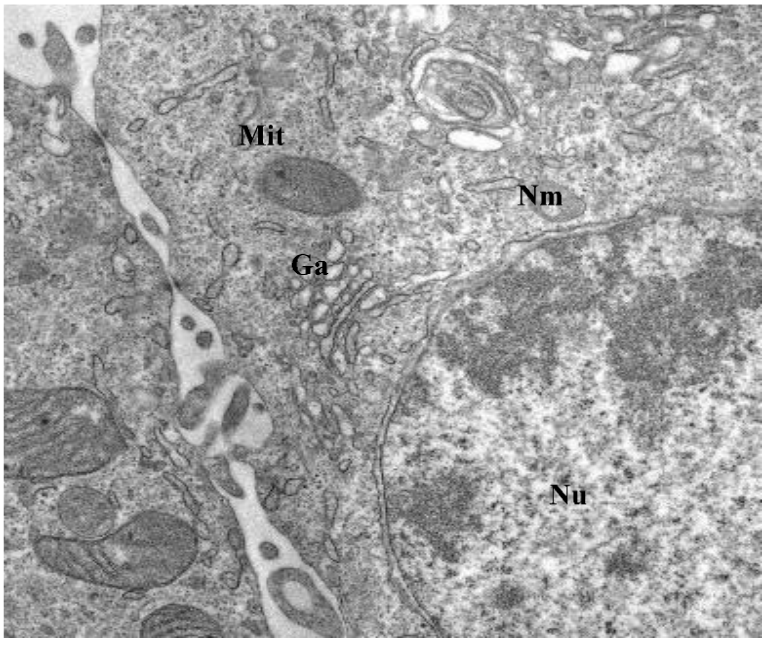


*Figure 5*. HT-29 CRC cell line. Mit: mitochondria; Nu: nucleus; Nm: nuclear membrane; Ga: Golgi apparatus.

According to Repetto et al. (2005), the advantages and disadvantages of *in vitro* experimentation methods with cell lines are:

| Advantages | Disadvantages |
|---|---|
| Ethical and morally more acceptable | Greater difficulties in testing poorly soluble, volatile, gaseous, and easily degradable substances |
| Improved accuracy and reproducibility of results, without interference | Possibility of erroneous results due to tests under unsuitable conditions |
| Absolute control of the duration of the contact period | Complexity for extrapolation to *in vivo* assays |
| Easily objectifiable and quantifiable | Sensitive technique, since cell growth must be performed under strict aseptic conditions |
| Facilitate the study of the mechanisms of toxic action | |
| Lower cost | |
| Avoid interferences with other organs | |

### 2.5.3. *In vitro* cellular assays

Several *in vitro* assays/techniques have been developed to evaluate the anticancer activity of different compounds in cancer development. *In vitro* assays for viability/antiproliferation, apoptosis, gene and protein expression, and several others are commonly used in cancer drug discovery studies (Ediriweera et al., 2018).

i) The MTT assay is a sensitive and quantitative colorimetric method for measuring cell survival and proliferation. It is based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazol bromide by mitochondrial enzyme dehydrogenase in a dark blue compound (formazan), allowing the mitochondrial functionality of treated cells to be determined (Figure 6). MTT reduction depends on cellular metabolic activity due to the flow of NADPH. Formazan crystals accumulate in the cell due to its inability to pass through the plasma membrane (insoluble in water), which generates the need to smooth the cells to release it. Formazan crystals must be solubilized with organic solvents, including DMSO, isopropanol-HCl and ethanol. The amount of living cells is directly proportional to the amount of formazan produced and is

quantified by colorimetry. Besides the MTT tetrazolium, other tetrazolium compounds such as MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2Htetrazolium-5-carboxanilide) and WST can also generate formazan upon reduction by oxidoreductase or dehydrogenase (Twentyman and Luscombe, 1987; Ediriweera et al., 2018).

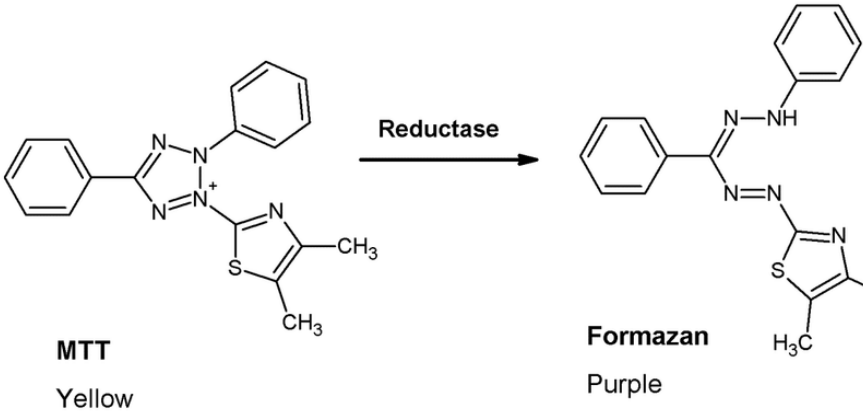


*Figure 6*. Metabolic reduction of MTT to formazan by the action of mitochondrial enzyme dehydrogenase (Ediriweera et al., 2018)

ii) LDH is a colorimetric method suitable for the measurement of cell death and lysis. It is based on the measurement of the activity of the enzyme lactate dehydrogenase (LDH), present in the cytoplasm of living cells, being released into the environment due to the permeabilization of the membrane of damaged cells. The amount and intensity of color due to solubilized formazan can be measured spectrophotometrically. The increase in LDH activity in the culture supernatant is proportional to the number of lysate cells (Fotakis et al., 2006).

iii) The molecular mechanisms related to cancer cell apoptosis involves certain biochemical events such as DNA fragmentation, chromatin condensation, cell organelle degradation and protein cleavage, among others. Detection of the activation of caspases has become a very common *in vitro* technique to confirm apoptosis (Elmore, 2007). Caspase-3 is a member of the caspase family that plays an important role as an indicator of apoptosis, or programmed cell death. It can process procaspase 2, 6, 7, and 9 and specifically split most of the caspase-related substrates known to date, including many key proteins such as the poly nuclear enzyme (ADP-ribose) polymerase (PARP), the

activated deoxyribonuclease caspase inhibitor (ICAD), and gelsolin and fodrin, which are proteins involved in the regulation of apoptosis (Kamada et al., 1998). After activation, caspase-3 cuts a wide variety of cellular proteins, causing morphological and functional changes in the cells that carry out apoptosis. The caspase-3 colorimetric assay is based on the hydrolysis of the substrate peptide acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, which results in the release of the p-nitroaniline fraction (pNA) (Elmore, 2007).

Figure 7 shows a whole overview about techniques described.

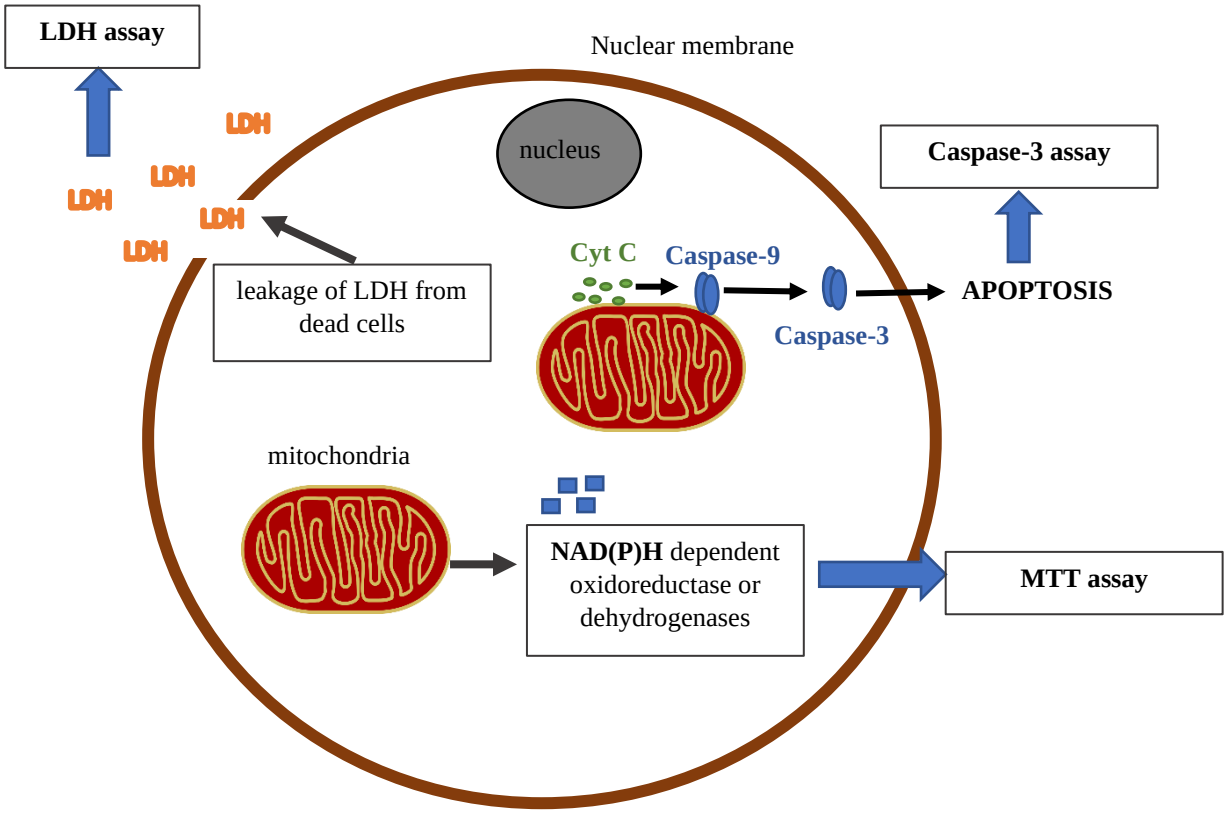


*Figure 7.* Illustration of assays and techniques available for the detection of cancer cell viability/proliferation and apoptosis (Ediriweera et al., 2018).

### 2.5.4. Mechanisms of PUFA on cancer

The effects of *n*-3 and *n*-6 PUFA on cancer cells proliferation have been shown to be variable. Although *n*-3 PUFA are usually thought to have inhibitory effect, and *n*-6 PUFA stimulate the growth of tumor cells, studies of evidence indicate that *n*-6 PUFA also have anticancer activity (Chen and Istfan, 2000). On the other hand, Davidson et al. (2004) concluded that the chemopreventive effect of fish oil is due to the direct action of *n*-3 PUFA and not to a reduction in the content of *n*-6 PUFA. For that reason, both *n*-3

and *n*-6 PUFA influence colon carcinogenesis by different mechanisms. Marventano et al. (2015) reviewed the main mechanisms of PUFA on cancer and assessed that a state of chronic inflammation may generate a series of effects, such as oxidative stress and DNA damage, which may increase cancer risk. PUFA have been shown to act by modulating a variety of molecular pathways involved in cell proliferation, apoptosis and metastasis, and which are strictly connected with tumor development and progression changing the expression of eicosanoids and oncogenes and tumor suppressor genes (Calviello et al., 2007; Roynette et al., 2004). Specifically, there are two molecular pathways which are crucial in the development and progression of CRC, the COX-2, and β-catenin pathways. Both they are factors strictly related to the transcription of genes involved in colon tumorigenesis, whose codified proteins act as regulators of cell cycle progression, apoptosis, angiogenesis, local invasion or metastasis. Besides these two pathways, Table 1 summarizes other mechanisms proposed as responsible for the apoptosis and antitumor activity of DHA and ARA on HT-29 CRC cell line.

Table 1. PUFA mechanisms action on HT-29 CRC cells

| Mechanisms | PUFA | Doses | Reference |
|---|---|---|---|
| lipid peroxidation | ARA and DHA | 50-200 µM | Chen and Itsfan, 2000 |
| | | 20-50 µM | Hofmanová et al., 2005 |
| | | 100 µM | Habermann et al., 2009 |
| | | 150 µM | Hossain, 2009 |
| down-regulation of Bcl-2 | DHA | 50-200 µM | Chen and Itsfan, 2000 |
| | | 10 µM | Calviello et al., 2005 |
| | | 150 µM | Hossain, 2009 |
| | | 50-200 µM | Habermann et al., 2010 |
| activation of PPAR gamma | DHA | 50-200 µM | Habermann et al., 2010 |
| | | 50 µM | Tylichová et al., 2017 |
| down-regulation of PGE2<br>down-regulation of COX-2 expression<br>up-regulation genes alter Erk/MAP kinase signaling<br>down-regulation of inhibitor of caspase-8 activation<br>activation of Bax and Bak<br>alteration in the expression and cellular location of GRP78<br>modulation of the WNT/β-catenin pathway | DHA | 10-30 µM | Calviello et al., 2004 |
| | | 60 µM | Giros et al., 2009 |
| | | 30 µM | Fasano et al., 2012 |
| | | 15-25 µM | Murad et al., 2018 |

## 2.6. Proteomics

The main objective of the proteomics study is the detailed characterization of the proteome. The proteome is a functional translation of the genome, directly associated to the cell phenotype, being a rich source of biomarkers. Proteomic could be key to deeply understand CRC by means of newly identified biomarkers. Proteomics has been used to determine differential expression profiles in normal or disease states, which might be responsible for abnormal cell proliferation. The identification of proteins that are characteristic for cancer formation and progression can potentially uncover diagnostic, or prognostic markers, or novel drug targets, and could help to understand the mechanisms underlying tumor formation. Diverse studies identified L-FABP, mortalin, hnRNP K and the chaperonin t-complex proteins as overexpressed in CRC. Another study revealed a significant increase in the glycolytic pathway (Warburg effect) and different effects on the gluconeogenesis, glucuronic acid and tricarboxilic acid pathways during the progression of CRC (Reymond et al., 2004; Barderas et al., 2010). Molecular and genetic studies have demonstrated that the CRC is related to alterations in the expression of genes controlling cell proliferation and apoptosis and increasing cell motility, such as the oncogene K-RAS, the tumor suppressor genes APC, DCC, and p53, as well as DNA repair genes (Wisniewski et al., 2015).

The study of the proteome, called proteomics, now evokes not only all the proteins in any given cell, but also the set of all protein isoforms and modifications, the interactions between them, the structural description of proteins and their higher-order complexes, and for that matter almost everything 'post-genomic' (Tyers and Mann, 2003). The basic tool in proteomics is liquid chromatography-mass spectrometry (MS), as method of identifying and characterizing the proteins in a sample, and making possible massive protein quantification, either absolute o relative. Quantitative information is a fundamental necessity to interrogate the highly dynamic global proteome of living organisms. Protein level and peptide level fractionation, combined with current high-resolution MS-based platforms can identify and quantify proteins within a given biological sample, depending on the complexity of the starting material (Jiménez et al., 2010). Protein expression profiles of cell lysates using 2D gel electrophoresis (2DE) coupled with MS has been the traditional and most widely established proteomic approach (Skandarajah et al., 2005). Today, liquid chromatography coupled to tandem MS (LC-MS/MS) -based proteomics aims to provide large-scale quantitative information

on protein abundances (Frese et al., 2019). There are two common strategies available: shotgun proteomics which is operated in data-dependent acquisition (DDA) mode and targeted proteomics where the MS instrument is operated in selected reaction monitoring (SRM) mode (Gillet et al., 2012). However, both strategies have limitations for example, they lack the throughput to routinely quantify large fractions of a proteome. Currently, the recently developed data-independent acquisition (DIA) mode allows to solve that drawback. DIA mode consists of consecutive scans of fragment ion spectra for all the precursors contained in predetermined isolation windows. One of the main approaches based on DIA mode is the sequential window acquisition of all theoretical mass spectra (SWATH)-MS, which was described by Gillet et al., (2012). SWATH consists on combining precursor data from survey scans with stepped m/z fragmentation windows, and then matching the resulting fragment ions to peptides and proteins using a previously generated MS/MS spectral library, so fragment ion chromatograms can be in-silico extracted and used for label-free protein quantitation. SWATH-MS is showing outstanding precision and accuracy even when used for proteome-wide quantitation (Huang et al., 2015; Selevsek et al., 2015). SWATH performance is comparable to that of selected reaction monitoring (SRM), the golden standard for protein and small molecule quantitation (Selevsek et al., 2015). The next step after the quantified and identified proteins by SWATH-MS, is to measure gene expression levels affected in an experiment. Although lists of genes provide valuable information, they alone cannot explain the complex mechanisms that are involved in the given condition. For solving this problem, various common techniques are used to leverage the knowledge contained in various pathway databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, Bio-Carta, NCI-PID, iPathwayGuide (iPG), and PANTHER, among others. Pathway analysis approaches use available pathway databases and the given gene expression data to identify the pathways which are significantly impacted in each condition (Nguyen et al., 2019). Ahsan and Draghici (2017) reviewed the useful iPG tools in systems biology approach. In this regards, iPG analyzes a list of differentially expressed genes and identifies the signaling pathways and Gene Ontology terms that are significant between the two phenotypes, or even, it identifies the diseases and drugs that are associated with any of the pathways involved, as well as the mechanisms that would explain all measured gene expression changes throughout the system. These results from iPG allow efficiently generate hypotheses, identify biomarkers, elucidate molecular

mechanisms, discover drug targets, and measure therapeutic response via the given comparison of two or more conditions.

# JUSTIFICATION, HYPOTHESIS AND OBJECTIVES

# 3. JUSTIFICATION, HYPOTHESIS AND OBJECTIVES

## 3.1. JUSTIFICATION

The World Health Organization (WHO) estimated in 2018 that cancer was the second leading cause of death before the age of 70 in most countries. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervical and thyroid cancer are the most common among women. CRC is one of the most widespread, with an estimated 1.8 million new and 881,000 deaths in 2018. This represents about 1 in 10 cancer cases and deaths. Numerous studies confirm the direct relationship between consumption of a *n*-3 PUFA-rich diet and a low incidence of CRC. The role of fats and oils in human nutrition is one of the main areas of interest and research in the nutrition field. There is currently a great concern and need for information on the benefits of certain FA in our health. One of the products of lipid metabolism are MAG which are became highly bioavailable by the organism. In this regard, there is an increasing amount of research to produce new functional ingredients such as structured lipids from purified *sn*-2 MAG of nutritional interest. Knowledge about the importance of these compounds and FA that make them up has steadily increased in human health over the last few years and several studies show their positive role in CVD, various mental illnesses, and cancer. Therefore, studies to determine the antitumor effects of all PUFA, both *in vitro* and *in vivo*, are needed.

The purpose of this doctoral thesis is, on one hand, developing strategies for obtaining highly purified PUFA, e.g. DHA and ARA, followed by the evaluation and characterization of their chemopreventive potential against CRC cells. This activity will be checked in the two main structural forms they present (FFA and MAG), with the aim of elucidating the benefits associated with their intake. On the other hand, the effects produced by these PUFA in the metabolic pathways of CRC cells was evaluated to obtain useful information about changes in protein expression and associated mechanisms in different cell stages, with the aim of improving methodologies for diagnosis and treatment of CRC diseases.

## 3.2. HYPOTHESIS AND OBJECTIVES

The general hypothesis of this doctoral thesis is that ARA and DHA, both as FFA and MAG, *in vitro* supplied to HT-29 CRC cells, will induce characteristics antitumor effects by affecting different metabolic pathways.

To demonstrate this hypothesis, the general objectives were designed **to**:

1º. Purify several bioactive PUFA, both as MAG and FFA, by different chromatographic techniques and the UC methodology (Chapter I and II).

2º. Evaluate the antitumor capacity of different PUFA as both FFA and MAG against HT-29 CRC cell line, by means of MTT and LDH tests (Chapter II and III).

3º. Evaluate the apoptosis phenomenon by checking Caspase-3 activity (Chapter II and III).

4º. Study the significant effects exercised by such PUFA on the metabolic pathways in HT-29 CRC cells, according to proteomic analyses, i.e. SWATH/MS analysis (Chapter II and III).

Based on the general hypothesis and its objectives, the thesis was divided into three chapters.

Hypothesis for CHAPTER I: Highly purified PUFA as both MAG and FFA can be obtained by means of two sequential chromatographic column steps using two different stationary phases, while the UC methodology can be suitable for highly pure FFA-PUFA obtainment.

Hypothesis for CHAPTER II: ARA- and DHA-based FFA will have *in vitro* anticancer activities due to the downregulation of specific proteins that will cause the phenomenon of apoptosis on HT-29 CRC cells.

Hypothesis for CHAPTER III: DHA- and ARA-MAG will cause a damage in membrane cell and an antiproliferative activity and, also, some protein will be affected by ARA- and DHA-MAG-treatment on HT-29 CRC cells. So, the high bioavailability of MAG in the human body will have a positive effect on the prevention of colon cancer.

Therefore, the objectives designed for the demonstration of these three hypotheses are aimed **to**:

CHAPTER I:

1º. Select commercially available edible oils having bioactive PUFA.

2º. Design a suitable open column chromatography process using $AgNO_3$ as stationary phase to purify diverse PUFA.

3º. Update the UC method for highly pure PUFA obtainment.

4º. Evaluate the rate of acyl migration within the TAG structure by proton magnetic resonance spectroscopy ($^1$H-NMR).

CHAPTER II:

1º. Purify ARA and DHA as FFA according to fractionation of fatty esters by column chromatography process using $AgNO_3$ as stationary phase.

2º. Evaluate the antitumor effects and cell membrane damages influenced by ARA and DHA as FFA against the HT-29 CRC cells by means of the MTT and LDH assays.

3º. Evaluate the apoptosis phenomenon by means of the Caspase-3 activity assay.

4º. Study the protein abundance changes induced by DHA and ARA in the global proteome of HT-29 CRC cells by proteomic analyses, i.e. SWATH/MS analysis.

5º. Assess the metabolic pathways affected by DHA and ARA in HT-29 CRC cells.

CHAPTER III:

1º. Purify ARA- and DHA-based MAG according to Chapter I.

2º. Evaluate the antitumor actions of these PUFA-MAG against HT-29 CRC cells by means of the MTT and LDH assays.

3º. Check the apoptosis phenomenon induced by these MAG on HT-29 CRC cells by means of the Caspase-3 activity assay.

4º. Study the protein abundance changes induced by DHA- and ARA-based MAG according to SWATH/MS analysis.

5º. Assess the metabolic pathway affected by both DHA- and ARA-MAG in HT-29 CRC cells.

# ARTICLES

## 4. ARTICLES

### CHAPTER I. PUFA purification process from edible oils

**Article 1º**

**Title:** Purification process for MUFA- and PUFA-based monoacylglycerols from edible oils.

**Authors:** María José González-Fernández, Rebeca Pilar Ramos-Bueno, Ignacio Rodríguez-García, José Luis Guil-Guerrero

**Journal:** Biochimie

**Year of publication:** 2017

**Impact index:** 3.362

**Quartile:** Q2 (Biochemistry & Molecular Biology)

**Article 2º**

**Title:** Highly concentrated very long-chain PUFA obtainment by Urea complexation methodology

**Authors:** María José González-Fernández, Dmitri Fabrikov, Svetlana Lyashenko, Francisca Ferrón-Carrillo, José Luis Guil-Guerrero

**Journal:** Environmental Technology & Innovation

**Year of publication:** 2020

**Impact index:** 2.800

**Quartile:** Q2 (Biotechnology & Applied Microbiology)

### CHAPTER II. Proteomics study reveals that docosahexaenoic and arachidonic acids exert different in vitro anticancer activities in colorectal cancer cells

**Article 3º**

**Title:** Proteomics Study Reveals That Docosahexaenoic and Arachidonic Acids Exert Different In Vitro Anticancer Activities in Colorectal Cancer Cells

**Authors:** Ignacio Ortea, María José González-Fernández, Rebeca Pilar Ramos-Bueno, José Luis Guil-Guerrero

**Journal:** Journal of Agricultural and Food Chemistry

**Year of publication:** 2018

**Impact index:** 3.571

**Quartile:** Q1 (Food Science & Technology)

**CHAPTER III. SWATH differential abundance proteomics and cellular assays show in vitro anticancer activity of arachidonic acid- and docosahexaenoic acid-based monoacylglycerols in HT-29 colorectal cancer cells**

**Article 4º**

**Title:** SWATH differential abundance proteomics and cellular assays show in vitro anticancer activity of arachidonic acid- and docosahexaenoic acid-based monoacylglycerols in HT-29 colorectal cancer cells

**Authors:** María José González-Fernández, Dmitri Fabrikov, Rebeca Pilar Ramos-Bueno José Luis Guil-Guerrero, Ignacio Ortea

**Journal:** Nutrients

**Year of publication:** 2019

**Impact index:** 4.171

**Quartile:** Q1 (Nutrition & Dietetics)

# CHAPTER I

# 5. CHAPTER I. PUFA purification process from edible oils

## 5.1. Abstract

Both *n*-3 and *n*-6 PUFA develops several positive physiological actions, and thus these have utility as pure compounds for several reasons: i) they could be used as healthy supplements for animals and humans; ii) they have stablished physiological effects in animal experimentation models; and iii) they are used as standards for chromatographic use. These reasons led us to the design several methodologies to purify these PUFA.

This chapter contains two studies:

In the first one, six edible oils, namely: olive, linseed, sunflower, evening primrose, DHASCO® and ARASCO® have been processed. First, the oils were hydrolyzed by means of an enzymatic process using porcine pancreatic lipase and then the reaction products were fractionated by using a liquid chromatography column containing $SiO_2$ as stationary phase to purify the MAG-enriched fraction. A second chromatography process was performed using $AgNO_3$ coated $SiO_2$ as stationary phase, to obtain the different MUFA- and PUFA-based MAG from the corresponding oils. Overall, MAG based on OA, LA, ALA, GLA, ARA and DHA have been isolated in high yields and purities (92.6, 97.4, 95.3, 90.9, 100.0 and 95.3% purity, respectively). Positional distribution was determined by means of $^1$H-NMR, which revealed a mix of 1(3) and 2-MAG in variable proportions in the different MAG.

In the second study, DHA-, EPA-, and ARA-concentrates were obtained at 98.0, 43.0 and 82.8% of total FA by the UC method at high urea:FA ratios (7:1) at 4 ºC. A final washing step was incorporated to remove ethyl carbamates in the final product. Five oil sources were used: commercially available tuna oil, DHASCO®, ARASCO®, and MARINOL®, and the oil from the microalga *Nannochloropsis*. This study clearly shows the importance of the proper selection of the oil source to achieve high-purity PUFA concentrates by the UC method.

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Research paper

# Purification process for MUFA- and PUFA-based monoacylglycerols from edible oils

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ABSTRACT

Important health benefits have been attributed to monoacylglycerols (MAGs) due to their various physiological functions, owing to which they become candidates for use as functional foods in order to prevent the onset of certain diseases such as colon cancer. In this work, six edible oils, namely: olive, linseed, sunflower, evening primrose, DHASCO® and ARASCO® have been processed to obtain different MUFA- and PUFA- based MAGs. First, the oils were hydrolyzed by means of an enzymatic process using porcine pancreatic lipase and then the reaction products were fractionated by using a liquid chromatography column containing silica gel as stationary phase in order to purify the MAGs-enriched fraction. A second chromatography process was performed using silver nitrate coated silica gel as stationary phase, in order to obtain the different MUFA- and PUFA-based MAGs from the corresponding oils. Overall, MAGs based on oleic, linoleic, α-linolenic, γ-linolenic, arachidonic and docosahexaenoic acids have been isolated in high yields and purities (92.6, 97.4, 95.3, 90.9, 100 and 95.3% purity, respectively). Positional distribution was determined by means of $^{1}$H NMR, which revealed a mix of 1(3) and 2-MAGs in variable proportions in the different MAGs.



## 1. Introduction

Today there is a great interest on the influence of several polyunsaturated fatty acids (PUFAs) on health such as eicosapentaenoic (EPA, 20:5n3), arachidonic (ARA, 20:4n6) and docosahexaenoic acids (DHA, 22:6n3). Although the optimal intake of PUFAs has not been officially set, FAO/WHO [1] establishes that the acceptable range for the total PUFAs (n-6 and n-3 fatty acids) is between 6 and 11% E (energetic percentage of fat) while an adequate intake to prevent deficiency is between 2.5 and 3.5% E. Such molecules have a prominent influence on several diseases such as cardiovascular risks, which have been a major concern over the last years, and have led to the development of diverse technologies for purifying different PUFAs to perform pharmacological essays [2]. In this way, PUFAs omega-3 (n-3) and omega-6 (n-6) have become popular as dietary supplements, leading to an increase in the global demand for high-purity PUFAs, for clinical and nutritional applications [3]. Essential FAs (EFAs) are FAs that the body cannot synthesize but that are necessary to maintain health, so they must be obtained through diet. Linoleic acid (LA, 18: 2n6) and α-linolenic acid (ALA, 18:3n3) are the EFAs that give rise to the n-6 and n-3 fatty acid families, respectively. Their absence in a normal diet has been described as responsible for the development of a wide variety of diseases such as cardiovascular, inflammatory, viral infections, certain cancers and autoimmune disorders [1]. Furthermore, it has been shown that the administration of several PUFA-enriched oils is effective in the prevention and treatment of the previously indicated diseases [4].

Monoacylglycerols (MAGs) are nonionic molecules made up of hydrophilic and hydrophobic groups which can exist in three isomeric forms, one regioisomer (*sn*-2) and two enantiomers (*sn*-1 and *sn*-3) [5,6]. Because of that, these molecules exhibit excellent emulsifying properties, being widely applied in food, cosmetic, pharmaceutical, and chemical industries [7]. Structured MAGs with a particular FA in *sn*-2 location could be considered as one of the main ways for the production of new functional ingredients with

*Abbreviations:* ALA, α-linolenic acid; ARA, arachidonic acid; DAGs, diacylglycerols; DHA, docosa-hexanoic acid; EFAs, essential fatty acids; FAs, fatty acids; FAMEs, fatty acid methyl esters; FFAs, free fatty acids; GLA, γ-linolenic acid; LA, linoleic acid; MAGs, monoacylglycerols; NMR, nuclear magnetic resonance; OA, oleic acid; PUFAs, polyunsaturated FAs; SFAs, saturated FAs; TAGs, triacylglycerols; TLC, thin layer chromatography.

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nutritional interest. This is so because in the metabolism of lipids in humans, the FAs located in the *sn*-1 and *sn*-3 positions of the glycerol backbone of MAGs are hydrolyzed by pancreatic lipase, while the FA esterified in the *sn*-2 position remains unreacted, being absorbed by the intestinal mucosa as 2-MAGs, which results in a higher bioavailability for metabolic needs [8]. In this way, the positional distribution of FAs in dietary triacylglycerols (TAGs) determines whether FAs are absorbed or not as 2-MAGs, and therefore influences the composition of chylomicrons [9].

Ramos-Bueno et al. [10] revealed that MAGs with FAs in *sn*-2 position have specific inhibitory actions on human colon cancer cells through different mechanisms. Other studies have also demonstrated a reduction of cell proliferation and even induction of apoptosis caused by different PUFA-based MAGs such as DHA, docosapentaenoic acid (DPA, 22:5n3) and eicosapentaenoic acid (EPA, 20:5n3) on colon cancer cells [11a], and DHA-MAGs on lung cancer cell line [11b]. Both studies showed a decreasing of NFκB activation leading to a reduction in COX-2 expression levels. In addition, MAGs containing MUFA and saturated FAs (SFAs) such as oleic acid (OA, 18:1n9), palmitic acid (PA, 16:0) and stearic acid (SA, 18:0) have been related to the attenuation of the activity of MRP2 (multidrug resistance-associated protein 2) in Caco-2 cells in lower concentration [12]. Furthermore, epidemiological studies [13] suggest that there is an association between the quantity and quality of dietary fat and the risk of colon cancer, being this fact reinforced by previous studies that have shown an application over the MAGs as antitumour agents [10].

Owing to these reasons, MAGs have a beneficial value on human health, and therefore, the development of different techniques for the production and purification of 2-MAGs have great interest. The main methodologies for this purpose are enzymatic alcoholysis followed by thin layer chromatography (TLC), ethanolysis followed by liquid-liquid extraction and enzymatic hydrolysis, among others. Nevertheless, chromatography processes using silver nitrate coated silica gel as stationary phase have been previously developed in order to purify several PUFA-esters [14,15]. It has been found that they are easily scalable, and also that the silver contamination in the final purified esters is negligibly. Thus, considering that both chromatographic processes applied in this work present clear similarities, the same previous features can be expected for the process developed in this work. Table 1 summarizes the main processes developed until now for the purification of MAGs.

The main objective of this work was the purification of different MUFA- and PUFA-based MAGs. To this end, several oils have been hydrolyzed applying enzymatic reaction followed by consecutive chromatographic procedures, with the aim of achieving high purity values. Several edible oils have been used for this purpose: olive, linseed, sunflower, evening primrose, DHASCO® and ARASCO®.

## 2. Materials and methods

### 2.1. Samples

Olive, sunflower (*Helianthus annuus* L.), linseed (*Linum usitatissimum* L.) and evening primrose (*Oenothera biennis* L.) oils were obtained in local markets in Almeria (Spain), and DHASCO® (40% DHA, a mixture of the oil extracted from the unicellular alga *Crypthecodinium cohnii* and high oleic sunflower oil) and ARASCO® (40% ARA, a mixture of an oil extracted from the unicellular fungi *Mortierella alpina* and high oleic sunflower oil) oils were supplied by Market Bioscience Corporation (Columbia, MD, USA).

### 2.2. Hydrolysis reaction

Enzymatic hydrolysis with porcine pancreatic lipase (E.C. 3.1.1.3) was carried out according to the methodology described by López-López et al. [16]. Briefly, the oil (4 g) was weighed into a glass bottle (1 L) and hydrolyzed by adding porcine pancreatic lipase (1.6 g) (triacylglycerol acylhydrolase, EC 3.1.1.3) (Sigma-Aldrich, St. Louis, MO, USA) in 160 mL of Tris (1M, pH 8.0), 40 mL of a solution of sodium cholate (0.1% w/v) and 16 mL of a solution of calcium chloride (22% w/v). This mixture was stoppered and placed into a water bath (37 °C, 120 rpm) for 30 or 45 min according to the assayed oil (30 min for olive, sunflower, linseed and evening primrose oils; while DHASCO® and ARASCO® oils was maintained for 60 and 45 min, respectively, in agreement with previously reported procedures [17]). Then, the bottle containing the mixture was cooled in a water bath at 20 °C, and 6 M hydrochloric acid (10.5 mL) was added. The mixture was then extracted with *n*-hexane (3 × 200 mL), stirred for 5 min and centrifuged (3500 rpm, 5 min). Finally, the *n*-hexane phase containing the hydrolysis products was separated and the solvent removed *in vacuo*. The hydrolysis products were kept under nitrogen at 4 °C until further purification.

### 2.3. MAGs purification by column chromatography

After silica gel activation (105 °C, overnight), 12 g were suspended in *n*-hexane and the slurry used for packing a glass column (17 cm height x 2 cm diameter). Then, 750 mg of the hydrolysis products from the different oils were applied on the top of the chromatographic column. Mixtures of increasing polarity of *n*-hexane:acetone (H:A, Table 2) were used as eluent, and several 15 mL-volume amounts of solvents were added depending on the kind of FA to achieve an adequate separation of MAGs [17]. MAG fractions were collected separately, and the solvent removed by evaporation under vacuum at room temperature.

### 2.4. $AgNO_3$-$SiO_2$ column chromatography

The stationary phase, a mixture of silica gel:$AgNO_3$ (10:1 w/w, for olive, linseed and sunflower oils; and 10:2 w/w, for the remaining samples) was prepared according to a previous study [14]: 4 g of $AgNO_3$ were diluted into 125 mL of ethanol in an opaque flask and stirred (1 h). Then, 20 g of silica gel were suspended in the solution of ethanol (125 mL) and incubated for 30 min. Later, the ethanol was evaporated in a rotary evaporator under vacuum at 60 °C in the dark. The mixture was activated by heating overnight at 105 °C and was stored in the desiccators until use. The chromatography column (7 cm height x 0.5 cm diameter) was packed by adding 800 mg of the stationary phase suspended in *n*-hexane. The output of the chromatography column was equipped with a cotton plug to retain solids. Then, the previously obtained MAGs were applied to the chromatography column (40 mg maximum) diluted in mixtures of *n*-hexane and acetone. The column was eluted with *n*-hexane:acetone mixtures of increasing polarity following the sequences shown in Table 3, which are the result of an optimization process to maximize the purity and recovery. The mobile phase flow was kept at a rate of 1–2 mL/min approximately. The eluates were collected separately in labeled test tubes and the solvent removed *in vacuo*. All fractions were analyzed by gas liquid chromatography (GLC).

### 2.5. GLC analyses

About 1 mg of each sample was weighed into test tubes with screw cap, and then 1 mL of *n*-hexane and 1 mL of freshly prepared transesterification reagent (methanol and acetyl chloride 20:1 v/v) were subsequently added. Then, the tubes were placed in a thermoblock at 100 °C for 30 min. After that, the mixtures were cooled

**Table 1**
Summary of the previously used methodology for monoacylglycerols purification and production.

| Methodology | Purification method | Chemical form | Source | Purity (%) | Yield (%) | Reference |
|---|---|---|---|---|---|---|
| Enzyme alcoholysis | – | DHA-MAG | Cod liver oil | 83.9 | 96.4 | [33] |
| | Column chromatography | *sn*-2 MAG | Fish oil | 96.0 | 85.0 | [28] |
| | Solvent extraction | | | 89.0 | 77.0 | |
| | Solvent extractions/Flash chromatography (FC) | *sn*-2 MAG | Soybean oil | 95.0 | 60.0 | [31] |
| | TLC | *sn*-2 MAG | Canarium oil | – | 74.0 | [34] |
| | TLC + Crystallization | *sn*-2 OA-MAG | Oleic acid-rich oil | 97–98 | 33.6 | [5] |
| Enzyme hydrolysis | Column chromatography | EPA-MAG | Cod liver oil | 98.0 | – | [22] |
| | | DHA-MAG | | 94.0 | | |
| | TLC | *sn*-2 ARA | ARASCO® oil | 53.0 | – | [21] |
| | | *sn*-2 DHA | DHASCO® oil | 47.0 | | |
| | Column chromatography | *sn*-2 SDA-MAG | *E. plantagineum* seed oil and MARINOL | 56.9 | 100 | [15] |
| | | *sn*-2 DHA-MAG | | 63.6 | | |
| Enzyme glycerolysis | – | MAG | Camellia oil | – | 82.0 | [23] |
| | Molecular destillation | MAG | Palm oil | 96.6 | 96.8 | [35] |
| | _ | MAG | Soybean oil | – | 24.02 | [24] |
| | _ | EPA + DHA-MAG | Tuna oil | 71.0 | 50.0 | [36] |
| | Short path distillation (SPD) | n-3 PUFA-MAG | Sardine oil | 91.0 | 94.0 | [37] |
| | _ | MAG | Soybean oil | – | 80.0 | [38] |
| Urea adducts | HPLC | ARA-MAG | Microbial lipids | 99.0 | – | [39] |
| Solvent-free system via glycerolysis | – | EPA-MAG | Menhaden oil | 25.9 | – | [40] |
| | | DHA-MAG | | | | |
| Esterification of glycerol, via lipase-catalyzed | – | MA-MAG | Sardine oil | 68.0 | – | [41] |
| | | PA-MAG | | | | |
| | | OA-MAG | | | | |

**Table 2**
Sequence of solvents applied as mobile phase in the chromatographic process of purification of MAGs fractions. *n*-hexane (H):acetone (A) (v/v) ratios.

| Starting oils | Solvents (H:A) |
|---|---|
| Olive, sunflower, linseed | 25 × 15 mL (90:10) |
| | 10 × 15 mL (80:20) |
| Evening primrose, DHASCO®, ARASCO® | 20 × 15 mL (90:10) |
| | 10 × 15 mL (70:30) |

at room temperature and 1 mL of distilled water was added to each one. Samples were shaken and centrifuged (2500 rpm, 3 min) and the upper *n*-hexane layer collected and stored in numbered vials at −20 °C until GLC analysis [18].

The equipment used for FA methyl esters (FAMEs) analysis was a GLC device (Focus, Thermo Electron, Cambridge, UK) equipped with flame ionization detector (FID) and an Omegawax 250 capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness) (Supelco, Bellefonte, PA, USA). The oven temperature program was 90 °C (1 min), 10 °C/min to 100 °C (3 min), 6 °C/min to 260 °C (5 min). The injector temperature was 250 °C with split ratio 50:1 and injector volume was 4 μL. The detector temperature was 260 °C. The flow of carrier gas (nitrogen) was 1 mL/min. Peaks were identified by retention times obtained for known FAME standards (PUFA No. 1, 47033; methyl linoleate 98.5% purity, L6503; and methyl stearidonate 97% purity, 43959 FLUKA) from Sigma, (St. Louis, USA), and FA contents were estimated by using methyl pentadecanoate (15:0; 99.5% purity; 76560 Fluka) from Sigma as internal standard.

### 2.6. $^{1}H$ NMR analysis

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance 300 MHz spectrometer (Bruker BioSpin GmbH, Germany). All samples were dissolved in deuterated chloroform and spectra were acquired at 298 K. Chemical shifts are reported as ppm from tetramethylsilane as a standard.

## 3. Results and discussion

The experimental scheme of the whole process starting from the

**Table 3**
Sequence of solvents applied as mobile phase in the chromatographic process of purification of MUFA- and MUFA-enriched MAGs. *n*-hexane (H): acetone (A) (v/v) ratios.

| Volume (mL) | Solvents (H:A) | Volume (mL) | Solvents (H:A) | Volume (mL) | Solvents (H:A) |
|---|---|---|---|---|---|
| Olive | | Sunflower | | Linseed | |
| 15 × 1 | 95:5 | 10 × 1 | 90:10 | 6 × 1 | 90:10 |
| 15 × 1 | 90:10 | 6 × 1 | 80:20 | 6 × 1 | 80:20 |
| 6 × 1 | 87.5:12.5 | 10 × 1 | 70:30 | 6 × 1 | 70:30 |
| 6 × 1 | 85:15 | 10 × 1 | 60:40 | 6 × 1 | 60:40 |
| 6 × 1 | 50:50 | 10 × 1 | 50:50 | 6 × 1 | 50:50 |
| Evening primrose | | ARASCO® | | DHASCO® | |
| 12 × 1 | 95:5 | 15 × 1 | 95:5 | 8 × 1 | 95:5 |
| 12 × 1 | 90:10 | 15 × 1 | 85:15 | 8 × 1 | 90:10 |
| 12 × 1 | 85:15 | 3 × 1 | 80:20 | 8 × 1 | 85:15 |
| 4 × 1 | 80:20 | 10 × 1 | 70:30 | 6 × 1 | 80:20 |
| 12 × 1 | 70:30 | 6 × 1 | 50:50 | 6 × 1 | 70:30 |
| 5 × 1 | 50:50 | | | 6 × 1 | 50:50 |

original oils for the purification of the targeted MUFA- and PUFA-based MAG species is shown in Fig. 1. This combined enzymatic hydrolysis process followed by two chromatographic processes is a novel approach for purifying most MAGs addressed in this work. Until now, MAGs have been produced through TAGs hydrolysis, glycerol and FAs esterification, and glycerolysis of TAGs (Table 1). The main disadvantages found in all these procedures are possible lipase deactivation (due to addition of ethanol or the glycerol formed by the hydrolysis of MAGs), and acyl-migration, which is mainly caused by the acidity of the silica gel, the use of some acidic solvents and high temperatures and by the use of aqueous medium [17,19,20]. Only EPA- and DHA-MAGs have been previously purified by chromatography column, using cod liver oil as raw material [21]; however, this work lacks on reliable data on the *sn*-2 and *sn*-1/3 proportions, since regiospecificity was assessed only by TLC, and yields are unreported.

### 3.1. FA profiles of the oils before and after enzymatic hydrolysis reaction

The FA profiles of the whole oils used in this study are summarized in Table 4. OA was found as the main component in olive oil, in which it reaches 78.5% of total FAs. Sunflower oil contains LA in high percentages (58.3%). ALA was particularly abundant in linseed oil, accounting for 55.7% of total FAs [22]. Evening primrose oil, which contains mainly LA and GLA, was used as raw material to purify GLA-MAG, as in this oil GLA reaches 10.2% of the total Fas, and there is an absence of ALA, which hinders the purification process of GLA as it has the same chain length and number of double bonds. ARASCO® oil, which is extracted from single cells, contains ARA in a 43.3% of total FAs content, and was used to purify ARA-MAG. Finally, DHASCO® oil (45.0% DHA of total FAs), was used to purify DHA-MAG.

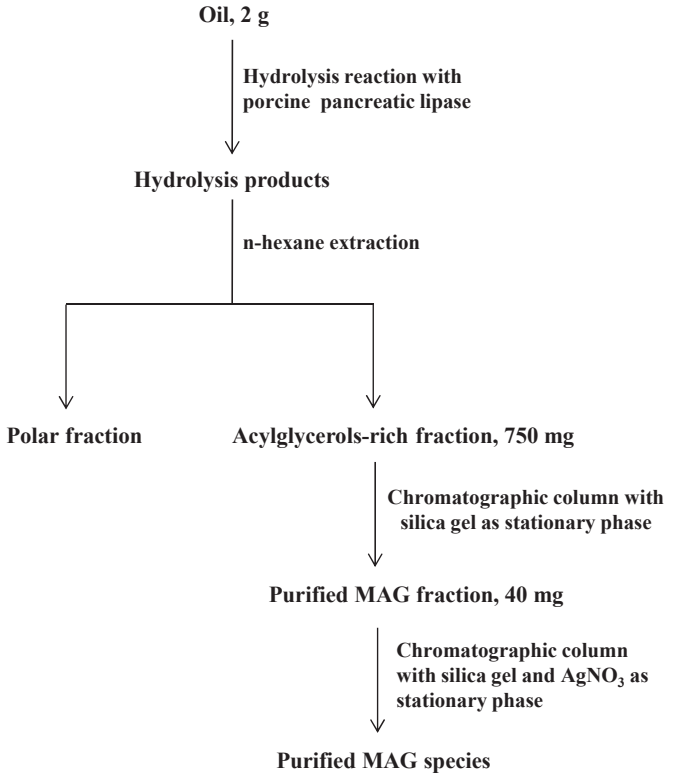


**Fig. 1.** Experimental scheme of the whole process from the original oils for the purification of the targeted MUFA- and PUFA-based MAG species.

The percentages of 2-MAGs and 1(3)-MAGs of each oils obtained after the enzymatic hydrolysis through using the first chromatographic column are summarized in Table 4. Such profiles agree with previous studies showing that the *sn*-2 position tends to be particularly rich in PUFA, while SFAs are concentrated in the extreme positions and MUFAs are distributed among all the positions in similar proportions [22,23].

As it is widely known, the production of MAGs through the enzymatic hydrolysis reaction is influenced by several factors, e.g. time and temperature reaction, and the type of lipase. In this work, the reaction time with porcine pancreatic lipase ranged from 30 to 45 min, depending on each oil, such values being selected after extensive previous experience in this reaction. However, other lipases have been used with the same purposes, such as Novozym 435 [24] or *C. Antarctica* lipase B [25]. Pancreatic lipase, also known as triacylglycerol acylhydrolase (EC 3.1.1.3), is an important enzyme for the digestion of dietary fat. Previous works revealed that this enzyme shows a higher affinity for the ester bonds of the *sn*-1/3 positions in contrast to only 22% of the FAs in the *sn*-2 position [26]. Mu and Høy [27] estimated that after a hydrolysis reaction using this enzyme, approximately 75% of the FAs in the *sn*-2 position kept their original location, despite the tendency for the migration of the acyl groups towards the *sn*-1/3 positions.

### 3.2. FA profiles obtained after silica gel column chromatography

Silica gel column chromatography was optimized to separate the MAG fraction from the mixture of the hydrolysis products. It was found that such column chromatography increases the percentage of some FAs in the MAG fraction, as is summarized in Table 4. That is, OA is found in naturally occurring oils mainly at the *sn*-2 position, as occurs in olive, cocoa butter, palm oil, peanut and canola oils [25]. In this study, although the starting oil contained 78.5% OA, an OA-MAG enriched-fraction (87.3% OA of total FAs) was obtained after this chromatography. LA and ALA followed a similar trend; i.e., LA ratio increased its concentration from 58.3 to 86.1% of total FAs, while ALA percentage was improved from 55.7 to 61.3%, considering the previously described process. GLA was weekly enriched, from 10.2 to 12.1% of total FAs. ARA from ARASCO® and DHA-MAG from DHASCO® oils were also improved with respect to the whole oils (Table 4); that is, ARA increased from 42.9 to 48.42%, while DHA percentage was improved from 44.4 to 58.0% of total FAs. All observed enrichments were due to the higher lipase affinity for saturated FAs than for PUFAs. Anyway, a second purification process to increase the percentage of purity is necessary.

Other authors used TLC for the separation of MAGs from the hydrolysis products. In the case of linseed oil, Guil-Guerrero et al. [22] obtained 44.4% of ALA in *sn*-2 position, in contrast with the 61.3% obtained using column chromatography. Regarding GLA, there was an increase in the concentration up to 12.1% with the column chromatography, while only a 10.7% was obtained by TLC [22]. Thus, after hydrolysis reaction, PUFA percentages in the *sn*-2 position in the recovered fractions were advantageous by using silica gel column chromatography methodology with respect to TLC method.

The silica gel column chromatography used in this work allows the separation of the different hydrolysis products according to the strength of their interactions with the stationary phase, the polarity of the mobile phase (*n*-hexane:acetone ratio), the nature of the stationary phase (silica gel) and the polarity of the compounds to separate. The more polar compounds show a stronger interaction with the stationary phase, are adsorbed more intensely and elute at a late stage. On the other hand, the less polar compounds, those with a weaker interaction with the stationary phase, elute faster.

**Table 4**
Regioespecific FA profiles of olive, sunflower, linseed, evening primrose, ARASCO® and DHASCO® oils obtained after enzymatic reaction and chromatographic column with silica gel as stationary phase (FA% of total FA area).[a]

| FAs | Olive | | | Sunflower | | | Linseed | | | Evening primrose | | | ARASCO® | | | DHASCO® | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | oil | sn-2 | sn-1,3 | oil | sn-2 | sn-1,3 | oil | sn-2 | sn-1,3 | oil | sn-2 | sn-1,3 | oil | sn-2 | sn-1,3 | oil | sn-2 | sn-1,3 |
| Lauric (12:0) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 5.3 ± 0.1 | 2.2 ± 0.05 | 6.7 ± 0.2 |
| Myristic (14:0) | – | – | – | – | – | – | – | – | – | – | – | – | 0.5 ± 0.0 | – | 0.4 ± 0.5 | 11.4 ± 0.3 | 2.8 ± 0.1 | 15.8 ± 0.4 |
| Palmitic (16:0) | 8.80 ± 0.3 | 1.6 ± 0.5 | 12.3 ± 0.2 | 6.76 ± 0.1 | – | 10.1 ± 0.2 | 5.62 ± 0.0 | 1.3 ± 0.7 | 7.8 ± 0.2 | 6.4 ± 0.0 | 0.7 ± 0.0 | 9.2 ± 0.1 | 9.6 ± 0.1 | 1.5 ± 0.4 | 13.6 ± 0.4 | 6.8 ± 0.1 | 1.1 ± 0.1 | 9.6 ± 0.2 |
| Palmitoleic (16:1n7) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 3.2 ± 0.0 | 1.8 ± 0.0 | 3.8 ± 0.1 |
| Stearic (18:0) | 2.9 ± 0.1 | 0.3 ± 0.1 | 4.1 ± 0.1 | 3.5 ± 0.0 | – | 5.2 ± 0.0 | 5.27 ± 0.0 | 1.2 ± 1.0 | 7.5 ± 0.6 | 1.8 ± 0.0 | 0.3 ± 0.1 | 2.6 ± 0.1 | 9.2 ± 0.1 | 1.0 ± 0.3 | 13.2 ± 0.3 | 0.8 ± 0.0 | – | 1.1 ± 0.1 |
| Oleic (18:1n9) | 78.5 ± 0.0 | 87.3 ± 3.5 | 74.3 ± 2.4 | 31.4 ± 0.0 | 14.0 ± 0.5 | 40.1 ± 2.8 | 20.0 ± 0.0 | 16.0 ± 6.2 | 22.0 ± 2.1 | 5.6 ± 0.1 | 6.2 ± 0.1 | 5.2 ± 0.0 | 20.8 ± 0.1 | 27.2 ± 4.1 | 17.2 ± 2.1 | 24.2 ± 0.1 | 32.1 ± 0.2 | 20.3 ± 0.2 |
| Vaccenic (18:1n7) | – | – | – | – | – | – | – | – | – | 0.6 ± 0.0 | – | 0.9 ± 0.1 | – | – | – | – | – | – |
| Linoleic (18:2n6) | 9.7 ± 0.0 | 9.6 ± 0.7 | 9.7 ± 2.1 | 58.3 ± 0.0 | 86.1 ± 2.5 | 44.4 ± 2.7 | 13.5 ± 0.0 | 21.1 ± 4.4 | 10.4 ± 2.0 | 75.5 ± 0.0 | 80.9 ± 0.3 | 72.9 ± 0.1 | 7.2 ± 0.0 | 14.2 ± 1.5 | 3.6 ± 0.7 | 1.3 ± 0.0 | 1.1 ± 0.0 | 1.3 ± 0.0 |
| α-linolenic (18:3n6) | – | – | – | – | – | – | – | – | – | – | – | – | 2.9 ± 0.1 | 4.4 ± 0.4 | 2.15 ± 0.3 | – | – | – |
| γ-linolenic (18:3n3) | – | – | – | – | – | – | 55.7 ± 0.0 | 61.3 ± 7.2 | 52.2 ± 1.1 | 10.2 ± 0.0 | 12.1 ± 0.1 | 9.2 ± 0.1 | – | – | – | – | – | – |
| Eicosenoic (20:1n9) | – | – | – | – | – | – | – | – | – | – | – | – | 0.5 ± 0.0 | – | 0.8 ± 0.0 | – | – | – |
| Arachidonic (20:4n6) | – | – | – | – | – | – | – | – | – | – | – | – | 43.3 ± 0.1 | 48.4 ± 6.3 | 41.5 ± 1.5 | – | – | – |
| Eicosapentaenoic (20:5n3) | – | – | – | – | – | – | – | – | – | – | – | – | 1.4 ± 0.0 | – | 2.1 ± 0.0 | – | – | – |
| Docosapentaenoic (22:5n3) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0.5 ± 0.1 | 0.6 ± 0.0 | 0.4 ± 0.1 |
| Docosahexaenoic (22:6n3) | – | – | – | – | – | – | – | – | – | – | – | – | 1.2 ± 0.0 | – | 1.8 ± 0.0 | 45.0 ± 0.4 | 58.0 ± 0.1 | 38.4 ± 0.7 |

[a] SD was routinely less than 5% of means (n = 3).

**Table 5**
MAGs purities and yields obtained after the whole process.

| MUFA- and PUFA-based MAGs | Purity (%) | Yield (%) |
|---|---|---|
| OA | 92.6 ± 0.8 | 79.6 ± 2.7 |
| LA | 97.4 ± 1.3 | 86.1 ± 1.3 |
| ALA | 95.3 ± 3.3 | 80.3 ± 2.8 |
| GLA | 90.9 ± 1.5 | 84.1 ± 4.3 |
| AA | 100.0 ± 0.0 | 87.9 ± 3.0 |
| DHA | 95.3 ± 2.1 | 67.3 ± 1.8 |

Mean ± SD (n = 3).

Thus, the first eluates contain the less polar molecules, the TAGs. They are followed by diacylglycerols (DAGs) and the last eluates are the most polar fractions, those containing free FAs (FFAs) (the 90:10 H:A fraction). On the other hand, the elution order of the different MAGs depends on the type of FA which esterifies the glycerol, as the number of double bonds on the FA increases the polarity of the MAG. Therefore, the choice of the eluent is a critical point in order to get the success of the chromatographic process.

### 3.3. MAGs purification

Different MAGs purifications were attempted by column chromatography using $AgNO_3$-silica gel as stationary phase (Fig. 1). In all cases, the SFA-based MAGs eluted first, followed by MUFA- and PUFA-based MAGs. Consequently, the number and geometric configuration of the double bonds determine the elution order. In this way, it can be achieved a high degree of purity of each targeted MAG from the different oils by the adequate choice of the eluting mixtures (Table 3).

The purity and yields obtained in the isolation of MAGs are summarized in Table 5. The higher purity was obtained for ARA-MAG (100%) and LA-MAG (97.4%). The remaining 2-MAGs showed lower purities, DHA-MAG 95.3%, ALA-MAG 95.3%, OA-MAG 92.6% and GLA-MAG 90.9%, all purities are expressed as FA-MAG % referred to total FA-MAGs percentage, which were detected by GC analyses. The results obtained in this work are remarkable. For instance, the oils having significant amounts of GLA esterifying the *sn*-2 position are considered as valuable active compounds [28]. In this process, from 12.1% GLA in *sn*-2 position in evening primrose oil, it has been reached up to 90.9% after the chromatography column using silica gel-silver nitrate as stationary phase.

MAG yields reached also significant values (Table 5), ranging from 67.3% for DHA-MAG to 87.9% for ARA-MAG.

The chromatographic method developed in this work provides higher purity and yield values when compared to the previous methodology using solvent extraction [29]. Furthermore, it allows obtaining high-value product as are MAGs, which have well defined potential health benefits. Furthermore, this chromatographic method could be easily scaled due to the well-known characteristics of the $AgNO_3$-silica gel column chromatography [15]; it is easily adaptable (in this work, it has been successfully employed to purify MAGs from diverse oils), being such process robust and cheap

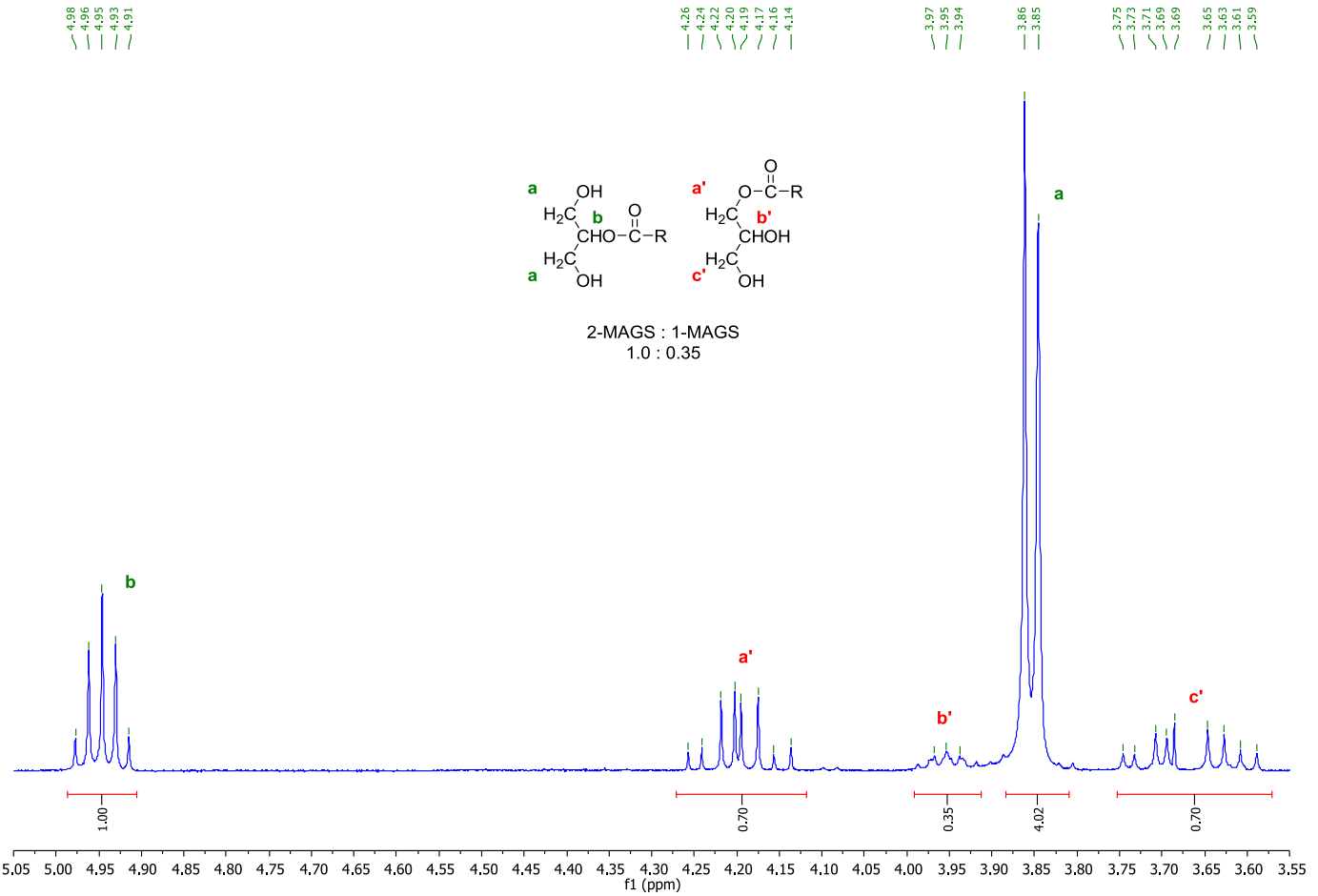


**Fig. 2.** [1]H NMR spectra of monoacylglycerols (MAGs) purified from linseed oil after chromatographic purification containing high purity ALA-MAGs. a, b, a', b' and c' denote glycerol proton assignments as illustrated.

considering that the solvents can be recovered by fractionating distillation, and that the column is reusable several times [15].

### 3.4. $^1$H NMR analysis

The degree of acyl-migration in the different processes was analyzed by $^1$H NMR spectroscopy (Fig. 2). For *sn*-2 products, there are two signals due to the glycerol protons, a doublet at 3.855 ppm due to the four hydrogens on methylene ($CH_2$-1 and $CH_2$-3) and a quintuplet due to the only one hydrogen present on the central CH-2 at 4.95 ppm. On the other hand, the lack of symmetry of *sn*-1 products leads to three different signals. Thus, while $CH_2$-3 produces an ABM system centered at 3.66 ppm and $CH_2$-2 a multiplet centered at 3.95 ppm, the acyl substituted $CH_2$-1 offers the most deshielded signal as another ABM system at 4.20 ppm. As can be seen in Fig. 2, integration of all the signals gives a straightforward measure of the ratio of both isomeric MAGs. Especially useful are the signals of the acyl CH, as they do not present any overlap and the measure is completely accurate. In the example of Fig. 2, the integral ratio b/2a' offers a relative proportion of 1:0.35 for *sn*-2:*sn*-1/3. Several studies indicate that migration of the acyl group of MAGs tends to balance 2-MAG:1-MAG until the 1:9 ratio [17,30]. This ratio depends on multiple factors; e.g., temperature, solvent polarity, the length of the FA chain, reaction time and acidity of silica gel, among others. In general, higher rates of acyl group migration result in lower purification yields [31]. Great migration rates of the acyl group have been detected in ARA-, DHA-, GLA- and OA-MAGs. On the other hand, LA-and ALA-MAGs show higher ratios of *sn*-2:*sn*-1/3 (1:0.35 and 1:0.53, respectively). This fact might be related to the acidity of the silica gel used for the column chromatography, which promotes acyl-migration from the *sn*-2 position to the most stable *sn*-1/3. Similar results were obtained by Rincón-Cervera et al. [17], who indicated that after silica gel chromatography only 39.4% of MAGs of the *E. plantagineum* seed oil remains as *sn*-2 location and 60.6% becomes 1(3)-MAGs, while in Marinol© oil the 54.2% of MAGs remains in *sn*-2 position.

Compton et al. [31] showed that the 2-MAGs could be separated from the hydrolysis products of the TAGs using silica gel chromatography column by means of a gradient of hexane/acetone without causing acyl-migration, resulting in 60% yield and 97% purity for 2-MAGs. However, the different MAG species were not isolated in that work, and therefore the results can only be marginally compared.

## 4. Conclusions

The process developed in the present work, which uses enzymatic hydrolysis of oils followed by two chromatographic processes, can be a good alternative in order to obtain purified MAGs fractions. Furthermore, all solvents used in the purification processes (*n*-hexane and acetone), are commonly used in the alimentary industry as extraction solvents, according to the Committee on Food Chemicals Codex [32], where extraction solvents are regulated for the production of food stuffs and food ingredients. In this way, the obtained MAGs are suitable for alimentary or pharmaceutical purposes. Moreover, this method improves purification and yields when compared to other processes, such as solvent extraction. However, this method has demonstrated to have high acyl-migration rates in some oils, being LA- and ALA-MAGs the more enriched in *sn*-2 position.

All studied oils have evidenced to be raw sources for the purification of the different MAGs, due to the high content of the targeted FAs. Although the process has been successfully implemented, more research is needed to avoid the acyl-migration phenomena detected, for instance essaying alternative stationary phases for column chromatography.

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# Highly concentrated very long-chain PUFA obtainment by Urea complexation methodology

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ABSTRACT

The intake of *n*-3 very long chain PUFA (VLCPUFA) develops several positive physiological actions. Thus, such compounds are currently used as healthy supplements for animals and humans, in animal experimentation, and as standards for chromatographic use. The urea complexation methodology is an old technique for obtaining PUFA concentrates; however, improvements can still be implemented for improving results using this methodology. In this work, highly concentrated eicosapentaenoic acid (EPA, 20:5*n*-3), docosahexaenoic acid (DHA, 22:6*n*-3) and arachidonic acid (ARA, 20:4*n*-6) have been successfully obtained using such procedure. For this, five oil sources were used: commercially available tuna oil, DHASCO®, ARASCO®, and MARINOL®, and the oil from the microalga *Nannochloropsis*. Overall, after optimizing the urea:fatty acid ratio, DHA-, EPA-, and ARA-concentrates were obtained at 98.0, 43.0 and 82.8% of total fatty acids. This work clearly shows the importance of the proper selection of the oil source to achieve highly-purity VLCPUFA concentrates by the urea complexation method.



## 1. Introduction

Polyunsaturated fatty acids (PUFA) are bioactive food components that can be classified in several groups, mainly as *n*-6 and *n*-3 PUFA. Both PUFA series induce well-documented protection against the development of several diseases and health disorders, such as cancer, atherosclerosis and cardiovascular ones (Saini and Keum, 2018; Ortea et al., 2018; Setyawardhani et al., 2015).

The *n*-3 PUFA series include $\alpha$-linolenic acid (ALA, 18:3*n*-3), which is and essential FA found in green vegetables and some oils (rapeseed, soybean). From ALA, *n*-3 very long chain PUFA (VLCPUFA) derivatives are produced in the body, such as eicosapentaenoic (EPA, 20:5*n*-3) and docosahexaenoic (DHA, 22:6*n*-3) acids (Sarr et al., 2019; Bougnoux et al., 2010). DHA is one of the predominant PUFA in the structural phospholipids of the human brain and retina, and it accumulates rapidly in the foetal neural tissues, thus being essential for the growth of the brain, as well as for visual and congenital development (Rey et al., 2019; Thomazeau et al., 2016). Both EPA and DHA have beneficial effects in insulin resistance; they repress lipogenesis and increase resolvins and protectins generation, ultimately leading to reduced inflammation

*Abbreviations:* ALA, $\alpha$-linolenic acid, 18:3*n*3; ARA, arachidonic acid, ARA, 20:4*n*6; DHA, docosahexaenoic acid, 22:6*n*3; EPA, eicosapentaenoic acid, 20:5*n*3; FA, fatty acid; MUFA, monounsaturated FA; NUCF, non-urea complex fraction; PUFA, polyunsaturated fatty acids; SFA, saturated FA; UCF, urea complex fraction; VLCPUFA, very long chain PUFA

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(Siriwardhana et al., 2012). These PUFA are present in foods of marine and animal origin, and fish oil constitutes one of the richest and cheapest source: EPA and DHA content in fish oil varies from 5 to 26 and 6 to 26% of total FA, respectively (Alkio et al., 2000). However, global consumer needs cannot be supplied by the current global fish harvest. The more promising alternative are microalgae, which are *n*-3 VLCPUFA producers, and thus a suitable raw source of such compounds that could prevent overexploitation of world fishing grounds (Ryckebosch et al., 2012).

The more common PUFA of the *n*-6 series included in foods are: (i) linoleic acid (LA, 18:2*n*-6), which is abundant in foods of animal origin and in several seed oils, such as those from sunflower and soy; (ii) arachidonic acid (ARA, 20:4*n*-6), which is a substrate of specific lipid oxygenases to produce bioactive inflammatory mediators. This VLCPUFA induces important healthy physiological actions, especially for childhood. In this regard, the application of ARA to infant diets is particularly important to ensure optimal conditions for developing membrane-rich systems such as those of the brain (Vali et al., 2003); (iii) $\gamma$-linolenic acid (GLA, 18:3*n*-6), which is found in few seed oils, and induces several health benefits (Bougnoux et al., 2010).

The study of highly purified *n*-3 and *n*-6 PUFA for establishing their various healthy actions is continuously increasing, while the use of fish oil as a food additive is limited due to problems associated with its typical fishy smell, unpleasant taste, and poor oxidative stability (Mendes et al., 2007). Thus, the methodologies to increase the concentration of the various PUFA contained in raw oils is timely. The most common available methods for producing *n*-3 PUFA concentrates include winterization, low-temperature crystallization, molecular distillation, urea complexation and open-column chromatography with silver nitrate as stationary phase (Magallanes et al., 2019; González-Fernández et al., 2017; Liu et al., 2006). Such methodologies are commonly based on differences in the polarity and spatial configuration of the FA contained in the extracts.

Urea complexation is considered a simple, quick and efficient technique, whose aim is the separation of FA according to their degree of unsaturation. PUFA are double bonds-containing non-linear molecules, whereas saturated FA (SFA) and monounsaturated FA (MUFA) have a linear structure and, hence, can be easily trapped by the cavity of the urea complex and crystallize out at cooling forming the urea complex fraction (UCF) (Magallanes et al., 2019). These complexes are mainly composed by SFA- and MUFA-based crystals, which can subsequently be removed by filtration, while the liquid or non-urea complex fraction (NUCF) becomes selectively PUFA-enriched (Salimon et al., 2012). One of advantages of the urea complexion method is the stability of complexed crystals; thus, filtration can be affected at higher temperatures than that used for winterization (Liu et al., 2006). So, urea complexation is a scalable and simple process because of its environmentally friendly operating conditions, and due to the use of inexpensive and renewable materials (urea and ethanol or methanol as solvent) (Hayes, 2006).

This work was designed for obtaining highly concentrated very VLCPUFA, i.e., DHA, EPA and ARA, from commercially available oils by the urea complexation methodology. The scope of this research included a study on the influence of the U:FA ratio on both VLCPUFA purity and VLCPUFA yield.

## 2. Material and methods

### 2.1. Samples

Several oils were used for VLCPUFA concentration: (i) DHASCO® (40% DHA, a mixture of oil extracted from the unicellular alga *Crypthecodinium cohnii* and high oleic sunflower oil); (ii) ARASCO® (40% ARA, a mixture of an oil extracted from the unicellular fungi *Mortierella alpina* and high oleic sunflower oil); (iii) MARINOL®, a natural EPA- and DHA-rich fish oil concentrate; (iv) *Nannochloropsis* oil, which is obtained from this microalga, and is mainly used as food to cultivate rotifers because of its high EPA content; (v) Tuna oil, a DHA-rich fish oil. ARASCO®, MARINOL®, DHASCO®, and tuna oil were supplied by Martek Bioscience Corporation (Columbia, MD, USA), while the microalgae come from bioreactors located at the University of Almeria, from which the oil was extracted with *n*-hexane.

### 2.2. Fatty acids extraction and fractionation with urea

Oil direct saponification and fractionation with urea was carried out according to Spurvey and Shahidi (2000). Briefly, 1 g of sample was mixed with 2.64 mL of an ethanol:water solution (96:4, v/v), and 230 mg of KOH and 0.440 mL of distilled water was added. The mixture was heated for 1 h at 60 °C, with constant agitation under argon atmosphere, and after that was mixed with *n*-hexane to remove the unsaponifiable phase. The saponifiable solution was then acidified to pH 1 with HCl and the FA molecules were extracted with *n*-hexane (3 × 15 ml). The extract was neutralized washing with water and the total volume was evaporated under vacuum and dissolved in *n*-hexane. The saponified FA fraction was crystallized with urea using methanol as solvent. The urea:fatty acid (U:FA) ratio was tested at 3:1, 4:1, 5;1, 6:1 and 7:1 (w/w), and the urea:methanol ratio was always 1:3 (w/v). Previously, urea was dissolved in methanol al 60 °C before to be mixed with the various FA mixtures. The temperature of reaction was 4 °C overnight. Later, the PUFA-containing the liquid phase was filtered and HCl 10% (v/v) was added. Finally, PUFA were recovered with *n*-hexane to be analysed by GC (Guil-Guerrero et al., 2001).

PUFA yields were calculated according to the following equation:

$$\text{PUFA yield\%} = (\text{PUFA}_1/\text{PUFA}_0) \times 100 \quad (1)$$

Were $\text{PUFA}_0$ correspond to the PUFA (g) in the starting oil, while $\text{PUFA}_1$ correspond to the PUFA amounts (g) in the resulting extract. This equation was applied to the main PUFA targeted in this study.

**Table 1**
FA profiles of ARASCO®, DHASCO®, MARINOL®, *Nannochloropsis* and Tuna oils (FA% of total FA area).

| | ARASCO® | *Nannochloropsis* | DHASCO® | MARINOL® | Tuna |
|---|---|---|---|---|---|
| 8:0 caprylic | nd | nd | 0.3 ± 0.0 | nd | nd[a] |
| 10:0 CA | nd | nd | 1.4 ± 0.0 | nd | nd |
| 12:0 LaA | nd | nd | 5.3 ± 0.1 | nd | nd |
| 14:0 MA | 0.7 ± 0.0 | 8.0 ± 0.1 | 11.4 ± 0.3 | 2.4 ± 0.0 | 4.2 ± 0.1 |
| 16:0 PA | 9.8 ± 0.1 | 18.0 ± 0.2 | 6.7 ± 0.1 | 11.2 ± 0.0 | 20.9 ± 0.2 |
| 16:1*n*-7 POA | nd | 9.3 ± 0.2 | 3.1 ± 0.0 | 2.5 ± 0.0 | 4.3 ± 0.0 |
| 17:0 (margaric) | nd | 2.2 ± 0.5 | nd | 0.8 ± 0.0 | 1.1 ± 0.1 |
| 18:0 EA | 9.4 ± 0.06 | 3.6 ± 0.0 | 0.7 ± 0.0 | 3.4 ± 0.0 | 5.1 ± 0.0 |
| 18:1*n*-9 OA | 21.0 ± 0.1 | 9.6 ± 0.1 | 24.2 ± 0.1 | 8.3 ± 0.0 | 13.8 ± 0.1 |
| 18:1*n*-7 VA | nd | 3.5 ± 0.1 | nd | 1.4 ± 0.0 | 2.2 ± 0.0 |
| 18:2*n*-6 LA | 7.4 ± 0.0 | 1.3 ± 0.0 | 1.2 ± 0.0 | 1.2 ± 0.0 | 2.0 ± 0.0 |
| 18:3*n*-6 GLA | 3.1 ± 0.1 | 1.1 ± 0.1 | nd | nd | nd |
| 18:3*n*-3 ALA | nd | 1.0 ± 0.0 | nd | 1.0 ± 0.0 | 0.9 ± 0.0 |
| 18:4*n*-3 SDA | nd | 2.8 ± 0.1 | nd | nd | nd |
| 20:0 (arachidic) | 1.0 ± 0.0 | nd | nd | 0.5 ± 0.0 | 0.5 ± 0.0 |
| 20:1*n*-9 GOA | 0.7 ± 0.0 | 0.9 ± 0.0 | nd | 1.1 ± 0.0 | 1.2 ± 0.0 |
| 20:4*n*-6 ARA | 43.5 ± 0.1 | 1.3 ± 0.0 | nd | 3.1 ± 0.0 | 2.4 ± 0.0 |
| 20:4*n*-3 ETA | nd | 1.0 ± 0.1 | nd | 0.4 ± 0.0 | 0.4 ± 0.0 |
| 20:5*n*-3 EPA | 1.6 ± 0.0 | 22.7 ± 0.4 | nd | 7.2 ± 0.0 | 7.9 ± 0.1 |
| 22:0 (behenic) | nd | 0.7 ± 0.0 | nd | 0.4 ± 0.0 | nd |
| 22:4*n*-6 (DTA) | nd | 1.3 ± 0.0 | nd | 0.4 ± 0.0 | 0.4 ± 0.0 |
| 22:5*n*-6 (*n*-6 DPA) | nd | nd | nd | 2.7 ± 0.0 | 1.7 ± 0.0 |
| 22:5*n*-3 (*n*-3 DPA) | nd | 2.7 ± 0.0 | 0.5 ± 0.1 | 1.8 ± 0.0 | 1.4 ± 0.1 |
| 22:6*n*-3 DHA | 1.4 ± 0.0 | 8.0 ± 0.0 | 45.0 ± 0.4 | 49.1 ± 0.1 | 28.3 ± 0.4 |
| 24:1*n*-9 (nervonic) | nd | 0.5 ± 0.0 | nd | 0.5 ± 0.0 | 0.6 ± 0.0 |

[a]nd: no detected.

### 2.3. Oil transesterification and FA analyses

FA determination was carried out after direct derivatization to FA methyl esters through the transesterification according to previous works (Lepage and Roy, 1984; Rodríguez-Ruiz et al., 1998). FAME were analysed in a Focus GC (Thermo Electron. Cambridge. UK) equipped with flame ionization detector and an Omegawax 250 capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness) (Supelco, Bellefonte, PA, USA) The temperature of the oven was: 90 °C (1 min), 10 °C/min to 100 °C (3 min), 6 °C/min to 260 °C (5 min), and the injector temperature was 250 °C with split ratio 50:1 and a volume of 4 μL. The detector temperature was 260 °C and the flow of carrier gas was 1 mL/min (González-Fernández et al., 2017). The peak area of the internal standard was used as a reference to compute the mass of every FA in the resulting chromatograms, and results were computed as FA percentages of total FA area. Peaks were identified by retention times obtained for known FA methyl esters standards (PUFA No. 1, 47033) from Sigma, (St. Louis, USA).

### 2.4. Statistical analysis

Statistical analyses were performed using the statistical package software Statgraphics Centurion XVI (StatPoint Technologies Inc., Warrenton, VA, USA). Data in Table 1 and Fig. 1 1 represent the mean of three complete independent experiments ± SD (error bars in Fig. 1). The comparison of means was made using a one-way ANOVA followed by a Minimal Significant Difference (Fisher's LSD procedure). $P < 0.05$ was regarded as statistically significant.

## 3. Results and discussion

The FA profiles of the whole oils used in this study are summarized in Table 1. ARASCO® oil, which is obtained from cultured single cells, contains ARA at a 43.5% of total FA, and it was used to purify ARA. *Nannochloropsis* oil (22.7% EPA of total FA) was used in this study to concentrate EPA. Several oils were tested to concentrate DHA: (i) DHASCO® oil (45.0% DHA of total FA); (ii) MARINOL® oil, which is a fish oil concentrated, containing 49.1% DHA of total FA, and; (iii) Tuna oil (28.3% DHA of total FA).

The influence of some operational variables – FA profiles, U:FA ratio, time and crystallization temperature – on the concentration of several PUFA have been previously described. The main effort has been devoted to upgrading fish and

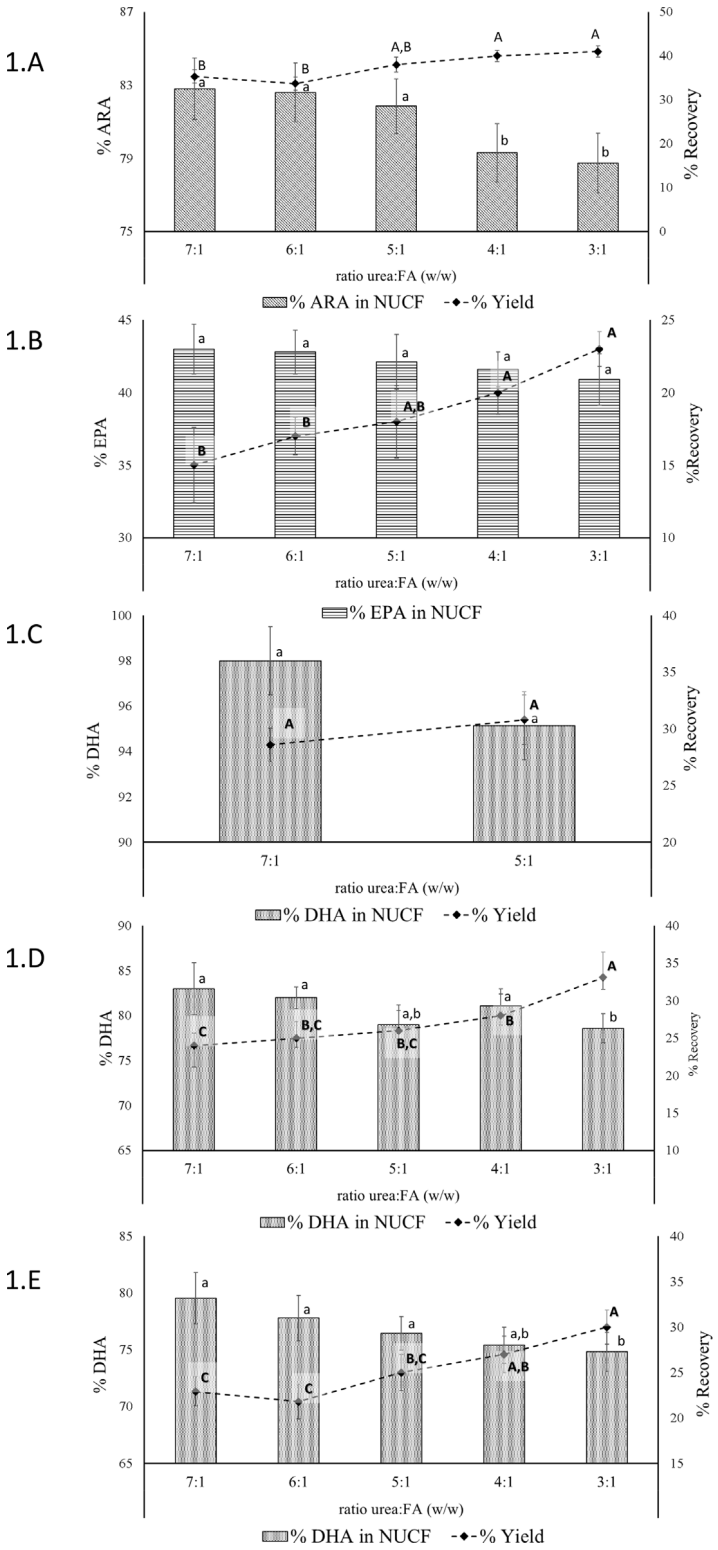


**Fig. 1.** Very long-chain PUFA concentration by the urea concentration method effected at different urea:fatty acid ratio. (1A) ARASCO® oil; (1B) *Nannochloropsis* oil; (1C) DHAsco® oil; (1D) MARINOL® oil; (1E) Tuna oil. Data were analysed using a one-way ANOVA followed by a Minimal Significant Difference (Fisher's LSD procedure). Within each series, points sharing the same superscript letter are not statistically different ($P < 0.05$). Lowercase and uppercase letters refer to fatty acid purity and yield, respectively. Data represent the mean of three complete independent experiments ± SD (error bars).

**Table 2**
Overview of the previously used urea complexation methodology for very long-chain PUFA purification.

| Referencia | PUFA | Purity (%) | Recovery (%) | Ratio U:FA | T °C | Time (h) | Source |
|---|---|---|---|---|---|---|---|
| Cohen and Cohen (1991) | EPA<br>ARA | 81.9<br>6.6 | –<br>– | 4:1 (w/w) | $4^{o}$ to $-15^{o}$ | 12 | *Porphyridium cruentum* |
| Medina et al. (1995) | EPA<br>DHA | 25.6<br>59.9 | 82.1 | 4:1 (w/w) | 4 | – | Cod liver oil |
| Giménez Giménez et al. (1998) | EPA<br>ARA | 45.1<br>30.7 | 67.7<br>61.8 | 4:1 (w/w) | 28 | – | *Porphyridium cruentum* |
| Senanayake and Shahidi (2000) | DHA | 97.1 | 32.5 | 3:1 (w/w) | 4 | 24 | *Crypthecodinium cohnii* oil |
| Guil-Guerrero et al. (2001) | EPA<br>ARA | 57.6<br>34.0 | 79.5<br>73.2 | 4:1 (w/w) | 28 | – | *Porphyridium cruentum* |
| Gámez Meza et al. (2003) | EPA<br>DHA | 46.2<br>40.3 | 78.0 | 1.6:1 (w/w) | 5 | 8 | Sardine oil |
| Liu et al. (2006) | DHA<br>EPA | 85.0 | 25.1 | 15:1 mol/mol | −5 | 20 | tuna oil |
| Mendes et al. (2007) | DHA | 89.4 | 49.9 | 4:1 (w/w) | 24 | 24 | *Crypthecodinium cohnii* CCMP0316 |
| Zhang et al. (2012) | EPA<br>DHA | 36.4<br>24.2 | 53.4 | 0.75:1 (w/w) | 65 | 0,5 | Fish oil |
| Latyshev et al. (2014) | EPA<br>DHA | 7.8<br>6.5 | 32.5<br>32.0 | 3:1 (w/w) | $0^{o}$ to $-20^{o}$ | 3 | Liver oil of the squid *Berryteuthis magister* |
| Lin et al. (2014) | EPA<br>DHA | 65.6 | 46.8 | 1.9:1 (w/w) | −1 | – | Sardine oil |
| Thammapat et al. (2015) | EPA<br>DHA | 88.0 | 26.0 | 4:1 (w/w) | −20 | – | Asian catfish oil |
| Mu et al. (2016) | DHA<br>EPA | 63.6<br>22.2 | 91.9<br>30.2 | 1.6:1 (w/w) | −8 | 16 | Tuna oil |
| Shan et al. (2016) | EPA<br>DHA | 85 | – | 1.5:1 (w/w) | $25^{o}$ | 1 | Tuna oil |
| Zheng et al. (2018) | EPA<br>DHA | 71.3 | 82.3 | 2.38:1 (w/w) | 15 | 2,5 | Seal oil |

algal oils regarding DHA and EPA purities (Zheng et al., 2018; Mu et al., 2016). Table 2 shows the main features on PUFA purification processes using the urea complexation methodology. Zheng et al. (2018) concluded that the optimal conditions for obtaining over more than 70% of EPA+DHA from fish oil were at 2.38:1 U:FA ratio upon crystallization at 15 °C for 2.5 h, whereas Thammapat et al. (2015) achieved 88% EPA+DHA at 4 °C, and 4:1 U:FA ratio. In all cases after the urea complexation process two fractions were obtained: the crystals, where most SFA and MUFA remained as inclusion compounds, and the mother liquor, which was selectively enriched in PUFA. This selective enrichment has been previously noted by several authors, e.g., Crexi et al. (2012) found an increase of 31.4% in unsaturated FA content and a decrease of 75% in SFA in the NUCF.

In this work, using ARASCO® oil, ARA was concentrated to 82.8% of total FA in the NUCF layer using the 7:1 U:FA ratio, whereas at the remaining U:FA ratios ARA achieved 78.8–82.6% of total FA (Fig. 1A), while ARA purities at the 3:1 and 4:1 ratios had significant differences with respect to other ratios ($P < 0.05$). However, ARA recoveries were slightly higher using the 3:1 (41.0%) and 4:1 (40.0%) U:FA ratios, followed by the 5:1 (38.0%), 7:1 (35.3%) and 6:1 (33.7%) U:FA ratios. Other authors also achieved an upgraded ARA extract through the urea complexation method. For instance, Zhu et al. (2002) using *M. alpina* oil obtained a 6.2-fold increase in ARA% in the resulting extract, and ARA was obtained at 57.1% of total FA, a lower figure than that achieved in the present work. Differences in ARA purities obtained in both works were due to the presence of other interfering PUFA in *M. alpina* oil, thus the selection of suitable oils when PUFA concentration through the urea method will be made is a crucial fact (Guil-Guerrero et al., 2001; Giménez Giménez et al., 1998).

When applying the urea complexation method to *Nannochloropsis* oil, EPA purity increased from 22.7 to 43.0% of total FA at the 7:1 U:FA ratio, whereas at the remaining U:FA ratios tested, EPA purity slightly decreased (Fig. 1B). This effect was due to the presence of DHA in this oil, which contains more double bounds than EPA, so EPA and DHA concentrate together but the last to a greater extent than the previous. On the other hand, EPA recovery improved by using lower U:FA ratio, i.e. 23.0 and 20.0% EPA yield at 3:1 and 4:1 ratios, respectively, whereas a 7:1 U:FA ratio ratios induced a lower recovery: 15.0% EPA yield. In this regard, Zheng et al. (2018), concluded that the U:FA ratio influences the FA recoveries obtained by the urea complexation method, being lower as the U:FA ratio increases.

DHA was concentrated from DHASCO®, MARINOL® and tuna oils (Fig. 1C, 1D y 1E, respectively). DHASCO® oil gave best results for the 7:1 U:FA ratio, at which it was obtained an exceptional increase of DHA (98.0% of total FA) with respect to the initial percentage (45.0% of total FA), and a yield of 28.6% was obtained. The recovery was higher using the 5:1 U:FA ratio (30.8%), at which 95.1% purity was obtained. Senanayake and Shahidi (2000) obtained similar results for *C. cohnii* oil and reported an enrichment from 47.4 to 97.1% at 4 °C after crystallization at the 3:1 U:FA ratio, while the recovery was 32.5%. Our results showed that high purity DHA can be easily obtained by the urea complexation methodology, and similar to other results obtained by using open-column chromatography, as reported by González-Fernández et al. (2017). In this regard, Vali et al. (2003) performed PUFA purification by combining the urea complexation method and silver silica gel column chromatography. However, as demonstrated here, to purify DHA as free FA just a single urea complexation processing is needed, and this method should be chosen instead column chromatography, since it is more easily scalable and reproductible than open column chromatography. By using MARINOL® and tuna oils (Fig. 1D and E), DHA purities were lower than that obtained from DHASCO® oil. This way, the 3:1 U:FA ratio provided concentrates containing 78.6

and 74.8% DHA of total FA, respectively, and by applying higher U:FA ratios the purity of DHA slightly increased in both oils. This was because they contain other interfering PUFA such as EPA and ARA, and urea preferably selects ARA and EPA to form complexes rather than DHA. In this regard, Senanayake and Shahidi (2000) reported that higher U:FA ratios (7:1, 6:1 and 5:1) causes that more DHA was complexed by urea, and this fact also explains that at such high U:FA ratios, DHA recoveries decreased in both MARINOL® and tuna oils.

Given that the objective of this work was to increase VLCPUFA purities, process yields were not very high. This drawback can be easily solved by performing additionally urea complexation steps using the remaining FA from the UCF, for obtaining new DHA concentrates at decreasing purities, although still having commercial interest (data not shown).

We used 4 °C for U:FA ratio complexation throughout the study. Such temperature has been widely reported as the best for obtaining the highest PUFA purity by using the urea complexation methodology (e.g., Zheng et al., 2018). Another aspect to be considered when applying this method is the formation of ethyl carbamates. Previously, Canas and Yurawecz (1999) warmed on that the urea complexation methodology produces ethyl carbamates, which are potent carcinogens for humans, including gene mutations and DNA damage, and it occurs naturally in alcoholic beverages and most fermented foods. Animal studies have shown that ethyl carbamates increase the incidence of tumours in several tissues, including lung, liver, and blood vessels (Ryu et al., 2015). However, Vázquez et al. (2017) reported that at room temperature and by the incorporation of a final washing step in the urea complexation process, ethyl carbamates diminish in the final product to undetectable levels. Therefore, the NUCF in our study were treated with HCl 10% (v/v), so, an absence of such compounds is expected.

## 4. Conclusions

The urea complexation method, when applied to suitable PUFA-rich oils, can be the right tool to achieve highly concentrated VLCPUFA. In this work, ARA, DHA and EPA concentrates from ARASCO®, DHASCO® and *Nannochloropsis* oils have been obtained at high U:FA ratios (7:1). As shown here, this process is easy to perform and reproductible, allowing to obtain highly concentrated bioactive VLCPUFA. Besides laboratory and research uses, these could be used as low-caloric food supplements for improving health. Future research should focus on optimizing the handling of the remaining urea crystal fractions to develop a whole process in which the different objectified PUFA are fully recovered, at different purities that would logically have different economic values as a function of the fatty acid purities.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**María José González-Fernández:** Conceptualization, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Dmitri Fabrikov:** Investigation, Resources, Visualization. **Svetlana Lyashenko:** Investigation, Visualization. **Francisca Ferrón-Carrillo:** Investigation, Visualization. **José Luis Guil-Guerrero:** Conceptualization, Software, Validation, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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# CHAPTER II

# 6. CHAPTER II. Proteomics study reveals that docosahexaenoic and arachidonic acids exert different *in vitro* anticancer activities in colorectal cancer cells

## 6.1. Abstract

Two PUFA, DHA and ARA, as well as derivatives, such as eicosanoids, regulate different activities, affecting transcription factors and, therefore, DNA transcription. This study has attempted to determine the *in vitro* anticancer activities of these molecules linked to the gene transcription regulation of HT-29 CRC cells. We applied the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test along with lactate dehydrogenase and caspase-3 assays; proteome changes were assessed by "sequential windowed acquisition of all theoretical mass spectra" quantitative proteomics, followed by pathway analysis, to determine the affected molecular mechanisms. In all assays, DHA inhibited cell proliferation of HT-29 CRC cells to a higher extent than ARA and acted primarily by downregulating proteasome particles, while ARA presented a dramatic effect on all six DNA replication helicase particles. The results indicated that both DHA and ARA are potential chemopreventive agent candidates.

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# Proteomics Study Reveals That Docosahexaenoic and Arachidonic Acids Exert Different *In Vitro* Anticancer Activities in Colorectal Cancer Cells

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Supporting Information

**ABSTRACT:** Two polyunsaturated fatty acids, docosahexaenoic acid (DHA) and arachidonic acid (ARA), as well as derivatives, such as eicosanoids, regulate different activities, affecting transcription factors and, therefore, DNA transcription, being a critical step for the functioning of fatty-acid-derived signaling. This work has attempted to determine the *in vitro* anticancer activities of these molecules linked to the gene transcription regulation of HT-29 colorectal cancer cells. We applied the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test along with lactate dehydrogenase and caspase-3 assays; proteome changes were assessed by "sequential windowed acquisition of all theoretical mass spectra" quantitative proteomics, followed by pathway analysis, to determine the affected molecular mechanisms. In all assays, DHA inhibited cell proliferation of HT-29 cells to a higher extent than ARA and acted primarily by downregulating proteasome particles, while ARA presented a dramatic effect on all six DNA replication helicase particles. The results indicated that both DHA and ARA are potential chemopreventive agent candidates.

**KEYWORDS:** *ARA, colorectal cancer, DHA, proteomics, PUFA, SWATH*

## INTRODUCTION

Colorectal cancer (CRC) has one of the highest mortality rates. The fact that large regional differences exist in the incidence and mortality of CRC suggests a close relationship between genetic and environmental factors, with diet and lifestyle being two of the most determinant for CRC incidence. Diet is widely recognized as a critical factor for cancer risk and mortality.[1]

Free fatty acids (FFAs) assume a critical part in many biological roles. Apart from serving as precursors of a wide range of signaling and structural molecules, they are used to obtain energy.[2] Although many of the circulating FFAs are bound to albumin, the small fraction of unbound FFAs in the aqueous phase mediate physiological activity and are most sensitive to changes in health and disease; they are also transported across membranes.[3]

Previous data have recognized the importance of certain polyunsaturated fatty acids (PUFAs) for the prevention of CRC, while the results from epidemiological studies have suggested a negative correlation between both CRC development and PUFA intake.[4] Some studies have also found that, *in vitro*, some PUFAs increase the cytotoxicity of different chemotherapeutics in brain, lung, breast, sarcoma, lymphocytic, and colon human cell cultures.[5] The degree of *in vitro* anticancer activity of various fatty acids (FAs) has been related to their chemical structure, both of which increase such actions. Montaung et al.[6] evaluated the cytotoxic capacity of most of the FAs of interest in a combined manner in prostate cancer cells.

$\omega$-3 PUFAs have been correlated with CRC preventive effects, while $\omega$-6 PUFAs have shown a positive association with CRC, although some authors have undermined this archetype by proposing more complex functions for PUFAs.[7,8] Because of the nutrition and health claims made on $\omega$-3 PUFAs, they are widely available in a "nutraceutical" formulations.[9] Although $\omega$-6 PUFAs show a positive association with CRC risk,[9] some studies have also found that arachidonic acid (ARA, 20:4$\omega$6) inhibits the growth of some cancer cell lines, such as Caco-2, but not HT-29.[10] In addition, eicosanoids, biologically active molecules derived from ARA, have been proposed as mediators involved in intestinal epithelial homeostasis; other studies have linked hydroxyeicosatetraenoic acids (HETEs), metabolized from ARA by the lipoxygenase (LOX) pathway and cytochrome P-450 isozymes, with cancer-related processes, such as cell apoptosis and proliferation, angiogenesis, or metastasis.[11−13] However, because of the contradictory effects of the manipulation of the ARA cascade,[12,14] the mechanisms that mediate these effects remain unclear.

Different mechanisms have been suggested to participate in the anticancer activities of $\omega$-3 PUFAs, such as apoptosis promotion, inhibition of cell transformation, and repression of cell proliferation. Many of them have been associated with the repression of eicosanoid production from $\omega$-6 PUFAs.[15] Previous studies have tested the inhibition of HT-29 cell proliferation produced by some $\omega$-3 FAs. Docosahexaenoic acid (DHA, 22:6$\omega$3) strongly and dose-dependently promotes apoptosis and enhances many cellular responses that decrease cell proliferation.

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Because DHA is promptly integrated into cancer cell membranes, considerable research has been conducted on membrane-initiated phenomena. For instance, DHA has been described to take part in the promotion of apoptosis and the inhibition of both *in vivo* and *in vitro* proliferation. However, the role of DHA on cell cycle regulation has been scarcely studied on cancer cells, and therefore, the mechanisms of action of DHA are not yet entirely understood.[16]

As for ARA, previous studies have debated whether the increased ARA metabolism found in cancer cells is an initiator or an output of tumor progression. Studies on COX-2 and its inhibitors have strongly supported the notion that the induction of COX-2 is a critical step in carcinogenesis and tumor growth. A previous study has also shown that unesterified ARA is an important signal for apoptosis and that the apoptosis promotion by non-steroidal anti-inflammatory drugs and other inhibitors of ARA metabolism results from ARA accumulation.[17] Bishayee and Sethi[18] have stated that the ARA pathway, a metabolic process, plays a key role in carcinogenesis. Hence, previous studies have considered the ARA pathway metabolic enzymes phospholipase A2s, cyclooxygenases (COXs), and LOX, together with the metabolic derivatives of these enzymes, such as prostaglandins and leukotrienes, to be new targets for the prevention and treatment of cancer.

This work has attempted to determine the *in vitro* anticancer activities of DHA and ARA based on the regulation of gene transcription in HT-29 CRC cells. To do so, we performed different cell assays and studied protein abundance changes induced by DHA and ARA in the global proteome of these cells by means of a quantitative proteomics approach based on liquid chromatography−mass spectrometry (LC−MS) called "sequential windowed acquisition of all theoretical fragment ion mass spectra" (SWATH-MS),[19] followed by pathway analysis.

## MATERIALS AND METHODS

**Oil Samples.** Two commercial oils, DHASCO and ARASCO, 40% DHA and 40% ARA, respectively, obtained from the Market Bioscience Corporation (Columbia, MD, U.S.A.), were used as a source for obtaining DHA and ARA FFAs. DHASCO and ARASCO are produced by combining high oleic sunflower oil with an oil obtained from a unicellular alga (*Crypthecodinium cohnii*) and a fungi (*Mortierella alpina*), respectively.

**Purification Process.** ARA and DHA as FFAs were purified from 40 mg of DHA ethyl ester or ARA ethyl ester according to a previously described chromatography process,[20] using mixtures of *n*-hexane and acetone as eluting solvents in the sequence shown in Supplementary Table S1 of the Supporting Information, optimized for maximizing PUFA purity, and at flow rates of approximately 1−2 mL/min. Eluting fractions were collected in labeled tubes and analyzed directly using gas−liquid chromatography (GLC) to determine the purity grade. Subsequently, the ethyl esters were acidified according to the approach by Vázquez and Akoh.[21] FFAs were obtained in this manner.

**GLC Analyses.** A GLC device (Focus, Thermo Electron, Cambridge, U.K.) combining a flame ionization detector with a capillary column (30 m × 0.25 mm inner diameter × 0.25 μm, Omegawax 250, Supelco, Bellefonte, PA, U.S.A.) were used for FA ethyl ester analysis. The temperature of the oven was programmed as follows: 90 °C for 1 min, increase to 100 °C in 1 min, then 3 min at 100 °C, increase of 6 °C/min until reaching 260 °C, and finally a step of 260 °C during 5 min. The injector temperature was set to 250 °C, with a split ratio of 50:1 and an injector volume of 4 μL. The detector temperature was set to 260 °C. The nitrogen carrier gas flow was 1 mL/min.

**Cell Assays.** The cell line used for the assays was HT-29 (colon cancer), kindly provided by the Technical Instrumentation Facility of the University of Granada (Granada, Spain) and incubated at 37 °C and 5% $CO_2$ in Gibco RPMI medium 1640 (Thermo Fisher Scientific, Waltham, MA, U.S.A.) with penicillin−streptomycin (100 mg/mL), 1-glutamine (2 mM), sodium pyruvate (1 mM), and 5% fetal bovine serum. Cell cultures and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test were conducted as detailed in previous works.[22]

The final concentration of the purified acyl species (acetone-diluted ARA FFA or DHA FFA) added to the medium was in the range of 50−600 μM. The final concentration of acetone added to culture medium was 1% (v/v). Control cells were also cultured with a medium containing 1% acetone. Lactate dehydrogenase (LDH) assay (the *In Vitro* Toxicology Assay Kit, lactic dehydrogenase base, Sigma TOX 7, Sigma-Aldrich, St. Louis, MO, U.S.A.) was conducted the same as for the MTT assay but with a cell density of $5 \times 10^3$ cells/well, and measures were performed following the instructions of the manufacturer. The results were obtained by determining the absorbance at 490 nm with a reference filter at 690 nm at 48 and 72 h. To measure caspase-3 activity, we used the Caspase-3 Assay Kit (Sigma CASP-3-C, Sigma-Aldrich, St. Louis, MO, U.S.A.), following the instructions from the manufacturer and determining the absorbance (450 nm) at 90 min, with a cell density of $1 \times 10^7$.

**Proteomics Analysis.** *Sample Preparation.* HT-29 cells from three different groups, DHA (600 μM DHA, added to the culture medium at 24 h), ARA (600 μM, added at 24 h), and control (no acyl species added), were recovered and lysed. Protein extracts were obtained from six biological replicates for each group, and protein content was solubilized in 0.2% RapiGest SF (Waters, Milford, MA, U.S.A.) after precipitation. The protein content in each sample was determined using a nanofluorimetric assay (Qubit Protein Assay, Thermo Fisher Scientific). A total of 40 μg of protein was digested with trypsin (Promega, Madison, WI, U.S.A.) as previously described[23] in two steps, incubating at 37 °C for 2 h in the first step and for 15 h in the second step. RePliCal iRT peptides (PolyQuant GmbH, Bad Abbach, Germany) were spiked into each sample to calibrate the peptide retention times in the SWATH runs.

*Creation of the Spectral Library.* To build the spectral library, equal mixtures of the six biological replicates from each condition were pooled and analyzed using a shotgun data-dependent acquisition (DDA) approach by nanoliquid chromatography coupled to tandem mass spectrometry (nLC−MS/MS) as described elsewhere.[24] The equipment used was a Sciex Triple TOF 5600+ (Redwood City, CA, U.S.A.) coupled to an Eksigent Ekspert nLC415 (Sciex) equipped with a reversed-phase capillary column (Acclaim PepMap C18, Thermos Fisher Scientific). Detailed LC and MS parameters used can be found in the Supporting Information.

DDA runs were searched against a human Swiss-Prot protein database (20 200 entries, appended with the sequences from the RePliCal iRT peptides) using Protein Pilot (version 5.0.1, Sciex), setting a false discovery rate (FDR) threshold of 0.01. SWATH Acquisition MicroApp (version 2.0, Sciex) was used for building a peptide spectral library containing the peptides identified in the database search with a confidence score above 99%.

*SWATH Data Acquisition and Analysis.* A total of 2 μL of each of the six biological replicates from each group was run in the same LC parameters and MS equipment described above for the DDA runs but using a SWATH data-independent acquisition (DIA) method instead. MS parameters used in the SWATH method are detailed in the Supporting Information. Briefly, it consisted of the acquisition of 60 MS/MS events with precursor isolation windows of variable width comprising the $m/z$ range of 350−1250. The width of each of the $m/z$ 60 windows was adjusted according to the ion frequency found in the previous shotgun runs.

Protein quantitative data were extracted from the SWATH raw files as previously described.[24] Briefly, SWATH Acquisition MicroApp was used for extracting the ion chromatogram traces from the SWATH raw files and using the previously generated spectral library and the following parameters: 10 peptides/protein, 7 fragment ions/peptide, extraction windows of 20 min and 20 ppm, peptide FDR of 1%, and confidence score threshold of 95%. The iRT peptides spiked in each sample were used for realigning the retention times of the quantified peptides.


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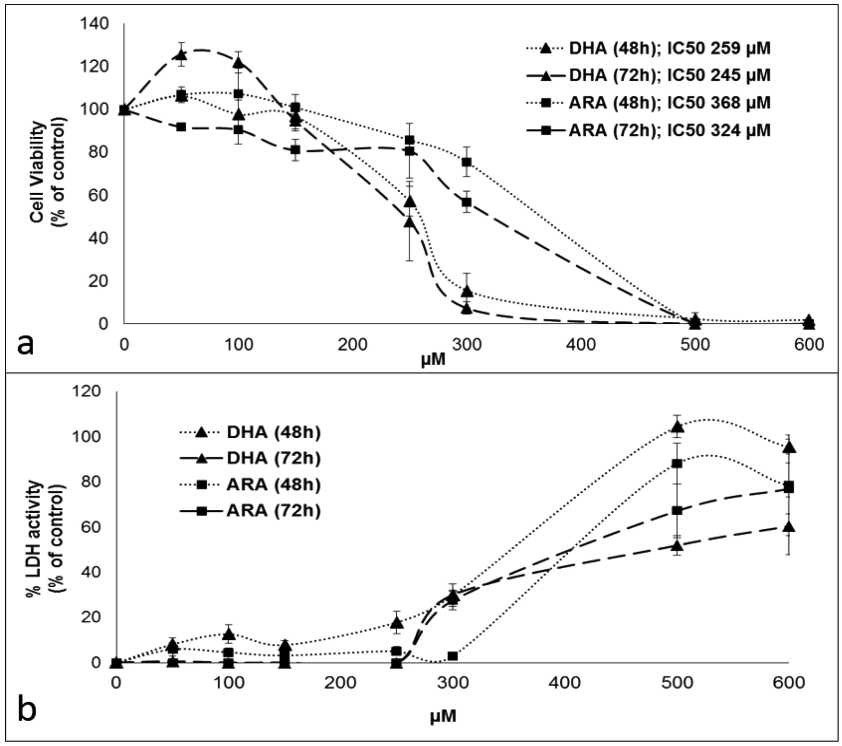


**Figure 1.** Effect of DHA (92.1%) and ARA (98.8%) on proliferation and cell membrane integrity of HT-29 cancer cells treated with 1% acetone (control) or different concentrations of FFAs (50, 100, 150, 250, 300, 500, and 600 $\mu$M) for 48 and 72 h. The data represent the means of three complete independent experiments ± SD (error bars). (a) DHA and ARA FFAs inhibit the viability of HT-29 cancer cells. Dose−response and $IC_{50}$ values were determined by the MTT assay using an enzyme-linked immunosorbent assay (ELISA) microplate reader. (b) Cell membrane damage; dose-dependent LDH activity plot.

*Pathway Analysis.* The significantly affected pathways were studied using Advaita Bio's iPathwayGuide (http://www.advaitabio.com/ipathwayguide), which considers over-representation of significant proteins, pathway topological information, and position and role of every protein.[25] Pathways obtained from the "Kyoto Encyclopedia of Genes and Genomes" (KEGG) database were used.

**Statistical Analysis.** For cell assays (MTT, LDH, and caspase-3 assays), statistical significance ($p < 0.05$) was determined by analysis of variance (ANOVA), followed by the assessment of differences using STATGRAPHICS Plus, version 5 (Statistical Graphic Corp., Warranton, VA, U.S.A.). Figures 1 and 2 show mean data for three independent

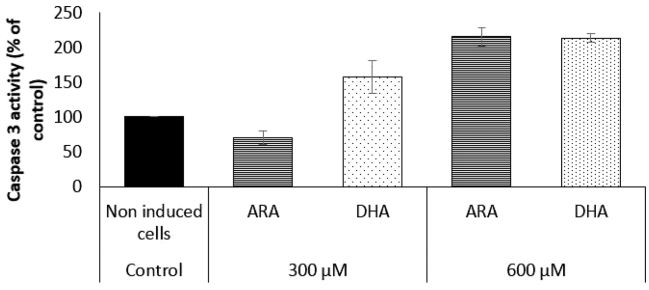


**Figure 2.** Caspase-3 activity test. Bar histogram displaying caspase-3 activity in HT-29 colon cancer cells after 90 min of incubation with DHA and ARA at 300 and 600 $\mu$M compared to untreated cells (control). The data represent the means of three complete independent experiments ± SD (error bars).

experiments ± standard deviation (SD). Normalization of the protein abundance signal as measured by SWATH was carried out using MarkerView (version 1.2.1, Sciex), and testing for differential abundance was performed at the protein level by applying Student's $t$ test. The qvalue R package was used to obtain a FDR $q$ value estimation to check for multiple testing underestimation of $p$ values.[26] For pathway analysis, a $p$ value was calculated for each reported pathway by iPathwayGuide using Fisher's method, and $p$ values were adjusted for multiple comparisons using the FDR approach.

## RESULTS AND DISCUSSION

**Antiproliferative Actions of DHA and ARA.** The purity of the assayed PUFAs was found to be 98.1 and 99.2% (FA percentage of the total FA area) for DHA and ARA, respectively. The cell proliferation inhibition exercised by both FFAs on HT-29 CRC cells was evaluated by means of the MTT test, which measures cell survival and proliferation. The effects of DHA and ARA on HT-29 cell proliferation are shown in Figure 1a. As depicted in the figure, significant concentration-dependent inhibitory effects were found from the two studied compounds. The half-maximal inhibitory concentrations ($IC_{50}$) at 48 and 72 h for DHA were 259 and 245 $\mu$M, respectively, while for ARA, the same values were 368 and 324 $\mu$M, respectively.

For the physiological relevance of these concentrations, a previous study found the serum concentration of DHA in rats to be 97.3 $\mu$g/mL (297 $\mu$M) after 4 weeks of DHA supplementation.[27] The present $IC_{50}$ for DHA may thus be relevant to tumor progression *in vivo*, while uncertainty remains for ARA; it would be necessary to experiment *in vivo* to determine the concentrations of such PUFAs attainable in serum without producing adverse effects.

Das and Das,[28] using the MTT assay, found a significant cell viability reduction of SH-SY5Y (55% reduction) and C6G (54% reduction) cells after a 24 h incubation with 100 $\mu$M DHA. In addition, Ramos-Bueno et al.[22] demonstrated that acyl species ARA and DHA derived from ARASCO and DHASCO oils,


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respectively, had relatively high *in vitro* anticancer activity on HT-29 cells. Chen et al.,[29] using the MTT test, showed that Hep G2-MV2E1-9 cells lost more than 80% viability after a pretreatment with 50 $\mu$M ARA.

**LDH Assay.** A LDH assay was conducted to assess the release of the LDH enzyme into the culture medium after cell membrane damage caused by both DHA and ARA. The concentrations for performing the tests were 0−600 $\mu$M, at which the tested compounds showed different *in vitro* antiproliferative activities from those detected by the MTT assay.

DHA induced more active damage at 300 $\mu$M and 48 h of exposure than ARA (Figure 1b), although at 500 $\mu$M and after 72 h of exposure, ARA was more effective in this action than DHA. Overall, LDH values were lower than those obtained in the MTT assay. In this regard, Ramos-Bueno et al.[22] indicated that, when comparing the effects measured by LDH and MTT assays, the former was attained at higher levels of cell damage. Such differences could be attributable to the specificity of LDH testing compared to the MTT assay. A previous study showed that LDH leakage occurred only when reactive oxygen species related to cell death appeared, which were responsible for mitochondrial damage.[22] Overall, the MTT assay showed cell growth inhibition at lower concentrations of FFA than the LDH test, because MTT reduction is not an indicator of cell death but rather constitutes a measure of relative metabolic activity, as demonstrated in a previous study on HT-29 cells.[30] Concerning ARA, our results agree with previous findings,[29,31] which have shown the same PUFA-stimulated LDH activity in some other cells (e.g., Hep G2-MV2E1-9 and Sertoli).

**Caspase-3 Assay.** The activation of caspase-3, specifically through self-cleavage, has been shown to be the event that commits cells to apoptotic death.[32] As shown in Figure 2, DHA induced caspase-3 activation at 300 $\mu$M, while ARA did not, and both PUFAs exercised a similar induction of apoptosis at 600 $\mu$M. Several studies have suggested that DHA displays a notable protective effect on CRC, which could at least be partly due to its pro-apoptotic activity. The mechanisms affected by DHA for promoting apoptosis in CRC are still largely unknown, however. Previous works have demonstrated the apoptotic activity exerted by DHA in several cancer cell lines,[33,34] while a variety of authors have tested whether DHA induced apoptosis in CRC cells.[4,35,36] Habermann et al.,[37] using an *in vitro* model and HT-29 and LT97 cells, proposed a generic mechanism for the apoptosis promoted by DHA that was based on inducing reactive oxygen species as a result of DHA double bonds, which led to apoptosis. Narayanan et al.[38] found that DHA-regulated genes and transcription factors induced apoptosis in human colon cancer cells. Zhang et al.[39] have also proposed that both DHA and ARA trigger the apoptosis of colon cancer cells through a mitochondrial pathway; they found that DHA in particular induced dramatic activities in both caspase-3 and caspase-9.

**Proteomics Analysis.** DIA MS strategies are showing great power for the study of abundance differences in complete proteomes. Precision and accuracy of the DIA approach of SWATH-MS[19] has been described to match that of selected reaction monitoring, the gold standard in protein quantitation, even when applied to the quantitation of whole proteomes.[40]

*Shotgun Analysis and Spectral Library.* Equal amounts of all samples from each group were pooled and analyzed by DDA LC−MS. All of the data sets were combined, and 15 406 peptides and 2140 proteins were identified using the ProteinPilot search engine using a FDR threshold of 1% (Supplementary Table S2 of the Supporting Information). This search result was then used for building a spectral library, which comprised 2002 proteins.

*Protein Abundance Changes.* Protein quantitative data were extracted from the SWATH raw files, as described in the Materials and Methods. In total, 7653 peptides and 1882 proteins were confidently quantified (Supplementary Table S3 of the Supporting Information). To assess the global impact of the overall quantified proteins, principal component analysis (PCA) was performed on the whole protein data set. PCA was able to separate the two tested groups from each other and from the control group (Figure 3a), thus showing a large and different effect of the specific FFA (DHA or ARA) on HT-29 colon cancer cell proteomes.

*Effects of DHA and ARA on HT-29 Colon Cancer Cell Proteomes.* Supplementary Tables S4 and S5 of the Supporting Information show the results from the differential abundance testing for both comparisons (the DHA group to the control and the ARA group to the control, respectively), including a $p$ value and the corresponding FDR-adjusted $q$ value for each of the 1882 quantified proteins. A total of 1015 proteins showed changes in expression when we addressed the impact of the DHA extract and considered a non-adjusted $p$ value threshold of 0.05. Notably, as a result of the $p$ value distribution (Supplementary Figure S1a of the Supporting Information), the $q$ values obtained from the FDR $p$ value correction were lower than the corresponding non-adjusted $p$ values (Supplementary Figure S1b of the Supporting Information), which meant that we had more significant tests (significant proteins) if we chose a $q$ value threshold rather than a $p$ value threshold to consider a protein to be significantly regulated (Supplementary Figure S1c of the Supporting Information).

For downstream analyses, we considered a restrictive scenario, namely, $p$ value and fold-change thresholds of 0.01 and 2.0, respectively, to have more confidence in selecting the proteins that presented real expression changes. When we applied these cutoff values, 284 proteins showed changes in expression, with 45 proteins being upregulated and 239 proteins being downregulated (Figure 3b and Supplementary Table S6 of the Supporting Information).

When we addressed the changes caused by the ARA extract on HT-29 cancer cells and applied the same $p$ value and fold-change cutoff values, only 73 proteins were found to present changes in expression, with 27 proteins being upregulated and 46 proteins being downregulated (Figure 3c and Supplementary Table S7 of the Supporting Information). The $p$ value distribution, $q$ value to $p$ value correspondence, and number of significant proteins according to several cutoff values for ARA to control differential abundance tests are shown in Supplementary Figure S2 of the Supporting Information. When we compared the 284 and 73 proteins that we found were regulated as a consequence of adding DHA and ARA to the media (respectively), only 43 proteins were commonly regulated in both conditions (Figure 3d). DHA was responsible for a larger effect on HT-29 cancer cells than ARA, because it is derived from the number of affected proteins and the larger fold changes that are found.

**Pathway Impact Analysis.** After FDR correction of the $p$ values, three pathways were found to be significantly affected by DHA (Figure 4), with the proteasome pathway (KEGG: 03050) presenting the deepest effect. The proteasome is a protein-degrading large complex that participates in different critical cellular functions, including cell cycle regulation, inflammatory and stress responses, apoptosis, signal transduction, cell differentiation, and antigen processing. The 26S proteasome is the most characteristic conformation, consisting of two 19S regulatory particles


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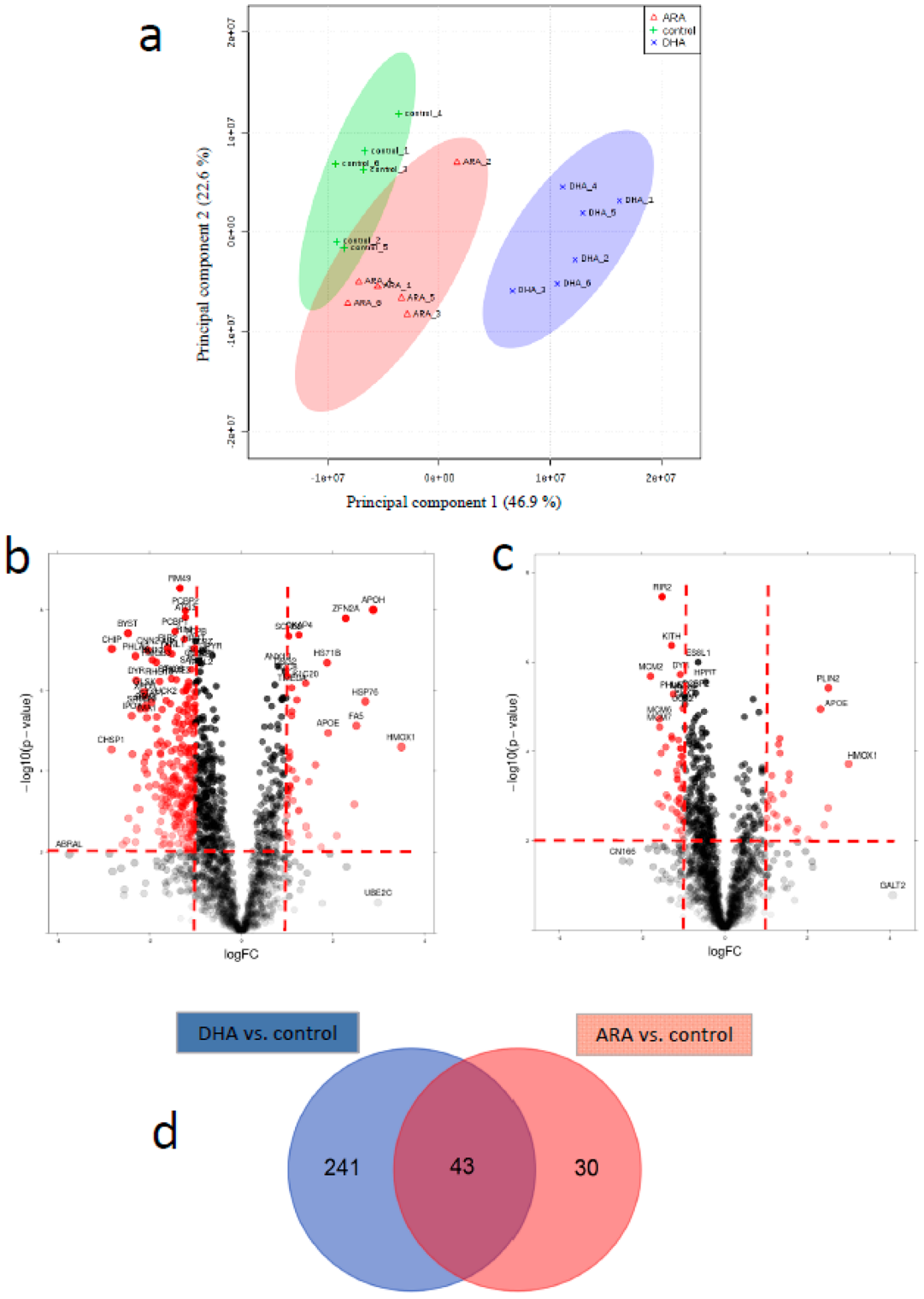


**Figure 3.** Quantitative proteomics results. (a) PCA of all 1882 quantified proteins. Using principal components 1 and 2, samples from the two tested groups were separated from each other and from the control group. (b) Volcano plot for DHA versus the control and (c) ARA versus the control, showing $p$ value of <0.01 and fold change of ≥2.0 (or ≤0.5) thresholds. Proteins above these cutoffs are shown in red and were considered significant proteins for the subsequent downstream analyses. The most extreme proteins are labeled with the protein name. (d) Venn diagram showing the number of significant proteins specifically for each comparison and common to both comparisons.

(which combine two base particles, PA700 lid and PA700 base) and a 20S proteolytic particle, and it is responsible for the degradation of ubiquitinated-labeled proteins.[41] The 11S regulator particle or PA28 is a second type of proteasome particle. It consists of two different subparticles, PA28-$\alpha$ and PA28-$\beta$, which have been described to be greatly upregulated by IFN-$\gamma$ and have a role in generating antigenic peptides that are presented in the MHC-I pathway.[42] We found up to 18 proteins from the proteasome pathway to be downregulated as a consequence of the addition of DHA extract, including members of



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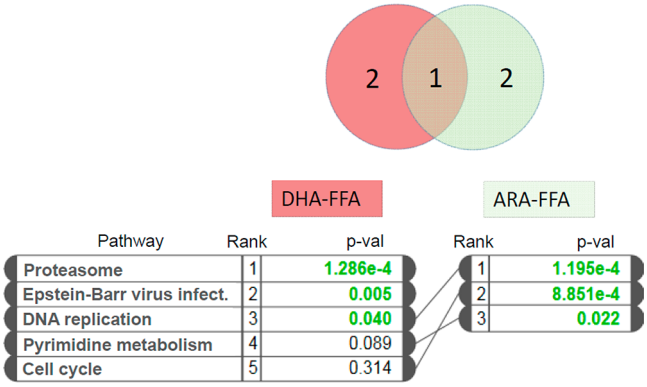


**Figure 4.** Pathway impact analysis. Pathways with significant impacts and their associated corrected $p$ values for the differentially expressed proteins for the comparisons of DHA versus the control and ARA versus the control.

most of the proteasome regulatory particles (PA700 lid particle, PA700 base particle, PA28-$\alpha\beta$, and PA28-$\gamma$), in addition to the 20S core particle (Figure 5). PA28-$\gamma$ is localized in the nucleus and has been described to have a role in apoptosis and cell cycle regulation.[43] Many proteasome inhibitors, including natural substances, such as polyphenol compounds, have been found to hinder cell proliferation, selectively promote apoptosis in proliferating cells, and inhibit angiogenesis.[44,45] These qualities make these agents attractive drug candidates for treating different kinds of cancer, and some have even been studied in human clinical trials.[44,46] According to our results, DHA-derived FFA could be included among these candidates. Even though the mechanisms of action are not completely understood, various studies have demonstrated that proteasome inhibitors alter the balance of pro- and antiapoptotic proteins in human cancer cells, generating an accumulation of tumor-suppressor proteins and the induction of apoptosis.[47] Some authors have suggested that

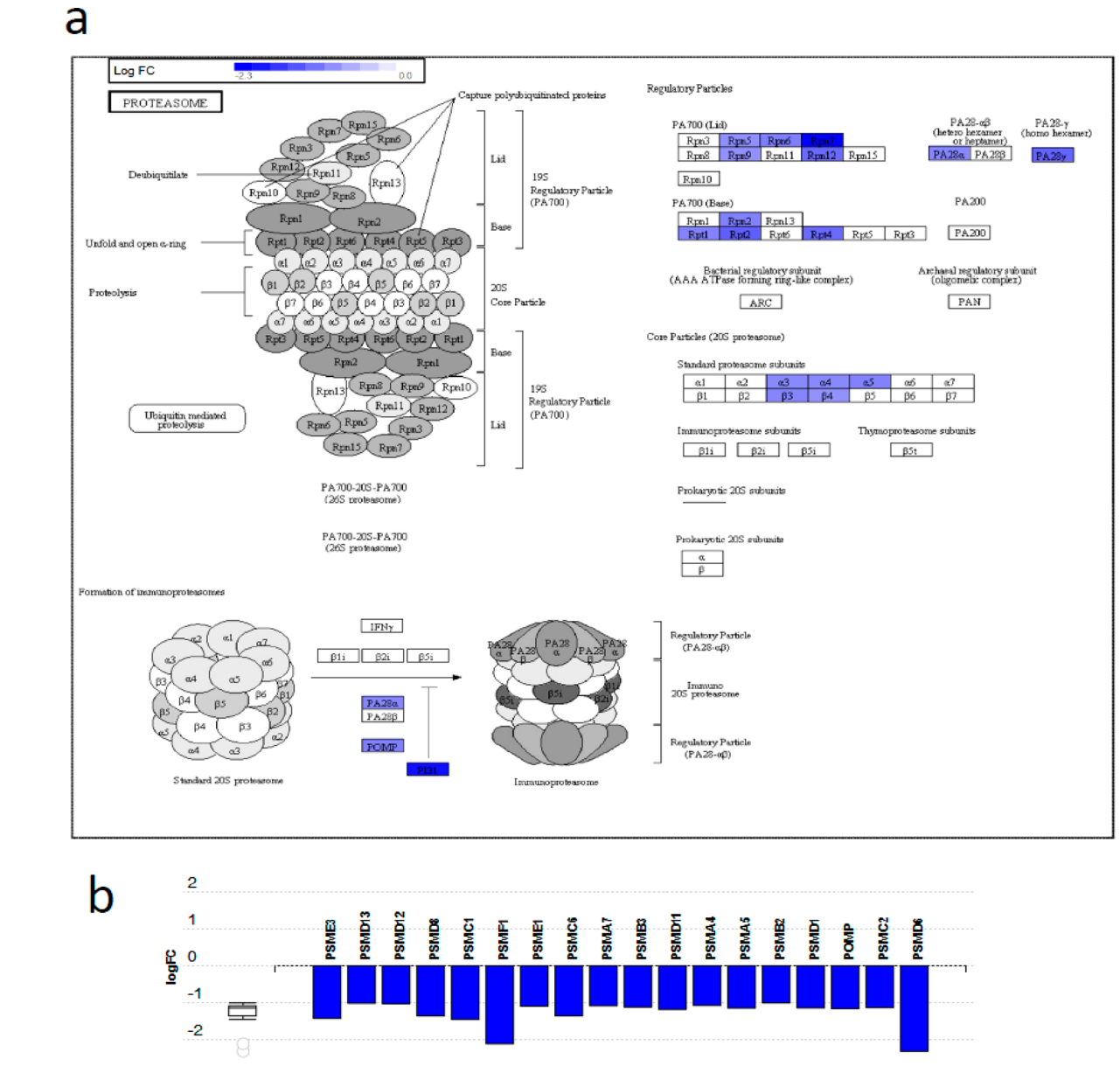


**Figure 5.** Effect of DHA extract on HT-29 cancer cell proteasome pathway (KEGG: 03050). (a) Proteasome pathway diagram overlaid with the measured fold change of the gene. (b) Gene perturbation bar plot for the proteasome pathway; differentially expressed genes are represented with negative values in blue and positive values in red.



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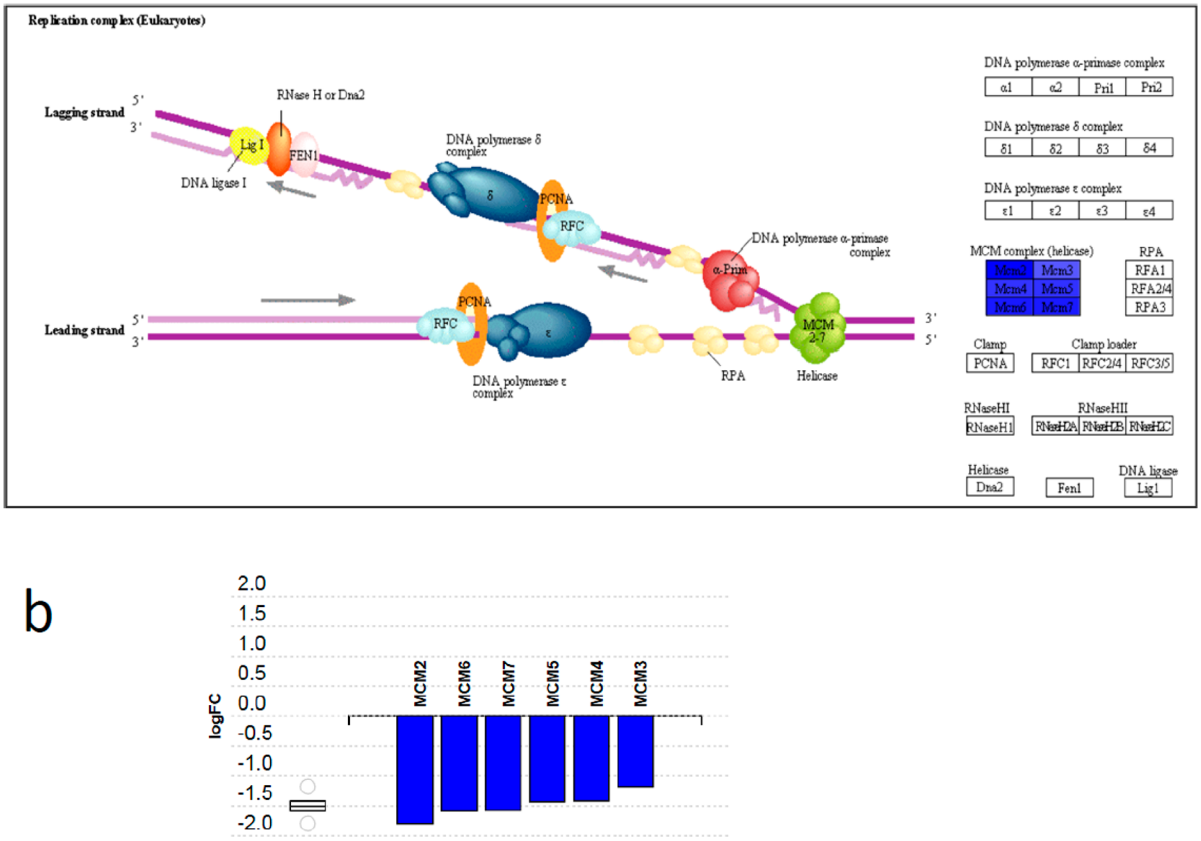


**Figure 6.** Effect of ARA extract on the HT-29 cancer cell DNA replication pathway (KEGG: 03030). (a) DNA replication pathway diagram overlaid with the measured fold change of the gene. (b) Gene perturbation bar plot for the DNA replication pathway; differentially expressed genes are represented with negative values in blue and positive values in red.

proteasome inhibitors promote cell death by means of a p53-dependent mechanism, by inducing the expression of some of the genes related to apoptosis, such as PUMA or Bax.[48] Others, however, have pointed to a p53-independent mechanism of proteasome inhibitor-induced apoptosis. Noxa, one pro-apoptotic protein, has been linked to this apoptosis mechanism, because several proteasome inhibitors have been found to induce it in a p53-independent manner in cancer cells of different origins.[49] Our data could not clearly demonstrate a p53-dependent nor -independent mechanism of apoptosis. No quantitative information was obtained from our SWATH-MS analysis for the main pro- and antiapoptotic proteins, PUMA, NOXA, Mcl-1, p21, or Bcl-2. A few other pro-apoptotic factors and tumor-suppressor proteins were found to be slightly upregulated, such as apoptosis-inducing factor 1 (AIFM1, with a fold change of 1.65) and tumor protein p53-inducible protein 11 (P5i11, with a fold change of 1.40). A few other antiapoptotic proteins were found to be downregulated, including apoptosis inhibitor 5 (API5, with a fold change of 0.70), which has been described as a suppressor of E2 promoter binding factor-dependent apoptosis *in vivo* and *in vitro* in multiple tissues,[50] and TNF-$\alpha$-induced protein 8 (TFIP8, with a fold change of 0.61), which is an inhibitor of TNF-mediated apoptosis.[51]

Notably, we found PRKC apoptosis WT1 regulator protein (PAWR), a protein described as pro-apoptotic in certain cancer cells, such as prostate,[52] to be downregulated as an effect of the DHA treatment on HT-29 cells (fold change of 0.52). However, when we analyzed the differentially expressed proteins in the context of the p53 signaling pathway in iPathwayGuide, we found that three key proteins were regulated by DHA (Supplementary Figure S3 of the Supporting Information): TSP1 (thrombospondin 1, gene THBS1), which mediates antiangiogenic properties, presenting a fold change of 2.05; CDC2 (cyclin-dependent kinase 1, gene CDK1), which regulates the cell cycle and whose inhibitors are being explored as chemotherapeutic agents in cancer,[53] presenting a fold change of 0.24; and p53R2 (RIR2, ribonucleotide reductase regulatory subunit M2, gene RRM2), involved in DNA synthesis, presenting a fold change of 0.33. Interestingly, none of the proteins related to apoptosis within the p53 signaling pathway were affected by DHA (Supplementary Figure S3 of the Supporting Information). This behavior could point to a p53-independent mechanism of apoptosis as a consequence of treating HT-29 cells with DHA, although more research would be required to maintain this argument. When we added ARA FFA to the HT-29 cells, only one protein in the p53 signaling pathway, p53R2 (gene RRM2), resulted in regulation (downregulation).

According to our pathway analysis, two other pathways, Epstein−Barr virus (EBV) infection (KEGG: 05169) and DNA replication (KEGG: 03030), were also found to be significantly affected by the addition of DHA, although the effect was not as evident as for the proteasome pathway, which presented much higher $p$ values. EBV is a ubiquitous human herpesvirus that has been associated with oncogenesis in Burkitt lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, gastric adenocarcinoma, and breast cancer.[54] A total of 16 proteins from the EBV


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infection pathway were affected by DHA in our study, with 14 proteins downregulated, most related to DNA transcription factors (Supplementary Figure S4a of the Supporting Information). EBV makes cancer cells more aggressive;[54] therefore, we hypothesize that the downregulation of the proteins related to this pathway (which we found as a consequence of adding DHA) could have had the opposite effect, that is, reducing CRC cell viability. For DNA replication, DHA was found to produce the underexpression of five out of the six helicase [also called the mini-chromosome maintenance (MCM) complex] particles: Mcm2, Mcm3, Mcm4, Mcm5, and Mcm7 (Supplementary Figure S4b of the Supporting Information).

DNA replication was the top-ranked pathway when assessing the effect of ARA extract; in that case, all six helicase particles (Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7) were found to be downregulated (Figure 6), presenting larger fold changes than for DHA. DNA helicase is the protein complex that unwinds the duplex parental DNA at the replication fork, ahead of the DNA synthetic machinery.[55] The DNA replication procedure is likewise linked to the cell cycle and to DNA processes to support genomic integrity,[56] and therefore, the downregulation of the helicase proteins that our results showed was expected to transfer an inhibitory effect to these processes. In fact, the cell cycle pathway (KEGG: 04110), which also occurred as a result of the downregulation of the six MCM particles, and the pyrimidine metabolism (KEGG: 00240) pathway were the two other significant pathways affected by the addition of ARA (Supplementary Figure S5 of the Supporting Information), although the pyrimidine metabolism pathway presented a much higher $p$ value than the DNA replication and cell cycle pathways. Interestingly, neither the proteasome pathway nor the EBV pathway were significantly affected by ARA, because none of the proteins included in these pathways were found to have been differentially expressed.

We have established that both ARA FFA and DHA FFA exercise different *in vitro* anticancer activities on CRC. At 300 $\mu$M, DHA induced a stronger decrease of CRC cell proliferation than ARA but DHA also induced fewer cytotoxic effects. Pathway analyses of the SWATH-MS-generated proteomics data revealed that both FAs had an impact on the cell cycle by means of downregulating the protein particles that form the helicase complex, but DHA also affected the proteasome system, because many proteins from the different proteasome subunits were also underexpressed.

The present study shows that DHA and ARA are significantly inhibiting the growth of cancer HT-29 cells depending upon the time and concentration, which suggests that both of them may be helpful for hindering the development of aggressive adenocarcinoma.

On the basis of these findings, further studies will need to be conducted to determine if a combination of PUFAs with regular anticancer molecules would represent a reasonable approach to CRC treatment. The use of next-generation proteomics and other "omics" approaches will allow for analyzing the effects of natural compounds on global patterns of gene expression and protein composition of the cell, thus improving the knowledge that we have about the biological roles of nutraceuticals.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b00915.

Detailed LC and MS parameters used for both data-dependent acquisition and SWATH methods, differential abundance test results for DHA versus the control (Figure S1), differential abundance test results for ARA versus the control (Figure S2), HT-29 cells p53 signaling pathway showing protein changes observed as a result of treatment with DHA (Figure S3), HT-29 cells EBV infection and DNA replication pathways showing the effect of DHA treatment (Figure S4), HT-29 cells cell cycle and pyrimidine metabolism pathways showing the effect of ARA treatment (Figure S5), and gradient used in the chromatographic purification of both FFAs, DHA and ARA (Table S1) (PDF)

2140 proteins that were identified using the ProteinPilot search engine and a FDR threshold of 1% (Supplementary Table S2) (XLSX)

SWATH-based protein quantification data for all of the samples (Supplementary Table S3) (XLSX)

Differential abundance test for DHA versus the control (Supplementary Table S4) (XLSX)

Differential abundance test for ARA versus the control (Supplementary Table S5) (XLSX)

Differential expressed proteins for DHA versus the control (Supplementary Table S6) (XLSX)

Differential expressed proteins for ARA versus the control (Supplementary Table S7) (XLSX)

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### Notes

The authors declare no competing financial interest.

The LC−MS raw files associated with this paper are available at www.proteomexchange.org (ProteomeXchange Consortium), data set id PXD008653.

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# CHAPTER III

# 7. CHAPTER III. SWATH differential abundance proteomics and cellular assays show *in vitro* anticancer activity of arachidonic acid- and docosahexaenoic acid-based monoacylglycerols in HT-29 colorectal cancer cells

## 7.1. Abstract

CRC is one of the most common and mortal types of cancer. There is increasing evidence that some PUFA exercise specific inhibitory actions on cancer cells through different mechanisms, as a previous study on CRC cells demonstrated for two very long-chain PUFA. These were DHA and ARA in FFA form. In this work similar design and technology have been used to investigate the actions of both DHA and ARA as MAG molecules, and results have been compared with those obtained using the corresponding FFA. Cell assays revealed that ARA- and DHA-MAG exercised dose- and time-dependent antiproliferative actions, with DHA-MAG acting on cancer cells more efficiently than ARA-MAG. SWATH-MS massive quantitative proteomics, validated by parallel reaction monitoring and followed by pathway analysis, revealed that DHA-MAG had a massive effect in the proteasome complex, while ARA-MAG main effect was related to DNA replication. Prostaglandin synthesis also resulted inhibited by DHA-MAG. Results clearly demonstrated the ability of both ARA- and DHA-MAG to induce cell death in CRC cells, which suggests a direct relationship between chemical structure and antitumor actions.

*nutrients*

MDPI

*Article*

# SWATH Differential Abundance Proteomics and Cellular Assays Show In Vitro Anticancer Activity of Arachidonic Acid- and Docosahexaenoic Acid-Based Monoacylglycerols in HT-29 Colorectal Cancer Cells

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**Abstract:** Colorectal cancer (CRC) is one of the most common and mortal types of cancer. There is increasing evidence that some polyunsaturated fatty acids (PUFAs) exercise specific inhibitory actions on cancer cells through different mechanisms, as a previous study on CRC cells demonstrated for two very long-chain PUFA. These were docosahexaenoic acid (DHA, 22:6*n*3) and arachidonic acid (ARA, 20:4*n*6) in the free fatty acid (FFA) form. In this work, similar design and technology have been used to investigate the actions of both DHA and ARA as monoacylglycerol (MAG) molecules, and results have been compared with those obtained using the corresponding FFA. Cell assays revealed that ARA- and DHA-MAG exercised dose- and time-dependent antiproliferative actions, with DHA-MAG acting on cancer cells more efficiently than ARA-MAG. Sequential window acquisition of all theoretical mass spectra (SWATH) – mass spectrometry massive quantitative proteomics, validated by parallel reaction monitoring and followed by pathway analysis, revealed that DHA-MAG had a massive effect in the proteasome complex, while the ARA-MAG main effect was related to DNA replication. Prostaglandin synthesis also resulted as inhibited by DHA-MAG. Results clearly demonstrated the ability of both ARA- and DHA-MAG to induce cell death in colon cancer cells, which suggests a direct relationship between chemical structure and antitumoral actions.

**Keywords:** colorectal cancer; proteomics; SWATH; docosahexaenoic acid; arachidonic acid; HT-29 cells; monoacylglycerols

## 1. Introduction

The fat content of a normal diet consists mainly of triacylglycerols (TAG) (about 90% of total ingested lipids) and small amounts of sterols and phospholipid esters, as well as fat-soluble vitamins (A, D, E, and K) [1]. The fatty acid (FA) distribution on the glycerol backbone of TAG influences their absorption, distribution, and tissue uptake [2]. Free FA (FFA) and *sn*-2-monoacylglycerol (*sn*-2 MAG), the two hydrolysis products of dietary TAG, are absorbed from the lumen into polarized enterocytes in the small intestine. Polyunsaturated FAs (PUFAs) are better absorbed when they are esterified at

the *sn*-2 position of the glycerol molecule, while the type of FA at the remaining locations also influences their intestinal absorption [2–5].

Arachidonic acid (ARA, 20:4*n*6) is a long-chain PUFA (LCPUFA) belonging to the *n*-6 family. This molecule is the precursor of the biosynthetic pathway leading to the production of prostaglandin PGE2, thromboxanes and other metabolites involved in the regulation of various physiological processes, e.g., inflammation and immune function [6]. Docosahexaenoic acid (DHA, 22:6*n*3) is one of the predominant LCPUFAs in the structural phospholipids of the human brain and retina, and it accumulates within the fetal neural tissue during pregnancy and the postnatal period [7]. At present, fish oil constitutes the main source of DHA, although alternatively it can be industrially obtained from several microorganisms, mainly from marine origin, e.g., the unicellular microalga *Crypthecodinium cohnii*, which contains large amounts of DHA [8].

The *n*-6/*n*-3 ratio is commonly used as an index to evaluate the nutritional quality of dietary FA, and it has particular relevance on human health. *n*-3 LCPUFAs help to reduce inflammation, while most *n*-6 LCPUFAs tend to increase it. An imbalanced *n-6*/*n*-3 ratio contributes to the development of diseases, while an adequate balance helps to maintain and even improve health [9,10].

Different mechanisms have been proposed for the anticancer actions of the various LCPUFAs, including suppression of neoplastic transformation, inhibition of cell proliferation and enhancement of apoptosis [11]. This last phenomenon is morphologically characterized by a decrease in cell and nuclear volume, chromatin condensation and DNA fragmentation, and the presence of lipid bodies, without changes in organelle integrity [12]. Morin et al. [13,14] demonstrated a reduction in cell growth and the induction of apoptosis caused by DHA-MAG, eicosapentaenoic acid (EPA, 20:5*n*3)-MAG, and docosapentaenoic acid (DPA, 22:5*n*3)-MAG, in colon and lung cancer cell lines. In addition, MAG derived from monounsaturated FAs (MUFAs) such as oleic acid (OA, 18:1*n*9), and saturated FAs (SFAs) such as palmitic acid (PA, 16:0), have been related to the decrease in the activity of MRP2 (protein 2 associated with multidrug resistance) in Caco-2 cells at low concentrations [15]. Also, Philippoussis et al. [3] showed that murine T-cells undergo a rapid apoptosis following treatment with different MAG types.

One of the aspects to be considered in the performance of MAG as a cancer inhibitor is the mechanisms for entering into cells. Whereas Schultess et al. [16] stated that MAGs are absorbed by passive diffusion, Ho and Storch [5] suggested that they require a protein-mediated process. Little is known about these input mechanisms, although different enzymes related to biosynthesis and lipid degradation are supposed to be involved in this process. For instance, Acyl-CoA synthetases (ACS) play a critical role in the transport of FA into cells by making this transport unidirectional [17]. Mashek and Coleman [18] found that the overexpression of ACS4, which is located on the mitochondrial-associated membrane in hepatocytes, increases EPA incorporation into cell lipids by 67% during a 3-h labeling period in COS-1 cells. MAG-lipase and $\alpha$/-$\beta$-hydrolase domain 6 (ABHD6) and ABHD12 are enzymes which are also involved in intracellular degradation of MAG in many tissues. Poursharifi et al. [19] showed that the suppression of ABHD6 caused the accumulation of 1-MAG-containing saturated FA in pancreatic islets and INS-1 cells and also in white and brown adipose tissues, while the changes in 2-MAG content was modest. Gadja and Storch [20] suggest an important role on this subject for a liver-FA binding protein (LFABP), which is part of a complex along with microsomal TAG transport protein (MTP), CD36, and ApolipoproteinB48 (ApoB48), and is responsible for budding of prechylomicron transport vesicles (PCTV) from the endoplasmic reticulum (ER). Circulating FA and MAG enter the enterocyte via the basolateral membrane where they can be bound to LFABP, and bloodstream-derived FA and MAG are primarily oxidized or incorporated into phospholipids. Taken together, all this information supports the fact that for MAG there is a mediated transport across the cell membrane of enterocytes.

Currently, there is a growing research effort focused on the production of functional nutrients such as structured lipids. However, few studies have focused on assessing the effects of ARA- and DHA-MAG in colon cancer cells and the related molecular mechanisms. In a previous study focused on FFA, we demonstrated that DHA-FFA inhibited HT-29 cells proliferation to a higher extent than ARA-FFA did, with either proteasome or DNA replication, respectively, being the main mechanisms

affected [21]. Here, we used the same cellular model, study design, and technology to investigate the actions of DHA and ARA-MAG in colorectal cancer (CRC) cells, and the results are compared with those reported for the corresponding FFA. For this objective, cell viability and cell cytotoxicity assays have been performed, and the biological pathways that are affected by these two MAGs have also been studied by means of sequential window acquisition of all theoretical mass spectra (SWATH) – mass spectrometry (MS) global protein quantitation followed by pathway analysis.

## 2. Materials and Methods

### *2.1. Oil Samples and Purification of MAG*

DHASCO® (40% DHA, a mixture of the oil extracted from the unicellular alga *Crypthecodinium cohnii* and high oleic sunflower oil) and ARASCO® (40% ARA, a mixture of an oil extracted from the unicellular fungi *Mortierella alpina* and high oleic sunflower oil) oils were supplied by Martek Bioscience Corporation (Columbia, MD, USA). Purification of ARA- and DHA-MAG was carried out according to the methodology described by González-Fernández et al. [22] based on a chromatography process. Briefly, both DHASCO® and ARASCO® oils were subjected to an enzymatic hydrolysis with porcine lipase. Then, MAGs were separated from the remaining hydrolysis products (mainly FFA and glycerol) using an open chromatography column with silica gel as stationary phase and a hexane/acetone mixture as mobile phase. Once MAG mixtures were obtained, another open chromatography column with silver nitrate as stationary phase was used to purify either DHA-MAG or ARA-MAG. Liquid chromatographic fractions were collected in test tubes and analyzed by gas chromatography (GC) to determine the purity grade according to Rodríguez-Ruiz et al. [23]. To this end, about 1 mg of each fraction was weighed into test tubes next to 1 mL of *n*-hexane and 1 mL of freshly prepared transesterification reagent (methanol and acetyl chloride 20:1 v/v). Then, the tubes were placed in a thermoblock at 100 °C for 30 min. After that, the mixtures were cooled at room temperature and 1 mL of distilled water was added. Samples were shaken and centrifuged (2500 rpm, 3 min) and the upper *n*-hexane layer collected and stored in numbered vials at –20 °C until GC analysis. The equipment used for FA methyl esters (FAME) analysis was a GC device (Focus, Thermo Electron, Cambridge, UK) equipped with flame ionization detector (FID) and an Omegawax 250 capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness) (Supelco, Bellefonte, PA, USA). The oven temperature program was 90 °C (1 min), 10 °C/min to 100 °C (3 min), 6 °C /min to 260 °C (5 min). The injector temperature was 250 °C with split ratio 50:1, and injector volume was 4 μL. The detector temperature was 260 °C. The flow of carrier gas ($N_2$) was 1 mL/min. Peaks were identified by retention times obtained for known FAME standards (PUFA No. 1, 47033; methyl linoleate 98.5% purity, L6503; and methyl stearidonate 97% purity, 43959 FLUKA) from Sigma, (St. Louis, USA), and FA contents were estimated by using methyl pentadecanoate (15:0; 99.5% purity; 76560 Fluka) from Sigma as internal standard.

### *2.2. Cell Assays*

All assays were accomplished using the HT-29 colon cancer cell line, which was supplied by the Technical Instrumentation Service of University of Granada (Granada, Spain). Cell cultures and cell assays, that is, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, lactate dehydrogenase (LDH) assay and caspase-3 activity, were performed as previously described [21]. The number of cells used in the cell assays ranged from $5 \times 10^3$ for LDH assay to $1 \times 10^7$ for caspase-3 assay, as previously described [21,24].

### *2.3. SWATH-MS Differential Abundance Proteomics Analysis*

HT-29 cells, cultured in media supplemented with 600 μM of either DHA- ($n = 6$) or ARA-MAG ($n = 6$) at 24 h, and the same cells with no acyl species added (control group, $n = 6$) were recovered and lysed. Protein extracts were obtained, and protein was precipitated with TCA/acetone for removing contaminants. Then, 40 μg protein for each sample were subjected to trypsin digestion, and

massive protein relative quantitation was assessed following a SWATH approach as described in Ortea et al. [21]. Briefly, this approach consisted on three steps: (i) an MS/MS peptide library was built from the peptides and proteins identified in data-dependent acquisition (DDA) shotgun nanoLC-MS/MS runs from the samples, using Protein Pilot software (v5.0.1, Sciex) with a human Swiss-Prot protein database (20,200 protein entries, appended with the RePliCal iRT peptides (PolyQuant GmbH, Bad Abbach, Germany) and downloaded from UniProt on March 2017). Main settings used in Protein Pilot were iodoacetamide as Cys alkylation, trypsin as enzyme, TripleTOF 5600 as instrument, and thorough ID as search effort. The false discovery rate (FDR) was set to 0.01 for both peptides and proteins; (ii) each sample was analyzed with a variable SWATH LC-MS method; and (iii) protein quantitative data for the proteins contained in the peptide library were obtained from the SWATH runs by extracting the corresponding fragment ion chromatograms using the MS/MS$^{ALL}$ with SWATH Acquisition MicroApp (v.2.0, Sciex). Peptide retention times were calibrated in all the SWATH runs using the RePliCal iRT peptides, spiked into each sample according to manufacturer's instructions. To be confident on the proteins being identified and quantified, only those showing confidence scores above 99% and FDR below 1% were included in the analysis. For both kinds of LC-MS analysis, DDA and SWATH, a hybrid Q-TOF mass spectrometer (Triple TOF 5600+, Sciex, Redwood City, CA, USA) coupled on-line to nano-HPLC (Ekspert nLC415, Eksigent, Dublin, CA, USA) was used. For higher sensitivity, both DDA and SWATH runs were performed at nano-flow (300 nL/min) in a 25 cm long × 75 μm internal diameter column (Acclaim PepMap 100, Thermo Scientific, Waltham, MA, USA) using a 120 min gradient from 5% to 30% B (A: 0.1% FA in water; B: 0.1% in ACN).

### *2.4. Pathway and Gene Ontology (GO) Analysis*

Advaita Bio's iPathwayGuide (Advaita Corporation, Plymouth, MI, USA) was used for analyzing the significantly impacted pathways and for GO analysis. We considered a restrictive scenario, namely a differential expression threshold of log (fold change) 1.0 (that is, fold change 2.0) and adjusted *p*-value 0.01, in order to have more confidence in selecting the proteins that presented real expression changes. Data were analyzed in the context of pathways obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Release 78.0+/06-02, Jun 16).

### *2.5. Validation by Parallel Reaction Monitoring (PRM) Analysis*

Several protein changes corresponding to the main significant affected pathways were subjected to validation using targeted quantitation by micro-HPLC PRM on a Triple TOF 5600+ (Sciex). Skyline software (v4.2.0) [25] was used for designing and optimizing a PRM acquisition method for all of the targeted proteins. Two to six proteotypic peptides for each protein were selected according to the following criteria: (i) enzyme: trypsin [KR/P] with 0 missed cleavages; (ii) 7–16 amino acid residues; (iii) carbomidomethylation of cysteines as structural modification; (iv) excluding peptides containing methionine; and (v) excluding the N-terminal amino acids. Transitions were filtered according to the following criteria: (i) +2 and +3 precursor charges; (ii) *y* and *b* product ion types; (iii) product ions from (m/z > precursor) – 1 to last ion; (iv) method match tolerance 0.055 m/z; (v) a maximum of 10 product ions; and (vi) resolving power of 15,000 for MS/MS filtering. The HPLC gradient consisted of 5%–22% buffer B (A: 0.1% FA in water; B: 0.1% FA in ACN) at 5 μL/min for 45 min, plus 5 min at 95% B and another 6 min at 5% B for re-equilibration. The column used was a 15 cm long × 300 μm internal diameter C18 column (Dionex Benelux B.V., Amsterdam, Netherland). Five to six individual samples (4 μg per sample) from each of the groups, DHA-MAG, ARA-MAG, and control, and two blank samples, were analyzed with the developed PRM, and the resulting chromatograms for all the monitored peptides were imported into Skyline and manually curated. Three injection replicates were used for calculation of the coefficients of variation (CV) for each of the monitored peptides.

### *2.6. Statistical Analysis*

For cell assay results, statistical significance was determined using generalized linear models (GZLMs) using Statgraphics Plus 4.0 (Statistical Graphics Corp., Rockville, MD, USA). For SWATH-MS protein quantitation, data were analyzed following Ortea et al. [21]. Briefly, quantitative data were normalized for inter-run variability, and differences in protein abundance were assessed by applying a Student's *t*-test, checking for multiple testing underestimation of *p*-values by obtaining a q-value estimation for FDR using the qvalue R package [26]. For impact pathway and GO analysis, iPathwayGuide software calculated a *p*-value using a hypergeometric distribution. The *p*-values were adjusted using FDR correction for pathways and Bonferroni correction for GO analysis. For PRM validation of protein changes, statistical analysis was performed using the 'group comparison' function in Skyline. Briefly, Skyline performed pairwise group comparisons for each protein using a Student's *t*-test on the log2 transformed summed transition peak area for all the peptides from that protein, adjusting the *p*-values for multiple testing with the Benjamini-Hochberg correction.

## 3. Results

### *3.1. DHA- and ARA-MAG Showed Differential and Concentration-Dependent Effects on HT-29 Cell Viability, Cell Membrane Integrity, and Apoptosis*

The present study was conducted using the well-established HT-29 human colon cancer cell line. The purities of the assayed MAGs were 98.0% and 98.7% for DHA and ARA, respectively. According to González-Fernández et al. [22], during MAG purification process an acyl migration occurs quickly, so the concentrations of 1(3)-MAG balance with 2-MAG at one point that depends on several causes, e.g., solvent type, pH, and temperature. Although the purified forms are mainly 2-MAG and these are those added to cell cultures, in the culture medium the acyl-migration continues [27]. For this reason, we generically refer to MAG instead of 2-MAG.

First, we tested the actions of ARA- and DHA-MAG in HT-29 cells. Cell cultures were treated with different concentrations (50–600 μM) of ARA- and DHA-MAG for 48 and 72 h and then the MTT assay was performed to measure cell viability and cell proliferation (Figure 1a). After 48 and 72 h of treatment, the MTT assay revealed concentration-dependent inhibitory effects on HT-29 cells for both assayed acyl species. Cell growth inhibition was exercised much better by DHA-MAG, with $IC_{50}$ values of 135 and 115 μM for 48 and 72 h, respectively, while for ARA-MAG these values were 236 and 169 μM.

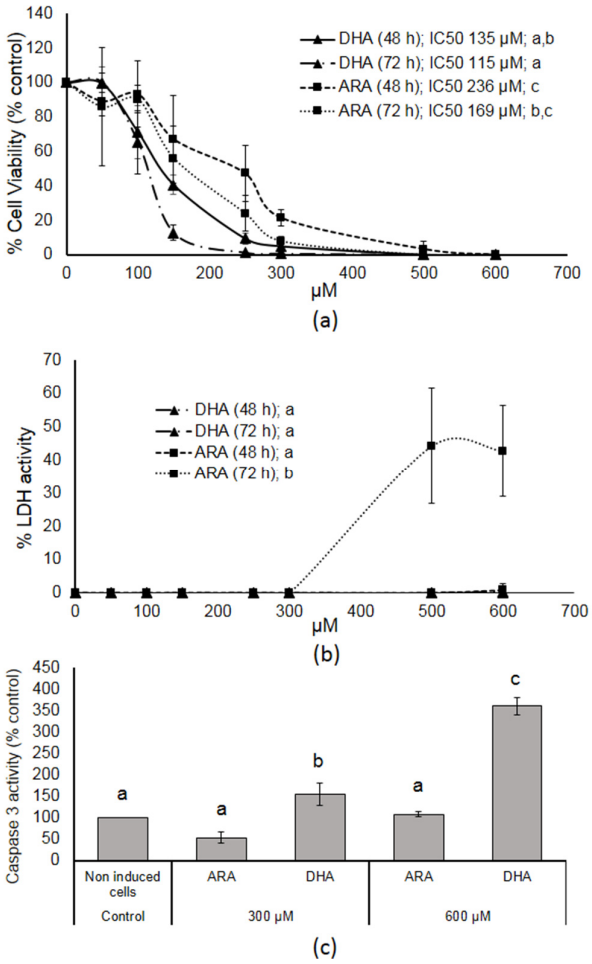



**Figure 1.** Plots showing results of cell assays. (**a**) Dose-dependent viability of HT-29 cells after exposure to docosahexaenoic acid (DHA)- and arachidonic acid (ARA)-monoacylglycerol (MAG). (**b**) Dose-dependent lactate dehydrogenase (LDH) release from HT-29 colon cancer cells after exposure to DHA- and ARA-MAG. (**c**) Dose-dependent caspase-3 activity from HT-29 colon cancer cells in comparison with untreated cells (control). Data represent the mean of three complete independent experiments ± SD (error bars). Data were analyzed using generalized linear models (GZLMs). There are no significant differences ($p < 0.05$) among series sharing the same letter.

The actions of ARA- and DHA-MAG on cell membrane integrity measured by the LDH assay after 48 and 72 h of treatment, are shown in Figure 1b. The test assesses the release of the LDH enzyme into the culture medium after cell membrane damage caused by MAG. The tested concentrations ranged from 50 to 600 µM. No effect of DHA-MAG on the amount of LDH release was noted, while a 40% increase in LDH activity after 72 h treatment was detected at the highest ARA-MAG concentrations.

To clarify whether ARA- and DHA-MAG were able to reduce cancer cell viability by promoting apoptotic cell death, a classical marker of apoptosis, caspase-3, was determined. In this study, caspase activation was evaluated in cells treated with ARA- and DHA-MAG at 300 and 600 µM for 90 min (Figure 1c). Caspase-3 activity is expressed as the percentage of activity compared to that of the untreated samples. As shown, a significant increase (up to 361%) of caspase-3 activity in the HT-29 cells was observed after 90 min exposure to DHA-MAG, while ARA-MAG did not show remarkable effects as compared to the respective untreated controls.

### *3.2. SWATH Quantitation of 1882 proteins Showed That DHA- and ARA-MAG Differentially Affect the Whole Proteome of HT-29 Cells*

Samples were analyzed by DDA nanoLC-MS/MS, and runs were searched against a human protein database using Protein Pilot software. As a result, after integrating all three data sets, 2140 proteins and 15,406 peptides were identified (FDR < 1% at both protein and peptide levels); the list of identified proteins is shown in Table S1. The identified MS/MS spectra were compiled into a spectral library containing 2002 proteins. Using this library, chromatographic traces were extracted from the SWATH runs for 7653 peptides, corresponding to 1882 proteins. SWATH-based quantification normalized data for these 1882 proteins in all the samples is shown in Table S2.

Tables S3 and S4 show the results for the differential abundance tests for DHA-MAG vs. control and for ARA-MAG vs. control, respectively. For subsequent analyses, we considered a restrictive scenario, namely $p$-value < 0.01 and two-fold change (FC), in order to have more confidence in selecting the proteins presenting actual expression changes. When addressing the changes in HT-29 cell proteome caused by DHA-MAG, a total of 896 proteins showed changes in expression (189 up-regulated and 707 down-regulated) (Figure 2a). Applying the same $p$-value and FC thresholds, only 70 proteins revealed a differential abundance as a consequence of the exposure to the ARA-MAG supplemented medium, 21 proteins being up-regulated and 49 down-regulated (Figure 2b). When looking for the largest effects, extreme FCs (above 5.0) were found in 119 and six proteins ($p$-value < 0.01) for DHA- and ARA-MAG, respectively (Tables S3 and S4, respectively). Therefore, it is clear than DHA-MAG produces a deeper effect than ARA-MAG on HT-29 cancer cells. When comparing the differentially abundant proteins ($p$-value < 0.01 and FC ≥ 2.0) in both tested groups, only 45 proteins were commonly being affected. Multivariate analyses of SWATH-based data including all 1882 quantitated proteins showed a complete separation of all groups: DHA-MAG, ARA-MAG, and control (Figure 2c and Figure S1).

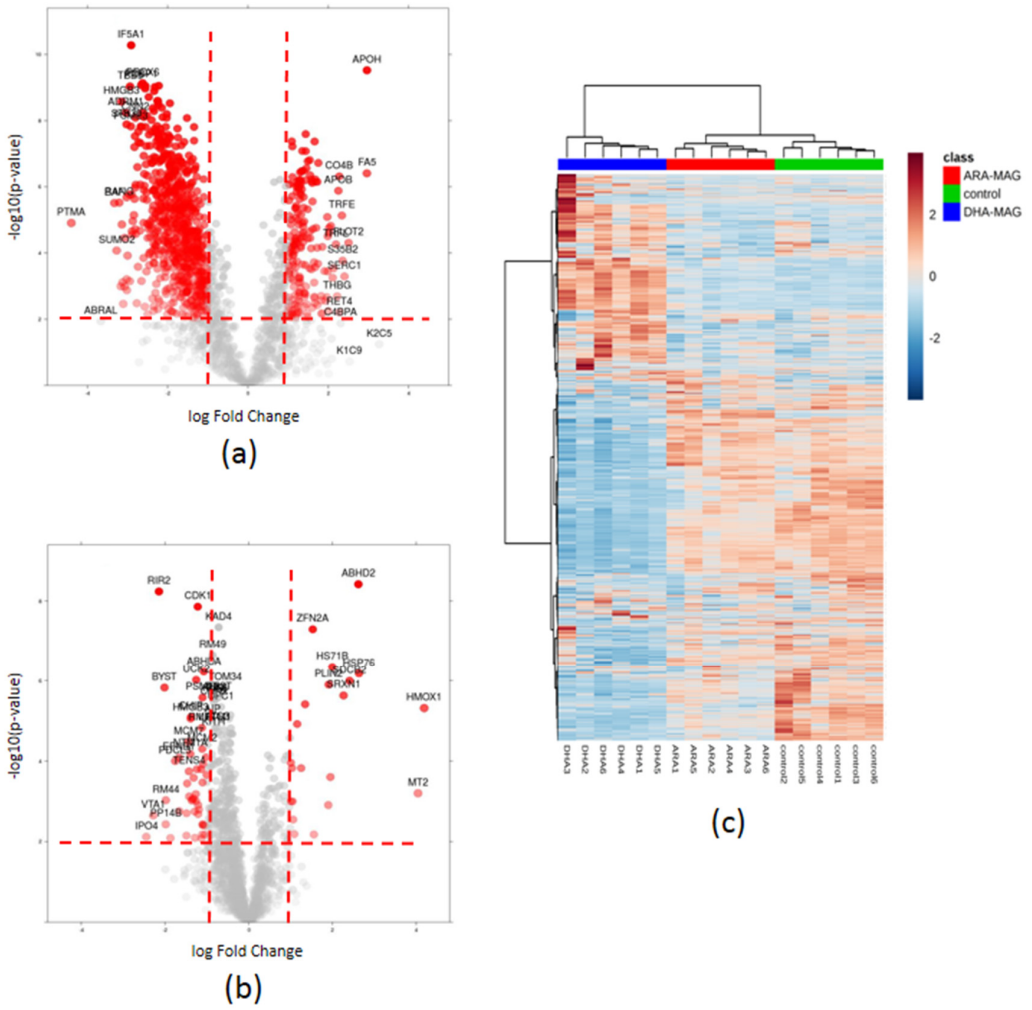



**Figure 2.** Quantitative proteomics result overview. Volcano plot showing proteins with differential expression as a result of the addition of (**a**) DHA-MAG; and (**b**) ARA-MAG. Red dotted lines show *p*-value < 0.01 and two-fold change cut-offs; proteins above these thresholds are shown in red. (**c**) Heat map including all 1882 quantified proteins; samples treated with DHA-MAG and ARA-MAG are separated from each other and from the control group.

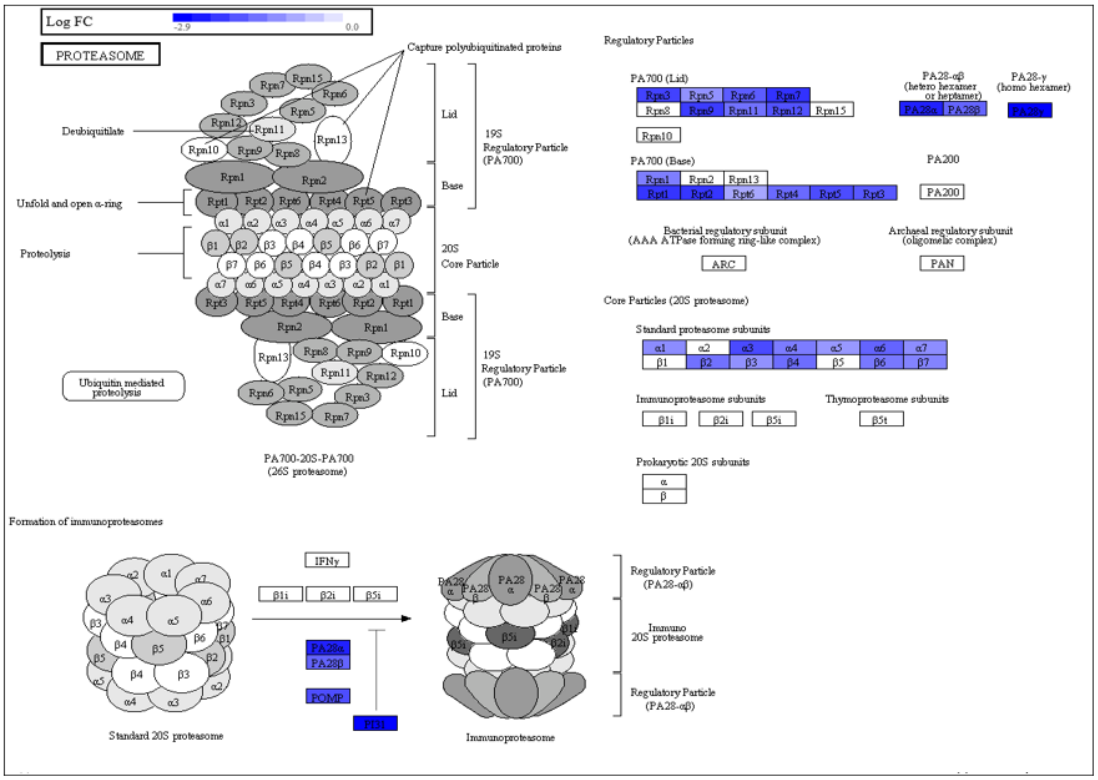
### *3.3. Pathway and GO Analysis Showed Different Mechanisms of Action of DHA- and ARA-MAG on HT-29 cells*

The affected pathways and GO components were analyzed using iPathwayGuide software. Significantly impacted pathways and over-represented GO groups according to this analysis are shown in Figure 3. After FDR correction, only one pathway was found to be significantly impacted by DHA-MAG, namely the proteasome pathway (KEGG: 03050), with 30 proteins down-regulated (Figure 4). On the other hand, two pathways resulted as significantly affected by ARA-MAG, DNA replication (KEGG: 03030), with a DNA polymerase and three helicase subunits down-regulated (Figure 5); and pyrimidine metabolism (KEGG: 00240).

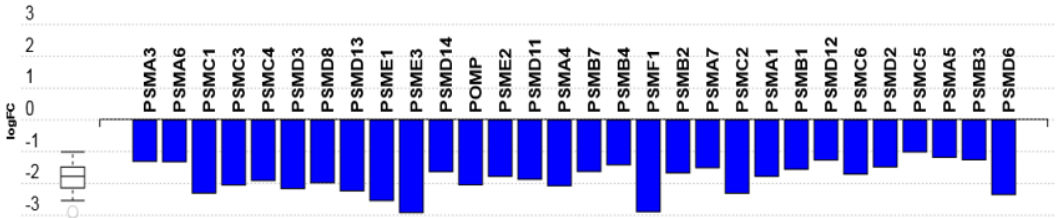
The biological processes GO component most affected by DHA-MAG was nucleobase-containing compound biosynthetic process (adjusted *p*-value $2.66 \times 10^{-4}$) (Figure 3b), with 239 proteins presenting differential abundance (Table S5). For ARA-MAG, the only significantly over-represented biological processes according to our GO analysis were those related to the G1/S transition of the cell cycle (Figure 3b), presenting eight proteins being regulated (Table S5). Regarding GO analysis of cellular components, DHA-MAG regulated proteins were mainly related to cytosol

(adjusted $p$-value $2.05 \times 10^{-20}$), but also from extracellular and nuclear origin (Figure 3c and Table S6), while no cellular component resulted as over-represented as a consequence of ARA-MAG regulation.


**Figure 3.** Pathway impact and gene ontology (GO) analysis for DHA-MAG and ARA-MAG. (**a**) Significantly impacted pathways and their associated adjusted $p$-values. (**b**,**c**) GO analysis showing significant (adjusted $p$-values < 0.05) (**b**) biological processes or (**c**) cellular components.




(a)




(b)

**Figure 4.** Effect of DHA-MAG extract on HT-29 cells' proteasome pathway. (**a**) Proteasome pathway diagram highlighting regulated proteins. (**b**) Gene perturbation bar plot for affected proteins of the proteasome pathway.

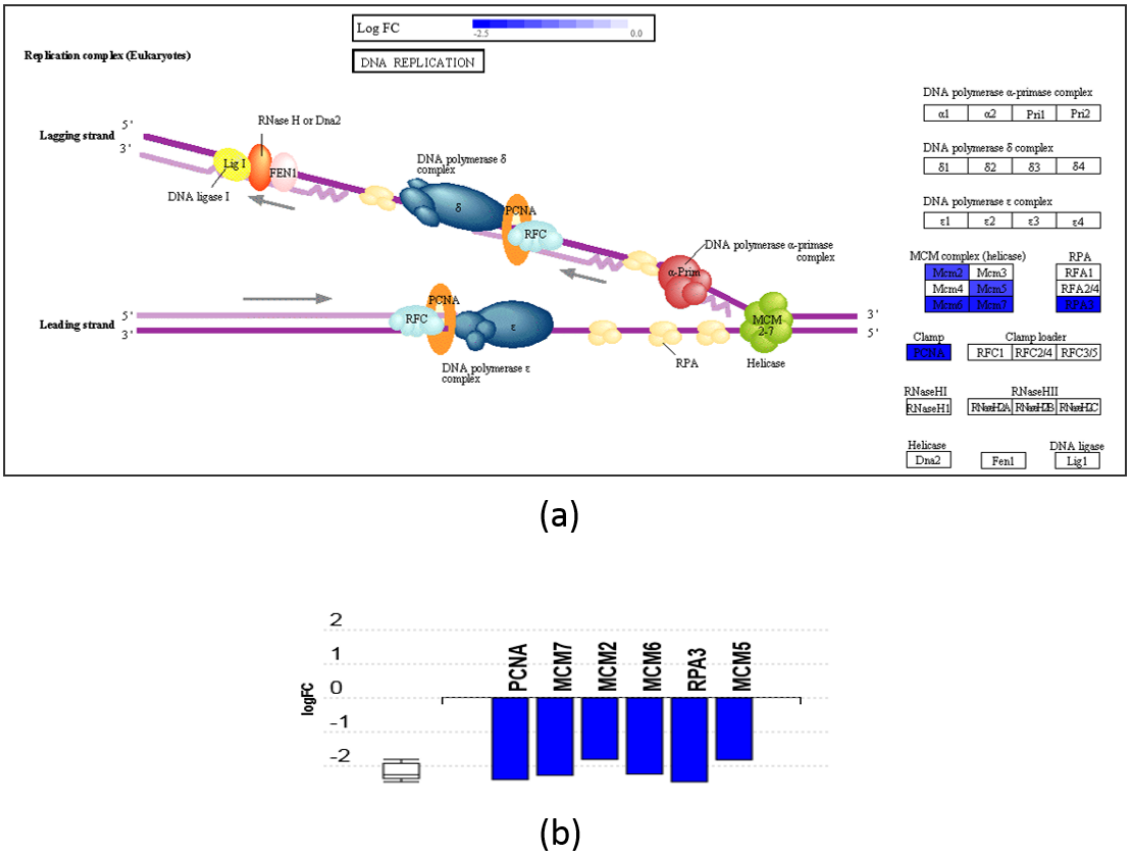



**Figure 5.** Effect of ARA-MAG extract on HT-29 cells' DNA replication pathway. (**a**) DNA replication pathway diagram highlighting regulated proteins. (**b**) Gene perturbation bar plot for affected proteins of the DNA replication pathway.

### *3.4. SWATH Proteomics Analysis was validated by PRM*

We developed a micro-HPLC PRM method to validate several of the protein changes previously found with the SWATH quantitative analysis workflow. Specifically, nine proteins were included in the PRM assay (Table 1): the most relevant pathways, as found in the pathway analysis, were represented by proteins MCM2 and MCM7 (helicase proteins from DNA replication pathway) and PSMF1, PSME3, and PSA3 (proteasome pathway); AIFM1 (apoptosis-inducing factor 1), as an apoptotic marker which we had found up-regulated in the SWATH analysis only in DHA-MAG treated cells but not in ARA-MAG, was also included in the validation assay; three other proteins showing SWATH-measured differences according to the compound used (ABHD2, up-regulated in ARA-MAG and not-significant in DHA-MAG; SRXN1, up-regulated in ARA-MAG and down-regulated in DHA-MAG; and TEBP, down-regulated in DHA-MAG and not-significant in ARA-MAG) were also included for validation of the SWATH results. After optimization of the method for these nine proteins, a total of 27 proteotypic peptides were used for targeted PRM validation (Table S7). As an indication of the precision of the PRM assay, measured CVs were below 20% for all the peptides except for peptide TFVDYAQK (CV of 22%); median CV for the entire peptide set was 9.0% (Figure S2 and Table S7). The effect of both compounds, DHA-MAG and ARA-MAG, on the nine targeted proteins, as measured by PRM, is shown in Figure 6.

**Table 1.** Differential abundance for the proteins subjected to validation. Fold-changes and statistical significance are shown for both SWATH and Parallel Reaction Monitoring (PRM) analyses.

| Protein ID | Protein name | ARA-MAG to control | | | | | |
|---|---|---|---|---|---|---|---|
| | | SWATH | | | PRM validation | | |
| | | FC | *q*-value | Significant change | FC | adj. *p*-value | Significant change |
| MCM2 | DNA replication licensing factor MCM2 | 0.46 | $8.3\times10^{-0}$ | ↓ | 0.56 | $2.1\times10^{-2}$ | ↓ |
| MCM7 | DNA replication licensing factor MCM7 | 0.37 | $6.4\times10^{-4}$ | ↓ | 0.57 | $2.9\times10^{-2}$ | ↓ |
| PSMF1 | Proteasome inhibitor PI31 subunit | 0.44 | $7.4\times10^{-3}$ | ↓ | 1.00 | $1.0\times10^{+0}$ | no |
| PSME3 | Proteasome activator complex subunit 3 | 0.47 | $1.3\times10^{-4}$ | ↓ | 0.81 | $3.7\times10^{-2}$ | ↓ |
| PSA3 | Proteasome subunit alpha type-3 | 1.05 | $3.6\times10^{-1}$ | no | 1.18 | $2.2\times10^{-1}$ | no |
| ABHD2 | Abhydrolase domain-containing protein 2 | 6.14 | $2.4\times10^{-6}$ | ↑ | 5.81 | 0 | ↑ |
| SRXN1 | Sulfiredoxin-1 | 4.81 | $1.3\times10^{-4}$ | ↑ | 5.12 | 0 | ↑ |
| TEBP | Prostaglandin E synthase 3 | 0.97 | $3.6\times10^{-1}$ | no | 1.03 | $8.7\times10^{-1}$ | no |
| AIFM1 | Apoptosis-inducing factor 1, mitochondrial | 1.24 | $6.7\times10^{-2}$ | no | 1.42 | $1.3\times10^{-1}$ | no |
| | | DHA-MAG to control | | | | | |
| | | SWATH | | | PRM validation | | |
| Protein ID | Protein name | FC | *q*-value | Significant change | FC | adj. *p*-value | Significant change |
| MCM2 | DNA replication licensing factor MCM2 | 0.29 | $4.8\times10^{-6}$ | ↓ | 0.24 | 0 | ↓ |
| MCM7 | DNA replication licensing factor MCM7 | 0.21 | $2.6\times10^{-6}$ | ↓ | 0.16 | 0 | ↓ |
| PSMF1 | Proteasome inhibitor PI31 subunit | 0.13 | $1.8\times10^{-5}$ | ↓ | 0.50 | $2.1\times10^{-2}$ | ↓ |
| PSME3 | Proteasome activator complex subunit 3 | 0.13 | $1.4\times10^{-7}$ | ↓ | 0.24 | 0 | ↓ |
| PSA3 | Proteasome subunit alpha type-3 | 0.40 | $6.7\times10^{-7}$ | ↓ | 0.31 | 0 | ↓ |
| ABHD2 | Abhydrolase domain-containing protein 2 | 1.35 | $1.0\times10^{-1}$ | no | 0.97 | $8.4\times10^{-1}$ | no |
| SRXN1 | Sulfiredoxin-1 | 0.40 | $9.7\times10^{-5}$ | ↓ | 0.38 | $7.0\times10^{-4}$ | ↓ |
| TEBP | Prostaglandin E synthase 3 | 0.14 | $2.0\times10^{-7}$ | ↓ | 0.08 | 0 | ↓ |
| AIFM1 | Apoptosis-inducing factor 1, mitochondrial | 2.34 | $1.6\times10^{-6}$ | ↑ | 1.82 | $1.1\times10^{-3}$ | ↑ |

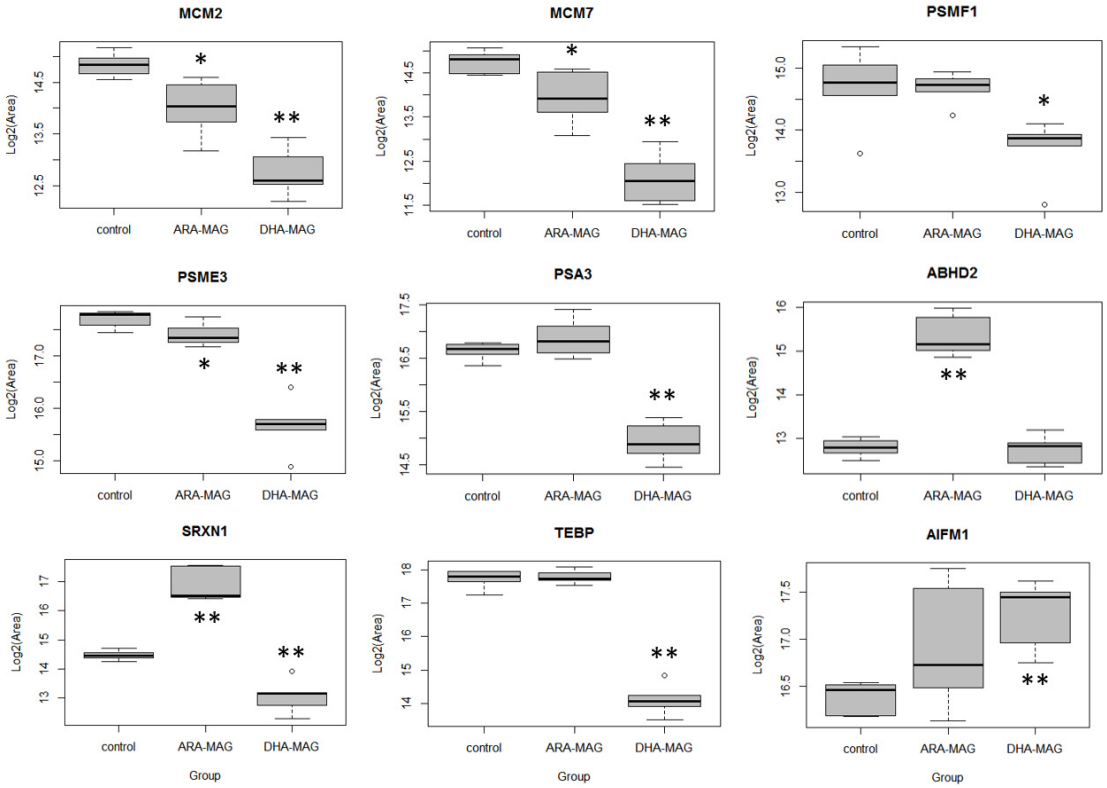



**Figure 6.** Differential expression of nine proteins from DHA-MAG- and ARA-MAG-treated HT-29 colorectal cancer cells by PRM quantification (*adjusted *p*-value <0.05, **adjusted *p*-value <0.01; group comparison to control).

## 4. Discussion

$IC_{50}$ values obtained by the MTT assay for ARA- and DHA-MAG are lower than those obtained for both LCPUFAs in the FFA form applied to the same cell line [21]. These differences may be related to the existence of a protein-mediated transport for PUFA across cell membranes. In this regard, a specific protein-mediated process has been reported for intestinal Caco-2 cells, which allows the entry of LCPUFA into such cells [5], and both acyl forms, i.e., FFA and MAG, establish competition for the same membrane-associated protein transporters (FAT, FATP, CD36, FABP). The findings of this work outline other ones from Ramos Bueno et al. [24], who demonstrated that unspecific oil-derived ARASCO®- and DHASCO®-MAG induced noticeable in vitro antitumor activity on HT-29 cells. Previous studies demonstrated that DHA significantly decreases cancer cells proliferation [28]; while Pompeia et al. [12] found dose- and time-dependent ARA-induced cytotoxicity in leukocytes, i.e., ARA at 10–400 μM induced apoptosis, while at concentrations above 400 μM the noted effect was necrosis. Several studies have discussed the relationship between FAs and mitochondrial permeability transition. Such relation is modulated by a variety of effectors of cell death, including reactive oxygen species (ROS), which are important messengers in normal-cell signal transduction and cell cycle being produced by mitochondria after stimulation of the TNF$\alpha$ receptor [29,30]. Moreover, Scorrano et al. [29] showed that ARA is a powerful permeability-transition inducer in MH1C1 cells, causing a release of cytochrome c followed by cell death.

The LDH test is a colorimetric method suitable for the measurement of cell membrane integrity. It is based on the measurement of LDH enzyme activity, whose increase in the culture supernatant is proportional to the number of lysed cells [31]. Until now, several authors have studied cell membrane permeability to determine non-cytotoxic concentrations of MAG on different cell lines, such as Caco-2 and HT-29 cells, and no significant toxicity measured by the LDH assay was observed [15,24,32,33]. In this study, toxicity was found only after 72-h treatment at the highest ARA-MAG concentrations.

This might be linked to the differential spatial configuration of both ARA- and DHA-MAG. The activities of both ARA and DHA were previously checked in the FFA form, with a higher activity being noted for ARA than for DHA [21,34], thus, after the intracellular hydrolysis of both MAGs, the action of the ARA-MAG would still prevail. The low activity could be due to the fact that both ARA- and DHA-MAG cannot be integrated effectively into cell membranes, since MAGs are low-polarity compounds and therefore they cannot establish effective chemical links with membrane proteins on these structures [35]. In this regard, Dommels et al. [36] stated that the cytotoxic effects mediated by some PUFAs, e.g., ARA and EPA, are due to the peroxidation products generated during lipid peroxidation and cyclooxygenase activity. However, MAGs are known to be surface-active compounds, so they might show minor cytotoxic effects on cells by disrupting cell membranes [32].

Caspase-3 is considered to be the most important effector of apoptosis and a marker for both intrinsic and extrinsic pathways [37]. An important aspect to consider is the chemical structure of the different MAGs, which affects the potency to induce apoptosis; in this regard, Philippoussis et al. [3] concluded that SFA-based MAG had no effect on such phenomenon, while PUFA-MAGs were highly potent to induce apoptosis in T-cells. The apoptotic activity noted here for DHA agrees with previous findings [3,38,39], and the potency of both LCPUFA-MAGs was higher than that previously reported for both FFA-based LCPUFAs [21]. Our findings demonstrated that DHA-MAG induces apoptotic cell death via activation of caspase-3. The non-activation of caspase by ARA-MAG may be due to the chemical structure of MAG, since in the FFA form such activity was detected [21]. These results completely agree with those from Ho and Storch [5], who suggested the existence of a protein-mediated process for MAG transport through cell membranes. Accordingly, LCPUFA-based MAGs could reach high concentrations inside cells and would be able to perform effective apoptosis actions, as reported here.

SWATH, one of the recently developed data-independent acquisition (DIA) MS strategies [40], shows outstanding precision and accuracy even when used for proteome-wide quantitation [41]. SWATH performance is comparable to that of selected reaction monitoring (SRM), the golden standard for protein and small molecule quantitation [41]. SWATH consists of acquiring MS/MS data in stepped m/z fragmentation windows, and then matching the resulting fragment ions to peptides and proteins using a previously generated MS/MS spectral library, so fragment ion chromatograms can be in-silico extracted and used for label-free protein quantitation. Here we have used a SWATH v2.0 method, with variable Q1 isolation windows according to the ion density found in previous DDA runs, which has been described to improve peptide identification and quantification [42]. The results found in our SWATH analysis, with DHA-MAG producing a deeper effect than ARA-MAG on the HT-29 cancer cell proteome, suggest that the decrease of cell viability and increase of apoptosis observed should be produced by means of different mechanisms depending on the MAG tested. For comparing these results to those found in our previous work [21], it has to be noted that both experiments (cell cultures and cell assays) were run in parallel, and the analytical LC-MS methods and bioinformatics analysis performed have been exactly the same. All LC-MS runs were combined in one dataset, normalized for inter-run variability, and analyzed all together (SWATH extraction, pathways, and GO analysis). When comparing the results with those previously obtained for DHA- and ARA-FFA [21], 284 (45 up-regulated and 239 down-regulated) and 73 (27 up-regulated and 46 down-regulated) proteins, respectively, were reported for the FFA forms. Therefore, these previous results also showed a deeper effect for DHA than for ARA. Within the DHA-derived molecules, MAG affected the HT-29 cell proteome more globally than FFA did (896 vs. 284 differentially expressed proteins, respectively), although there is a protein core of 237 proteins that are common to both DHA forms (Figure S3). Taking all these figures together, these results indicates that (i) DHA and ARA (in both forms, FFA and MAG) differentially affect the whole proteome of HT-29 cells, suggesting that the decrease of cell viability and increase of apoptosis observed should be produced by means of different mechanisms depending on the molecule tested; and (ii) MAG have a much deeper effect than FFA only for DHA forms, not for ARA. As an additional interesting result, we found PTGES3 (prostaglandin E synthase 3, TEBP, accession Q15185) was strongly down-regulated in DHA-MAG (fold-change 0.14, that is, seven times less abundant in DHA-MAG than in the control group) (Table

S3). This protein, also down-regulated as an effect of DHA-FFA (fold-change 0.5, that is, two-fold less abundant in DHA-FFA than in control) [21], belongs to the prostaglandin biosynthesis pathway, and catalyzes the conversion of prostaglandin H2 to prostaglandin E, that is, the next step to the transformation of arachidonic acid to prostaglandin H2, which is catalyzed by COX-2. COX-2 is involved in regulation of apoptosis and proliferation of colorectal, liver, pancreatic, breast, and lung cancer cells [43], and although we do not have quantitative data for COX-2 in our results, the strong down-regulation of PTGES3 we found could mean that one of the antitumor activities of DHA could be effected by means of the prostaglandin cascade, since it plays an important role in antigen presentation and immune activation in cancer [44].

The significantly affected pathways were analyzed using iPathwayGuide software, which implements an 'impact analysis' approach, taking into consideration not only the over-representation of differentially expressed (DE) genes in a given pathway (i.e., enrichment analysis), but also topological information such as the direction and type of all signals in a pathway, and the position, role, and type of each protein [45]. Only the proteasome pathway resulted as significantly impacted by DHA-MAG in our analysis. The proteasome is a large protein complex which main action is degrading ubiquitinated-labeled proteins [46], and plays an important role in the regulation of many cellular processes such as cell cycle, cell differentiation, signal transduction, inflammatory response, and antigen processing. In Figure 4, a diagram of the different particles and proteins constituting the proteasome are shown, highlighting the proteins that we have found to be significantly regulated as an effect of DHA-MAG on HT-29 cancer cells. In our results, we found 30 proteins from the proteasome pathways to be significantly down-regulated, comprising all the main particles: 11 proteins from the 20S core particle and 14 proteins from the 19S regulatory particle, in both lid and base subunits. In addition, the PA28-$\alpha\beta$ and PA28-$\gamma$ are also down-regulated. PA28-$\gamma$ has been found in the nucleus and plays an important role in the regulation of apoptosis and cell cycle progression [47]. Interestingly, proteasome was the main pathway that was found to be affected by DHA-FFA in our previous study using the same workflow [21]. In that case, 18 proteins from the proteasome complex resulted as down-regulated. Since the number of DE proteins reached 30 in the case of DHA-MAG, we can conclude that both forms of DHA affect the proteasome, but the MAG form induces its massive switch-off. Interestingly, proteasome inhibitors, such as natural polyphenol compounds, have been tested in clinical trials as drug candidates for treating different cancers, due to their ability to induce apoptosis and reduce cell proliferation [48,49]. According to the strong down-regulation of the proteasome particles we have found in our study, we suggest DHA-derived MAG, in addition to DHA-FFA as previously reported, as one of these candidates that deserve further studies as an anticancer effector.

Two pathways resulted as significantly affected by ARA-MAG: DNA replication and pyrimidine metabolism. Regarding the DNA replication pathway, POLE3, one of the proteins conforming the DNA polymerase ε, and three proteins from the helicase (MCM2, MCM3, and MCM7) were found to be significantly down-regulated (Figure 5). In addition, the remaining helicase proteins, MCM4, MCM5, and MCM6, which are not significant according to our threshold (above two-fold change), are also affected to a certain extent, since corresponding fold-changes (case to control) found are 0.51, 0.55, and 0.51, respectively. The DNA helicase protein complex is responsible for unwinding the duplex DNA helix ahead of the DNA synthetic machinery at the replication fork [50]. Since DNA replication is linked to cell cycle progression and to DNA repair processes, it would be expected that the down-regulation of the helicase-constituting proteins and POLE3 would have an inhibitory effect on these other related processes. Actually, our results show that cell cycle pathway (KEGG: 04110), even though is not significant in our pathway analysis (adjusted $p$-value of 0.214), is perturbed by the addition of the ARA-MAG extract, since several proteins belonging to this pathway are regulated by it (Figure S4a). The other pathway that resulted as significantly impacted according to our pathway analysis was the pyrimidine metabolism pathway (KEGG: 00240) (adjusted $p$-value 0.041), where ARA-MAG produces the under-expression of several proteins (Figure S4b). When comparing to the results obtained in the previous study using ARA-FFA, these two pathways, DNA replication and

pyrimidine metabolism, together with cell cycle, also resulted as significantly impacted in the pathway analysis [21].

Interestingly, DHA-MAG also affects proteins from the DNA replication pathway (Figure S5), showing four helicase proteins being down-regulated, although our pathway analysis does not report this pathway as statistically significant due to the high global number of affected proteins. DNA replication turned out to be significantly impacted in our previous study using DHA-FFA, and therefore we can say that both forms of DHA induce a down-regulation of helicase proteins in addition to the deep effect on the proteasome. In contrast, ARA-MAG, as was the case also for ARA-FFA [21], does not induce a strong effect on the proteasome pathway ($p$-value of 0.661) since only two of the proteins included in this pathway were found to be regulated, PSMF1 and PSME3, with fold-changes (case to control) of 0.44 and 0.47, respectively (Figure S6).

For validating the SWATH-derived results, we developed a micro-HPLC PRM method which included 27 proteotypic peptides from a total of nine proteins. Interestingly, these nine proteins covered all the possible differences (up-regulated, down-regulated, or non-significant changes) for both comparisons (DHA-MAG to control and ARA-MAG to control) as found in the SWATH analysis. PRM [51], being a high-resolution (HR) MS targeted proteomics approach, has significantly greater sensitivity, accuracy, and precision than non-targeted discovery measurements, and more specificity than non-HR targeted methods, and therefore it is commonly used as a tool for validating protein abundance changes in all kinds of quantitative proteomics applications [52]. The results from the PRM assay were consistent with those previously found with the SWATH discovery approach (Table 1) for both comparisons (DHA-MAG to control and ARA-MAG to control), in terms of significance, fold-change, and direction of change (up- or down-regulation). Actually, the fold-changes were very similar in both measures, SWATH and PRM. As some examples, MCM2 fold-changes for the DHA-MAG to control comparison are 0.29 and 0.24, 0.40 and 0.38 for SRXn1, or 2.34 and 1.82 for AIFM1, for SWATH and PRM, respectively. For the comparison of ARA-MAG to control, some example fold-changes are 0.46 and 0.56 for MCM2, 4.81 and 5.12 for SRXN1, or 1.05 and 1.18 for PSA3 (Table 1). Only protein PSMF1 showed a different behavior when comparing SWATH and PRM analyses, and only for the ARA-MAG to control comparison (Table 1). While it had been found to be down-regulated by SWATH, PRM analysis found a fold-change of 1.0 (and, accordingly, no statistically significant difference). When inspecting the PRM results for this protein, we found that this issue could be explained by a discordance between the measures for the two peptides that were monitored for this protein: one of the peptides (ALIDPSSGLPNR) showed a fold-change of 0.56, while the fold-change for the other peptide (LPPGAVPPGAR) was 1.41 (Table S7). On the other hand, this discordance between the two peptides was not found in the comparison DHA-MAG to control, where the protein was found to be significantly down-regulated in both PRM and SWATH measures.

An interesting finding of the PRM analysis was the validation of the strong down-regulation of protein TEBP, prostaglandin E synthase 3, by DHA-MAG: measured fold-changes were 0.14 and 0.08 (SWATH and PRM, respectively), with adjusted $p$-values close to zero (Table 1), while ARA-MAG did not affect this protein. Prostaglandin E synthase 3 is one of the main proteins in the prostaglandin biosynthesis, converting prostaglandin H2 in prostaglandin E. Prostaglandin H2, the rate-limiting step in the formation of prostaglandins, is the product of prostaglandin G/H synthase 2, or COX-2, which has been related to colorectal cancer and whose inhibition (e.g., by nonsteroidal anti-inflammatory drugs) has been linked to tumor cell apoptosis, inhibition of proliferation, and reduction of colorectal cancer risk [53,54]. Therefore, it could be proposed that, apart from the proteasome pathway, one of the mechanisms contributing to the anticancer activity of DHA-MAG in HT-29 cells is carried out through the inhibition of prostaglandin synthesis, counteracting COX-2.

It should be noted here that an increase in lipid droplet accumulation, as a consequence of a potential saturation of the cells by lipids, could have a role in some of the effects found. However, we have demonstrated, and this is the main finding of this work, that, depending on the MAG, the molecular mechanisms working in the background are different, with DHA-MAG deeply affecting the proteasome, with 30 proteins being strongly regulated, while ARA-MAG, with only two proteins affected, does not. Actually, the conclusion of our study is that DHA- and ARA-MAG, while

differentially affecting the whole proteome of HT-29 cancer cells by means of different mechanisms are not affected by the possibility of lipid droplets playing a role on the effect seen.

In summary, we have demonstrated that both ARA- and DHA-MAG showed concentration-dependent inhibitory effects on HT-29 cell viability, with a clear ability of DHA-MAG to induce cell death. The biological interpretation of SWATH-MS-generated proteomics data, validated by the quantification of nine relevant proteins by PRM, revealed that DHA-MAG outperforms the effect previously described for DHA-FFA, having a massive effect on the proteome of HT-29 cancer cells, with the proteasome complex being completely shut down. The strong down-regulation of prostaglandin E synthase 3, validated by PRM, also suggest a significant role of the prostaglandin synthesis in the anticancer activity of DHA-MAG in these colorectal cancer cells. On the other hand, although the effect of ARA-MAG is reduced in comparison to that of DHA-MAG, mainly in terms of inducing cell death, it still produces concentration-dependent inhibitory effects on HT-29 cell viability, as revealed by the MTT test. According to the proteomics experiments, this decrease of cell viability could be effected through inhibition of DNA replication and G1/S cell cycle transition, as it was previously described for ARA-FFA. Even though the MAG concentration used in the proteomics experiments (600 μM) could be considered relatively high, previous studies have reported higher (above 1700 μM) FA concentrations reached in human volunteers [55] and therefore, in the case of developing drugs based on MAG (and the corresponding FFA) for the treatment of cancer, 600 μM would be physiologically achievable, and it even would not represent negative effects on health, although, of course, further research in preclinical models and also in the clinical setting should be needed.

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Table S1**:** List of identified proteins in the integrated data set. FDR was set to 1% at both peptide and protein levels. Table S2: SWATH-based protein quantification data for all the samples. 1882 proteins were quantified by SWATH-MS and measures were normalized for inter-run variability as described in Material and Methods section. Table S3**:** Differential abundance test for DHA-MAG vs. control. Fold changes, resulting *p*-values, and FDR analysis q-values for each of the 1882 quantified proteins are shown. Table S4: Differential abundance test for ARA-MAG vs. control. Fold changes, resulting *p*-values, and FDR analysis q-values for each of the 1882 quantified proteins are shown. Table S5: GO analysis: biological processes. Differential expressed proteins found in each biological process, together with all the proteins in that process, and the adjusted *p*-value, are shown for both comparisons, DHA-MAG vs. control and ARA-MAG vs. control. Table S6: GO analysis: cellular components. Differential expressed proteins found in each cellular component group, together with all the proteins in that component, and the adjusted *p*-value, are shown for both comparisons, DHA-MAG vs. control and ARA-MAG vs control. Table S7: Peptides selected for PRM validation. Fold-changes and adjusted *p*-values are shown for the comparisons DHA-MAG to control and ARA-MAG to control, together with the coefficient of variation (CV) measured using three technical replicates for each of the peptides. Figure S1: Multivariate analysis including all 1882 quantified proteins, showing the complete separation of the samples from each group (DHA-MAG, ARA-MAG, and control). (a) Group-averaged heat map; (b) partial least-squares discriminant analysis (PLS-DA); and (c) hierarchical cluster analysis (Spearman distance). Figure S2: Coefficient of variation (CV) for the 27 peptides monitored in the PRM validation, calculated using three injection replicates. Median CV for the PRM assay was 9%, with all peptides but one showing a CV below 20%. Figure S3: Differentially expressed proteins found as effect of DHA- and ARA-MAG, together with the previous results for DHA- and ARA-FFA [21]. Figure S4: Effect of ARA-MAG extract on HT-29 cells (a) cell cycle pathway (KEGG: 04110), highlighting protein perturbation according to our quantification results and showing coherent cascades; and (b) pyrimidine metabolism pathway (KEGG: 00240), highlighting significantly regulated proteins. Figure S5: Effect of DHA-MAG extract on HT-29 cells DNA replication pathway (KEGG: 03030). (a) DNA replication pathway diagram highlighting significantly regulated proteins. (b) Gene perturbation bar plot for DNA replication pathway affected proteins. Differentially expressed genes are represented with negative values in blue and positive values in red. In this case all proteins highlighted are under-regulated. Figure S6**:** Effect of ARA-MAG extract on HT-29 cells proteasome pathway (KEGG: 03050). (a) Proteasome pathway diagram highlighting significantly regulated proteins. (b) Gene perturbation bar plot for Proteasome pathway affected proteins. Differentially expressed proteins are represented with negative values in blue and positive values in red. In this case all proteins highlighted are under-regulated. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014874.

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# CONCLUSIONS

# 8. CONCLUSIONS

## 8.1. Conclusions related to chapter I

Highly purified PUFA can be efficiently obtained by different methodologies. The first one was focused on purifying PUFA as MAG by means of a two-steps process: i) enzymatic hydrolysis of appropriate oils, and ii) two-column sequence chromatographic process. Although the process has been successfully implemented and pure ARA- and DHA-MAG were obtained, more research is needed to avoid the acyl-migration phenomena detected, for instance by checking alternative stationary phases to be used in the column chromatography processes. Another process was designed based on the UC methodology, and by using appropriate PUFA-rich oils, highly concentrated PUFA were obtained. Future research should focus on optimizing the handling of the remaining urea crystal fractions to develop a whole process in which the different targeted PUFA could fully recovered at different purities, which could be used in different application according to the various FA purities.

## 8.2. Conclusions related to chapter II

DHA- and ARA-based FFA exercise different *in vitro* antitumor activities depending upon the time and concentration on HT-29 CRC cells, as revealed by the MTT test. DHA and ARA caused cell membrane damage noticed by the release of the LDH enzyme into the culture medium. Pro-apoptotic activity of DHA was confirmed by Caspase-3 assay. In all assays, DHA inhibited cell proliferation of HT-29 CRC cells to a higher extent than ARA. SWATH-MS-generated proteomics data revealed that both PUFA had an impact on the proteasome system and the cell cycle pathway, particularly on the helicase complex.

## 8.3. Conclusions related to chapter III

Both ARA- and DHA-MAG showed concentration-dependent inhibitory effects on HT-29 CRC cell viability, as revealed by the MTT test, with a clear ability of DHA-MAG to induce cell death noticed by Caspase-3 assay. The release of the LDH enzyme into the culture medium proved cell membrane damage caused by ARA. The interpretation of SWATH-MS-generated proteomics data revealed that the decrease of cell viability is effected through inhibition of DNA replication and G1/S cell cycle transition, as it was previously described for ARA-FFA. DHA-MAG outperforms the

effect previously described for DHA-FFA, having a massive effect on the proteome of HT-29 CRC cells. According to proteomics experiments, DHA-MAG was able to exercise a strong down-regulation of prostaglandin E synthase 3, suggesting a significant role of the prostaglandin synthesis in the activity of DHA-MAG on HT-29 CRC cells.

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# ANNEX
# Other scientific contributions

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# Various acylglycerols from common oils exert different antitumor activities on colorectal cancer cells

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**ABSTRACT**

Colorectal cancer is one of the leading causes of death in Western countries; therefore, the implementation of healthy dietary habits in order to prevent its occurrence is a desirable action. We show here that both free fatty acids (FFAs) and some acylglycerols induce antitumoral actions in the colorectal cancer cell line HT-29. We tested several C18 polyunsaturated fatty acid-enriched oils (e.g., sunflower and *Echium*) as well as other oils, such as arachidonic acid-enriched (Arasco®) and docosahexaenoic acid-enriched (Marinol® and cod liver oil), in addition to coconut and olive oils. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test indicated inhibitory effects on HT-29 cells viability for FFAs, and monoacylglycerol and diacylglycerol (DAG) species, while the lactate dehydrogenase test proved that FFAs were the more effective species to induce membrane injury. Conversely, all species did not exhibit actions on CCD-18 normal human colon cells viability.

Furthermore, transmission electron microscopy showed the presence of necrosis and apoptosis, while the monoacylglycerol lipase (MAGL) inhibition test demonstrated high activity for 2-monoacylglycerols derived from Arasco and sunflower oils. However, different monoacylglycerols and DAGs have also the potential for MAGL inhibition. Therefore, checking for activity on colon cancer cells of specifically designed acylglycerol-derivative species would be a suitable way to design functional foods destined to avoid colorectal cancer initiation.

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## Introduction

The identification of cytotoxicity-inducing agents, specifically targeting malignant cells while sparing normal cells, is of major interest. Epidemiological studies indicate that *n*−3 polyunsaturated fatty acids (PUFAs) reduce the incidence and growth of colorectal cancer, by considering evidence on the decrease in cell proliferation in normal healthy persons submitted to oral fish oil supplementation (1). The related mechanism for tumor growth inhibition is linked to the enzyme cyclooxygenase (COX), whose activity catalyzes the conversion of arachidonic acid (AA, 20:4*n*−6) to prostaglandins (PGs). PGs are important mediators of signal transduction pathways and are involved in cellular adhesion, growth, and differentiation. Overexpression of an isoform of COX, COX-2, has been reported in 90% of colon tumors and premalignant colorectal adenomas (2). In this context, *n*−3 PUFAs, such as docosahexaenoic acid (DHA, 22:6*n*−3), which are the typical components of fish oil, have also been shown to inhibit COX-mediated AA metabolism (3). In addition, other mechanisms have been proposed to explain the effect of *n*−3 PUFAs on this cancer; for instance: 1) the negative correlation of DHA intake with tumor growth could be related to the reduction of kinases activities (4); 2) the growth inhibitory and cytotoxic effects of PUFAs with methylene-interrupted double bonds such as AA and eicosapentaenic acid (EPA, 20:5*n*−3), which are due to peroxidation products that are generated during lipid peroxidation and COX activity (3); 3) the suppression of cell proliferation by functional ligand of PPAR$\gamma$1, such as is produced by EPA (5).

Conversely, *n*−6 PUFAs promote colorectal tumorigenesis via production of prostaglandin E2 ($PGE_2$), which can stimulate cellular proliferation in the colon. Hence, the mechanism responsible for the inhibitory effects of *n*−3 PUFAs on colorectal tumors may partly be related to inhibition of $PGE_2$ synthesis from AA (3).

So far, in vitro models for research on the effect of lipids on progression of colon cancer cells have been mainly limited to individual fatty acids (FAs). However, recent observations provide a similar role to some glycerolipids, that is, 2-acylglycerol (2-AG) and 2-AG hydrolysis inhibitors exert antiproliferative activity and reduction in prostate cancer cell invasiveness (6). 2-AG is

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onemonoacylglycerol (MAG) that is an endocannabinoid (7,8). FA amide hydrolase and MAG lipase (MAGL) hydrolyze 2-AG to free fatty acids (FFAs) and glycerol. MAGL is upregulated in aggressive human cancer cells and primary tumors as FFAs provide for the synthesis of oncogenic signaling lipids, which promote cancer proliferation. Hence, MAGL inhibitors impair colorectal cancer tumorigenesis through enhancing antitumorigenic cannabinoid pathways and decreasing FFA-derived protumorigenic lipid signals (9). Interestingly, in colorectal tumor tissues, MAGL activity is significantly higher than in normal tissues, as occurs in breast cancer, and exerts promotion in tumor progression (10).

According to the above research, the elucidation of the cytotoxic activity on colon cancer of the different FAs as well as their combinations with glycerol would suggest possible courses of action regarding therapeutics thereof. Previous findings indicate that in vitro, supplementation with fish oil or olive oil leads to an induction of apoptosis and cell differentiation in HT-29 cells (11). Other authors attempted to inhibit the growth of the HT-29 cell line using several oils, all in their lipase-digested form (12). However, there is a lack of knowledge about the effects of the high diversity of AG derivatives from common oils on colon cancer cells.

This research is focused on the study of the antitumor activities of several oils as well as their AG and FA derivatives on the HT-29 colon cancer cell line. The results of this study can draw conclusions in order to implement dietary habits for the prevention of colon tumors. Findings are relevant because very little research exists about colon cancer prevention by means of dietary oils. The tested species were the molecular forms into which the ingested oils were enzymatically transformed in vivo.

## Materials and methods

### Samples

Several oils were evaluated in this study (Table 1): Marinol®, 42.9% DHA, which is an enzymatically modified marine oil, purchased from Lipid Nutrition (Wormerveer, The Netherlands); Dhasco®, 40% DHA, a mixture of the oil extracted from the unicellular alga *Crypthecodinium cohnii* and high oleic sunflower oil, which was acquired from Martek Biosciences Corporation (MBC) (Columbia, MDArasco®, 40% AA (20:4*n*−6), a mixture of an oil extracted from the unicellular *Mortierella alpina* and high oleic sunflower oil was also obtained from MBC; *Echium plantagineum* seed oil, obtained from Aromantic (Forres, United Kingdom); coconut oil, from Sigma-Aldrich (St. Louis, MO); and olive oil purchased from local markets. All oils were hydrolyzed to FFA, MAG, and diacylglycerol (DAG) species, which were obtained by enzymatic hydrolysis using lipases by the procedures described below.

### Hydrolysis reaction

Standard hydrolysis was accomplished following the method of López-López et al. (13). Fifty milligrams of different oils were hydrolyzed by adding 40 mg (100–400 U/mg protein) of porcine pancreatic lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) and 4 ml of 1 M Tris (hydroxymethyl)aminomethane solution (both from Sigma-Aldrich) (pH 8.0). The mixture was shaken and after that, 0.4 ml of 22% (wt/vol) calcium chloride (Riedel-de Haën, Seelze, Germany) and 1 ml of 0.1% (wt/vol) sodium cholate (Sigma-Aldrich) were added. Then, flasks were placed in a water bath at 37°C and were shaken at 120 rpm for 30 min. Arasco and Dhasco were shaken for 45 and 60 min, respectively, in order to optimize the hydrolysis process. Afterward, 2 ml of 6 M hydrochloric acid and 2 ml of diethyl ether were added. The mixture was transferred to test tubes, which were shaken in a vortex at 2,500 rpm for 30 s and centrifuged at 1,500 *g* for 3 min. The upper ether layer, containing the hydrolysis products, was then collected from each tube. The extraction was repeated twice more in order to improve the recovery and then, ether extracts were combined and dried under vacuum.

### Purification of FFA and AG species by thin layer chromatography

The different products of hydrolysis were separated by preparative thin layer chromatography (TLC) as previously described (14). The solution, composed of a mixture of *n*–hexane/diethyl ether/acetic acid (65:35:1, vol/vol/vol), was applied on an activated silica gel plate (105°C, 60 min) (20 × 20 cm, 0.2-$\mu$m thickness). Then the plate was dried at room temperature, and bands corresponding to MAGs, DAGs, FFAs, and triacylglycerols (TAGs) were revealed under iodine stream, which were scraped off. All compounds were obtained by adding 2 ml of diethyl ether in each tube and shaken in a vortex and centrifuged at 1,000 *g* for 3 min, and after phase separation the clear diethyl ether top layer was collected (three times). After that, three ether aliquots were scraped up and evaporated under vacuum. All samples were diluted in acetone at low concentrations for the bioassay test and stored at –20°C until use, or methylated with 1 mg of internal standard (heptadecanoic acid, 17:0) for further gas-liquid chromatography (GLC) quantitative analyses.

**Table 1.** Fatty acid profiles of the oils and their enzymatic hydrolysis products essayed on HT-29 cells.

| | Fatty acid profiles at the different *sn* locations of essayed oils (FA% of total FA%)[a] | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Echium plantagineum* | | | Dhasco© | | | Marinol© | | | Arasco© | | | Olive oil | | | Coconut oil | | | Cod liver oil | | | Sunflower | | |
| Fatty Acids | oil | *sn*-2 | *sn*-1,3 | oil | *sn*-2 | sn-1,3 | oil | *sn*-2 | sn-1,3 | oil | *sn*-2 | sn-1,3 | oil | *sn*-2 | sn-1,3 | oil | *sn*-2 | sn-1,3 | oil | *sn*-2 | sn-1,3 | oil | *sn*-2 | *sn*-1,3 |
| 6:00 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0.6 | 0.1 | 0.9 | – | – | – | – | – | – |
| 8:00 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 7.8 | 0.1 | 11.7 | – | – | – | – | – | – |
| 10:00 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 5.8 | 15.6 | 0.9 | – | – | – | – | – | – |
| 12:00 | – | – | – | 5.4 | 16.7 | 0.2 | – | – | – | 0.9 | – | – | – | – | – | 44.7 | 29.7 | 52.2 | – | – | – | – | – | – |
| 14:00 | – | – | – | 11.7 | 5.5 | 14.8 | 3.1 | 2.9 | 5.1 | – | 0.5 | 1.3 | – | – | – | 17.4 | 19.5 | 16.4 | 4.5 | 7.7 | 2.9 | – | – | – |
| 16:00 | 7.7 | 1 | 11.1 | 6.7 | 5.2 | 7.5 | 16.1 | 15 | 21.3 | 12.5 | 1.2 | 19.5 | 12.9 | 12.7 | 13 | 9.3 | 13.1 | 7.4 | 13.8 | 19 | 11.2 | 6.9 | 0.5 | 10 |
| 16:01 | – | – | – | 3.3 | 1.2 | 4.4 | – | – | – | – | – | – | 0.7 | 2.1 | 0 | – | – | – | 7.1 | 8 | 6.7 | – | – | – |
| 17:00 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 1.4 | 0.2 | 2 | – | – | – |
| 18:00 | 3.9 | 0.4 | 5.7 | – | – | – | 3.2 | 0 | 1.5 | 8 | – | 6.4 | 2.1 | 3.5 | 1.4 | 1.5 | 4.5 | 0 | 2.2 | 0.6 | 3 | 3.7 | 0.2 | 5.4 |
| 18:1*n*−9 | 14.9 | 16.8 | 14 | 25.6 | 32.2 | 22.3 | 12 | 5.8 | 21 | 22.3 | 25.5 | 31.5 | 77.7 | 71.7 | 80.7 | 10.5 | 14.2 | 8.7 | 18.6 | 8 | 23.9 | 29.8 | 30.1 | 29.6 |
| 18:1*n*−7 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 3.5 | 1.4 | 4.6 | – | – | – |
| 18:2*n*−6 | 14.8 | 19.4 | 12.5 | 1.2 | 0.1 | 1.8 | 1.2 | 0.5 | 0.6 | 7.9 | 12.8 | 5.1 | 6 | 7.6 | 5.2 | 2.4 | 2.9 | 2.2 | 2.1 | 1.6 | 2.3 | 59.7 | 68.9 | 55.1 |
| 18:3*n*−3 | 32.7 | 20.4 | 38.9 | – | – | – | 0 | 0 | – | 1.9 | 6.1 | 2.6 | 0.6 | 1.8 | 0 | – | – | – | 0.3 | 0.1 | 0.4 | – | – | – |
| 18:3*n*−6 | 11.3 | 21.3 | 6.3 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 1.5 | 3.2 | 0.7 | – | – | – |
| 18:4*n*−3 | 14 | 20.8 | 10.6 | – | – | – | 0.9 | 0.3 | 1.1 | – | – | – | – | – | – | – | – | – | 2.5 | 4.6 | 1.5 | – | – | – |
| 20:1*n*−9 | 0.7 | 0 | 1.1 | – | – | – | 1.1 | 2.2 | 1.2 | – | – | – | – | – | – | – | – | – | 8.3 | 2 | 11.5 | – | – | – |
| 20:4*n*−6 | – | – | – | – | – | – | 2.6 | 3.7 | 1.4 | 43.7 | 53 | 30.6 | – | – | – | – | – | – | 0.6 | 0.9 | 0.5 | – | – | – |
| 20:4*n*−3 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0.7 | 0.5 | 0.8 | – | – | – |
| 20:5*n*−3 | – | – | – | – | – | – | 7.3 | 7.3 | 5.4 | 0.5 | – | – | – | – | – | – | – | – | 9.3 | 13.7 | 7.1 | – | – | – |
| 22:1*n*−11 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 7.8 | 3.9 | 9.8 | – | – | – |
| 22:4*n*−6 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0.3 | 0.5 | 0.2 | – | – | – |
| 22:5*n*−6 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 1 | 0.3 | 1.4 | – | – | – |
| 22:5*n*−3 | – | – | – | – | – | – | 2.5 | 4 | 1.8 | – | – | – | – | – | – | – | – | – | 1.4 | 2 | 1.1 | – | – | – |
| 22:6*n*−3 | – | – | – | 44.4 | 40 | 46.6 | 42.9 | 55.9 | 25.6 | 0.5 | – | – | – | – | – | – | – | – | 12.7 | 20.9 | 8.6 | – | – | – |

[a]SD among triplicates was routinely less than 5% of means (n = 3).

### Preparation of FA ethyl esters

Derivation of the different oils was carried out according to the method proposed by Lepage and Roy (15): >10 mg of different oils were placed in a test tube with 1 ml of *n*–hexane and 1 ml of a mixture of ethanol/acetyl chloride (20:1, vol/vol). The tube was placed at 100°C for 30 min and then was cooled to room temperature and 1 ml of distilled water was added. The tube was shaken and centrifuged (2,500 rpm, 3 min) and the upper *n*–hexane layer was collected and evaporated. The residue was dissolved in ethanol and stored at –20°C until use.

### FA profiles

Samples were mixed with a solution of 100 $\mu$l of internal standard (heptadecanoic acid 17:0, 10 mg/ml) and derivatized with ethanol according to the procedure described previously. The upper *n*–hexane layer was collected for GLC analysis. Resulting FA ethyl esters (FAEEs) were analyzed in a Focus GLC (Thermo Electron, Cambridge, UK) equipped with a flame injection detector and an Omegawax 250 capillary column (30 m × 0.25 mm i.d. × 0.25 $\mu$m film thickness; Supelco, Bellefonte, PA). The temperature program was 1 min at 90°C, heating until 200°C at a rate of 10°C/min; constant temperature at 200°C (3 min), heating until 260°C at a rate of 6°C/min; and constant temperature at 260°C (5 min). The injector temperature was 250°C with split ratio of 50:1. Injection volume was 4 $\mu$l. Detector temperature was 260°C. Nitrogen was used as carrier gas (1 ml/min).

### Cell cultures

HT-29 (colon cancer cells line) and CCD-18 (colonic human myofibroblast cells line) were used to determine different antitumor activities. Cultures were supplied by the Technical Instrumentation Service of University of Granada, Granada, Spain. First, it was checked for the absence of *Mycoplasma* and bacteria. Then, cells were grown at 37°C and 5% $CO_2$ humidified atmosphere in medium RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.125 mg/ml amphotericin, and 100 mg/ml penicillin-streptomycin. All cultures were plated in 25 $cm^2$ plastic tissue culture flasks (Sarstedt, Nümbrecht, Germany). All culture media and reagents were purchased from Sigma-Aldrich.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the lactate dehydrogenase (LDH) assay were conducted under similar culture conditions. In both trials, the oils and their derivatives were supplied to cells dissolved in acetone and then in the culture medium in designed concentrations (0–200 $\mu$g/ml) as indicated below. The amounts of acetone used to dissolve the checked compounds were the same as the control plate (medium plate).

### Cell viability test: MTT assay

In the MTT assay, cells were divided into 96-well microtiter plates, adjusted to $1 \times 10^4$ cell/well and cultivated in RPMI-1640 medium at 37°C, 5% $CO_2$ for 24 h prior to adding the different extracts dissolved in medium. Forty-two hours later, MTT solution, 5 mg/ml density, was added to determine the viability of cells. Results were determined by measuring the absorbance at 570 nm on an enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo Electron Corporation, Sant Cugat del Vallés, Barcelona, Spain).

### LDH cytotoxicity assay

LDH cytotoxicity assay (Cytotoxicity Detection Kit PLUS, Roche, Mannheim, Germany) was carried out using a lower cell density than MTT ($5 \times 10^3$ cell/well). This parameter was determined in preliminary experiments to improve the procedure on HT-29 cells. Cells treatment in the LDH assay was the same as MTT, until adding the extracts and its incubation (48 h). In this test, all steps were carried out according to the manufacturer's instructions. Cell death dates were quantified by measuring the absorbance at 450 nm with a reference filter at 690 nm. A "high control" was used to estimate the total LDH content treating cells with lysis solution to release all LDH. The percentage of cell death was determined using the following equation:

$$\%\ \text{of LDH activity} = \frac{\text{Experimental value–low}}{\text{High control–low control}} \times 100.$$

All parameters represent absorbance values of the triplicate samples and controls. Low control indicates the LDH activity released from the untreated cells and high control is the maximum releasable LDH activity in the cells, while experimental values designate LDH activities released from treated cells with the cytotoxic agents.

### MAGL inhibition test

MAGL inhibition (MAGLI) assay (MAGL Inhibitor Screening Assay kit, Cayman Chemical Co., Ann Arbor, MI) was carried out using similar culture conditions used in the previous assays. Cells were incubated in a plate reader at density of $1 \times 10^4$ cell/well. After 24-h

incubation (37°C, 5% $CO_2$), reagents and inhibitors were added to each well. The inhibitors were assayed in the higher concentration (200 $\mu$g/ml) and it was incubated for 48 h. Then, the reaction was initiated by adding MAGL substrate to all the wells being used; the plate was shaken and was incubated for 1 h at room temperature. The samples were assayed in triplicate and the absorbance was measured at 410 nm using an ELISA plate reader. The percent inhibition for each sample was calculated by the following equation :

$$\% \text{ Inhibition } = \left(\frac{\text{IA} - \text{inhibitor}}{\text{IA}}\right) \times 100$$

where IA is 100% initial activity wells (wells without inhibitor) and inhibitor is wells that contain the assayed inhibitors.

### Transmission electron microscopy

Cells were cultured on flat-bottom microplates at $1 \times 10^5$ cell/well at 37°C and 5% $CO_2$ humidified atmosphere. The assay was carried out as previously described MTT assay until MTT reagent addition, and multiplying reagent amounts by 10. After 72-h incubation, the culture medium was removed and replaced with 1% paraformaldehyde and 1.5% glutaraldehyde in 0.05 M sodium cacodylate buffer. Fixation was done at 4°C temperature for 60 min. Afterward, the cells were postfixated with 1% osmium tetroxide and 1% potassium ferricyanide at 4°C for 60 min, and the block was stained with 1% uranyl acetate. The samples were dehydrated by increasing ethanol concentrations.

The embedding procedure was made with epoxy resin (Epon 812) and different mixtures of absolute ethanol (1:1, 1:3 ethanol:epoxy), decanted, and changed to pure resin. Then, epoxy 812 embedding medium was polymerized for 48 h at 60°C. The whole procedure was realized in a flat-bottom microplate. After the epoxy was polymerized, areas of the sheet of epoxy-embedded cells were selected and cut. The pieces were stuck on the top of a block of resin polymerized without a sample and sections were cut using an ultramicrotome (Ultramicrotome Leica Ultracut S, Leica, Mannheim, Germany). Semithin sections (2–3 $\mu$m) were stained with toluidine blue and examined under light microscopy (Olympus BX51, Tokyo, Japan). These sections were used as a reference to trim blocks for thin sectioning. The appropriate blocks were then sectioned with diamond knife at 70–80 nm, and the ultrathin sections were placed on copper grids, stained with uranyl acetate and lead citrate to enhance contrast, and viewed with a Zeiss Libra 120 transmission electron microscopy (TEM; Carl Zeiss AG, Jena, Germany).

The attention was fixed on ultrastructural changes typical of apoptosis, such as chromatin condensation, nuclear fragmentation, nuclear dissolution, mitochondrial swelling, and the formation of apoptotic bodies.

### Statistics analysis

All values represented the mean ± SD of three complete independent experiments made in triplicate. Statistical significance ($P < 0.05$) was determined by analysis of variance, followed by assessment of differences using STATGRAPHICS Plus version 5 (Statistical Graphic Corp., Warrenton, VA).

## Results and discussion

The HT-29 cell line was selected in order to elucidate to what extent the various FAs, either individually or more or less esterified with glycerol, exerted antitumoral actions. This cell line was originally isolated from a moderately differentiated human adenocarcinoma, thus it provides a useful in vitro system for studies on both cell growth and cell differentiation in early stages of colorectal cancer (16).

### Fatty acid profiles of oils and hydrolysis products

The FA profiles of all assayed oils and their hydrolysis products are shown in Table 1. Most tested oils are naturally occurring ones, while others are products of the enrichment in specific FAs, that is, Arasco – AA-enriched oil, and both Marinol and Dhasco – DHA-enriched oils. Despite the diversity of FAs observed, some of them are present in all tested oils, that is palmitic acid (PA, 16:0), oleic acid (OA, 18:1*n*–9), and. linoleic acid (LA, 18:2*n*–6). However, each one of the tested oils had characteristic FAs. That is, in coconut oil, saturated even-chain FAs were predominant, such as lauric acid (LA, 12:0) and myristic acid (MA, 14:0); in olive oil OA was the prominent FA; and in sunflower oil LA was the main FA. Other oils were C18 PUFA enriched, such as *E. plantagineum* oil, which had LA, $\alpha$-linolenic acid (18:3*n*–3), $\gamma$-linolenic acid (GLA, 18:3*n*–6), and stearidonic acid (SDA, 18:4*n*–3). Finally, cod liver oil had a high PUFA complexity, comprising among others PA, OA, gondoic acid (20:1*n*–9), cetoleic acid (22:1*n*–11), EPA, and DHA. Furthermore, as a general trend, after the hydrolysis reaction, most PUFAs were placed in the *sn*–2 position of TAGs, as it occurs with LA; GLA in *E. plantagineum*; and SDA, EPA, AA, and DHA in their respective oils.

### Effects of oils and FAEEs on HT-29 cells viability

The effects of oils and FAEEs on HT-29 cells viability are shown in Figs. 1 and 2, respectively. Considering oils, in the assayed range of concentrations, 0–200 $\mu$g/ml, only olive oil showed a moderate effect, that is, >100 $\mu$g/ml. This result is in agreement with previous findings for this oil using the same cell line, in which a minor effect on tumor progression was noted (11). However, the more unsaturated DHA-enriched oils, cod liver oil, Marinol, and Dhasco, showed a negligible effect, which clearly differs from the results of Llor et al. (11), who described apoptosis in cells by adding either olive or fish oils to HT-29 cells. Differences between both studies could be related to the followed methodology, that is, Llor et al. (11) added fish oil/olive oil supplements daily to the culture medium until achieving the apoptotic effect, while in our study single doses at lower concentrations were supplied.

It is known that the unsaponifiable fraction of olive oil has cytotoxic effect on HT-29 cells. Moreover, this fraction selectively induces apoptosis in HT-29 colon cancer cells by a JNK-p53-dependent mechanism (17), as well as induces significant changes in the cytoskeleton of this human colon cancer cell line (18). The effect on cell proliferation and apoptosis exerted by olive oil in HT-29 cells has been considered due to some components of the skin of olives, which contains pentacyclic triterpenes, that is, maslinic and oleanolic acids (19).

The influence of the FAEEs on HT-29 cells viability (Fig. 2) was closely related to that obtained from different oils. Similarly, olive oil exerted some activity, although to a lesser extent than the previously tested oils. This residual activity could be attributed to some compounds of the unsaponifiable fraction, which could have been extracted along with FAEEs after transesterification of TAGs.

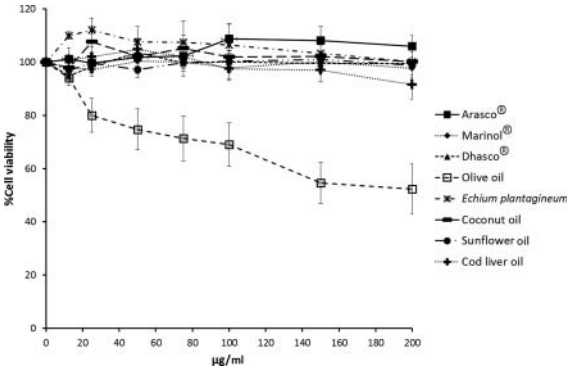


**Figure 1.** MTT assay for HT-29 cells following 48-h treatment with several oils. Data represent the mean of three complete independent experiments ± SD (error bars). Concentrations of olive oil ≥25 $\mu$g/ml were different ($P < 0.05$) from the control. Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

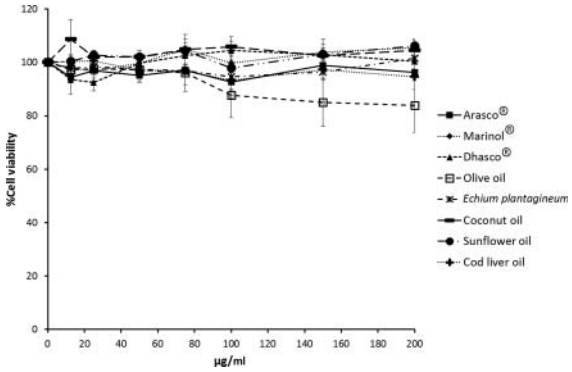


**Figure 2.** MTT assay for HT-29 cells following 48-h treatment with several ethyl esters species. Data represent the mean of three complete independent experiments ± SD (error bars). Concentrations of olive oil ≥100 $\mu$g/ml were different ($P < 0.05$) from the control. Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

### Effects of the hydrolyzed oils on HT-29 cells viability

Several in vivo studies have shown that oils having high percentages of *n*–6 PUFAs, for example LA, may enhance colorectal carcinogenesis via stimulation of colonic cell proliferation (20), while fish oil, which contains high percentages of *n*–3 PUFAs, have inhibitory effects on tumor cell growth. However, in a more complex natural context comprising different FFAs and several AG species, there is some uncertainty about the predominating effects. Thus, the cell viability assay by using enzymatically obtained hydrolyzed oils (HOs) is of great interest, given that this is the natural form in which all these oils will contact with any cancer cell in a physiological environment. The effects on HT-29 cells viability of the HOs derived from the previously tested oils is shown in Fig. 3. It is interesting to note that all assayed products completely inhibited cell viability at 150 $\mu$g/ml, except the hydrolyzed olive oil, which inhibited cell viability at 200 $\mu$g/ml. In the assay conditions, all tested products were composed of more or less a heterogeneous mixture of FFAs along with some AG species—this fact yields uncertainty about the effects of single FAs. In any case, considering that the bioavailability of AGs and their derivatives is partial, particularly for those composed of long chain FAs (21), one should consider the possibility that some active lipid species can reach colon areas and thus they can act against tumors in the intestinal lumen. This is more achievable in early stages of carcinogenesis, when cancer cells partially retain their morphology (as happens in HT-29 cells) and thus they can effectively absorb nutrients from the lumen.

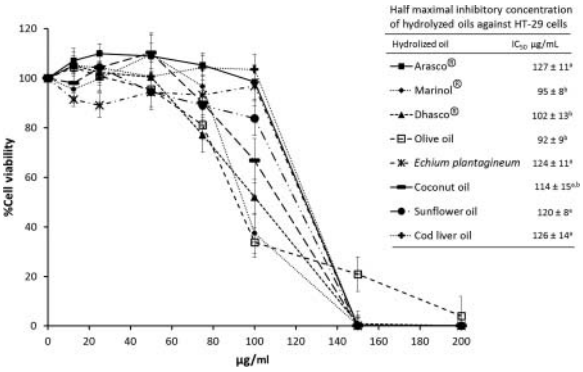


**Figure 3.** MTT assay. Dose-response plot and $IC_{50}$ values for HT-29 cells following 48-h treatment with several hydrolysis product species. Data represent the mean of three complete independent experiments made in triplicate ± SD (error bars). Values not sharing the same superscript letters are significantly different from one another by Duncan's multiple range test ($P < 0.05$). Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

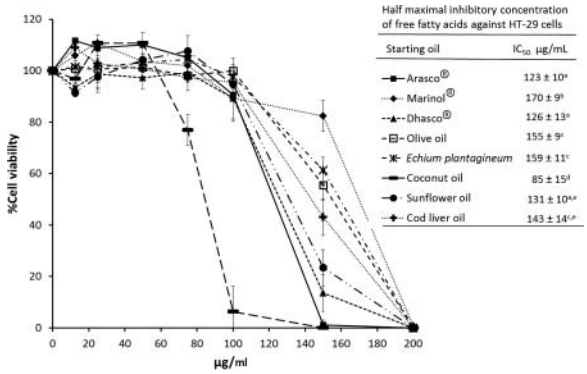


**Figure 4.** MTT assay. Dose-response plot and $IC_{50}$ values for HT-29 cells following 48-h treatment with several free fatty acids species. Data represent the mean of three complete independent experiments made in triplicate ± SD (error bars). Values not sharing the same superscript letters are significantly different from one another by Duncan's multiple range test ($P < 0.05$). Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Previous results (3,11) have indicated that the number of double bonds in the carbon atom chain of the FAs is critical for the antiproliferative and cytotoxic effects of *n*–3 and *n*–6, which act in the early downregulation of COX-2. In this sense, all tested oils contain variable amounts of PUFA, with olive and coconut oils in the bottom of the range (Table 1; however, these showed actions on cell viability similar to those exercised by the more unsaturated oils, thus indicating that there are other mechanisms for cells inhibition, as discussed below. On the other hand, our results agree with a previous study, which pointed out that several vegetable oils, such as corn, soybean, safflower, olive, and coconut oils, all in their lipase-digested form, inhibited HT-29 cells growth (12).

### *Effects of the different lipid classes on HT-29 cells viability*

In order to establish to what extent the AG species exerted inhibition on HT-29 cells viability, after the lipase-hydrolysis reaction, the HOs were separated by TLC into FFAs, MAGs, and DAGs, which were separately tested (Figs. 4–6, respectively). The FFA fraction obtained from all previously tested oils completely inhibited HT-29 cells growth at 200 $\mu$g/ml (Fig. 4), in a more intense form than the completely hydrolyzed fractions, with the FFA fraction from coconut oil being the most active. Previously, it has been noted that capric (CA,10:0) and LaA (12:0), which are the major components of coconut oil, potentially enhanced Ca absorption in vivo (22), as well as that Ca at 1,000 $\mu$M was effective in inducing apoptosis in Caco-2 and IEC-6 cell lines, due to oxidative stress induction via a p53-independent pathway (23). Probably, some FFAs from coconut oil act through this mechanism, thus the noted cytotoxic effect could be due to an interaction among all FAs. This effect slightly differs from those shown by MAGs (Fig. 5). When DAGs were tested (Fig. 6), the species derived from sunflower oil were also inactive, in parallel with olive and coconut oils; only the C20 and C22 PUFA oils (Arasco, Marinol, Dhasco, and cod liver oil) were able to inhibit cell proliferation similar to MAGs. Probably, differences in activity might be related to the different degree of unsaturation. On the other hand, the activity of both MAGs and DAGs might be exercised through the

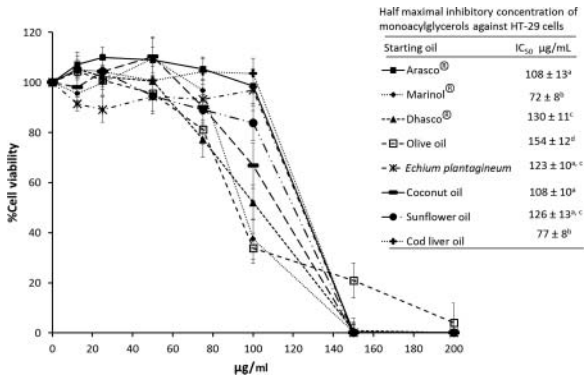


**Figure 5.** MTT assay. Dose-response plot and $IC_{50}$ values for HT-29 cells following 48-h treatment with several 2-monoacylglycerol species. Data represent the mean of three complete independent experiments made in triplicate ± SD (error bars). Values not sharing the same superscript letters are significantly different from one another by Duncan's multiple range test ($P < 0.05$). Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

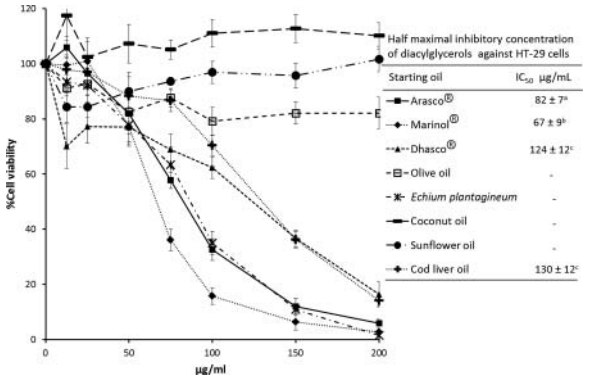


**Figure 6.** MTT assay. Dose-response plot and $IC_{50}$ values for HT-29 cells following 48-h treatment with several diacylglycerols species. Data represent the mean of three complete independent experiments made in triplicate ± SD (error bars). Values not sharing the same superscript letters are significantly different from one another by Duncan's multiple range test ($P < 0.05$). Abbreviation: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

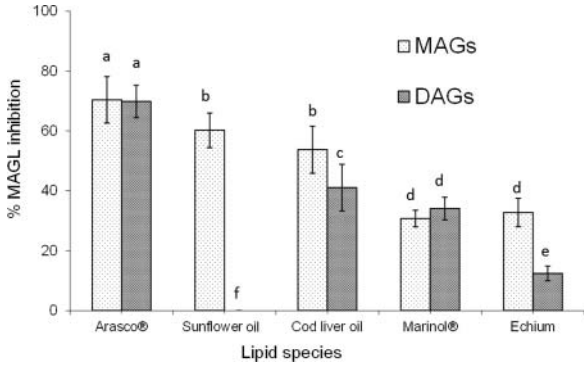


**Figure 7.** Bar histogram displaying the inhibitory effect induced by either monoacylglycerols or diacylglycerols on HT-29 cells growth following monoacylglycerol lipase inhibition test. Data represent the mean of three complete independent experiments made in triplicate ± SD (error bars). Bars not sharing the same superscript letters are significantly different from one another by Duncan's multiple range test ($P < 0.05$). Abbreviations: DAG, diacylglycerol; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase.

inhibition of the MAGL; thus, species derived from olive, coconut, and sunflower oils marginally could exert this activity, especially DAGs, because they lack adequate PUFA to carry out MAGLI.

Probably, among the tested species, MAGs are the more interesting ones. As is previously reported, the absorption of MAGs is favored because they have specific transporters (21). Thus, PUFAs in the *sn*–2 position of TAGs are incorporated into the de novo synthesized TAGs, and they could reach several tumors via blood to exert the expected inhibitory actions. Furthermore, lipases expressed by cancer cells (24) could produce some acyl species that would inhibit their own growth.

### *Effects of the different lipid species on CCD-18 cells viability*

Parallel to the tests described previously, the MTT cell viability assay was run for all oils and their derivatives in CCD-18 cells (normal human colon myofibroblasts). In stark contrast to the above results, the cells did not deviate from its normal growth pattern in any case (data not shown), thus indicating no influence on cell viability. This finding indicates that AG species and FFAs act only on cancer cells viability.

### *MAGLI test*

The MAGLI screening assay kit was performed to scrutinize activity for the more prominent AG species showing activity in the MTT assay test (Fig. 7). This activity was measured at 200 $\mu$g/ml, concentration at which the differential activity of different AGs is well differentiated. Surprisingly, no major differences were found between MAGs and DAGs for C20 or higher PUFAs-containing AG species, either AA-enriched (Arasco), DHA-enriched (Marinol), or *n*–3-rich oil (cod liver oil). The order of activity was Arasco > cod liver oil > Marinol, which means that for the inhibition of this enzyme the AA-enriched MAGs and DAGs are highly effective, followed by AGs composed of a wide range of PUFAs, and finally by both DHA-enriched forms of glycerides. However, for C18-based AGs a different situation was found, that is, the LA-enriched MAGs derived from sunflower oil were highly efficient, although the DAGs from this oil were ineffective. Furthermore, the AGs from the C18 PUFA-enriched *Echium* oil showed higher inhibitory activity for MAGs than for DAGs. These findings suggest that the C18 PUFA-based AGs had lesser activity than the C20–24 ones, as well as that both the degree of unsaturation and the *n*–6 structure were important for this activity, although the *n*–3 AG species also exerted MAGLI.

The MAGLI exercised by DAGs could be done per se, though it might also be explained that HT-29 cells express DAG lipase (24). Considering that cells were incubated for 48 h in the presence of DAGs, MAGs would be present when the assay was carried out with the colorimetric substrate. Therefore, although the net effect of DAGs is the inhibition of MAGL, the way by which this is done would require additional experimentation to be clarified. In any case, we must consider that the MAGs derived from LA-enriched sunflower oil were active, whereas DAGs were ineffective; therefore, it is likely that the hydrolysis of DAGs was marginal, and thus the inhibitory activity would be exercised by the

own DAGs. On the other hand, for DAGs the results of the MAGLI test more or less agreed with the results of MTT essay; in both trials, DAGs from sunflower oil were ineffective, while DAGs from *Echium* oil showed low activity in the MAGLI essay and an absence of action in the MTT cell viability test.

The above-described results agree with previous reports, in that tumor growth and invasion were significantly inhibited in vitro and in vivo by pharmacological and small interfering RNA-mediated MAGL knockdown (9). Moreover, the present study is similar to the study by Philippoussis et al. (25), who indicated that OA-based MAGs induced cell death in several human leukemic cell lines by rapid apoptosis. Our results indicate that in colorectal cancer cells, in addition to the OA-based MAGs, other PUFA-based MAGs had higher apoptosis activity than the previously tested species. Similarly, our results agree with the previous findings by Morin et al. (26), who showed that MAGs based on EPA, DHA, and docosapentaenoic acid (22:5*n*–3) were highly effective in decreasing cell proliferation, as well as in inducing apoptosis in HCT-116 cells.

## LDH assay

In order to characterize cytotoxic effects, we performed LDH leakage experiments using a cytotoxicity detection kit (Roche Molecular assay, Roche Diagnostics GmbH, Mannheim, Germany) for selected active compounds detected by the MTT assay. The concentration to perform the cytotoxic tests was 200 $\mu$g/ml, at which most tested compounds showed differential cytotoxicity. None of the compounds (Fig. 8) caused a great (>35.0% LDH released) membranolytic effect up to 48 h for all the cultured cells. The membrane damage caused by 48-h exposure to AGs resulted in LDH release from the cultured cells in the following order, for the more prominent species, FFAs: coconut > Arasco; MAGs: Arasco > Marinol > coconut; DAGs: Arasco > *Echium* > Marinol. Thus, HT-29 cells were more susceptible to membrane injury caused by FFAs than by other species, although MAGs and DAGs were effective, especially those derived from Arasco. On the other hand, in all cases, cell inhibitory actions measured by MTT test were higher than those detected by LDH assay. This is due to the fact that cytotoxic actions measured by the LDH test are always achieved at higher stages of cell injury than that is detected by the MTT test, which measures the metabolic viability of cells subjected to different culture conditions.

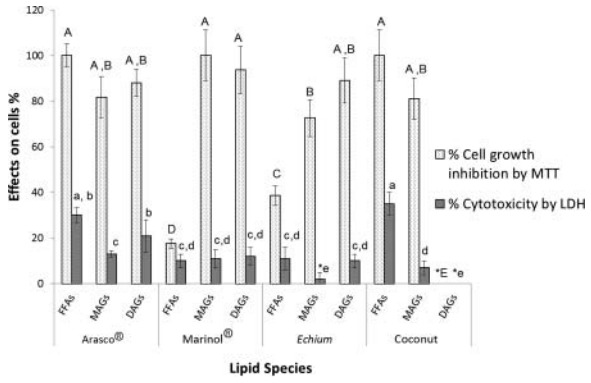


**Figure 8.** MTT and LDH assays. Dose-response plot for HT-29 cells following 48-h treatment with several acylglycerol species from different oils at 200 $\mu$g/ml. Data represent the mean of three complete independent experiments made in triplicate ± SD (error bars). $^{*}P > 0.05$ versus control. Within each test, bars not sharing the same superscript letters (MTT test in uppercase, LDH test in lowercase) are significantly different from one another by Duncan's multiple range test ($P < 0.05$). Abbreviations: DAG, diacylglycerol; FFA, free fatty acid; LDH, lactate dehydrogenase; MAG, monoacylglycerol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

## Transmission electron microscopy

This experiment was aimed at visualizing the mechanism for which the compounds tested here exerted negative influence on cell viability through analyzing cell images (Fig. 9). To this end, cells were incubated at 200 $\mu$g/ml with FFAs and selected AG species, as well as a control disposed without treatment. At the assayed concentration, all compounds induced similar morphological changes in HT-29 cells, although with differential intensity (Fig. 9). The main findings found in HT-29 cells are summarized in Table 2. Note that in control cells only minor alterations were present. Conversely, cells treated with either FFAs, MAGs, or DAGs displayed intense vacuolization and membrane-bound protrusions of the cytoplasm. The membrane blebs contained cytoplasmic material, ribosomes, and mostly dilated and swollen endoplasmic reticulum, and an intense deformation of the mitochondria and chromatin fragmentation. Other common findings were the numerous lipid droplets included in the cytoplasm, which were surrounded in some cases by endoplasmic reticulum. In addition, the membrane of some mitochondria appeared broken, and the content scattered. Breakdown of nucleus membrane was another common finding in most cases.

In all trials, the tested concentrations of both FFAs and AGs were toxic to the colon cancer cell line HT-29, as assessed by loss of membrane integrity and DNA fragmentation. These discoveries are indicative that necrosis and apoptosis occurred in all cases, and probably both processes occurred concomitantly. That is, in spite of the presence of blebbing and budding,

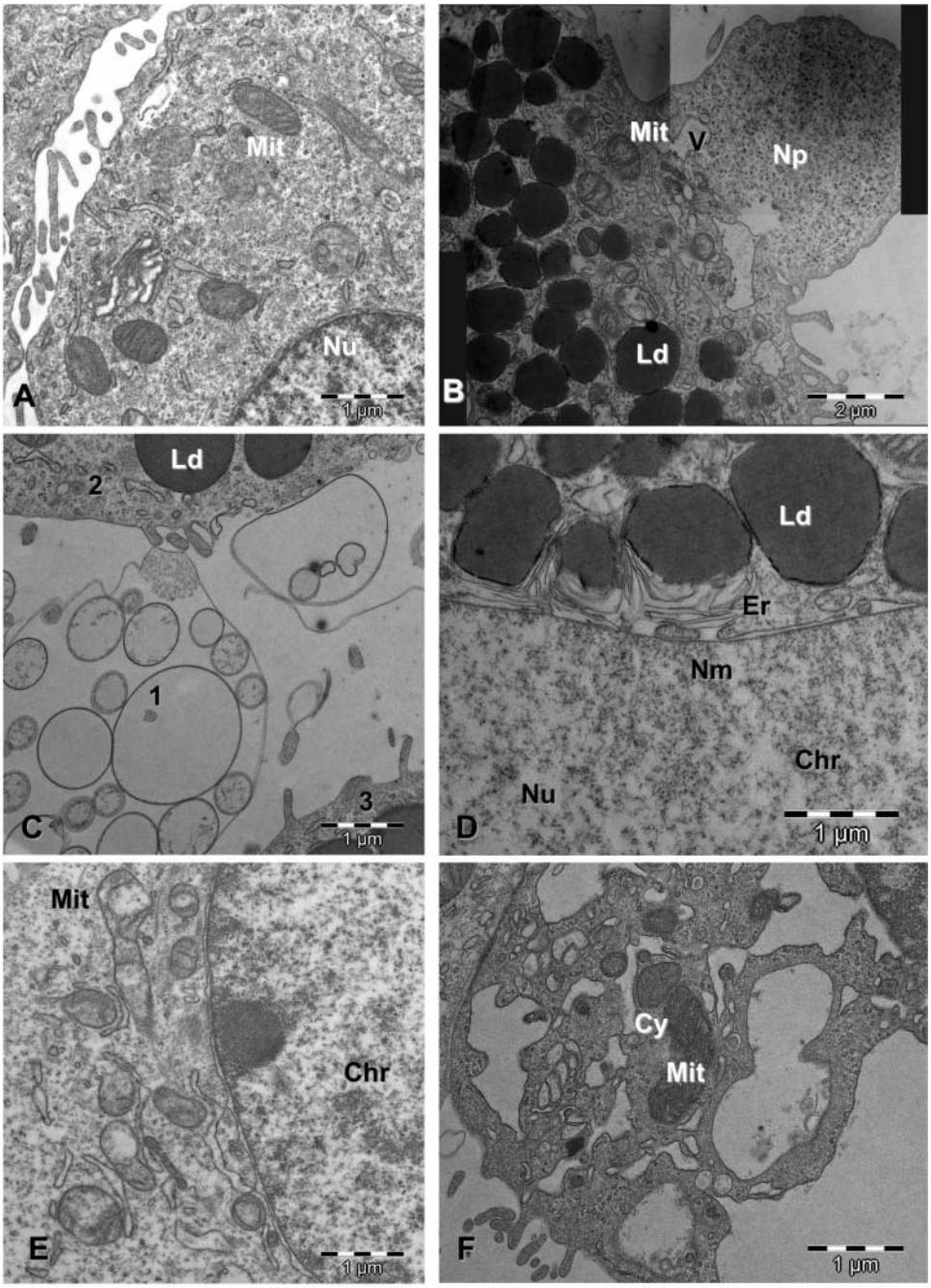


**Figure 9.** Morphological changes induced in HT-29 cells by diverse acylglycerol species at 200 $\mu$g/ml. A: Control cell, without cytotoxics added. Mit: mitochondria; Nu: nucleus. B: Cells treated with 2-MAG from cod liver oil. Np: cellular protrusion, including small vesicles (V) full of membrane and debris, characteristic of cells exposed to apoptotic inducers, and having numerous lipid droplets (Ld) in the basis. C: Cells exposed to 2-MAG from Marinol®. The cell labeled 1 has completed apoptosis and is dispersing as granular debris (the ghost cell stage); cells 2 and 3 still remain at early stages of apoptosis. This image clearly demonstrates that induction of apoptosis is asynchronous. D: Cells treated with 2-MAG from cod liver oil. Note the lipid droplets surrounded by endoplasmic reticulum (Er). Nm: nuclear membrane. E: Cells exposed to 2-MAG from Marinol. Notice the intense chromatin (Chr) condensation and broken amorphous Mit. F: Cells after exposition to DAGs from Marinol. Advance phase of cellular swelling (ghost cell). Notice the intense vesiculation and the amorphous Mit included in the rest of the cytoplasm (Cy). Abbreviations: DAG, diacylglycerol; FFA, free fatty acid; MAG, monoacylglycerol.

the simultaneous detection of apoptotic bodies and the rupture of the cell membrane and the release of the cell content are indicative of these phenomena. In good agreement with the present findings, induction of DNA fragmentation and loss of membrane integrity in leukemia cell lines after treatment with FAs have been reported (27). However, in the present study, the toxicity of the FA and AG species on HT-29 cells was

**Table 2.** Major alterations found in HT-29 cells induced by different acylglycerol species detected by transmission electron microscopy.

| | FFAs coconut oil | MAG Arasco® | MAG Marinol® | MAG cod liver oil | DAG Marinol® | Control |
|---|---|---|---|---|---|---|
| Lipid droplet | 3 | 1 | 2 | 3 | 2 | – |
| Chromatin condensation | 3 | 2 | 1 | 1 | 1 | 1 |
| Breakdown of nucleus membrane | 3 | 2 | 2 | 3 | 2 | 1 |
| Apoptotic body | 2 | 2 | 2 | 3 | 2 | 1 |
| Amorphous mitochondria | 3 | 3 | 2 | 3 | 3 | 1 |
| Broken mitochondria | 3 | 1 | 1 | 1 | 1 | – |
| Ghost | 3 | 1 | 1 | 2 | – | – |
| Breakdown of cell membrane | 3 | 2 | 2 | 3 | 2 | 1 |
| Cytoplasmic fragmentation | 3 | 2 | 1 | 3 | 1 | 1 |
| Membrane blebbing | 2 | 1 | 1 | 1 | 1 | 1 |

1: Rarely; 2: frequently; 3: very often.

not strictly related to carbon-chain length and the number of double bonds.

## Conclusions

Collectively, our study demonstrates that AG derivatives from commonly consumed oils in vitro constitute antitumor compounds that specifically induce cytotoxicity in colon cancer cells. TEM showed that both FFA and AG species exerted different inhibitory actions on the colon cancer cell line HT-29, as assessed by loss of membrane integrity and DNA fragmentation. These findings indicate that necrosis and apoptosis occur simultaneously. Conversely, any tested species produced effects on the viability of normal human colon myofibroblasts CCD-18 cells. Different AG derivatives exerted antitumor action in similar concentrations, as detected by different techniques, that is, FFAs from coconut oil by LDH and MTT tests; MAGs from Arasco by MAGLI test; MAGs from cod liver oil and Marinol by MTT assay; and DAGs from Marinol by MMT test, which were the more active species. Further research is needed to elucidate the multiple combinations of AG derivatives and PUFAs acting on different cancer cells through different mechanisms, as well as the accessibility of the different AG derivatives to reach colon tumors, either through the digestive lumen or via blood.

## Acknowledgments

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ORIGINAL PAPER

# Phytochemical Composition and Antitumor Activities of New Salad Greens: Rucola (*Diplotaxis tenuifolia*) and Corn Salad (*Valerianella locusta*)

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**Abstract** *D. tenuifolia* and *V. locusta*, two greens, were analyzed for active compounds and antitumor actions on colorectal cancer cells. Phenolics were determined by UHPLC-Orbitrap-MS; carotenoids and glucosinolates by HPLC-MS; and sterols and fatty acids by gas–liquid chromatography (GLC). For antitumor effects, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) tests were run on HT-29 colorectal cancer cells, and in CCD-18 untransformed enterocyte cells. Six main carotenoids were identified in both vegetables, while total carotenoids accounted for 3520 and 2970 $\mu g \cdot g^{-1}$ dry weight in *D. tenuifolia* and *V. locusta*, respectively. Six phenolics were detected in *D. tenuifolia* (68,600 $\mu g \cdot g^{-1}$ dry weight) and five in *V. locusta* (139,000 $\mu g \cdot g^{-1}$ dry weight). Three glucosinolates (GSL) were found in *D. tenuifolia* (1960 $\mu g \cdot g^{-1}$ dry wt. total). Low-polarity extracts from *V. locusta* and *D. tenuifolia* showed $IC_{50} \sim 150$ and ~200 $\mu g \cdot mL^{-1}$ on HT-29 cells, while both plants lacked actions on CCD-18 cells. *V. locusta* inhibited HT-29 cancer cells viability more efficiently than *D. tenuiofolia*, but induced less cytotoxicity. This work highlights the importance of functional foods for colorectal cancer prevention.

**Keywords** Bioactive compounds · Cytotoxicity · *Diplotaxis tenuifolia* · HT-29 colorectal cancer cells · *Valerianela locusta*

**Abbreviations**

| | |
|---|---|
| 4-MSB | 4-methylsulphinylbutyl (glucoraphanin) |
| AIF | All-ion fragment |
| ALA | α-linolenic acid |
| APCI | Atmospheric pressure chemical ionization |
| BSTFA | Tris (2-carboxyethyl) phosphine hydrochloride bis-(trimethylsilyl) trifluoroacetamide |
| EFA | Essential FA |
| ESI | Heated electrospray interface |
| FA | Fatty acid |
| FBS | Fetal bovine serum |
| FID | Flame ionization detector |
| GSL | Glucosinolate |
| LA | Linoleic acid |
| LDH | Lactate dehydrogenase |
| MTBE | Methyl tert-butyl ether |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| PUFA | Polyunsaturated fatty acid |

## Introduction

Food preferences in Western societies have recently shifted towards healthier options in contrast to previous high caloric diets inherent to processed foods. This way, new leafy vegetables are being consumed worldwide in salads and cut-fresh products [1]. Leaves of *Valerianella locusta* L. (Valerianaceae) and *Diplotaxis tenuifolia* L. (Cruciferae), more commonly known in Europe as corn salad and rocket or rucola respectively, are among the most representative

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vegetables of this trend [2]. Both species have been reported to have a positive influence on certain diseases such as diabetes, cardiovascular disorders and cancer [1]. Moreover, many studies show that cruciferous vegetables usually display strong inhibitory effects on several cancer types. Specifically, a diet rich in Brassica vegetables such as *Eruca sativa* has been related to prevent specifically colon carcinogenic processes [3]. *E. sativa* is a green which is commonly marketed and consumed together with *D. tenuifolia*, and both are known under the common name of rucola. The relationship between vegetables consumption and lower risk of suffering some diseases seems to be related to certain bioactive compounds they contain, such as carotenoids, phenols, glucosinolates (GSL) and fatty acids (FAs) [4].

Unlike other related species, a high lipid content has been reported in *D. tenuifolia* [5], and studies indicate suitable percentages of n-3 polyunsaturated FAs (PUFAs) in leaves of green vegetables [6], whose effects comprise the prevention of a large number of diseases such as cancer, inflammatory disorders and coronary heart disease [7]. Carotenoids are also powerful antioxidant molecules inherent to vegetables, whose consumption is related to the prevention of several cancer types, such as colorectal ones [3]. Lutein, which has been related to the prevention of several eye disorders, is the most abundant carotenoid in leafy vegetables, being β-carotene and β-cryptoxanthin also relevant due to their important role as protective agents and that they are metabolized to vitamin A [8]. Among phytochemicals, sterols have been shown to exert a beneficial influence on several health disorders such as heart diseases and colon cancer cell proliferation [9]. They are obtained mainly from fruits and vegetables, being β-sitosterol, stigmasterol and campesterol found in higher amounts than others [9].

Phenolic compounds possess important antioxidant and other potential health-promoting effects, including anti-inflammatory, antimicrobial and antitumor activities [10]. There are strong evidences pointing out that some flavonoids such as quercetin, kaempferol and isorhamnetin are responsible of the protective effects associated to these compounds [11]. Furthermore, glucosinolates (GSLs) have shown beneficial effects on human health. They are secondary metabolites with protective effects against several cancer types including colorectal cancer through the regulation of carcinogen-metabolizing enzymes [3, 12]. For instance, glucoraphanin (4-MSB) is hydrolysed after tissue disruption by the enzyme myrosinase to yield sulforaphane as its breakdown product, which is believed to exhibit beneficial effects on human health through a variety of mechanisms, being considered as a promising natural anticancer compound [12]. GSLs have been cited in *D. tenuifolia* [13] but not in *V. locusta* [14].

This work was designed to characterize the bioactive composition of two recently introduced greens in our diets, *D. tenuiofolia* and *V. locusta*. This paper constitutes the first report about the FAs and sterols composition of the leaves from both species. In addition, the antitumor effects of both plants on colorectal cancer cells, which is unknown for *V. locusta* and only partially established for *D. tenuifolia*, have been assessed.

## Material and Methods

Samples *V. locusta* and *D. tenuifolia* were purchased in local markets in Almería (Spain). Leaves of the two vegetables were washed with cold water and freeze-dried (LyoQuest, Telstar) for 48 h. Then, dried leaves were ground and kept at −70 °C until they were used for further processing.

**Moisture** The moisture content was determined by drying 5 g of sample in an air circulation oven at 105 °C until constant weight (24–48 h).

**Fatty Acids Analyses** Prior to derivation, 500 mg of freeze-dried sample of each leafy vegetable was treated with 7 mL of ethanolic sodium hydroxide (10 %, *w*/*v*) and this solution was maintained at 82 °C for 2 h, with constant stirring. Thereafter, the saponified solution was cooled at 4 °C and washed with water, removing by decantation the ether upper layer. Then, the soaps were acidified to pH 1 with HCl/$H_2O$ 1:1 (*v*/*v*) and FAs were extracted with n-hexane (3 × 10 mL), and the hexane was evaporated. Derivation to FA methyl esters (FAMEs) and gas liquid chromatography (GLC) analyses were carried out as previously reported [15].

**Carotenoids** Extraction and analyses were accomplished by means of HPLC-MS [15].

**Phenolics** Phenolic compounds were extracted following previous methodology [16]. Analyses were carried out by using a UHPLC-Orbitrap-MS as previously described [10].

**Sterols** Samples were processed according to previous reports [17], and analyzed using a Focus GLC (Thermo Electron, Cambridge, UK) equipped with a flame ionization detector (FID) and an Omegawax 250 capillary column (30 m X 0.25 mm i.d. X 0.25 μm film thickness; Supelco, Bellefonte, USA). The column temperature was programmed from 150 to 260 °C at 6 °C $min^{-1}$, then to 300 °C at 2.5 °C $min^{-1}$ and constant temperature at 300 °C for 7 min. Nitrogen was used as carrier gas (1 $mL \cdot min^{-1}$). A 50:1 split ratio was programmed, and injection volume was 4 μL. Temperatures in the injection port, transfer line and detector were set at 260, 280 and 220 °C, respectively. Sterols were identified using commercial standards, and 5α-cholestane was used as internal standard for quantification purposes.

**Glucosinolates (GLS)** Sample extraction was carried out according to previous work [18]. Analyses were performed by HPLC-MS; a Hewlett-Packard HP11100 with a C18 Phenomenex Luna column (250 × 4.6 mm, 5 μm particle size) was used, as well as a mobile phase based on acetonitrile (eluent A) and a water solution of ammonium formate 50 mM with formic acid pH 3.5 (eluent B) at a flow rate of 1 mL · min$^{-1}$. Injection volume was set at 20 μL. The gradient method for GSLs separation was carried out as follows: t = 0 min 90 % B; t = 30 min 0 % B; t = 35 min 0 % B; t = 40 min 90 % B; t = 45 min 90 % B.

The drying gas flow was 6 L · min$^{-1}$, the nebulizer pressure was 40 psig, the drying gas temperature was 325 °C, the vaporizer temperature was 450 °C, the capillary voltage was 2500 V and the corona current was 3 mA. The interface between the LC and MS was APCI (atmospheric pressure chemical ionization) positive (fragmentor 100 V). Peak identification was based on comparison of HPLC retention times and mass spectra with chemical standards of glucosativin, glucoraphanin and glucoerucin. These compounds were identified by their UV spectra, molecular weight and their characteristic m/z fragments. Standard solutions were used for calibration with quantification purposes.

**Cell Assays** Freeze-dried samples were extracted by using with two different solvent systems, a mixture of distilled water-absolute ethanol (1:1, *v*/*v*) and a mixture of chloroform-methanol (1:1, *v*/*v*) using a sample:solvent ratio of 1:10 (*w*/*v*) [19].

Cell viability and selectivity were assessed using two cell cultures, the HT-29 colon cancer cell line and the CCD-18 untransformed colon fibroblast cell line, which were supplied by the Technical Instrumentation Service from the University of Granada (Spain). Cell cultures and MTT assay were accomplished as detailed in previous works [19].

Lactate dehydrogenase (LDH) assay (Cytotoxicity Detection Kit PLUS, Roche, Mannheim, Germany) was carried out using similar culture conditions than in the MTT assay, to allow a comparative study between both tests. A lower cell density ($5 \times 10^3$ cell/well) was determined in preliminary experiments to improve the procedure on cells HT-29. Cell treatment in the LDH assay was the same as in the MTT assay until the steps of the addition of extracts and the incubation (48 h). Data were acquired according to manufacturer's instructions. Results of cytotoxicity were quantified by measuring the absorbance at 450 nm with a reference filter at 690 nm. A "high control" was used to estimate the total LDH content, treating cells with lysis solution to release all LDH. The percentage of cell death was calculated using the following equation:

$$\%\,\text{of LDH activity} = \frac{\text{exp. value-low control}}{\text{high control-low control}} \times 100$$

All parameters are based on absorbance data from samples and controls by triplicate. Low control determined the LDH activity released from the untreated cells; high control determined the maximum releasable LDH activity in the cells and experimental value (exp. value) determined the LDH activity released from treated cells with the plant extracts. Assayed extract concentrations ranged from 12.5 to 1000 μg · mL$^{-1}$.

Different tested extracts as well as negative controls for MTT and LDH assays were evaluated in independent tests, being the results reported as mean ± S.D.

**Statistical Analysis** Data in tables and figures are expressed as the average ± SD of the analysis of five different sampled leaves of each variety analyzed in triplicate. All values are presented as mean ± SD. Statistical significance ($P < 0.05$) was determined by analysis of variance (ANOVA) followed by assessment of differences using STATGRAPHICS Plus version 5 (Statistical Graphic Corp., Warrenton VA, USA.

## Results and Discussion

### Moisture Content

Moisture contents of *D. tenuifolia* and *V. locusta* were 941 and 934 g · kg$^{-1}$, respectively, values in agreement with previous ones [20].

### Carotenoids Content

The following carotenoids were identified in both vegetables: neoxanthin, violaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene (Table 1, Online Resource 1), which eluted in order of decreasing polarity from polar oxycarotenoids to lipophilic hydrocarbons. Neoxanthin was the first detected carotenoid followed by violaxanthin, lutein and zeaxanthin. Then, β-cryptoxanthin eluted at 20.35 min and finally β-carotene at 30.25 min. Total carotenoid was 3520 and 2970 μg · g$^{-1}$ dry weight (dw) in *D. tenuifolia* and *V. locusta*, respectively. References reporting the carotenoid profile of *D. tenuifolia* are very scarce; Žnidarčič et al. [21] indicated a value of 1030 μg · g$^{-1}$ dw for *D. tenuifolia* leaves, which is lower than that found in this work. However, large differences are found for carotenoid content even in works reporting values for *E. sativa* (Table 1, Online Resource 1) due to different factors such as climate conditions, nutrient intake and growth practices among others, making difficult the comparison among studies. *V. locusta* showed also intermediate figures in relation to other ones previously reported (Table 1, Online Resource 1). The main contributor to both carotenoid profiles was neoxanthin, while β-carotene was at the bottom of the range. However, other works reported lutein as the most

prominent carotenoid in both vegetables (Table 1, Online Resource 1). Again, differences in carotenoid profiles can be related to the multiple conditions that affect the phytochemical content of vegetables, such as plant variety, soil type, agronomic treatments, storage variables, post-harvest treatments, and the employed methodology for the quantification of the target analytes [8]. Given that lettuce, which is the most common vegetable consumed as salad, provides 1230 $\mu g \cdot g^{-1}$ dw (Table 1, Online Resource 1), it is shown that in comparison, both greens assessed in this work become more suitable options as salad ingredients regarding carotenoids content.

## Phenolic Compounds

Phenolic profiles for *V. locusta* and *D. tenuifolia* are reported in Table 2 (Online Resource 2). Total phenolics accounted for 68,600 and 139,000 $\mu g \cdot g^{-1}$ dw in *D. tenuifolia* and *V. locusta*, respectively. Total amounts were in good agreement with previous works in the case of *D. tenuifolia*, and higher for *V. locusta* (Table 2, Online Resource 2). Six phenolic compounds were detected in *D. tenuifolia* leaves: chlorogenic acid, rutin, luteolin, tamarixetin, isorhamnetin-3-O-glucoside, and quercetin-3-0-galactoside/quercetin-3-0-glucoside, being rutin in the upper range (41,100 $\mu g \cdot g^{-1}$ dw). Kaempferol-3-O-rutinoside, isorhamnetin-3-O-glucoside and quercetin-3-0- glucoside have been previously identified in this species (Table 2, Online Resource 2).

Five phenolic compounds were detected in *V. locusta* leaves: chlorogenic acid, rutin, luteolin, kaempferol-3-O-rutinoside and genistein, with chlorogenic acid in the top with 116,100 $\mu g \cdot g^{-1}$ dw. The remaining quantified compounds represent only 16.5 % of the total amount. Using again lettuce as a reference green due to its large use in salads, it can be noted that phenolics of both analyzed species were much higher than those found in lettuce (Table 2), both in quantity and diversity, therefore an increased consumption of these crops may be linked with long-term, decreased incidences of several chronic diseases.

## Sterol Composition

Sterol composition for both greens is shown in Table 3 (Online Resource 3). Four compounds were detected and quantified. Total sterols accounted for 5140 and 10,480 $\mu g \cdot kg^{-1}$ dw in *D. tenuifolia* and *V. locusta*, respectively. Three of them -campesterol, stigmasterol, and β-sitosterol- are usually found in commonly consumed vegetables [22]. β-sitosterol was the predominant sterol in both vegetables (3730 and 9550 $\mu g \cdot kg^{-1}$ dw in *D. tenuifolia* and *V. locusta*, respectively), while stigmastanol was quantified at the bottom of the range (50 $\mu g \cdot kg^{-1}$ dw). Sterols amounts found in both vegetables were somewhat lower than those reported for other vegetables such as celery [22]. However, *V. locusta* contains high amounts of β-sitosterol, which possess the highest anticancer activity among all sterols [24].

## Fatty Acids

FA compositions are shown in Table 4 (Online Resource 4). α-linolenic acid (ALA, 18:3n-3) was the most abundant FA, accounting for 48.5 and 57.4 % of total FAs in *D. tenuifolia* and *V. locusta*, respectively. This PUFA belongs to the n-3 family and is considered an essential FA (EFA), being reported its protective effect against certain types of human cancer [25]. n-3 PUFAs are also involved in the biosynthesis of eicosanoids with anti-inflammatory activity [12]. Other main FAs found in *D. tenuifolia* and *V. locusta* were linoleic acid (LA, 18:2n-6), with 10.6 and 12.0 %, and palmitic acid (PA, 16:0), with 16.4 and 11.7 %, respectively. A peak compatible with heptadecenoic acid (17:1) was found at 17.69 min retention time (Rt) in the chromatogram of *D. tenuifolia* and *V. locusta* leaves. However, although this signal came from a compound contained in a saponified extract and thus it has a FA structure, such amount for this FA in greens is unusual, and it could be due to any unidentified branched or hydroxilated FAs [26] thus we classified this signal as "unidentified".

Both analyzed greens showed good percentages of n-3 PUFA, comparable to other figures found in lettuce (Table 4, Online Resource 4), and an n-6/n-3 ratio lower than that of the latter; therefore, the intake of the species studied in this work might provide derived n-3 health benefits to a greater extent than lettuce [7]. It highlights that both greens contain almost three times the amounts of FAs found in lettuce (Table 4, Online Resource 4).

## Glucosinolates (GSLs)

GSLs found in *D. tenuifolia* are shown in Table 5 (Online Resource 5). Three GSLs were detected and quantified by HPLC-MS: glucoraphanin, glucosativin and glucoerucin. These compounds have been previously identified as the most prominent GSLs in *D. tenuifolia* and *E. sativa* leaves (Table 5, Online Resource 5). The most abundant GSL found in this work was glucosativin (1030 $\mu g \cdot g^{-1}$ dw), accounting for 52.4 % of total GSLs. Total GSLs detected in this work (1960 $\mu g \cdot g^{-1}$ dw) was in good agreement with results revealed by Pasini et al. [23] (Table 5, Online resource 5).

## Antitumor Assays

To evaluate the effects of extracts on HT-29 and CCD-18 cells, both *D. tenuifolia* and *V. locusta* were extracted by using ethanol:water (1:1, *v*/*v*) and chloroform:methanol (1:1, *v*/*v*). These solvent mixtures have different polarities, thus they extract hydrophilic or lipophilic molecules and showing up activities of compounds having similar polarity. Lipophilic tomato extracts were composed by a mixture of glyceryl esters of FAs, mainly ALA, LA, PA and oleic (OA, 18:1n-9) acids, together with small amounts of carotenoids and other non-

polar bioactive compounds; conversely, ethanol:water extracts are composed by several carbohydrates types, mainly fructose and glucose [19]. Although both extracts from each vegetable showed inhibitory effects on HT-29 cells growth, the chloroform-methanol extracts were the most effective (Fig. 1). $IC_{50}$ values obtained for this extract were ~150 and ~200 $\mu g \cdot mL^{-1}$ in *V. locusta* and *D. tenuifolia*, respectively. Nonetheless, in both vegetables, ethanol-water extracts did not reach $IC_{50}$ values at the assayed concentrations, reaching the lowest values in *V. locusta*. Besides other active compounds such as phenolics, differences might be related to FAs content, that is, *V. locusta* was an n-3 rich plant, and these PUFAs have been associated to a lower risk of suffering cancer and metastasis [3]. Altogether, active compounds such as phenols and carotenoids are only partially soluble in water, thus fat can improve their dispersion, enabling contact with cell membranes, and then promoting cytotoxic effects on targeted cells. Focusing on the results of this work, *V. locusta* showed the highest phenolic and FA amounts, which was surely responsible for the noted effects on HT-29 cell growth inhibition.

In order to discern selectivity of both plant extracts in cell viability of normal colon cells, chloroform-methanol extracts were also assayed on the CCD-18 untransformed colon cell line at different concentrations, obtaining a lack of effects on cell viability in all cases (Fig. 1). Therefore, these extracts act selectively on tumour cancer cells. Although an absence of immediate effects on normal colon cells was obtained by inducing proliferation or decreasing viability; identifying the effects of both plant extracts on normal cells during a higher number of cell cycles might be helpful in order to discern the scope of such absence.

LDH is one of the marker enzymes used for the detection of colorectal cancer [3]. The LDH cytotoxicity assay was carried out to compare the release of the lactate dehydrogenase enzyme into the culture medium after cell membrane damage. Both MTT and LDH assays were carried out at similar culture conditions in order to compare results (Fig. 2). LDH assay was carried out with the most active extracts (chloroform-methanol 1:1, *v/v*) from both vegetables. As shown in Fig. 2, for *V. locusta*, LDH values at all tested concentration were lower than MTT values, while for *D. tenuifolia* LDH values were lower than MTT ones only for concentrations higher than 400 $\mu g \cdot mL^{-1}$. On the other hand, whereas MTT assay revealed no increased inhibitory cell growth effects when using higher extract concentrations (600, 800 and 1000 $\mu g \cdot mL^{-1}$), LDH test showed increasing cytotoxic activity in parallel with extract concentrations.

Differences in effects measured by both tests can be related to their respective sensitivities; that is, the MTT assay showed actions at an earlier stage than LDH because it detects any change in cells viability. Conversely, LDH leakage occurred at later stages, when reactive oxygen species related to cell

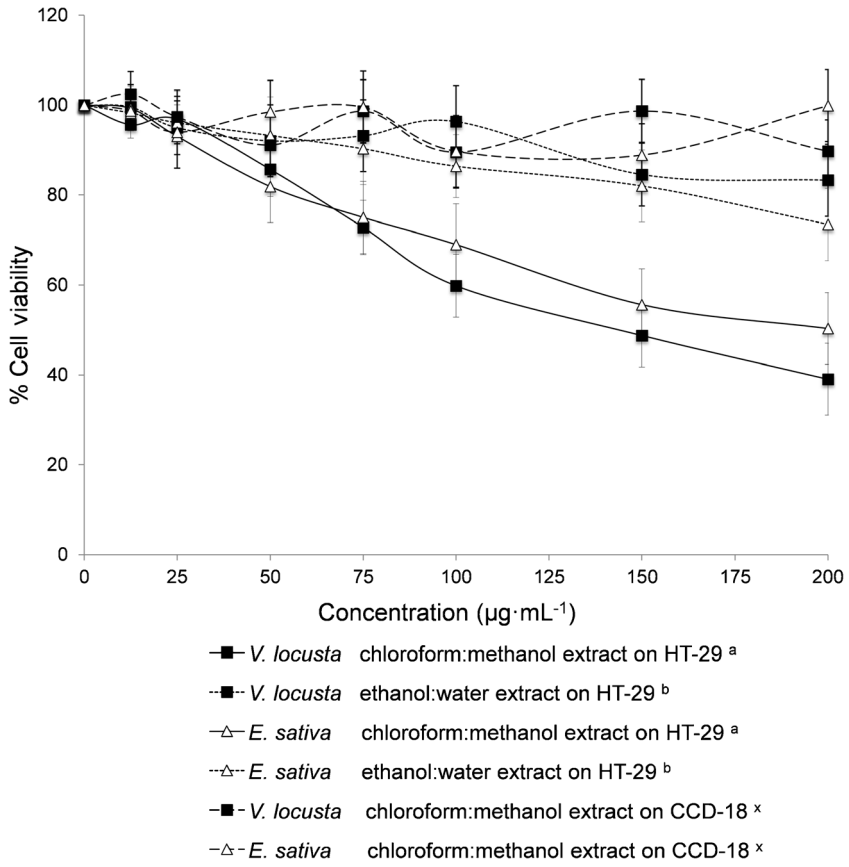


**Fig. 1** MTT assays. Dose–response plot for HT-29 and CCD-18 cells following 48-h treatment with plant extracts. Data represent the mean of five complete independent experiments made in triplicate ± SD (error bars). Within each cell line, series not followed by the same letter are significantly different from one another by Duncan's multiple range test ($P < 0.05$)

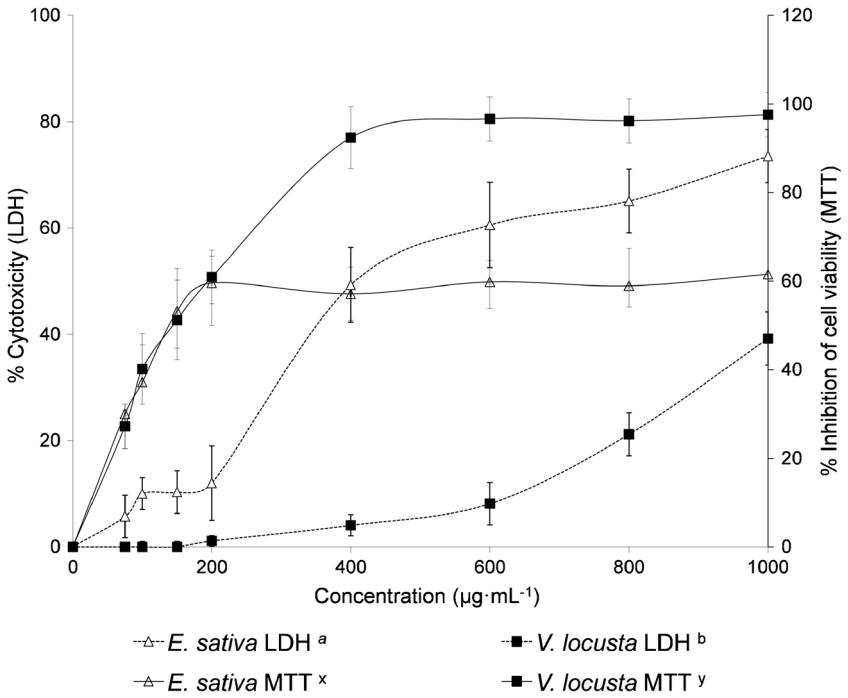


**Fig. 2** MTT assays (% inhibition of cell viability) and LDH release assay (% cytotoxicity) of chloroform-methanol (1:1, *v*/*v*) extracts from *V. locusta* and *E. sativa* on HT-29 cell line. Data represent the mean of five complete independent experiments made in triplicate ± SD (error bars). Within each test, series not followed by the same letter are significantly different from one another by Duncan's multiple range test ($P < 0.05$)

death appear, being responsible of the mitochondria damages [3]. Cytotoxicity outcomes detected in extracts from both plants seem to be related to their different phytochemical profiles; that is, *D. tenuifolia* extracts induce cytotoxicity due to mitochondria damages that lead to cell death even at low concentrations, being such activity probably mediated by isothiocyanates. Conversely, *V. locusta* extracts induce a strong decrease of cell viability; however, these extracts induce a lower cell death than *D. tenuifolia*, being effects probably due to synergistic action of several of its phytochemicals.

In relation to the anticancer activity found in both plants, it must be pointed out that the NCI (National Cancer Institute, USA) indicates that a crude plant extract is promising for further purification when its $IC_{50}$ values is lower than 30 $\mu g \cdot mL^{-1}$, looking for potential anticancer natural compounds [27]; therefore, the antitumor actions found in both species are not especially intense. However, considering that both plants are edible, it would not be logical to expect a very high anticancer activity, and the actions showed by such greens are in line with those effected by some other anticancer vegetables, such as that of tomatoes [19].

## Conclusions

We have established that both, *D. tenuifolia* and *V. locusta*, constitute a healthy source of bioactive compounds such as carotenoids, phenols, sterols, fatty acids and glucosinolates. Both vegetables are able to produce antitumor actions on colorectal cancer cells without causing damage to untransformed cells. At the assayed extract concentrations, both plants exert differential antitumor actions; *V. locusta* induced a decreasing of colorectal cancer cell viability higher than *D. tenuifolia*, but it also induced less cytotoxic effects.

The results of this work highlight the importance of consumption of functional foods that are sources of physiologically-active components, such as those found in *D. tenuifolia* and *V. locusta*, which constitute an appropriate source of phytochemicals for colorectal cancer prevention.

**Compliance with Ethical Standards**

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**Conflict of Interest** The authors declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects.

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ORIGINAL PAPER

# Fatty acid profiles and cholesterol content of seven insect species assessed by several extraction systems

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**Abstract** Species from Diptera (*Hermetia illucens* and *Lucilia sericata*), Coleoptera (*Tenebrio molitor* and *Zophoba morio*) and Orthoptera (*Locusta migratoria*, *Acheta domestica* and *Anacridium aegyptium*) were analyzed for fatty acid profiles and cholesterol content. The following solvent systems were tested for extraction: direct methylation ($CH_3OH/CH_3COCl$/hexane); n-hexane; acetone; ethanol/water; hexane/ethanol; and direct saponification (KOH and ethanol). Direct methylation was performed as control of extraction yields and to evaluate the possible use of these fats as biodiesel. Insect lipids were extracted by ethanol in a similar extent as did other tested organic solvents, while direct methylation of the biomass provided the highest yields. *L. sericata* and *Z. morio* contained high percentages of monounsaturated fatty acids; *A. aegyptium* and *L. migratoria* were two polyunsaturated fatty acid-enriched species, while *H. illucens* and *Z. morio* showed high proportions of medium-chain fatty acids. All extracted fats might be used in the alimentary industry, as evidenced by their low cholesterol content, as well as for biodiesel obtainment, as suggested by computed saponification, iodine and cetane values. Samples of *H. illucens* and *L. migratoria* showed exceptional cetane numbers (64.8 and 60.7, respectively), and all tested species except *A. aegyptium* exhibited an exceptional fatty acid profile for biodiesel production.

**Keywords** Insect · *Hermetia illucens* · *Locusta migratoria* · *Lucilia sericata* · *Anacridium aegyptium* · Alimentary industry · Fatty acids · Cholesterol · Biodiesel

## Introduction

Edible insects constitute a natural and inexpensive renewable food resource for humans [1, 2]. They are consumed mainly in countries with essential amino acid deficiency like some of Latin America, Asia and Africa [2]. Furthermore, certain insect species contain high proportions of fat [1, 3], thereby opening interesting possibilities with alimentary and industrial purposes, e.g., biodiesel obtainment, for which the fatty acid (FA) methyl esters (FAMEs) from some insect species have been previously found as suitable [3]. Although some insect species have been evaluated as FA sources assessed by different extracting solvent systems, essays have been performed with non-food-grade solvents [4]; thus, results can be marginally used by the food industry. Other studies have been focused on the use of insects for biodiesel production, such as those concerning *H. illucens*, [5], *T. molitor* [6] and *Z. morio* [7]; however, the FA profiles of insects could change according to feeding and/or lipid extraction methodologies; therefore, they should be evaluated by using different biomass sources and various extracting procedures.

This study was conducted to assess: (i) the FA composition and cholesterol content of several species of insects, some of them raised on farms and others captured in the

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wild; (ii) the influence of different food- and industrial-grade extracting systems on both fat yield and FA profiles; (iii) the cholesterol content of all tested insect species; and (iv) the obtaining of FAMEs from insects, to discuss their feasibility as biodiesel source.

## Materials and methods

### Samples

Insects were chosen according to the following criteria: easy to rearing and adequate development stage. Sampling characteristics (order, stage of development, origin, company and city) are exposed in Table ESM_1 (Online Resource 1).

### Lipids/fatty acids extraction systems

For analyses, an appropriate number of insects of each variety were homogenized. The resulting pastes were freeze-dried (LyoQuest, Telstar) for 48 h. The following solvent systems were tested for extractions, DirMet: direct methylation ($CH_3OH/CH_3COCl$/hexane; 1:0.05:0.5, v/v/v), effected at 100 °C for 10 min; Hex: pure n-hexane; Act: pure acetone; EtOH: ethanol/water (96:4, v/v); HexEtOH: hexane/ethanol (1:2.5, v/v); SAP: direct saponification, the latest effected at 60 °C for 2 h. All systems, excepting DirMet, were carried out in controlled temperature reactors. SAP system was carried out using ethanol (96 %) as extraction solvent; in a typical experiment, 1 g of lyophilized paste was treated with 7 mL of freshly prepared solvent containing 0.4 g of KOH for lipid extraction and simultaneous saponification. Extractions/saponification was carried out at 82 °C for 2 h, with constant agitation in an argon atmosphere. The obtained mixture was filtered through sintered glass filters (100–160 μm pore size) to remove the biomass residue. The residue was washed with 10 mL of ethanol (96 %), and the resulting filtrate was added to the combined filtrate; then, unsaponifiables were separated by extraction with hexane (3 × 50 mL). The hydroalcoholic phase, containing the soaps, was acidified to pH 1 with $HCl:H_2O$ (1:1, v/v), and the FAs were recovered by extraction with hexane (3 × 10 mL). The extract was washed with water to neutral pH, and the total volume was made up to 5 mL.

### Preparation of fatty acid methyl esters (FAMEs), DirMet system

Derivations of the samples by DirMet were carried out as follows: 300 mg of freeze-dried insect paste was placed into a test tube with 1 mL n-hexane and 2 mL of a mixture of methanol/acetyl chloride (20:1, v/v). The tube was placed in a thermoblock (Multiplaces, P. Selecta) at 100 °C for 30 min, after that, it was cooled at room temperature, and 1 mL of distilled water was added. Then, the tube was shaken and centrifuged (1300×*g*, 3 min) and the upper n-hexane layer, which contained the FAMEs, was collected and evaporated. This extract was dissolved in 1 mL of n-hexane and stored at −20 °C until use.

### Fatty acid analyses

FAMEs were analyzed using a Focus GC (Thermo Electron, Cambridge, UK) equipped with a flame ionization detector (FID) and an Omegawax 250 capillary column (30 m × 0.25 mm i.d. ×0.25 mm film thickness; Supelco, Bellefonte, USA). The temperature program was: 1 min at 90 °C, heating until 220 °C at a rate of 10 °C/min, constant temperature at 220 °C (2 min), heating until 250 °C at a rate of 10 °C/min and constant temperature at 250 °C (1 min). The injector temperature was 250 °C with a split ratio of 50:1. The injection volume was 4 μL. The detector temperature was 260 °C. Nitrogen was used as carrier gas (1 mL/min). Peaks were identified by retention times obtained for known FAME standards (PUFAs No. 1, 47,033, and methyl stearidonate 97 % purity, 43959 Fluka) from Sigma, (St. Louis, USA), while FA contents were estimated by using pentadecanoic acid (15:0; 98 % purity) from Sigma as internal standard.

### Cholesterol analyses

Cholesterol was determined in the oils extracted from freeze-dried insects with n-hexane. Oil (1 g) was saponified with 20 mL of aqueous sodium hydroxide 1 N (80 °C, 1 h). Then, it was allowed to reach room temperature and the unsaponifiable matter was isolated by three consecutive extractions with n-hexane (10 mL each). After centrifugation (1500 × *g*, 5 min), the upper hexane layers were collected and combined. Then, the solvent was evaporated under vacuum and the residue reconstituted in 1 mL n-hexane for further analysis. Cholesterol quantification was carried out by HPLC–DAD using a calibration curve with a cholesterol standard (≥99.0 %, Sigma-Aldrich, Germany). An HPLC–DAD Finnigan Surveyor (Thermo Electron, Cambridge, UK) equipped with a Hypersil Gold C18 column (250 × 4.6 mm, 5 μm i.d.), and UV–Vis detector was employed. A mixture of acetonitrile/isopropanol (65:35 v/v) was used as mobile phase in isocratic regime (1 mL/min). Injection volume was 1 μL and column temperature 30 °C. Detection was carried out at 210 nm [8].

### Quality indices for biodiesel production

Some biodiesel parameters were assessed for insect fats: saponification number (SN) and iodine value (IV), which

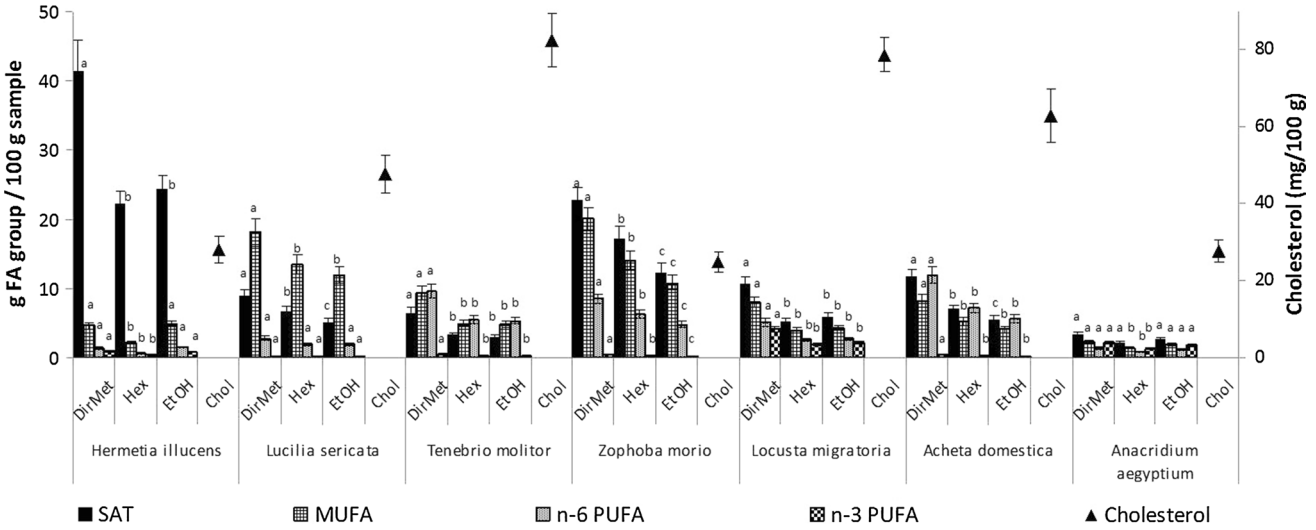


**Fig. 1** Fatty acid groups extracted by three different extracting systems and cholesterol content of seven insect species. Within each species, FA groups sharing the *same letter* indicate no differences in extraction ($P < 0.01$, Duncan's test). To know the complete results for all assessed extracting systems in relation to individual fatty acids and fatty acid groups, see supporting information 254 × 103 mm (150 × 150 DPI)

were calculated from FAME composition of oils with the help of Eqs. (1) and (2), respectively [9].

$$\mathrm{SN} = \Sigma(560 \times Ai)\big/\mathrm{MW}i \tag{1}$$

$$\mathrm{IV} = \Sigma(254 \times D \times Ai)\big/\mathrm{MW}i \tag{2}$$

where Ai is the FA percentage, D is the number of double bonds, and MWi is the molecular mass of each component.

CN was calculated from Eq. (3) [10].

$$\mathrm{CN} = 46.3 + 5458\big/\mathrm{SN} - 0.225 \times \mathrm{IV} \tag{3}$$

### Statistical analyses

Three different experiments, each performed in triplicate, were effected. Results in tables and figures represent the mean ± SD. A multifactorial analysis of the variance (ANOVA) was effected with the software package Statgraphics Plus 5.1 for Windows (Manugistics, Inc., Rockville, MA, USA) through the data obtained from each sample. $P < 0.01$ was regarded as significant.

## Results and discussion

### Fatty acid profiles and cholesterol content of insects

The organic extraction systems formed by hexane, acetone and ethanol optimally extract acylglycerides-containing lipids. Other tested extraction systems yield FAs-derived forms, as FAMEs (DirMet system) or FA soaps (SAP system).

The FA profiles of the various species analyzed here were noted strongly influenced by taxa. Diptera and Coleoptera were analyzed at larval stage, while Orthoptera ones were analyzed at nymph stage (Table ESM_1, Online Resource 1). Both Diptera species (Table ESM_2, Online Resource 2) accumulated saturated FAs (SFAs) and monounsaturated ones (MUFAs); i.e., *H. illucens* contained ~67 % lauric acid (LaA, 12:0) of total FAs, while *L. sericata* was an exceptional MUFA species (~59 % of total FAs). Coleoptera species assessed in this work could be considered as PUFA sources; i.e., *T. molitor* contained high amounts of n-6 PUFA (~39 % linoleic acid, LA, 18:2n-6), while in *Z. morio* this was approximately half of the latter (Table ESM_3, Online Resource 3). Orthoptera species (Table ESM_4, Online Resource 4) yielded good amounts of PUFAs; on total FAs, *A. domestica* contained LA at ~36 %; *L. migratoria* was an n-3-enriched species (~11.5 % ALA), while *A. aegyptium* included ALA and LA at ~22 and ~14 %, respectively. Considering FA groups (Fig. 1), SFAs were predominant in *H. illucens* (~85 % of total FAs), followed by *Z. morio* (~43 %); MUFAs highlighted in *L. sericata* (~59 %); n-6 PUFAs in *T. molitor* and *A. domestica* (39 and 36 %, respectively); and n-3 PUFAs in *A. aegyptium* and *L. migratoria* (23 and 11.5 %, respectively). Considering cholesterol contents (Fig. 1), species were found ranging from 24.8 in *Z. morio* to 82.6 mg/100 g dry wt. in *T. molitor*.

### Influence of extracting solvent systems in the fatty acid profiles and yields

The organic extraction systems formed by hexane, acetone and ethanol optimally extract glycerides-containing lipids.

In addition to triacylglycerols (TAGs), it has been probed in insects the presence of diacylglycerols (DAGs), which constitutes the lipids form of transport in such organisms, and they occur along with variable amounts of phospholipids (PLs), monoacylglycerols (MAGs), cholesterol and free FAs (FFAs) [11]. The latter compounds could be produced by cold active lipases, which have optimum activity at both low temperatures and water percentages. Other tested extraction systems yield exclusively FAs-derived forms, as FAMEs (DirMet system) or FA soaps (SAP system). The higher the yield of the latter's, the higher the presence of fats in the insect lipids, thus reducing unsaponifiable content in lipids.

The similarity of chemical properties between solvents and solutes should be a factor that determines extraction efficiency; thus, we tested four solvent systems having different *polarity index* (PI), whose values have been previously obtained [12, 13], i.e., hexane (PI = 0), ethanol/water (96:4, v/v, PI = 4.93), pure acetone (PI = 5.1), as well as a mixture of hexane/ethanol (96 %) (1:2.5, v/v, PI = 3.81). In a first approach, the lower PI, the maximum triacylglycerol yields in the fats. However, despite the differences in polarity described, few dissimilarities were found in the FA profiles and yields as a result of the different solvent systems used for fat extraction from insects. In all cases, the maximum yields were obtained by using DirMet system, although fats were recovered derivatized to FAMEs, and thus, this system allows accurately knowing total FA content in the different biomasses. A comparison about extraction yields for FA groups among DirMet, Hex and EtOH systems is displayed in Fig. 1. Notice that excepting for *Z. morio*, EtOH extracted fats in a similar extent to that was achieved by using Hex. However, for all FA groups, the DirMet system was the most efficient, being differences with respect to the remaining systems statistically significant ($P < 0.01$) for all species excepting for *A. aegyptium*, which contains low amounts of lipids. Moreover, it was confirmed that when the aim is to obtain FA esters, methodologies in which extraction and reaction run together, as occurs when applying the DirMet system, constitute potentially viable alternatives, due to their low cost and less environmental impact unlike other extraction systems [14].

Two species highlighted due to their exceptional FA content assessed by the direct methylation of the biomass, i.e., *H. illucens* and *Z. morio*, which contained 48.8 and 52.4 % FAMEs on dry biomass, respectively (Tables ESM_2 and ESM_3, Online Resources 2 and 3, respectively). However, *L. sericata* (Table ESM_2, Online Resource 2), *L. migratoria* and *A. domestica* (Table ESM_3, Online Resource 3) also showed high FA amounts, around 30 % FAs on dry matter.

All acylglycerol- and soaps-extracting systems exert extraction in a similar extent, and yields were typically in the 50–80 % range when comparing with DirMet system. On the other hand, all these extracts were mainly composed by FAs, especially in the case of *H. illucens*, *Z. morio* and *T. molitor*, which contained on average 95, 91 and 87 % FAs on total extracted oil, respectively (Tables ESM_2 and ESM_3, Online Resources 2 and 3).

## Assessment of differences in extraction among extracting systems

To discern differences among the different extraction systems, a multifactorial analysis of variance (ANOVA) has been carried out (Table ESM_4, Online Resource 4), for FA percentages and yields effected by the various solvent systems. All tested parameters influenced results, being the F-ratio—a ratio of the between-group estimate to the within-group estimate—equals to 1355.64, 9.83 and 7.63, for FAs, solvents and species, respectively ($P < 0.01$). Considering interaction among parameter, two of them were significant, i.e., the expected interaction between species and FAs ($F$-ratio 207.12, $P < 0.01$) and solvent systems with FAs ($F$-ratio 10.48, $P < 0.01$). To discern which means are significantly different from which others, Duncan's multiple range tests by solvent were performed, and Fisher's least significant difference (LSD) procedure was the method used to discriminate among the means. The least square (LS) means indicate that the mean for solvent system DirMet has significant differences ($P < 0.01$) with all others, which was due to FA yields.

## Possible use of insect fats

Three types of products have been extracted according to the different extraction systems: (i) by DirMet system: FAMEs, which might be used as biodiesel, (ii) by Hex, Acet, EtOH and HexEtOH systems: fats as mono-, di- and triglycerides along with unsaponifiables, which are suitable for use in foodstuffs, (iii) by SAP system: FA soaps, which could be used directly in the cosmetic industry, or after acidification to free FAs, in intra- and interesterification processes to be developed in the alimentary industry.

## Evaluation of these insect species for the production of biodiesel

The nature of FAs of insects has been considered as the main criteria when selecting insect species for biodiesel production [3]. In some cases, this factor can be adjusted by modifying the type of food ingested by insects. Moreover, insects present the ability to be fed by different sources of biomass, which is an environmental advantage, unlike traditional sources such as sunflower or palm oils among others, which present several drawbacks related to bioclimatic

**Table 1** Quality indices of FAMEs from selected insects for use as biodiesel

| | Diptera | | Coleoptera | | Orthoptera | | |
|---|---|---|---|---|---|---|---|
| | *Hermetia illucens* | *Lucilia sericata* | *Tenebrio molitor* | *Zophoba morio* | *Anacridium aegyptium* | *Locusta migratoria* | *Acheta domestica* |
| FAMEs % dry wt. | 48.8 | 30.7 | 26.4 | 52.4 | 9.4 | 28.2 | 32.6 |
| Saponification number (SN) | 243.1 | 194.0 | 191.8 | 197.4 | 194.4 | 191,0 | 192.3 |
| Iodine value (IV) | 17.6 | 75.0 | 98.3 | 63.0 | 88.2 | 63.0 | 90.9 |
| Cetane number (CN) | 64.8 | 57.6 | 52.6 | 59.8 | 54.5 | 60.7 | 54.2 |

conditions, the need of large areas of cultivation or a considerable amount of water [3, 15, 16].

Concerning *H. illucens*, 35.6 % LaA methyl ester of total FAMEs was reported [5], which is approximately half of what we have achieved (67 %); *T. molitor* was also evaluated as biodiesel source; however, a different FAME profile was obtained; i.e., PA, OA and ALA were the major FAs obtained in the present work, while the referenced authors indicated an ALA-based profile [6]; *Z. morio* was also assessed for biodiesel production [7]; however, FAMEs yield (33.8 %) was only ~2/3 compared to what we obtained (52.4 %). For the remaining species, this is the first report about their FA profiles for biodiesel production. According to the FA methyl esters proportion showed by the insect here evaluated, in addition to the previously described species, *L. sericata* (Table ESM_2, Online Resource 2), *L. migratoria* and *A. domestica* (Table ESM_4, Online Resource 4), which contained 30.7, 28.2 and 32.6 % FA methyl esters on dry wt, respectively, constitute potential sources of biodiesel, whose FA content might be improved after the appropriate studies to optimize variables related to their rearing and feeding.

Although LaA and similar saturated FAs are desired for good biodiesel quality, the presence of unsaturated FAs in FAMEs is also required, since they protect to FAMEs from solidification. However, highly unsaturated FAMEs are not suitable for biodiesel as the unsaturated molecules react with atmospheric oxygen and are converted to peroxide, cross-linking at the unsaturation site can occur, and thus, the material may get polymerized into a plastic-like body [13].

Previously, it has been shown that the calculated SN and IV are in good agreement with other experimentally determined respective values, and Eq. (3) predicts the CN of FAMEs of seed oils with reasonable accuracy [10]. Values for FAMEs%, SN, IV and CN of seven insect species are shown in Table 1. The calculated SN ranged from 191.1 (*L. migratoria*) to 243.1 (*H. illucens*), while IV ranged from 17.4 (*H. illucens*) to 98.3 (*T. molitor*). CN values varied from 52.6 (*T. molitor*) to 64.6 (*H. illucens*). CN denotes the ability of fuel to ignite quickly after being injected, which has better ignition quality for high CN values. In Europe, CN was set at a minimum of 40 in the year 2000. The current standard for diesel sold in European Union indicates a minimum CN of 51, while premium diesel fuel can have a CN higher than 60 [13]. Accordingly, *H. illucens* and *L. migratoria* seem to be exceptionally adequate for biodiesel obtainment, although all species have CN numbers higher than the minimum required. Other quality criteria refer to PUFA content; i.e., ALA and FAs containing four double bonds in FAMEs should not exceed the limit of 12 and 1 %, respectively [17]. In concordance, *A. aegyptium* should be discarded for biodiesel obtainment. In addition to the above criteria, the iodine value, which should be maintained <120, is overcome by all tested species. However, there are many quality requirements for biodiesel, as some metal content, mono-, di- and triglycerides and the total glycerol, etc. [18]. Therefore, a more exhaustive research is needed before deciding the use of these oils for biodiesel production.

## Possible use of insects in the alimentary industry

According to the main FA groups showed by insects, there are four main possible uses in the food industry: (i) MUFA species: Their oils are mainly composed by OA and POA (*L. sericata*) and OA (*Z. morio*). The presence of OA in dietary fats gives them favorable textural properties; OA-rich fats are commonly used as emulsifying agents and food additives, so these oils are interesting to both food and pharmaceutical industries [19]. The low cholesterol content they show (47.8 and 8.9 mg/100 g dry wt., respectively, Fig. 1) adds value to their use, since other animal fats show much higher cholesterol values, such as those from pigs, which contains 58 mg/100 g fresh muscle [20], i.e., ~300 mg/100 g dry muscle. (ii) n-3 PUFA species. These are *A. aegyptium* and *L. migratoria*, which have favorable both PUFA percentages and n-6/n-3 ratio (0.6 and 1.3,

respectively) (Table ESM_4, Online Resource 4). However, the oil content in *A. aegyptium* was low, although this content could be a by-product giving added value to insect processes developed in the industry; e.g., when removing fat from insect biomass for protein concentrates obtainment. In any case, all these fats are interesting for the food industry, given that PUFA-enriched oils cover a great number of edible uses, due to imbalances in the intake and metabolism of PUFA [21]. This fact has spurred the introduction of these oils for biomedical and nutritional products, fortified foods and health supplements [22]. Moreover, these species show also very low cholesterol content, 2.8 in *A. aegyptium* and 78.9 mg/100 g dry wt. in *L. migratoria*, thus providing added value to their use. (iii) SFA species for the alimentary industry. This is the case of *H. illucens* and *Z. morio*, which are sources of medium-chain triacylglycerols; thus, their oils could be used in similar applications to that coconut oil, e.g., in infant milk powders; as a confectionery fat, particularly in the preparation of ice creams; in imitation of chocolate coconut in place of cocoa butter; in the soap industries; in the pharmaceuticals, cosmetics, plastics, rubber substitutes, synthetic resins, etc. [23]. As previously exposed for other species, these fats have advantages over others as milk fats, since they have very low cholesterol content, i.e., 28.2 in *H. illucens* and 8.5 mg/100 g dry wt. in *Z. morio* (Fig. 1).

## Conclusions

Among the various solvent systems tested for fat extraction from several insect species, ethanol, a safe and biocompatible solvent, extracted lipids from insect biomass with both yields and FA profiles as did other more apolar solvents, as well as by the direct saponification of the biomass. However, for all insect species, the maximum FA extraction yield was obtained by the direct methylation of the biomass. All fats from the insect species here assessed might be used in the alimentary industry with different applications, considering both their low cholesterol content and the diverse FA profiles they show, existing n-3/6 PUFA, MUFA and medium-chain FA species. Two species, *Z. morio* and *H. illucens*, showed the highest fat percentage, ~50 % of dry biomass, while *H. illucens* and *L. migratoria* showed an exceptional FA profile for biodiesel obtainment, considering the appropriated CN computed (64.8 and 60.7, respectively) and other characteristics established by various standards for biodiesel sources.

#### Compliance with ethical standards

**Human and animal rights** All procedures were conducted according to the guidelines of Council Directive 86/609/EEC (European Communities 1986) on the protection of animals used for experimental and other scientific purposes, and the study was approved by the Bioethical Committee for Animal Experiments at the University of Almería.

**Conflict of interest** The authors declare that they have no conflict of interest.

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# Phytochemical composition and *in vitro* anti-tumour activities of selected tomato varieties

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## Abstract

**BACKGROUND: Previous studies indicated that tomato is a rich source of phytochemicals that act on different tumours. In this research, the phytochemical composition of selected tomato varieties was assessed by GLC and UHPLC/HPLC-MS, as well as their anti-tumour activities on HT-29 colorectal cancer cells.**

**RESULTS: Significant differences were found among tomato varieties; lycopene was high in *Racimo*, phenolics in *Pera*, sterols in *Cherry*, and linoleic acid predominated in all varieties. The MTT and LDH assays showed significant time- and concentration-dependent inhibitory/cytotoxic effects of all tomato varieties on HT-29 cells. Furthermore, the joint addition of tomato carotenoids and olive oil to HT-29 cell cultures induced inhibitory effects significantly higher than those obtained from each of them acting separately, while no actions were exercised in CCD-18 normal cells.**

**CONCLUSION: Tomato fruits constitute a healthy source of phytochemicals, although differences exist among varieties. *In vitro*, all of them inhibit colorectal cancer cell proliferation with *Racimo* variety at the top, and exercising a selective action on cancer cells by considering the lack of effects on CCD-18 cells. Furthermore, synergy was observed between olive oil and tomato carotenoids in inhibiting HT-29 cancer cell proliferation; conversely, phenolics showed no significant effects and hindered carotenoids actions.**


*Supporting information may be found in the online version of this article.*

**Keywords:** tomato varieties; *Lycopersicum esculentum*; HT-29 cancer cells; carotenoids; sterols; phenolics; cytotoxicity; olive oil; Mediterranean diet; fatty acids

## INTRODUCTION

Tomato is one of the most consumed vegetables worldwide, playing an important role in the human diet because of its well-documented health benefits. It is known as one of the main components in the so-called 'Mediterranean diet', and its phytochemicals are considered effective for the prevention of some cancer types and cardiovascular diseases.[1,2] Different mechanisms have been identified acting on cancer prevention exercised by active compounds of tomato, such as inhibition of inflammatory processes and interference with tumour angiogenesis.[2] Moreover, in addition to the whole fruit, tomato by-products have shown anti-tumour activity on HT-29 colorectal cancer cells.[3]

Tomato fruit is a good source of some carotenoids, namely lycopene, $\beta$-carotene and lutein. Lycopene has been reported as the most antioxidant tomato carotenoid, and it is considered as the main bioactive compound of tomato, which has anti-tumour properties.[4,5] In addition, previous studies have shown that lycopene is accumulated in different animal and human organs, thus improving its bioactivity.[6] Other bioactive compounds of tomatoes are vitamins C and E, dietary fibre, flavonoids, sterols and phenolics, whose consumption has been related to a decrease in cancer development.[7,8] Previous works indicate variable amounts of phenolics in tomato varieties, and caffeic acid, chlorogenic acid, ferulic acid, rosmarinic acid, rutin, quercetin and naringenin were the most abundant compounds.[9] Phenolics from tomato exert antioxidant activity, avoiding the production of reactive oxygen species and consequently preventing cellular damage, as well as cancer cell proliferation.[10] Sterols are also present in tomatoes, although in lower amounts than in other fruits and vegetables, and $\beta$-sitosterol, stigmasterol

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and campesterol are the main compounds.[11] Some studies revealed that sterols influence several health disorders, such as heart diseases and the inhibition of colon cancer cell proliferation.[11,12]

Among the previous detailed compounds, stand out carotenoids are better incorporated into human metabolism when they are consumed simultaneously with olive oil,[13] whose intake has also demonstrated healthy effects.[14] In the Mediterranean area, olive oil constitutes the main source of dietary fatty acids (FAs), and according to some studies, it provides intense activity against cancer cell proliferation, unlike other kinds of dietary fats.[15] For instance, a 25% reduction in colorectal cancer incidence has been estimated for people consuming olive oil,[16] while *in vitro* this oil is active against HT-29 cancer cell proliferation.[17] The direct relation between cancer and olive oil is probably due to the high amounts of oleic acid (OA, 18:1*n*9) it contains, which is involved in several mechanisms of carcinogenesis, for instance in the modification of cell membranes structure and functions and alteration of the immune system.[17] However, some other minor compounds from the unsaponifiable fraction of olive oil also have high cytotoxic activity.[18]

In line with the above exposed research, the aim of this study is based on the hypothesis that different tomato varieties, having different active compound profiles, will exercise selective anti-tumour effects on the HT-29 colorectal cancer cell line; likewise, such activity will be mainly due to carotenoids, and effects will be enhanced by olive oil. To accomplish such objectives, carotenoids, phenolics, sterols and FAs were determined in selected tomato varieties, and later on anti-tumour actions of different tomato extracts were checked *in vitro* on HT-29 cells. In addition, anti-tumour actions of olive oil jointly administered with tomato carotenoids and phenolics were tested on HT-29 colorectal cancer cell line, to assess possible synergism among all these components.

## EXPERIMENTAL

### Samples

Four common varieties of tomato (*Solanum lycopersicum* L.) were collected in agricultural cooperatives from Almería (Spain). Selected tomato varieties were: *Racimo* (round tomato), *Pera* (long tomato), *Cherry* (small tomato) and *Raf* (ribbed tomato), which correspond to recognised standard varieties in Europe.[19] All tomatoes were grown in greenhouse conditions, using similar soil and agricultural practices. Tomatoes were collected five times throughout 1 year, according to their suitable ripening stage, which was assessed by qualified staff. After picking, the fruits were packed in a portable refrigerator until they were transported to the laboratory (2–3 h). Before performing analyses, the samples were washed, firstly with running water and then with distilled water, and residual moisture was evaporated at room temperature. Within each collecting, five fruits per variety in adequate ripening stage were randomly selected and analysed for moisture, firmness and colour. Then, an appropriate number of fruits of each one were homogenised for analysis of total soluble solids content (TSS), titratable acidity (TA) and carotenoids. The remaining fresh tomato pastes were freeze-dried (LyoQuest, Telstar, Tarrasa, Spain) for 48 h, to perform other chromatography analyses and cytotoxicity assays. Extra virgin olive oil, 88% oleic acid and 0.2° acidity, was purchased at a local market in Almería.

### Chemical reagents

Standards of $\beta$-carotene, phenolic compounds (chlorogenic acid, rutin, quercetin, kaempherol and luteolin) and sterols (campesterol, 5$\alpha$-cholestane, stigmastanol, stigmasterol and $\beta$-sitosterol) and bis-(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS), dry pyridine, HPLC-grade solvents and analytical-grade solvents were purchased from Sigma (St Louis, MO, USA). Lycopene, lutein and $\beta$-cryptoxanthin standards were obtained from IMATRA, S.A (Barcelona, Spain). HPLC-grade acetic acid was purchased from J.T. Baker (Debenter, the Netherlands).

### Colour evaluation

The colour of all tomato samples was evaluated using a colorimeter (Spectrophotometer CM-3500D; Konika Minolta, Madrid, España). A CIE colour space co-ordinates, $L^*$, $a^*$, $b^*$ values were determined. The luminosity is represented by $L^*$ (0, black to 100, white), $a^*$ represents the variation of greenness to redness (−60 to 60) and $b^*$ indicates the variation of blueness to yellowness (−60 to 60). Each record was an average of three measurements on every tomato sample. One measure was taken in the equatorial zone and the other two at the distal area.

### Moisture

It was determined by drying a representative 2 g sample in an oven with air circulation at 100–105 °C for 40 h.[20]

### Fatty acids analyses

Derivation to fatty acid methyl esters (FAMEs) and gas liquid chromatography (GLC) analyses were carried out as previously reported.[20,21]

### Extraction and HPLC-MS analyses of carotenoids

Extraction and analyses were made using the same equipment and method described in previous reports.[20,21]

### Extraction and analyses of phenolic compounds

These were extracted following previous methodology.[22] UHPLC-Orbitrap-MS analyses were carried out by using an UHPLC-Orbitrap-MS, as previously described.[23]

### Extraction and analyses of sterols

Samples were processed according to previous reports,[24] and analysed using a Focus GLC (Thermo Electron, Cambridge, UK) equipped with a flame ionisation detector (FID) and an Omegawax 250 capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Supelco, Bellefonte, PA, USA). The column temperature was programmed from 150 °C to 260 °C at 6 °C min$^{-1}$, then to 300 °C at 2.5 °C min$^{-1}$ and constant temperature at 300 °C for 7 min. Nitrogen was used as carrier gas (1 mL min$^{-1}$). A 50:1 split ratio was programmed, and injection volume was 4 µL. Temperatures in the injection port, transfer line and detector were set at 260, 280 and 220 °C, respectively. Sterols were identified using commercial standards, and 5$\alpha$-cholestane was used as internal standard to quantify both the total and individual sterols.

### Tomato extracts for cell assays

Cell viability, proliferative and cytotoxicity assays were performed for each tomato variety in a single paste conformed by adding

proportional amounts of all previously collected and freeze-dried tomato pastes. The resulting pastes were mixed with either distilled water/absolute ethanol (1:1 v/v) or pure petroleum ether. In both cases a 1:10 ratio was used (tomato paste:solvent, w/v). The resulting solutions were homogenised and stored in darkness for 7 days at 4 °C under an inert atmosphere of nitrogen. Then, the extracts were filtered with suction and evaporated under vacuum to reduce volume and later were lyophilised. All tomato extracts were stored at −20 °C. The hydro-alcoholic and petroleum ether dry extracts and olive oil were dissolved in ethanol/water (1:1, v/v), dimethyl sulfoxide (DMSO) and acetone, respectively. Other similar dilutions were made with all compounds to be checked, while appropriate dilutions for testing the different blends of compounds were performed by mixing the previous solutions (1:1, v/v), to obtain in all cases 100 mg $mL^{-1}$ culture medium stock solutions. The different solutions were mixed with culture medium to achieve the different concentrations. All of them contained low solvent concentrations in the bioassay test: less than 0.1% of DMSO and 1% of ethanol and acetone. All solutions were stored at −20 °C until analysis.

### Cell cultures

These were performed using the HT-29 colorectal cancer cell line and the CCD-18 untransformed human colon fibroblast cell line, which were supplied by the Technical Instrumentation Service of Granada University, Granada, Spain. After checking for the absence of *Mycoplasma* and bacteria, cells were grown in RPMI 1640 medium (Sigma) supplemented with 5% fetal bovine serum, 2 mmol $L^{-1}$ L-glutamine, 1 mmol $L^{-1}$ sodium pyruvate, 0.125 mg $mL^{-1}$ amphotericin and 100 mg $mL^{-1}$ penicillin–streptomycin (Sigma–Aldrich (St Louis, MO, USA)). All cultures were plated in 25 $cm^2$ plastic tissue culture flasks (Sarstedt, Newton, NC, USA) and were incubated at 37 °C and 5% $CO_2$ until assayed.

### Cell viability and proliferation assays

These were accomplished by using the 3-(4,5-dimethytlthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which was effected as indicated in previous studies.[3] Antiproliferative effects were established by measuring cell growth at 48 and 72 h.

### LDH release assay

The lactate dehydrogenase (LDH) test (Cytotoxicity Detection Kit PLUS; Roche, Mannheim, Germany) was carried out using a lower cell density than MTT ($5 \times 10^3$ cells $well^{-1}$). This parameter was determined in preliminary experiments to improve the procedure on HT-29 cells. Cell treatment in the LDH assay was the same as MTT until adding the extracts and its incubation (48 h). In this test, all steps were carried out according to manufacturer's instructions. Cytotoxicity was quantified by measuring the absorbance at 450 nm with a reference filter at 690 nm. A 'high control' was used to estimate the total LDH content treating cells with lysis solution to release all LDH. The percentage of cytotoxicity was determined according to the following equation:

$$\text{LDH activity}\,(\%) = \frac{\text{exp. value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

All parameters represent absorbance values of the triplicate samples and controls. Low control indicates the LDH activity released from the untreated cells; high control is the maximum releasable LDH activity in the cells, while experimental values designate LDH activities released from treated cells with the cytotoxic agents. Different extracts and negative controls for MTT and LDH assays were evaluated in three independent assays made in triplicate, being the results presented as mean ± SD, as error bars.

### NMR spectra

$^1$H NMR spectra were obtained through the same equipment and method described in previous studies.[3]

### Statistical analysis

All values represent the mean ± SD of five complete independent experiments made in triplicate. A multifactorial analysis of the variance (ANOVA) and cluster analysis (Statgraphics Plus 5.1 for Windows; Manugistics, Inc., Rockville, MA, USA) was performed. $P < 0.05$ was considered as significant.

## RESULTS AND DISCUSSION

### Quality parameters

The CIELAB colour parameters from all tomato varieties are summarised in Table 1. The most related to tomato ripening is $a^*$, which explains the differences found between varieties unlike other colour parameters ($b^*$ and $L^*$).[25] In this work, $a^*$ values ranged from 11.6 in *Raf* to 26.7 in *Racimo*, while *Pera* and *Cherry* showed intermediate values, 25.0 and 21.7, respectively. On the other hand, $b^*$ values ranged from 24.9 to 31.7, while $L^*$ was above 37.9, as previously described.[26] The $a^*/b^*$ ratio indicates the maturity stage of tomatoes, which ranged from −0.4 for green tomatoes to 3.0 for over-ripe ones.[26] Accordingly, all studied samples showed moderate ripening stages, namely 0.9 for *Cherry* and *Pera*, 0.8 for *Racimo* and 0.4 for *Raf*, which corresponds with the degree of maturity preferred by consumers for each variety, according to producers' criteria. Obviously, differences in the degree of maturity of analysed tomatoes lead to changes in their phytochemical composition and cytotoxic activity, which are detailed below for the previously defined maturity stages.

Generally, the ripening of the fruits leads to carotenoid synthesis and degradation of chlorophylls and thus to a decrease in $L^*$, which reflects the darkening of the fruits due to the disappearance of greenness.

Soluble solids and total acids amounts were similar for all varieties, with *Racimo* variety at the bottom of the range. According to the $a^*/b^*$ ratio, the maturity stage of all tomatoes was *red*, excepting for *Raf* variety, which was light red.

### Carotenoids content

The separation and quantification of carotenoids were conducted by using a HPLC-MS system. The results indicated that all the varieties contained the same main four carotenoids in their composition: all-*trans*-lutein, all-*trans*-$\beta$-carotene, 9-*cis*-$\beta$-carotene and lycopene. Table 2 summarises total carotenoids and values for individual carotenoids of each variety. The components were eluted in order of decreasing polarity from polar oxycarotenoids to lipophilic hydrocarbons. Lutein was the first eluted compound, and then, all-*trans*-$\beta$-carotene was detected at 19.96 min and 9-*cis*-$\beta$-carotene eluted after it. Lycopene, the most abundant carotenoid, was detected at 21.48 min.

Total carotenoids ranged from 265 (*Raf*) to 409 (*Racimo*) mg $kg^{-1}$ dry weight (DW). *Raf* variety, which had the lower experimental $a^*/b^*$ ratio in this study, also had the lowest content of carotenoids.

**Table 1.** Quality parameters of four tomato varieties

| Tomato variety | Colour parameters (CIELAB) | | | | Moisture (g kg$^{-1}$) | Soluble solids °Brix) | TA (g kg$^{-1}$ DW) | Ripening stage |
|---|---|---|---|---|---|---|---|---|
| | *L** (D65) | *a** (D65) | *b** (D65) | *a**/*b** | | | | |
| *Cherry* | 379 ± 24$^{a}$ | 217 ± 25$^{a}$ | 249 ± 28$^{a}$ | 087 | 951 ± 12$^{a}$ | 5.5 ± 1.2$^{a}$ | 682 ± 39$^{a}$ | Red |
| *Racimo* | 421 ± 23$^{a}$ | 267 ± 26$^{b}$ | 317 ± 26$^{b}$ | 084 | 935 ± 17$^{a}$ | 4.6 ± 1.1$^{a}$ | 287 ± 26$^{b}$ | Red |
| *Raf* | 467 ± 26$^{b}$ | 116 ± 32$^{c}$ | 305 ± 34$^{b}$ | 038 | 937 ± 15$^{a}$ | 5.7 ± 1.0$^{a}$ | 506 ± 19$^{c}$ | Light red |
| *Pera* | 401 ± 20$^{a}$ | 250 ± 27$^{a,b}$ | 288 ± 22$^{b}$ | 087 | 958 ± 14$^{a}$ | 5.2 ± 1.1$^{a}$ | 321 ± 28$^{b}$ | Red |

Results are expressed as the average ± SD of the analysis of all tomatoes studied of each variety as detailed in Material and Methods section ($n = 5$ in triplicate).
Values not sharing the same superscript letters in the same column are significantly different from one another by Duncan's multiple range test ($P < 0.05$).
TA, total acids (measured as citric acid); DW, dry weight.

**Table 2.** Carotenoids content of four tomato varieties (mg kg$^{-1}$ dry wt)

| Variety | All-*trans*-lutein | All-*trans*-$\beta$-carotene | 9-*cis*-$\beta$-Carotene | Lycopene | Total |
|---|---|---|---|---|---|
| *Cherry* | 4 ± 1$^{a}$ | 23 ± 4$^{a}$ | 3 ± 2$^{a}$ | 313 ± 21$^{a}$ | 343 ± 23$^{a}$ |
| *Racimo* | 5 ± 2$^{a}$ | 4 ± 1$^{b}$ | 6 ± 2$^{a,b}$ | 394 ± 33$^{b}$ | 409 ± 37$^{b}$ |
| *Raf* | 37 ± 7$^{b}$ | 75 ± 6$^{c}$ | 9 ± 3$^{b}$ | 144 ± 21$^{c}$ | 265 ± 18$^{c}$ |
| *Pera* | 6 ± 1$^{a}$ | 15 ± 3$^{d}$ | 8 ± 3$^{b}$ | 343 ± 29$^{a,b}$ | 372 ± 29$^{a,b}$ |

Results are expressed as the average ± SD of the analysis of five different samples of each variety ($n = 5$ in triplicate).
Values not sharing the same superscript letters in the same column are significantly different from one another by Duncan's multiple range test ($P < 0.05$).

Lycopene was the main contributor to this profile in all varieties, which comprises more than half of the total carotenoids. The amounts of total carotenoids detected in this work are related to previous studies,[22] although some differences were found within the same tomato varieties, which can be explained by considering that harvesting might have been affected in different stages of maturity. Furthermore, other studies have shown lower amounts of carotenoids in mature tomatoes, accounting for 250.8 mg kg$^{-1}$ DW.[27]

As further discussed in a subsequent section and was indicated in previous reports, total carotenoids and lycopene amounts increased according to maturity stages, although cultivar/variety differences also influenced such variations.[28]

### Phenolic composition

Total and individual phenolic compounds are summarised in Table 3. A chromatogram of the *Raf* variety showing the mass spectra of identified phenolic compounds is shown in Supporting Fig. 1. The higher phenolics content was found in *Pera* (726), followed by *Racimo* (392), *Cherry* (304) and *Raf* (211 mg kg$^{-1}$ DW). Similar results were observed in a previous study,[29] in which *Daniela* and *Raf* varieties accounted for 343 and 234 mg kg$^{-1}$ DW, respectively, figures in the range of those reported in the present work. Otherwise, significant differences were found in the amount of chlorogenic acid, rutin and quercetin in *Pera* with respect to the other varieties. In all varieties, chlorogenic acid and rutin were the predominant phenolics, as was previously found in *Cherry* variety.[22]

### Sterols composition

Studies have revealed that the intake of appropriate amounts of phytosterols reduces the risk of heart diseases, being this action more effective than the reduction in the intake of saturated fats.[11] In addition, some reports indicated that phytosterols may have anti-tumour activity, including that exercised against colon cancer.[12]

The sterol profiles are given in Table 4. Campesterol, stigmasterol, stigmastanol and $\beta$-sitosterol were detected in all tomato varieties. Three of them – campesterol, stigmasterol, and $\beta$-sitosterol – are usually found in other vegetables.[30] All tomato varieties showed similar amounts of sterols, and the total ranged from 918 (*Racimo*) to 1570 (*Cherry*) mg kg$^{-1}$ DW. $\beta$-Sitosterol was in the upper range in all varieties, while stigmastanol was the minor component, and concentrations obtained in this work agree with previous results.[30] Differences in sterol contents in tomato fruits have been attributed to maturity stage,[31] with the stigmasterol/$\beta$-sitosterol ratio very changeable in tomato.

### Fatty acid profiles of tomato varieties

The FA profiles of analysed tomato varieties are given in Table 5. Total FAs were similar in all cases, ranging from 17 (*Raf*) to 24 g kg$^{-1}$ DW (*Racimo*). The most prominent FA was linoleic acid (LA, 18:2*n*6), which ranged from 49.2 (*Raf*) to 54.1 (*Cherry*) % of total FAs, followed by palmitic acid (PA, 16:0), while OA and $\alpha$-linolenic acid (ALA, 18:3*n*3) were found in similar percentages. These results are similar to those obtained in previous reports, which revealed a similar FA profile in different tomato cultivars.[9] The relatively high PUFA amounts found in this work are in good agreement to previous reports,[32] in which PUFA represents more than 50% of total FAs in tomato seeds. As expected, the total FAs in all tomato varieties was low, a result which agrees with previous reports,[33] and thus this fact prevents it being considered that such lipids are nutritionally relevant.[19] However, some FAs are important cytotoxic agents against colorectal cancer cells, as previously reported.[3]

**Table 3.** Phenolic compounds of four tomato varieties (mg $kg^{-1}$) dry wt

| Variety | Chlorogenic acid | Rutin | Quercetin | Luteolin/kaempherol | Total |
|---|---|---|---|---|---|
| *Cherry* | $54 \pm 7^{a}$ | $242 \pm 10^{a}$ | $1 \pm 15^{a}$ | $7 \pm 13^{a}$ | $304 \pm 26^{a}$ |
| *Racimo* | $60 \pm 10^{a}$ | $327 \pm 8^{b}$ | $1 \pm 15^{a}$ | $4 \pm 17^{a}$ | $392 \pm 31^{b}$ |
| *Raf* | $37 \pm 13^{b}$ | $167 \pm 6^{c}$ | $1 \pm 14^{a}$ | $6 \pm 16^{a}$ | $211 \pm 18^{c}$ |
| *Pera* | $167 \pm 8^{c}$ | $545 \pm 11^{d}$ | $12 \pm 10^{b}$ | $2 \pm 12^{a}$ | $726 \pm 34^{d}$ |

Results are expressed as the average ± SD of the analysis of five different samples of each variety ($n = 5$ in triplicate).
Values not sharing the same superscript letters in the same column are significantly different from one another by Duncan's multiple range test ($P < 0.05$).

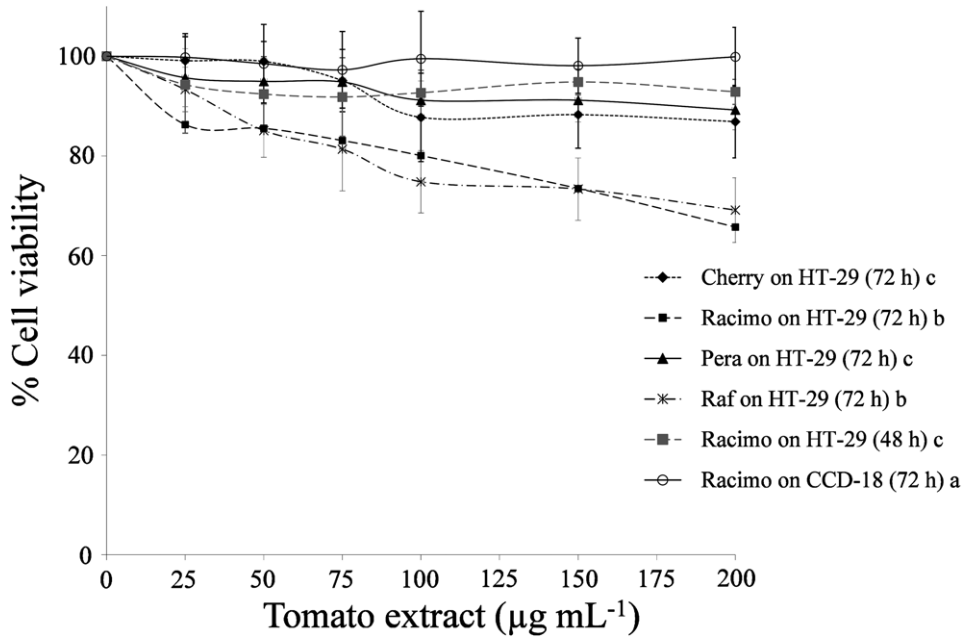


**Figure 1.** MTT assay. Concentration–response plot for the HT-29 colon cancer cell line following 48-h (*Racimo* tomato var.) and 72-h treatment (all tomato varieties), and for CCD-18 (72-h, *Racimo* var.) for extracts made with ethanol/water (1:1, v/v). Means of five independent experiments effected in triplicate and error bars, which represent standard deviations. Concentration–response curves not sharing the same superscript letters are significantly different from one another by Duncan's multiple range test ($P < 0.05$).

**Tomato extracts activities on HT-29 cancer cells viability**

To check the actions of tomato varieties on colorectal cancer cells viability, cell proliferation and cytotoxicity, fruit extracts were made by using two solvents, ethanol/water (1:1, v/v) and petroleum ether. Polarity differences allows them to extract compounds with a different ranges of solubility; thus, the biological activity of different tomato compounds was discerned depending on their hydrophilic or lipophilic characteristics.[3] $^{1}$H NMR analyses showed that petroleum ether extracts contained a mixture of glycerol FA esters (mainly derived of LA and OA), as well as lycopene and $\beta$-carotene in minor amounts, whereas the ethanol/water extract was mainly composed of carbohydrates, for instance fructose and glucose. All these results agree with a previous study.[3] The compounds contained in the petroleum ether extract were responsible for the high inhibitory effects shown below, which act individually or in a synergistic way. For instance, carotenoids are insoluble in water, but fat improves their dispersion and facilitates the contact with cell membranes.[34]

The HT-29 (colorectal cancer) and CCD-18 (normal colon enterocyte) human cell lines were used to check tomato extracts, at different concentrations and after 48 and 72 h incubation, by using the MTT cell viability assay. As depicted in Fig. 1 (ethanol/water extract) and Fig. 2 (petroleum ether extract), there were significant concentration-dependent inhibitory effects of both extracts, with the petroleum ether extract being the most active against cell viability (Fig. 2). The half maximal inhibitory concentration ($IC_{50}$) values obtained from the petroleum ether extract were below 150 µg $mL^{-1}$. On the other hand, the ethanol/water extracts of all tomato varieties did not reach $IC_{50}$ values at the assayed concentrations (Fig. 2). Previously, we referred to the high activity against HT-29 cells viability of petroleum ether extracts made with different tomato varieties,[3] as well as about the high amounts of vitamin C, fibre, selenium, FAs, lycopene and $\beta$-carotene of this variety.[20]

In this work, *Racimo* variety extracted with petroleum ether was the most active extract against HT-29 cells viability. As depicted in Fig. 2, there were significant time- and concentration-dependent inhibitory effects of this extract on HT-29 cells proliferation. When compared to untreated control, 150 µg $mL^{-1}$ of *Racimo* extract inhibited HT-29 cells proliferation by approximately 42% and 61% at 48 and 72 h respectively ($P < 0.05$). This fact could be attributed to the high contents in carotenoids (Table 2) and FAs (Table 4) detected in this variety. Thus, this cytotoxic activity could presumably be attributed to lycopene, which has a wide range of health benefits.[35] However, other compounds might also exert

  

**Table 4.** Sterols content of four tomato varieties (mg $kg^{-1}$ dry wt)

| Variety | Campesterol | Stigmasterol | Stigmastanol | $\beta$-Sitosterol | Total |
|---|---|---|---|---|---|
| *Cherry* | $180 \pm 12^{a}$ | $380 \pm 19^{a}$ | $10 \pm 1^{a}$ | $1000 \pm 33^{a}$ | $1570 \pm 13^{a}$ |
| *Racimo* | $100 \pm 5^{b}$ | $260 \pm 22^{b}$ | $38 \pm 5^{b}$ | $520 \pm 15^{b}$ | $918 \pm 8^{b}$ |
| *Raf* | $140 \pm 9^{c}$ | $510 \pm 34^{c}$ | $35 \pm 5$b | $710 \pm 23^{c}$ | $1395 \pm 10^{c}$ |
| *Pera* | $170 \pm 14^{a}$ | $400 \pm 23^{d}$ | $30 \pm 4^{b}$ | $650 \pm 41^{d}$ | $1250 \pm 9^{d}$ |

Results are expressed as the average ± SD of the analysis of five different samples of each variety ($n = 5$ in triplicate).
Values not sharing the same superscript letters in the same column are significantly different from one another by Duncan's multiple range test ($P < 0.05$).

**Table 5.** Fatty acid profiles of four tomato varieties

| | FA% of total FAs | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Variety | Palmitic (PA, 16:0) | Palmitoleic (POA, 16:1*n*7) | Stearic (SA, 18:0) | Oleic (OA, 18:1*n*9) | Vaccenic (VA, 18:1*n*7) | Linoleic (LA, 18:2*n*6) | $\alpha$-Linolenic (ALA, 18:3*n*3) | Total FAs (g $kg^{-1}$ DW) |
| *Cherry* | $12.4 \pm 3.5^{b}$ | – | $3.1 \pm 0.8^{a}$ | $15.8 \pm 1.1^{c}$ | $0.5 \pm 0.3^{a}$ | $54.1 \pm 3.8^{a}$ | $8.7 \pm 2.6^{c}$ | $23 \pm 3^{a}$ |
| *Racimo* | $18.0 \pm 1.5^{a}$ | $0.32 \pm 0.4^{a}$ | $3.9 \pm 0.8^{a}$ | $18.9 \pm 1.2^{a}$ | $0.5 \pm 0.2^{a}$ | $53.2 \pm 3.6^{a}$ | $4.7 \pm 2.1^{a}$ | $24 \pm 3^{a}$ |
| *Raf* | $22.5 \pm 1.0^{c}$ | – | $2.8 \pm 0.7^{a}$ | $9.0 \pm 1.1^{a}$ | – | $49.2 \pm 3.5^{a}$ | $16.0 \pm 2.7^{b}$ | $17 \pm 2^{b}$ |
| *Pera* | $19.7 \pm 1.1^{a}$ | – | $3.1 \pm 0.9^{a}$ | $11.7 \pm 1.2^{b}$ | $0.6 \pm 0.3^{a}$ | $49.4 \pm 2.6^{a}$ | $15.3 \pm 2.6^{b}$ | $21 \pm 2^{a}$ |

Results are expressed as the average ± SD of the analysis of five different samples of each variety ($n = 5$ in triplicate).
Values not sharing the same superscript letters in the same column are significantly different from one another by Duncan's multiple range test ($P < 0.05$)

antiproliferative activity on cancer cells, for instance phenolic compounds, which were previously tested in HeLa cells.[9]

### Selectivity of the extracts on different cells types

In order to find differences in cell viability between cancer and normal colon cells, the most active extract (*Racimo*) was assayed on the CCD-18 normal colon cell line at different concentrations. In all cases, negative effects on cell growth inhibition (Fig. 1 and Fig. 2) were obtained. Although this experiment confirms the absence of immediate effects on normal colon cells, by inducing proliferation or decreasing viability, further research, checking the effects of tomato extracts on normal cells during a higher number of cell cycles, might be helpful in order to discern the scope of such absence. The effects of tomato carotenoids on arresting of cell cycle progression and apoptosis induction in HT-29 cells were the mechanisms involved in such selectivity, as previously demonstrated.[3]

### LDH cytotoxicity assay

This assay was carried out to determine the ratio of LDH released into the culture medium after cell membrane damage over total LDH in the intact cells. Both the MTT and LDH assays were conducted at similar culture conditions in order to compare results (Fig. 3). Notice that LDH values for all tested concentration were lower than MTT ones, and the results were closely related at higher tested concentrations (100, 150 and 200 $\mu g\,mL^{-1}$). Such differences could be attributed to the specificity of the LDH test when compared to the MTT assay. Clarifying this issue, a previous research showed that LDH leakage occurs only when reactive oxygen species related to cell death appear, which are responsible for mitochondria damages.[36] On the contrary, MTT assay shows cell growth inhibition at lower concentrations of anti-tumour agents than LDH test. This is true because MTT reduction is not an indicator of cell death, but rather constitutes a measure of the relative metabolic activity, as demonstrated in a previous study focused in HT-29 cells.[37]

### Synergistic activity between olive oil and tomato carotenoids and/or phenolic extracts

Tomato fruits and olive oil are two of the main components of the Mediterranean diet, and their frequent consumption is related to a lesser incidence of several cancer types.[14,16] Accordingly, we designed different assays to determine possible synergism among the main active compounds of tomato fruits, carotenoids and phenolics, in conjunction with olive oil (1:1, w/w) against HT-29 cells. Results are depicted in Fig. 4 (phenolics) and Fig. 5 (carotenoids). As shown in Fig. 4, olive oil induces significant time- and concentration-dependent inhibitory effects on HT-29 cells proliferation. When compared to untreated control, 150 $\mu g\,mL^{-1}$ of olive oil inhibited HT-29 cell proliferation by approximately 9% and 55% at 48 and 72 h, respectively ($P < 0.05$). This effect has been attributed to the unsaponifiable fraction of olive oil, which selectively induces apoptosis in HT-29 colorectal cancer cells via the activation of caspase-3 by a p53-independent mechanism, as well as inducing significant changes in the cytoskeleton of this human cell line.[38] Conversely, it was found that the phenolic extract of tomato showed a moderate reduction on both cell viability and cell proliferation (Fig. 4), and even hinders the antiproliferative effect of olive oil when both are essayed jointly.

The effects on cell viability and cell proliferation of olive oil and carotenoids are displayed in Fig. 5. In similar form to olive oil, carotenoids exercise significant time- and concentration-dependent inhibitory effects on HT-29 cell proliferation; when compared to untreated control, 150 $\mu g\,mL^{-1}$ carotenoids inhibited HT-29 cell proliferation by approximately 19% and 45% at 48 and 72 h, respectively ($P < 0.05$). Furthermore, when both solutions were applied to the cells together, a strong synergistic action was noted, and when compared to untreated

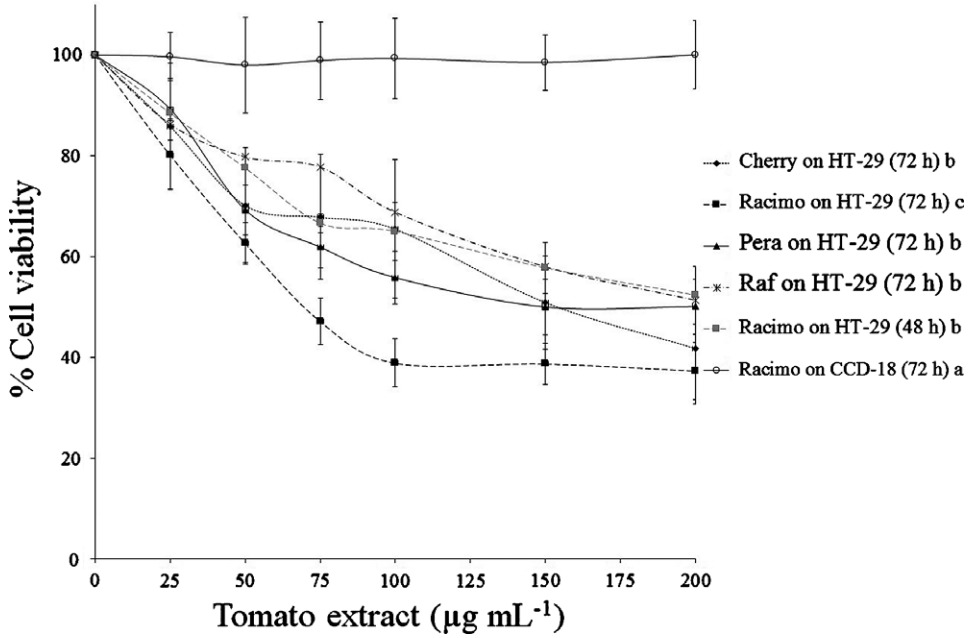


**Figure 2.** MTT assay. Concentration–response plot for the HT-29 colon cancer cell line following 48-h (*Racimo* var.) and 72-h treatment (all tomato varieties), and for CCD-18 (72-h, *Racimo* var.) made with petroleum ether. Means of five independent experiments effected in triplicate and error bars, which represent standard deviations. Concentration–response curves not sharing the same superscript letters are significantly different from one another by Duncan's multiple range test ($P < 0.05$).

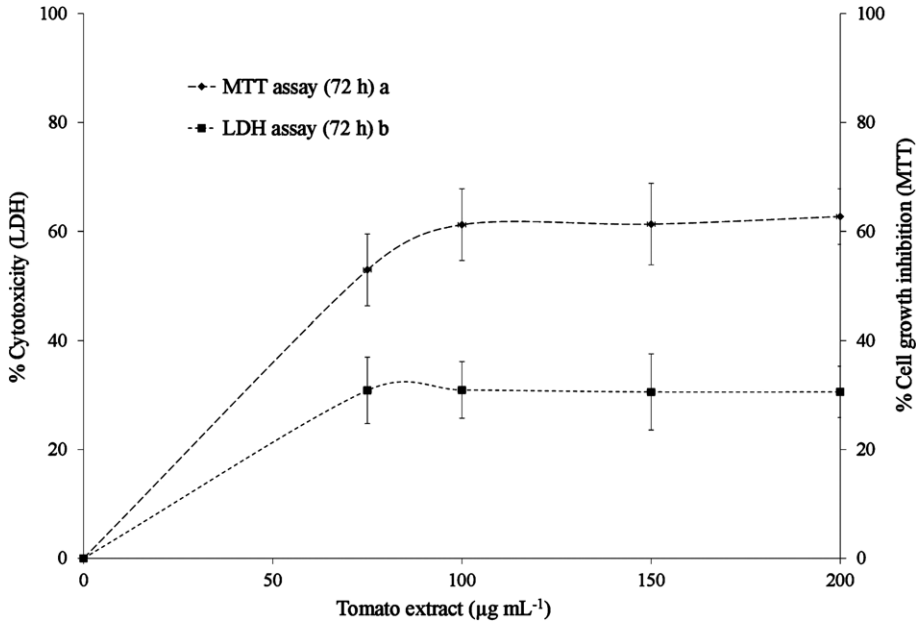


**Figure 3.** LDH release and MTT cell viability assays. Concentration–response plot for the HT-29 colon cancer cell line following 72-h treatment, for extracts of tomato *Racimo* var. made with petroleum ether. Means of five independent experiments effected in triplicate and error bars, which represent standard deviations. Concentration–response curves not sharing the same superscript letters are significantly different from one another by Duncan's multiple range test ($P < 0.05$).

control, 150 μg mL$^{-1}$ olive oil + tomato carotenoids (1:1, w/w), inhibited HT-29 cell proliferation by approximately 55% and 75% at 48 and 72 h, respectively ($P < 0.05$). This finding agrees with previous studies, where it is stated that benefits in cancer prevention due to both tested dietary components can be influenced by several factors, such as food matrix, processing and cooking methods, and interactions during digestion with other dietary compounds like fibre, lipids and so on.[13] Other MTT assays were effected by combining equal amounts of tomato phenolics and carotenoids with olive oil and 48 and 72 h; however, antagonistic effects were observed in HT-29 cells, both at 48 and 72 h. In addition, several MTT assays were conducted by using tomato carotenoids and the unsaponifiable fraction of olive oil, given that the latter has proved cytotoxicity;[38] however, cell grown inhibition was detected in a similar extent to that were exercised by both components acting individually (data not shown).

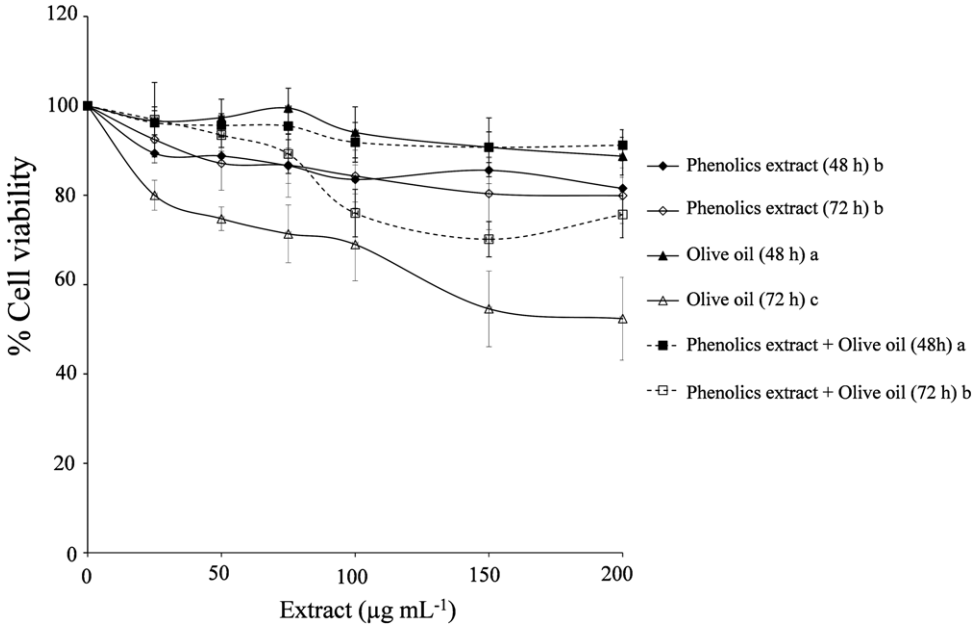


**Figure 4.** MTT assay. Concentration–response plot for the HT-29 colon cancer cell line following 48-h and 72-h treatment for phenolics extract obtained from tomato *Racimo* variety, olive oil and a blend of phenolics/olive oil (1:1, w/w). Means of five independent experiments effected in triplicate and error bars, which represent standard deviations. Concentration–response curves not sharing the same superscript letters are significantly different from one another by Duncan's multiple range test ($P < 0.05$).

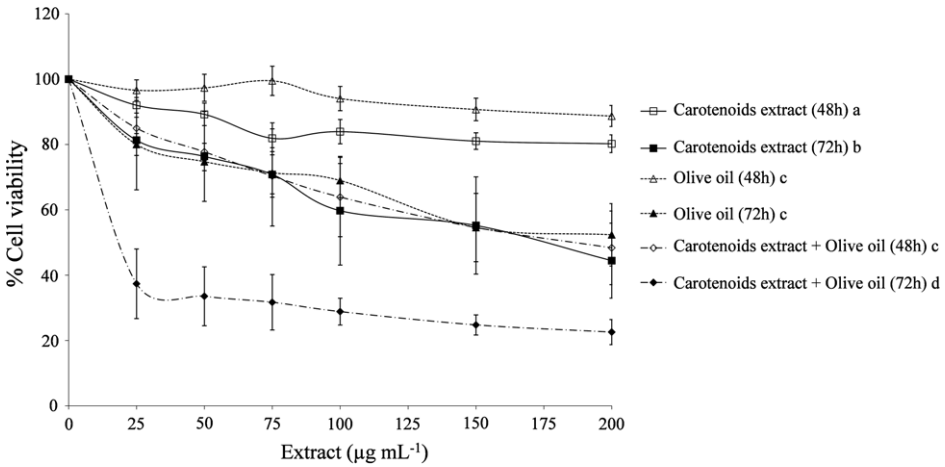


**Figure 5.** MTT assay. Concentration–response plot for HT-29 colon cancer cell line following 48-h and 72-h treatment for carotenoids extract obtained from tomato *Racimo* variety, olive oil and a blend of carotenoids/olive oil (1:1, w/w). Means of five independent experiments effected in triplicate and error bars, which represent standard deviations. Concentration–response curves not sharing the same superscript letters are significantly different from one another by Duncan's multiple range test ($P < 0.05$).

The concentrations of carotenoids and lipids checked in the assays performed in this study (Fig. 5), which induced anti-tumour actions in HT-29 cells, were within the range of those found in human plasma.[39] Furthermore, *in vitro* models show that absorption of lycopene by colorectal carcinoma cells is efficient,[40] and epidemiological studies demonstrated that dietary tomato carotenoids reduce the risk of occurrence of some cancer types,[7] for instance through actions on gene expression.[4] Thus, beneficial actions against colorectal cancer might be expected after the joint intake of both tomato carotenoids and olive oil. However, factors responsible for bioavailability of lycopene is not yet clear;[40] thus, *in vivo* models are needed to optimise effective anti-tumour actions by co-administration of designed amounts of tomato carotenoids and some selected fats, such as olive oil.

## CONCLUSIONS

The results of the present study demonstrate that tomato fruits constitute a healthy source of some bioactive compounds, with differences among varieties; that is, *Racimo* is a prominent source of lycopene; phenolics were high in *Pera* variety; sterols were low in all tested varieties; and concerning FAs, LA predominates in all varieties. Although significant differences among tested tomato varieties were detected, all of them *in vitro* inhibited HT-29 colorectal cancer cell proliferation, and exercising a selective action on cancer cells by considering the lack of effects on the CCD-18 normal colon cells. Furthermore, by supplying jointly both tomato carotenoids and olive oil, it has been demonstrated synergy *in vitro* between these two components in inhibiting HT-29 cell proliferation. Conversely, phenolics induced minor effects on HT-29 cells, and when

administered concurrently with olive oil and carotenoids, phenolics hinder the actions of the latter.

## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Short communication

# Sardinian Boraginaceae are new potential sources of gamma-linolenic acid

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ABSTRACT

The aim of this work was to establish the richness in γ-linolenic acid (GLA, 18:3n6) and stearidonic acid (SDA, 18:4n3) of several Sardinian Boraginaceae species. To this end, seeds of selected species were collected from their natural habitats and analysed. The highest GLA contents were found in the seed oils of two endemic *Borago* taxa, i.e. *B. morisiana* (24.4 and 24.6% GLA of total fatty acids for samples from San Pietro Island and Sardinia Island, respectively), and 22.9% GLA for *B. pygmaea*. Both *Borago* species contained more GLA than *B. officinalis* collected in the same ecosystems. SDA was found in significant amounts in *Echium plantagineum* seed oil from the Lattias Mountains (15% SDA of total fatty acids). It is notable that both *Borago* GLA-rich species are under threat of extinction, thus revealing the importance of the preservation of the natural Sardinian ecosystems for endangered species and human health.



## 1. Introduction

γ-Linolenic acid (GLA, *all-cis*-6,9,12-octadecatrienoic acid, 18:3n6) and stearidonic acid (SDA, *all-cis*-6,9,12,15-octadecatetraenoic acid, 18:4n3) are polyunsaturated fatty acids (PUFAs) belonging to the n – 6 and n – 3 series, respectively. Both FAs are produced in the body from their metabolic precursors linoleic acid (LA, *all-cis*-9,12-octadecadienoic acid, 18:2n6) and α-linolenic acid (ALA, *all-cis*-9,12,15-octadecatrienoic acid, 18:3n3), respectively, through the action of the enzyme Δ6-desaturase. GLA is further metabolized to dihomo-γ-linolenic acid (DGLA, *all-cis*-8,11,14-eicosatrienoic acid, 20:3n6), which undergoes oxidative metabolism by cyclooxygenases and lipoxygenases to produce anti-inflammatory eicosanoids (series-1 prostaglandins and series-3 leukotrienes) that are hormone-like bioactive compounds involved in the regulation of various physiological mechanisms in animals and humans (Guil-Guerrero, 2007; Horrobin, 1992; Meesapyodsuk & Qiu, 2012). In addition, GLA and its metabolites also affect the expression of various genes, having a significant role in immune functions and apoptosis (Kapoor & Huang, 2006), and several studies indicate that GLA possesses anti-cancer properties, including inhibition of cell proliferation and induction of apoptosis (Menéndez et al., 2001; Xu & Qian, 2015). Furthermore, recent works have attributed prominent health benefits to dietary supplementation with GLA, such as improved blood lipid profile and skin perspiration, showing promising effects in the treatment of dermatitis, skin hyperproliferation and osteoporosis among other syndromes (Kawamura et al., 2011; Tasset-Cuevas et al., 2013; Tso, Caldwell, Lee, Boivin, & DeMichele, 2012). Consequently, given its widely reported beneficial physiological actions, GLA is used increasingly in the cosmetic (Griinari & Bruheim, 2013) and food industries (Flider, 2005).

SDA is elongated and desaturated to eicosapentaenoic acid (EPA, *all-cis*-5,8,11,14,17, 20:5n3), which is the precursor of anti-inflammatory eicosanoids (series-3 prostaglandins and series-5 leukotrienes), and docosahexaenoic acid (DHA, *all-cis*-4,7,10,13,16,19, 22:6n3), which is required for the maintenance of normal brain function in adults (Guil-Guerrero, 2007). There is great interest in SDA-rich oils because the biosynthesis of EPA starting from SDA is much more efficient than from ALA (Guil-Guerrero, 2007). Both GLA and SDA sources are scarce in nature. Despite some microbiological cultures, GLA is primarily obtained

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from three plants: borage (*Borago officinalis* L., 21–23% GLA of total FAs); evening primrose (*Oenothera biennis* L., 9–12% GLA of total FAs); and blackcurrant (*Ribes nigrum* L., 15–20% GLA of total FAs) (Guil-Guerrero, García-Maroto, & Giménez-Giménez, 2001; Gunstone, 1992). The percentage of SDA in oils is low, with only *Buglossoides arvensis* (>14% SDA of total FAs) (Guil-Guerrero et al., 2001) containing significant amounts and, because of this, the oil from this species has been patented (Hebard, Coupland, Boughton, & Surette, 2008). The intake of oils rich in GLA and SDA reduces the amount of oil needed to achieve any health benefits compared to oils with lower amounts of these PUFAs as well as caloric intake. Furthermore, GLA and SDA purification processes can be carried out more easily when starting with GLA- or SDA-rich oils. Thus, the search for such oils is on-going. Boraginaceae species have been surveyed for seed oils with a high content of both GLA and SDA (Guil-Guerrero, 2007), and several endemic Boraginaceae species have been identified as potential new sources of GLA and SDA (Guil-Guerrero, Gómez-Mercado, Ramos-Bueno, Rincón-Cervera, & Venegas-Venegas, 2014; Guil-Guerrero, Rincón-Cervera, Gómez-Mercado, Ramos-Bueno, & Venegas-Venegas, 2013).

Interestingly, some of the best species, in terms of GLA and SDA contents, are being cultivated as SDA sources, e.g. *Echium plantagineum* and *Buglossoides arvensis*. If GLA is the target PUFA, *Borago officinalis* is still the most suitable option. However, some *Borago* species, occurring mainly in Tyrrhenian islands (Mediterranean Basin), remain unanalysed. These places are considered centres of relictual endemism of Boraginaceae and a source of some endemic *Borago* species (Médail & Quézel, 1999).

This paper focuses on the characterization of FA profiles of several Sardinian Boraginaceae species, which have remained unanalysed until now. Among them, two *Borago* species showed higher GLA contents than either wild or farmed *B. officinalis*.

## 2. Experimental procedures

### 2.1. Sample collection

Seeds were collected from their natural habitats (Table 1). Upon arriving at the laboratory, after cleaning and labelling, seeds were placed in a glass desiccator until analysis.

### 2.2. Oil extraction and transesterification and fatty acid analyses

Seeds were ground with the aid of a mortar, and then 150–200 mg were taken for further analysis. Extraction and trans-esterification were simultaneous, and FA analyses and quality control were carried out according to previous reports (Guil-Guerrero et al., 2013, 2014). Briefly, FA methyl esters (FAMEs) were analysed using a Focus GC (Thermo Electron, Cambridge, UK) equipped with a flame ionization detector (FID) and an Omegawax 250 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Supelco, Bellefonte, USA). Peaks were identified by retention times obtained for known FAME standards (PUFAs No. 1, 47033; methyl γ-linolenate 98.5% purity, L6503; and methyl stearidonate 97% purity, 43959 FLUKA) from Sigma, (St. Louis, USA), while FA contents were estimated by using methyl pentadecanoate (15:0; 99.5% purity; 76560 Fluka) from Sigma as internal standard.

FA compositions (FA% of total FAs area), expressed as mean value obtained from three different samples analysed in triplicate, are given in Table 2. Standard deviations (SD) were routinely less than 5% of means.

**Table 1**
Data on collection of Sardinian Boraginaceae species.

| Species | Sample location | Herbarium code | Geographical coordinates | Collection date |
|---|---|---|---|---|
| *Tribe Boragineae* | | | | |
| *Anchusa capellii* Moris | Cagliari: Esterzili. Sta. Vittoria mountain | HUAL 25607 | N 39.759 E 9.304 | 07/04/2014 |
| *Anchusa crispa* Viv. subsp. *maritima* (Vals.) Selvi & Bigazzi | Olvia-Tempio. Isola Rossa | HUAL 25609 | N 41.015 E 8.887 | 05/12/2013 |
| *Anchusa crispa* Viv. subsp. *maritima* (Vals.) Selvi & Bigazzi | Olvia-Tempio. Pirotto Li Fratti. Bahía delle Mimose | HUAL 25612 | N 40.945 E 8.826 | 05/12/2013 |
| *A. formosa* Selvi, Bigazzi & Bacchetta | Cagliari: Uta. Arcosu mountain | – | N 39.185 E 5.854 | 06/20/2013 |
| *Borago morisiana* Bigazzi *et* Ricceri | Isola S. Pietro. Carbonia Iglesias: Carloforte. Calavinagra | HUAL 25639 | N 39.163 E 8.242 | 07/03/2014 |
| *Borago morisiana* Bigazzi *et* Ricceri | Oristano: Laconi. Tanca de Cuccuru | HUAL 25965 | N 39.874 E 9.091 | 07/04/2014 |
| *Borago officinalis* L. | Cagliari. Near Lattias mountain. | HUAL 25637 | N 39.144 E 8.861 | 05/09/2013 |
| *Borago officinalis* L. | Medio Campidano: Gonnosfanadiga. Linas mountain | HUAL 25633 | N 39.435 E 8.624 | 05/12/2013 |
| *Borago pygmaea* (DC.) Chater & Greuter | Ogliastra: Gairo. Montarbu di Seui, Girolamo river | HUAL 25608 | N 39.839 E 9.455 | 07/04/2014 |
| *Tribe Echieae* | | | | |
| *Echium anchusoides* Bacchetta, Brullo & Selvi | Medio Campidano: Gonnosfanadiga. Linas mountain, near Punta Cammedda | HUAL 25603 | N 39.436 E 8.638 | 07/06/2014 |
| *Echium italicum* L. | Cagliari: Donori | HUAL 25601 | N 39.451 E 9.155 | 07/07/2014 |
| *Echium plantagineum* L. | Oristano: Assolo | HUAL 25602 | N 39.818 E 8.916 | 07/06/2014 |
| *Echium plantagineum* L. | Cagliari. Near Lattias mountains | HUAL 25640 | N 39.148 E 8.860 | 05/09/2013 |
| *Echium sabulicolum* Pomel | Carbonia-Iglesias. Buggerru: Costa Verde (Near Cala Domestica) | HUAL 25636 | N 39.364 E 8.398 | 05/10/2013 |
| *Tribe Cynoglosseae* | | | | |
| *Cynoglossum barbaricinum* Arrigoni & Selvi | Nuoro: Orgosolo. Monte Novo San Giovanni | HUAL 25604 | N 40.117 E 9.415 | 07/05/2014 |
| *Cynoglossum officinale* L. | Cagliari. Near Lattias mountain | HUAL 25634 | N 39.148 E 8.860 | 05/10/2013 |
| *Tribe Eritrichieae* | | | | |
| *Myosotis arvensis* (L.) Hill | Carbonia-Iglesias. Buggerru: Costa Verde (Near Cala Domestica) | HUAL 25635 | N 39.364 E 8.398 | 05/10/2013 |
| *Myosotis soleirolii* Godr. | Ogliastra: Villagrande Strisaili. Bruncu Spina | HUAL 25606 | N 40.016 E 9.301 | 07/05/2014 |

**Table 2**
Fatty acid profiles of seeds from Sardinian Boraginaceae.

| Species[b] | Fatty acids (FA% of total FAs)[a] | | | | | | | | | | | | | | | | | FA amount (g/100 g seed) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 12:0 | 14:0 | 16:0 | 16:1n7 | 18:0 | 18:1n9 | 18:1n7 | 18:2n6 | 18:3n6 | 18:3n3 | 18:4n3 | 20:0 | 20:1n9 | 22:0 | 22:1n9 | 24:0 | 24:1n9 | |
| *Tribe Boragineae* | | | | | | | | | | | | | | | | | | |
| 07 *Anchusa capellii* | – | – | 9.1 | – | 3.0 | 30.0 | – | 22.2 | 12.0 | 12.4 | 4.0 | – | 4.4 | 0.3 | 2.3 | 0.1 | 0.4 | 24.2 |
| 09 *Anchusa crispa* ssp. *maritima* | – | – | 9.2 | – | 2.6 | 26.5 | – | 23.7 | 12.3 | 12.9 | 3.6 | 0.3 | 4.1 | 0.2 | 4.0 | 0.2 | 0.5 | 16.9 |
| 12 *Anchusa crispa* ssp. *maritima* | – | – | 10.9 | – | 4.7 | 25.3 | – | 23.3 | 11.7 | 13.2 | 3.3 | 0.3 | 3.5 | 0.3 | 3.1 | – | 0.4 | 18.6 |
| - *Anchusa formosa* | – | – | 9.4 | – | 2.4 | 25.0 | – | 21.4 | 14.0 | 15.3 | 5.1 | 0.4 | 4.8 | – | 2.0 | – | 0.4 | 24.8 |
| 39 *Borago morisiana* | 0.2 | 0.8 | 13.2 | 0.2 | 5.9 | 16.6 | 0.3 | 33.0 | 23.4 | 1.1 | 0.8 | 0.5 | 3.1 | – | – | – | 1.1 | 24.2 |
| 65 *Borago morisiana* | 0.3 | 1.0 | 11.6 | 0.28 | 4.6 | 14.9 | 0.5 | 34.1 | 24.6 | 1.4 | 1.0 | 0.1 | 2.5 | 0.3 | 1.9 | – | 1.15 | 15.9 |
| 37 *Borago officinalis* | – | – | 10.0 | 0.2 | 8.7 | 22.0 | – | 31.5 | 19.2 | 0.4 | 0.3 | 0.5 | 3.6 | 0.3 | 1.8 | 0.1 | 1.3 | 27.9 |
| 33 *Borago officinalis* | – | – | 10.9 | – | 8.7 | 22.7 | – | 31.4 | 16.2 | 0.9 | 0.7 | 0.6 | 4.1 | 0.4 | 2.2 | – | 1.3 | 35.1 |
| 08 *Borago pygmaea* | – | – | 14.0 | – | 6.7 | 20.0 | – | 27.4 | 22.9 | 1.3 | 1.2 | 0.5 | 2.8 | 0.3 | 1.9 | 0.2 | 0.8 | 21.9 |
| *Tribe Echieae* | | | | | | | | | | | | | | | | | | |
| 03 *Echium anchusoides* | – | – | 8.4 | – | 3.9 | 16.6 | – | 19.5 | 8.8 | 32.7 | 9.4 | – | 0.6 | 0.3 | – | – | – | 25.4 |
| 01 *Echium italicum* | – | – | 8.5 | – | 3.9 | 13.9 | – | 13.4 | 5.6 | 43.4 | 10.4 | 0 | 0.4 | – | 0.3 | – | 0.2 | 16.6 |
| 02 *Echium plantagineum* | 2.9 | – | 27.9 | – | 4.5 | 8.3 | – | 26.2 | 7.4 | 3.9 | 15.0 | 3.7 | – | – | 0.3 | – | 0.2 | 2.05 |
| 40 *Echium plantagineum* | – | – | 9.6 | – | 3.2 | 16.8 | – | 21.1 | 9.3 | 30.9 | 8.3 | – | 0.7 | – | – | – | – | 18.8 |
| 36 *Echium sabulicolum* | – | – | 8.9 | – | 3.7 | 11.6 | – | 18.8 | 9.5 | 35.7 | 11.3 | – | 0.6 | – | – | – | – | 18.0 |
| *Tribe Cynoglosseae* | | | | | | | | | | | | | | | | | | |
| 04 *Cynoglossum barbaricinum* | – | – | 17.4 | – | 3.5 | 42.0 | – | 4.3 | 1.0 | 17.1 | 4.1 | 0.8 | 4.1 | 0.4 | 4.0 | – | 1.5 | 4.0 |
| 34 *Cynoglossum officinale* | – | 1.1 | 9.4 | – | 1.6 | 49.2 | – | 2.2 | 0.5 | 13.6 | 3.7 | 1.1 | 5.9 | 1.2 | 7.4 | 0.5 | 2.7 | 3.3 |
| *Tribe Eritrichieae* | | | | | | | | | | | | | | | | | | |
| 35 *Myosotis arvensis* | – | – | 10.9 | – | 2.7 | 25.1 | – | 27.5 | 6.1 | 12.0 | 8.3 | 0.6 | 3.5 | 0.4 | 2.2 | | 0.9 | 26.4 |
| 06 *Myosotis soleirolii* | – | – | 11.9 | – | 4.2 | 35.0 | – | 27.4 | 4.4 | 5.7 | 6.4 | 1.0 | 4.3 | – | – | – | – | 25.5 |

[a] SD was routinely less than 5% of means (n = 3).
[b] The two numbers preceding each species are the last two digits of the herbarium code shown in Table 1.

## 3. Results and discussion

Basic data for the collected samples are shown in Table 1, while the FA profiles of seed oils are reported in Table 2, grouped by tribes. This is because the FA profiles of seed oils from Boraginaceae species have taxonomic significance due to the differential activities of the enzyme Δ6-desaturase, which preferentially desatures LA to GLA, but also ALA to SDA, and determines PUFA profiles in Boraginaceae oils (García-Maroto, Mañas-Fernández, Garrido-Cárdenas, & López Alonso, 2006). Boragineae are characterized by high percentages of LA and GLA; Echieae by high percentages of n-3 PUFAs, i.e. ALA and SDA; Cynoglosseae by high oleic acid (OA, 18:1n9) percentages; and Eritrichieae by similar percentages of OA and LA. All these observations are in agreement with previous findings (Guil-Guerrero et al., 2001, 2013, 2014; Velasco & Goffman, 1999; Özcan, 2008).

The highest GLA percentage was found in the seeds of the two endemic *Borago* surveyed, i.e. *B. morisiana* (24.4 and 24.6% GLA for San Pietro Island and Sardinia Island respectively), and *B. pygmaea* (22.9% GLA). When comparing the amounts of total FAs/100 g seeds in samples of different origin, *B. morisiana* from San Pietro Island contained 24.2 g FAs/100 g seeds, while only 15.9 g FAs/100 g seeds were found in the same species from Sardinia Island. This could be attributed to differences in environmental conditions, although in both cases the plant was found on the margins of streams; San Pietro samples were however near the sea while those from Sardinia Island were in the mountains. Differences in temperature, humidity, soil composition and other abiotic factors could influence composition, although genetic differences between the two analysed populations might also contribute, despite the closeness of their FA profiles otherwise.

Seeds from other two *B. officinalis* populations were collected containing 19.2 and 16.2% GLA, respectively (Table 2). Thus, in the wild, the two endemic *Borago* taxa analysed surpassed the GLA% found in *B. officinalis*. However, after careful selection, *B. officinalis* seed oil can reach 21–23% GLA (Guil-Guerrero et al., 2001; Gunstone, 1992). Potentially, the two endemic *Borago* species analysed in this work might benefit from using the same techniques to achieve much higher GLA percentages than *B. officinalis*. Other species with high GLA percentages were the four *Anchusa* species surveyed (11.7–14.0% GLA of total FAs).

*Echium plantagineum* seed oil from the Lattias Mountains contained the highest SDA percentage (15% SDA of total FAs) followed by *E. sabulicolum* and *E. italicum* (11.3 and 10.4% SDA of total FAs respectively). Surprisingly, this *E. plantagineum* sample was very different from others samples collected in Assolo, (8.3% SDA) with also ten times more ALA. When comparing both FA profiles with figures reported for the same species (Guil-Guerrero, 2007), the sample from Assolo agreed with previous reports. Thus, the sample from the Lattias Mountains seems to constitute a different chemotype. Although both samples showed morphological characteristics compatible with *E. plantagineum*, they presented some morphological differences in habit and indument.

Considering the whole seed weight, total FA amount ranged between 4% in *Cynoglossum barbaricinum* and 35.4% in *B. officinalis* from the Lattias Mountains. Based on the FA percentage contained in the seeds of *B. morisiana* from San Pietro Island (24.2% of total seed weight) and the GLA percentage (24.4%), ca. 6 g GLA/100 g seeds would be expected in this species. *E. anchusoides* seeds produced the highest SDA amount (2.4 g SDA/100 g seeds). Considering that the oil content could be easily modified by agronomic practices or culture lines selection (Berti et al., 2007), GLA percentage or the amount of oil produced might be significantly increased. However, an increase in oil content does not necessarily lead to an increased GLA amount, so careful crop selection considering both factors would be necessary. Further studies to assess which climatic areas would be appropriate to grow these species are also required.

It is notable that *B. morisiana*, which was the best species for GLA production, is under threat of extinction; both populations, which are the only ones known, show a steady reduction in numbers of individuals (Bacchetta, Fenu, & Mattana, 2008). Thus, environmental authorities should take measures to preserve and improve their natural habitats: results from this work clearly point out towards potential uses as agronomic resources of some wild endangered species, as sources of beneficial FAs, to enhance the economic development of the areas where they grow.

In conclusion, both endemic *Borago* taxa from Sardinia analysed in this work contained more GLA than either farmed or wild *B. officinalis*. On this basis, these plants might be considered as suitable candidates for cultivation and selection with an interesting potential in terms of agronomic aptitudes and/or GLA percentage. Other target actions for research and development of new GLA-producing taxa could be hybridization and further selection between these species or between any of them with cultured borage as well as application of genetic engineering tools for Δ-6 desaturase gene transfer from these plants to other Boraginaceae species in order to increase the GLA percentage in their seed oils.

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JQS Journal of Quaternary Science QRA Quaternary Research Association

# Mammoth resources for hominins: from omega-3 fatty acids to cultural objects

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ABSTRACT: The mammoth is assessed here both for its cultural significance as well as a source of dietary *n*-3 (omega-3) fatty acids in Palaeolithic societies. For this, we analysed fats from several frozen mammoths found in the permafrost of Siberia (Russian Federation) and conducted a comprehensive literature review on the relationships of hominins with mammoths throughout the Stone Age. Different mammoth samples were included in this study, all very close to the Upper Palaeolithic. All samples were analysed by gas liquid chromatography-mass spectrometry and gas liquid chromatography-flame ionization detection. Hominins consumed mammoths throughout the Palaeolithic, while remains of this animal were used as building materials as well as to fabricate different tools and decorative objects, and thus it is possible to link cultural development and mammoth consumption. Based on the fatty acid profiles found, fat samples from two mammoths were in apparently good preservation, yielding α-linolenic acid percentages very close to values found in extant elephants, thus allowing an assessment of their feasibility as a source of essential fatty acids for Palaeolithic hunters. As demonstrated in this work, mammoths constituted a cultural resource in addition to contributing to fulfilling the *n*-3 fatty acid needs of Palaeolithic hominins in Europe. Copyright © 2018 John Wiley & Sons, Ltd.

KEYWORDS: docosahexaenoic acid; omega-3 fatty acids; subcutaneous fat; upper Palaeolithic; woolly mammoth.

## Introduction

The worldwide association of hominins with proboscideans is well documented, for instance, those of *Homo erectus* with elephants, which has been reported to be due to the dependence of humans for fat as a source of energy (Ben-Dor *et al.*, 2011). Such relations should include those developed by Palaeolithic hominins with mammoths, which appeared in Europe from African ancestors about 3 million years ago (Maglio, 1973; Lister, 1996; Lister and Sher, 2001). The most prominent species were *Mammuthus meridionalis* Nesti, 1825 from the Late Pliocene to Early Pleistocene; *M. trogontherii* Pohlig, 1885 from the Middle Pleistocene; and *M. primigenius* Blumenbach, 1799 (woolly mammoth), which appeared in Europe soon after 200 ka (Stuart *et al.*, 2000). During the Last Cold period the woolly mammoth was widely distributed, ranging throughout most of Europe, across northern Asia and into the northern half of North America. Its extinction is attributed largely to the global wave of extinction of megafauna (called the 'Mammoth complex') which took place before the Holocene. Such extinction has been attributed to 'overkill' by late Palaeolithic hunters, climatic and environmental changes, or a combination of the two factors (Stuart, 1991, 1999; Martin and Stuart, 1995).

The Palaeolithic diet of hominins in Europe was clearly dominated by animal foods (Svoboda *et al.*, 2005). Strong evidence for this contention is that the carbon and nitrogen isotope values of collagen indicate such dietary trends for Neanderthals (Bocherens *et al.*, 2005). Moreover, the mammoth $\delta^{15}N$ values from the Milovice site (Czech Republic), for instance, are higher than registered by other herbivores (Bocherens *et al.*, 1994, 1996, 1997; Richards *et al.*, 2000). This might be due to the mammoth's food preferences for particular plant species, whereas other herbivores were consuming a wider range of species. The mammoth's $\delta^{15}N$ values are informative, being similar to those found in Neanderthals, rather than other values shown by other prey species, implying that this mammal was one of the main dietary protein/fat sources for Palaeolithic hunters (Bocherens *et al.*, 2005).

The woolly mammoth was a monogastric herbivore, having a digestive physiology similar to other animals belonging to the Mammoth complex, e.g. woolly rhinoceros (*Coelodonta antiquitatis* Blum.), and Lena horse (*Equus lenensis* Russ.) (Boeskorov, 2004). Other components of this complex which were widely hunted were Pleistocene bison (*Bison priscus* Bojanus) and reindeer (*Rangifer tarandus* L.) (Nikolskiy and Pitulko, 2013). The selection of the prey to be hunted predictably had a great significance for Palaeolithic hunters, given that the fat-rich organs were especially needed for survival, as they provided much more energy than meat (Cordain *et al.*, 2000). It is well known that single-stomached animals, such as elephants, rhinoceroses and horses, can change the fatty acid (FA) profiles in their adipose tissue with the intake of fats of different FA composition (Doreau and Ferlay, 1994). Conversely, bison and reindeer, for instance, are ruminants and therefore hydrogenate the carbon–carbon double bonds of the FAs during digestion and, hence, polyunsaturated FAs (PUFAs) disappear, causing a lack of such nutrients in fat deposits (Wood *et al.*, 2008). This determined the availability of PUFAs for Palaeolithic hunters in Europe, as hominins there depended largely on animal resources for subsistence, and some uncertainty exists as to

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whether such food could supply the daily amounts of PUFAs needed for survival (Guil-Guerrero, 2017).

Two C18 long-chain PUFAs (LCPUFAs), linoleic acid (LA, 18:2*n*6), and α-linolenic acid (ALA, 18:3*n*3), are considered essential FAs (EFAs) because they cannot be biosynthesized by humans, and therefore they must be included in the diet. They play a unique metabolic role and are the most efficacious PUFAs for preventing skin lesions, the most obvious symptom of EFA deficiency (Brenna, 2016). Conversely, the C20–22 very-long-chain PUFAs (VLCPUFAs) can be biosynthesized by the consecutive action of several enzymes from their respective dietary EFA precursors: arachidonic acid (ARA, 20:4*n*6) from LA, and eicosapentaenoic acid (EPA, 20:5*n*3) and docosahexaenoic acid (DHA, 22:6*n*3) from ALA (Guil-Guerrero, 2007). ALA is especially abundant in all green organs of plants, some seeds, and in the fatty tissues of monogastric herbivores (Guil-Guerrero, 2007, 2017). Meanwhile, LA is widely distributed in seeds and animal fats, and it undergoes efficient bioconversion to ARA, and therefore *n*-6 deficiency is extremely rare. Today, ample evidence indicates that the bioconversion of ALA to EPA and DHA is very low, while such metabolites are scarce in terrestrial foods. Such bioconversion is rate-limited by the enzyme hepatic Δ6 desaturase, which rapidly declines with age of the animal (Bradbury, 2011). Thus, the maintenance of EPA and DHA status in older individuals may depend primarily upon dietary intakes of preformed EPA and DHA (Burdge and Calder, 2005). Both EPA and DHA are involved in the healthy performance of several physiological functions, for instance blood pressure and blood clotting, and the proper development and functioning of the brain and nervous system. Moreover, DHA is abundant in the grey matter of the brain (Guil-Guerrero *et al.*, 2007). All these factors suggest that EPA and DHA are 'conditionally essential' nutrients (Bradbury, 2011).

Seeking to clarify the sources of *n*-3 PUFAs for Palaeolithic and Mesolithic humans, we reported on the FA profiles of a relict Palaeolithic species of horse (Guil-Guerrero *et al.*, 2013). Later, we found that the subcutaneous fat of horses consumed by Palaeolithic hunters contained notable amounts of *n*-3 LCPUFAs (Guil-Guerrero *et al.*, 2014), while Mesolithic bison (*B. priscus* Bojanus) contained higher amounts of *n*-3 LCPUFAs than in bisons today (Guil-Guerrero *et al.*, 2015).

In this context, we hypothesize that mammoths constituted a notable source of *n*-3 PUFAs and were also cultural objects for Palaeolithic hunters during the last cold period, both in Eurasia and in America. This paper reports on the data available in the literature concerning the relationship between mammoths and humans in the Stone Age, as well as on the FA profiles of the fat of several mammoths found in the permafrost of Siberia (Russian Federation), to assess the cultural and nutritional significance of this pachyderm for Palaeolithic hunters.

## Samples

Five specimens of frozen mammoths (*M. primigenius*) from Siberia (Fig. 1) were included in this study (Table 1), which were relatively close in time, all from 29–45 000 years BP. Permission was received to examine the relevant specimens from museum collections. Samples from the frozen carcasses of mammoths were donated by the Museum of Mammoth, Institute of Applied Ecology of the North, North-eastern Federal University in Yakutsk; and the Yakutian Academy of Sciences, Yakutsk, Museum-Exhibition Center in Salekhard, Taimyr District museum in Dudinka (all of them in the Russian Federation).

The samples were kept in the freezers of the Zoological Institute Russian Academy of Sciences in Saint Petersburg. Sterile conditions were maintained during the dissection procedures and a special drill was used on the frozen carcasses. Samples were taken from layers beneath the skin in the best preserved areas (Table 1). Samples from all frozen mammals are available for inspection upon request.

## Methods

### *Oil extraction and transesterification*

Simultaneous oil extraction and transesterification was performed according to Rincón Cervera *et al.* (2012). From each sample, 50 mg was weighed in test tubes and n-hexane (1 mL) was added to each. FA methyl esters (FAMEs) were obtained after adding 1 mL of the methylation mixture, which was composed of methanol/acetyl chloride (20: 1, v/v), and then heated at 100 °C for 10 min. After cooling to room temperature, 1 mL of distilled water was added to each tube, after which the tubes were centrifuged at 3500 r.p.m. for 5 min. The upper hexane layer was removed for gas–liquid chromatography (GLC) analyses.

### *GLC analyses*

Firstly, FAMEs were analysed by using a Focus GLC (Thermo Electron, Cambridge, UK) equipped with flame injection detector (FID) and an OmegaWAX 250 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Supelco, Bellefonte, PA, USA). The temperature programme was 1 min at 90 °C, heating to 200 °C at a rate of 10 °C $min^{-1}$, constant temperature at 200 °C (3 min), heating to 260 °C at a rate of 6 °C $min^{-1}$ and constant temperature at 260 °C (5 min). The injector temperature was 250 °C with a split ratio 50: 1. The injection volume was 4 μL and the detector temperature was 260 °C. Nitrogen was used as the carrier gas (1 mL $min^{-1}$) and peaks were identified by retention times determined for known FAME standards (PUFA No. 1 from Sigma, St. Louis, MO, USA), while FA contents were estimated by using methyl pentadecanoate (15:0) as an internal standard.

All samples were subjected to a second round of analyses by GLC-mass spectrometry (GLC-MS) at the Scientific Instrumentation Centre of the University of Granada (Spain). Samples (2 μL) were injected into an Agilent 7890A gas chromatograph with an apolar column in split mode, coupled with a Quattro micro GLC mass spectrophotometer (Waters, Elstree, UK), with a positive electron impact source (70 eV) and full scan spectra acquisition. All FAs were detected and quantified by comparison of retention times and mass spectra with external standards, which were run at three different concentrations. Experiments for all samples were conducted at least in triplicate. Results are expressed as mean value ± SD in Table 2.

## Results and discussion

### *Mammoth hunting within the context of the Stone Age*

The most prominent data taken from the literature on mammoth hunting are summarized in Supporting Information Table S1. Mammoth remains from several sites show variable alterations by human butchering, and proof exists regarding the existence of an industry based on ivory and bone use. In most cases, the target mammoth species was *M. primigenius*, but there is also hunting evidence for *M. cf. chosaricus* in Eurasia (Hoffecker *et al.*, 1991) and *M. columbi* in North America (Sellards, 1952; Saunders, 1980, 1992; Holliday

**Figure 1.** The almost perfectly preserved ~34 000-year-old female mammoth Yuka, which was discovered in 2010 in Russia's Arctic Circle. This specimen yielded fat in two different states of preservation: a typical greyish-white, wax-like structure found in frozen fats called adipocere, in which the unsaturated fatty acids are more or less transformed to saturated fatty acids; and another fat in apparently good preservation state, showing a fatty acid profile very similar to that found in the African elephant.

*et al.*, 1994). Mammoth hunting was especially intense by the end of the Late Palaeolithic, during the Aurignacian and Gravettian phases. In the Middle Palaeolithic, mammoth remains sometimes dominated the faunal assemblage, e.g. in Grub-Kranawetberg, Austria (Bosch *et al.*, 2012), and in Bollschweil, near Freiburg in Breisgau, Germany (Conard and Niven, 2001).

The faunal remains appearing with mammoths belong to the 'Mammoth complex' – that is those from horse, bison, large bovids, woolly rhinoceros and reindeer. Other mammals belonging to this complex include the hyena, wolf, hare, fox and cave lion, as well as terrestrial molluscs (Bosch *et al.*, 2012), thus indicating adaptation of Stone Age humans to a high diversity of animal food resources. Notably, a high percentage of the mammals hunted by Palaeolithic hominins were monogastric, such as horse, cave bear and woolly rhinoceros.

The first period in the use of mammoths as animal food ranged from the Early Middle Palaeolithic to the Aurignacian (Table S1). During this period, mammoth hunting consisted almost exclusively of scavenging, although, given the age profile of most mammoth remains, which corresponds to juvenile individuals, it is possible that hunting and scavenging techniques were mixed (Péan and Patou-Mathis, 2003). There is also evidence from this period that Neanderthals occupied areas selected seasonally for mammoth acquisition, as well as for killing and butchering, followed by human transport of selected animal body parts. Several mammoth remains show bone and skeletal traces, such as: impact scars and bones broken when fresh; cut marks and fractures; and narrow linear incisions identified as stone tool cuts and chop marks (Hoffecker *et al.*, 1991; Conard and Prindiville, 2000; Conard and Niven, 2001; Smith, 2015; Pitulko *et al.*, 2016). Also, Neanderthals used mammoth bones to build dwellings, to be used as permanent or temporary settlements (Svoboda *et al.*, 2005; Germonpré *et al.*, 2008;Obada *et al.*, 2012), and in some cases 'symbolic' areas have been discovered, in which mammoth bones appear to have been coated with ochre (Demay *et al.*, 2012). The sites at which mammoth remains appear are interpreted as butchering or scavenging sites, where family groups developed diverse food-acquisition activities (Svoboda *et al.*, 2005).

The second mammoth hunting period is the more recent one, and it can be attributed to anatomically modern humans. During this period, bone tools reportedly appeared, and buildings were made with mammoth bones (Iakovleva and Djindjian, 2005; Bocherens *et al.*, 2015). Besides traces of cuts on the bones, ivory carvings appear; Cro-Magnon

**Table 1.** Samples characteristics of frozen mammoths.

| Sample code | Animal | Organ | Radiocarbon age ($^{14}$C a BP) | Reference |
|---|---|---|---|---|
| Y1, Y2 | Semi-adult mammoth 'Yuka' 7–8 years old, male | Fat from left hind leg | 34 300 | Guil-Guerrero *et al.* (2014) |
| L1 | Baby mammoth 'Lyuba' 2 months old, female | Fat from intestines | 41 900 | Guil-Guerrero *et al.* (2014) |
| L2 | Baby mammoth 'Lyuba' | Fat from hump | 41 900 | Guil-Guerrero *et al.* (2014) |
| L3 | Baby mammoth 'Lyuba' | Fat from abdominal wall | 41 900 | Guil-Guerrero *et al.* (2014) |
| L4 | Baby mammoth 'Lyuba' | Fat under the skin of belly | 41 900 | Guil-Guerrero *et al.* (2014) |
| O | Baby mammoth 'Oimyakon', female?, 1–1.5 years old | Fat from hump | 41 300 | Boeskorov *et al.* (2007) |
| KZ043 | Adult mammoth 'Malolyakhowski', female, 45–47 years old | Fat from the forearm | 28 600 | A. Tikhonov (unpubl. data) |
| Zh1 | Adult mammoth 'Zhenya', male, 15 years old | Fat under the ribs | 44 750 | Pitulko *et al.* (2016) |
| Zh2 | Adult mammoth 'Zhenya', | Fat from brain | 44 750 | Pitulko *et al.* (2016) |
| Zh3 | Adult mammoth 'Zhenya', | Fat from hump | 44 750 | Pitulko *et al.* (2016) |

**Table 2.** Fatty acid profiles of the subcutaneous fat of several mammoth samples (*Mammuthus primigenius*) and modern elephant (*Loxodonta africana*).

| | FA% of total FAs in animal fat | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Yuka | | Zhenya | | | Lyuba | | | | |
| Fatty acid | Oimyakon | KZ043 | Y1[a] | Y2[a] | Zh1 | Zh2 | Zh3 | L1[a] | L2[a] | L3[a] | L4[a] | *Loxodonta africana*[b] |
| 10:0 | – | 0.3 ± 0.0 | – | – | – | – | – | 0.5 ± 0.1 | 0.5 ± 0.0 | 0.5 ± 0.1 | 0.4 ± 0.1 | 1.2 |
| 11:0 | 2.1 ± 0.1 | 0.2 ± 0.0 | – | 0.2 ± 0.0 | – | – | – | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.1 | 0.1 ± 0.0 | – |
| 12:0 | 9.3 ± 0.4 | 0.6 ± 0.0 | – | 2.9 ± 0.1 | 1.8 ± 0.1 | – | – | 2.2 ± 0.2 | 2.1 ± 0.1 | 2.0 ± 0.3 | 2.5 ± 0.2 | 2.6 |
| 13:0 | 0.6 ± 0.1 | 0.2 ± 0.0 | – | 0.4 ± 0.0 | – | – | – | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.1 | – |
| 14:0 | – | 5 ± 0.2 | 7.1 ± 0.4 | 7.4 ± 0.3 | 10.1 ± 0.2 | 9.3 ± 0.5 | 8.4 ± 0.3 | 8.8 ± 0.3 | 7.4 ± 0.6 | 8.6 ± 0.6 | 9.9 ± 0.5 | 6.0 |
| 14:1 | 0.3 ± 0.0 | – | – | – | – | – | – | – | – | – | – | – |
| 15:0 | 3.2 ± 0.1 | 3.3 ± 0.2 | – | 2.4 ± 0.2 | 6.0 ± 0.2 | – | 5.2 ± 0.2 | 1.5 ± 0.1 | 1.3 ± 0.2 | 1.5 ± 0.2 | 2.0 ± 0.1 | – |
| 16:0 Palmitic | 49.7 ± 0.9 | 27.6 ± 0.7 | 39.1 ± 1.5 | 21.5 ± 1.1 | 41.7 ± 0.9 | 19.8 ± 0.7 | 42.6 ± 1.1 | 75.3 ± 2.1 | 75.4 ± 1.5 | 74.8 ± 1.6 | 70.6 ± 2.2 | 33.0 |
| 16:1*n*9 | 1.5 ± 0.1 | 3.5 ± 0.2 | 7.1 ± 0.5 | 1.6 ± 0.2 | – | – | – | 0.4 ± 0.1 | 0.9 ± 0.2 | 0.6 ± 0.1 | 0.6 ± 0.2 | 4.3 |
| 17:0 | 2.9 ± 0.2 | 4.9 ± 0.2 | 0.2 ± 0.0 | 2.9 ± 0.1 | 25.6 ± 0.6 | 41.9 ± 1.3 | 29.7 ± 0.7 | 0.9 ± 0.2 | 0.8 ± 0.1 | 0.7 ± 0.2 | 1.0 ± 0.1 | 0.5 |
| 17:1 | 0.8 ± 0.1 | – | – | 1.5 ± 0.1 | – | – | – | 0.2 ± 0.0 | 0.3 ± 0.1 | 0.3 ± 0.0 | 0.3 ± 0.1 | – |
| 18:0 | 4.0 ± 0.2 | 2.8 ± 0.3 | 14.3 ± 1.1 | 1.9 ± 0.2 | 5.4 ± 0.2 | 17.4 ± 0.3 | 5.8 ± 0.2 | 4.0 ± 0.3 | 2.9 ± 0.3 | 2.6 ± 0.2 | 4.4 ± 0.3 | 6.8 |
| 18:1*n*9 Elaidic | 0.2 ± 0.0 | – | – | – | – | – | – | 2.1 ± 0.3 | 1.4 ± 0.2 | 1.9 ± 0.3 | 4.3 ± 0.3 | – |
| 18:1*n*9 Oleic | 24.0 ± 0.9 | 45.0 ± 1.2 | 28.5 ± 1.8 | 51.9 ± 1.0 | 9.5 ± 0.8 | 11.6 ± 0.7 | 8.4 ± 0.6 | 3.0 ± 0.2 | 5.8 ± 0.6 | 4.9 ± 0.3 | 3.4 ± 0.4 | 30.5 |
| 18:1*n*7 | 0.5 ± 0.0 | 1.9 ± 0.2 | – | 2.1 ± 0.1 | – | – | – | – | – | – | – | – |
| 18:2*n*6 Linoleadic | – | – | – | – | 0.3 ± 0.0 | – | – | – | – | – | – | – |
| 18:2*n*6 Linoleic | 0.5 ± 0.0 | 1.2 ± 0.1 | 3.6 ± 0.3 | 1.0 ± 0.0 | – | – | – | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.3 ± 0.1 | 1.0 |
| 18:3*n*3 Linolenic | 0.4 ± 0.1 | 1.8 ± 0.1 | tr | 2.6 ± 0.2 | – | – | – | 0.08 | 0.09 | 0.08 | 0.09 | 2 |
| 20:0 | – | 0.1 ± 0.0 | – | – | – | – | – | – | – | – | 0.2 ± 0.1 | – |
| 20:1 | – | – | – | – | – | – | – | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.1 | – |
| 20:3*n*3 Eicosatrienoic | – | – | – | – | – | – | – | – | tr | 0.1 | tr | – |
| 20:2*n*6 | – | – | – | – | – | – | – | 0.04 | 0.03 | 0.03 | 0.04 | – |
| 20:3*n*3 | – | – | – | – | – | – | – | 0.07 | 0.08 | 0.07 | 0.04 | – |
| 24:1*n*9 Nervonic | – | – | – | – | – | – | – | – | – | – | 0.1 | – |
| 20:4*n*3 | – | 0.1 ± 0.0 | – | – | – | – | – | – | – | – | – | – |
| 20:4*n*6 Arachidonic | – | – | 0.01 ± 0.00 | – | – | – | – | 0.02 | – | – | 0.02 | – |
| 20:5*n*3 Eicosapentaenoic | – | – | – | – | – | – | – | 0.09 | | – | – | – |
| 22:5*n*3 Docosapentaenoic | – | 0.1 ± 0.0 | | | – | – | – | – | – | – | – | – |
| Total FAs | 15.0 ± 0.9 | 4.0 ± 0.3 | 0.6 ± 0.1 | 2.1 ± 0.2 | 4.0 ± 0.3 | 4.0 ± 0.5 | 3.0 ± 0.2 | 21.5 ± 0.7 | 23.6 ± 0.9 | 28.5 ± 1.2 | 18.5 ± 0.8 | – |
| Fatty acid groups | | | | | | | | | | | | |
| SAT | 71.7 | 45.0 | 60.7 | 39.6 | 90.6 | 88.4 | 91.6 | 93.6 | 90.6 | 91.0 | 91.1 | 50.1 |
| MUFA | 27.2 | 50.4 | 28.5 | 57.1 | 9.5 | 11.6 | 8.4 | 5.8 | 8.5 | 7.8 | 8.9 | 34.8 |
| PUFA | 1.0 | 3.2 | 3.6 | 3.6 | 0.3 | 0.0 | 0.0 | 0.2 | 0.1 | 0.2 | 0.1 | 3.0 |
| *n*3 | 0.4 | 2.0 | 0.0 | 2.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.0 |
| *n*6 | 0.5 | 1.2 | 3.6 | 1.0 | 0.3 | 0.0 | 0.0 | 0.2 | 0.1 | 0.1 | 0.1 | 1.0 |
| *n*6/*n*3 | 0.9 | 1.7 | 0.0 | – | 0.0 | 0.0 | – | 0.0 | 0.0 | 0.0 | 0.0 | 2.0 |
| Sum | 99.8 | 98.6 | 92.8 | 100.3 | 100.4 | 100.0 | 100.0 | 99.6 | 99.2 | 99.0 | 100.1 | 87.9 |

SAT, saturated; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.
[a]Guil-Guerrero *et al.* (2014).
[b]Nisson *et al.* (2014).

humans hunted mammoths not only as a food source, but also as a raw material for art objects (Table S1). For this period, evidence has been found for collective mammoth hunting made by several social units in cooperation using natural trapping in regions where this hunting technique was possible (Scheer, 2001; Svoboda *et al.*, 2005; Demay *et al.*, 2012). Alternatively, Stone Age hominins could have hunted mammoths, causing stampedes as a means of capturing animals that could not maintain contact with the herd (Germonpré *et al.*, 2014). Furthermore, two hunting strategies have been described as occurring at the same place, i.e. hunters chose the trapping of calves or adult mammoths, depending on different economic needs (Brugère, 2014). Moreover, 'passive hunting' in the winter, in the evening or at night, has been described (Obada *et al.*, 2012). To accomplish this, the group of hunters could attack several animals at the same time, to capture stragglers from the herd or as a scattered group. Furthermore, direct evidence of mammoth hunting exists; for example, a mammoth thoracic vertebra has a cone-shaped hole resulting from the penetration of a notched point, and fragments of quartzite flakes lodged in the hole were found (Orlova *et al.*, 2004; Zenin *et al.*, 2006). Also, the use of large dogs to capture young individuals has been cited (Germonpré *et al.*, 2008), as well to transport butchered mammoth pieces, rather than primarily for helping in hunting (Bocherens *et al.*, 2015).

The features described for this second period indicate the emergence of a better structured society, with the ability not only to produce art, but also capable of designing highly successful strategies for mammoth hunting; such a society therefore exerted a strong selective pressure on this food resource.

Although the use of mammoths by Palaeolithic hunters is undisputed, significant uncertainties persist as to what nutrients such prey would supply to our ancestors; could this pachyderm have been a source of *n*-3 PUFAs for Palaeolithic hominins? Brain, bone marrow, subcutaneous fat, viscera and meat would have been the targeted mammoth organs for Stone Age hunters.

## *Mammoth marrow as possible source of n-3 PUFA for Palaeolithic hunters*

The marrow has high variability in fat and energy content (Blumenschine and Madrigal, 1993), and it seems that it was consumed, as suggested by the discovery of mammoth bones fractured to access marrow at some Neanderthal sites (Thoms *et al.*, 2007; Yravedra *et al.*, 2014; Smith, 2015). There are no analyses of the fats from the marrow of mammoths, nor of marrow fat from their relatives (elephants), but analyses of the bone marrow from other mammals hunted by Palaeolithic hominins in Europe have shown that it lacks appreciable amounts of *n*-3 VLCPUFAs, although it contains on average 540 mg ALA per 100 g tissue; however, these values vary widely among species (Guil-Guerrero, 2017). Regardless, consumption of this tissue would have provided large amounts of energy to Palaeolithic hominins, given that it yields on average 484 kcal/100 g (Guil-Guerrero, 2017).

## *Role of the mammoth brain as an n-3 PUFA source*

Another target tissue for mammoth hunters would have been the brain. In this regard, the extraction and consumption of the brain of elephants in early Palaeolithic sites in Europe, Africa and Asia has been reported as very frequent (Agam and Barkai, 2016). Similarly, breaking of mammoth skulls by hominins has been reported (Germonpré *et al.*, 2008), and hunters carried certain body parts, especially the heads (Germonpré *et al.*, 2014); moreover, the systematic way in which the mammoth braincases were opened suggests that this was done by people to gain access to the fatty brain (Germonpré *et al.*, 2008). In fact, this organ would have been particularly attractive to any Stone Age humans, given that a mammoth weighting around 4 tonnes would have a brain mass of about ~3 kg, based on our estimates for the mammoth Zhenya (Table 1). Thus, the mammoth brain would have been an important terrestrial source of DHA and ARA, which are especially abundant in the brain of all mammals (Germonpré *et al.*, 2008; Guil-Guerrero, 2017). In mammals, the brain contains low amounts of ALA, ~200 mg per 100 g of tissue (Kuipers *et al.*, 2010). However, it contains ~10 g *n*-3 VLCPUFAs per 100 g FAs, and, based on ~9 g FAs per 100 g of brain tissue (Kuipers *et al.*, 2010), we can calculate that a typical brain of ~3 kg might contain ~27 g of *n*-3 VLCPUFAs. This might be enough to fulfil the daily needs of *n*-3 VLCPUFAs for good health for a group of 12–24 people, considering the size of the hominin groups during this period (Hayden, 2012), for about ~4–9 days, given a daily need for *n*-3 VLCPUFFAs of 250 mg $day^{-1}$ (European Food Safety Authority, 2010). Nevertheless, the brain is highly perishable due to its high VLCPUFAs and water content, and despite the low temperatures prevailing in the glacial or periglacial environment, it could have spoilt very quickly. Thus, Stone Age hunters would have needed to use other fatty organs of their prey displaying greater resistance to peroxidation and spoiling, such as subcutaneous fat (Guil-Guerrero *et al.*, 2013). However, we cannot rule out the use of primitive techniques of preservation, such as lactic acid fermentation, applied to highly perishable organs such as the brain. In this regard, fermentation provides an effective means of inhibiting lipid 'autoxidation', which leads to the food becoming rancid (Speth, 2017).

It bears noting that a mammal brain yields only 126 kcal per 100 g of tissue (Kuipers *et al.*, 2010); therefore, the total energy content of a mammoth brain weighing 3 kg would be ~3780 kcal. In this regard, it has been calculated that the total energy expenditure for Neanderthals was ~4500 kcal per day (Guil-Guerrero, 2017); thus, this organ could have only marginally met the energy needs of a single individual Palaeolithic hunter, and thus may have been eaten rather quickly by selected members of the hunter group, as a kind of ephemeral delicacy, and promptly replaced by other more energetic organs from the hunted animal.

## *Mammoth meat as a source of n-3 PUFA for Palaeolithic hominins*

The amount of meat in a medium-sized mammoth appears to be inexhaustible. However, there are serious limitations to large daily intakes of meat. We have reported that given the high energy needs of Stone Age hunters, protein-rich food, such as meat, should have been ingested to a lower extent than other fatty tissues. This is because of the potential toxicity caused by the consumption of such low-energy and high-nitrogen organs (Guil-Guerrero *et al.*, 2013, 2014, 2015; Guil-Guerrero, 2017). Regardless, it cannot be ruled out that Neanderthals developed some adaptations to a high-protein diet, as has been pointed out based on their 'bell'-shaped thorax and wide pelvis (Ben-Dor *et al.*, 2016).

It has been recently hypothesized that the food strategy at that time should have been to consume the toxicologically safe maximum amount of meat, energetically complemented by fat, to obtain the maximum amount of *n*-3 VLCPUFAs

(Guil-Guerrero, 2017). Achieving 4500 kcal, the previously estimated daily energy need at those times, would have been possible by consuming ~566 g of meat complemented by ~592 g of fatty tissues, such as subcutaneous fat. These quantities result from protein intake, for which the safe upper limit is ~162 g daily (Guil-Guerrero, 2017). By contrast, for all mammal species, the amount of ALA in meat is similar and very low, for instance 55 mg/100 g in the Palaeolithic-relict Galician horse (Guil-Guerrero *et al.*, 2013), and thus the meat could provide ~310 mg ALA daily, insufficient to fulfil the daily needs of *n*-3 LCPUFAs, as explained below.

Concerning *n*-3 VLCPUFAs, the most valuable FA within this group is DHA. Meat from mammals regularly hunted by Palaeolithic hominins in glacial and periglacial environments provides on average ~4 mg DHA/100 g (Guil-Guerrero, 2017) and, under the assumption of a similar amount of DHA in mammoth meat, the accepted upper limit of meat intake of ~566 g would provide, if bioavailable, ~23 mg DHA. By contrast, currently, in most developed countries, the daily intake of DHA ranges from 184 to 473 mg $day^{-1}$, while in low-income countries intake is about 96 mg $day^{-1}$ (Forsyth *et al.*, 2016). Thus, given the previously indicated daily need for *n*-3 VLCPUFAs, Stone Age hunters would have required another source of DHA to satisfy their daily needs.

## *Subcutaneous fat of mammoths as n-3 PUFA source for Palaeolithic hunters*

Another large tissue element of the mammoth was subcutaneous fat. For a mammoth of ~3.0 tons, ~5% subcutaneous fat and other similar fats distributed throughout the body would be a conservative figure, and thus a single specimen would have ~150 kg of stored subcutaneous fat, although large fluctuations throughout the year would be expected (Speth, 1991; Cordain *et al.*, 2002). The energy derived from the intake of 100 g of subcutaneous fat should be similar to that previously calculated for several mammals, which was on average ~660 kcal (Guil-Guerrero, 2017). This energy contribution was essential, because it would have reduced the daily intake of meat needed by Palaeolithic hominins, and the above-indicated daily energy need might be fulfilled by ~700 g of mammoth subcutaneous fat. Therefore, a medium-sized mammoth would have stored ~1 million kcal as fat, providing clean energy for a hunting group of 12–24 individuals for approximately 9–18 days, while the consumption of variable amounts of meat would have extended this figure for some days. Assuming the consumption of this fatty tissue, an important question is whether 700 g of mammoth subcutaneous fat could provide the recommended daily intake of *n*-3 PUFAs to maintain good health. Based on the similarity of the total FA content in the subcutaneous fat of all food-mammals (Guil-Guerrero, 2017), ~70% FAs should be included in this tissue, together with smaller amounts of water, protein and minerals.

Previously, we have reported on the *n*-3 PUFAs content in the subcutaneous fat of several frozen mammals recovered from the permafrost of north-eastern Siberia, in which the fat of two frozen mammoth carcasses was included. In these samples, the FAs were transformed to adipocere, and their original FA profiles could only be estimated (Guil-Guerrero *et al.*, 2014). Thereafter, we collected new samples from several other mammoths, and the well-preserved Yuka mammoth (Fig. 1) was re-examined in a search of new fatty areas with better preservation (Y2, Table 1). The FA profiles of all these samples are shown in Table 2. Given the previously discussed mechanisms for the β-oxidation of unsaturated FAs (Guil-Guerrero *et al.*, 2014, 2015), in the KZ043 and Y2 samples, oleic acid (OA, 18:1*n*9) percentages, at 45.0 and 51.9%, respectively, and the relatively low amounts of palmitic acid (PA, 16:0), suggest that the original FA profiles of such samples were similar to those now detected. Furthermore, the presence of LCPUFAs, i.e. LA + ALA, at 3.0 and 3.6%, respectively, reinforces this assumption. Also notable is the close similarity among the FA profiles of the subcutaneous fat of the two later samples of mammoths with others shown by modern elephants (*Loxodonta africana*, Table 2), suggesting that the FA profiles detected in these samples were in good condition. In this regard, Duncan and Garton (1968) reported a total PUFAs content of 2.5% in the subcutaneous fat of modern elephants; and recently, Nilsson *et al.* (2014) found ALA to comprise 2% of total FAs in the same tissue of the African elephant. These data agree with others discussed above for mammoths, and the *n*-3 PUFA contents in the subcutaneous fat of elephants and mammoths were probably at this level, although it is reasonable to assume that the FA profiles of such tissue of mammoths were affected by seasonal and climatic changes. In this regard, Meyer *et al.* (1998) indicated for free-ranging elephants that LA + ALA was 24.7% of total FAs in the subcutaneous fat; thus, for Proboscideans, fluctuations in the total PUFA contents of fatty tissues are expected in response to dietary changes. However, the good agreement between the percentages of ALA in the two well-preserved mammoth samples analysed here and those of the modern elephant is encouraging.

The remaining samples presented in Table 2 have FA profiles that have undergone some alteration, thus representing different stages of adipocere formation (Guil-Guerrero *et al.*, 2015). The high content of saturated 16:0, 17:0 and 18:0 FAs, which partially constitute β-oxidation and PUFA reduction products derived from the previous LCPUFAs content, supports this assumption. By contrast, the high percentage of 17:0 found in Zhenya brain tissue suggests that this FA was presumably a product of the degradation of C20 and/or C22 VLCPUFAs.

Under the assumption that the FA profiles detected in the subcutaneous fat of mammoth samples are similar to others present in Palaeolithic times, these could have been appropriate for Palaeolithic hunters; that is, given both the ALA percentage of total FAs in the subcutaneous fat of Yuka-2 (2.6%) and the 70% estimated total FA content in the subcutaneous fat, ~1.8% ALA could be expected for this tissue. Thus, the previously calculated intake of subcutaneous fat needed to meet the daily energy allowance might have provided ~13 g ALA. The daily needs of *n*-3 PUFAs for Stone Age humans might be assessed by considering that the available evidence indicates that >0.5% energy from ALA daily would have prevented deficiency symptoms, and that the energy derived from 1 g of fat catabolism is approximately 9 kcal (European Food Safety Authority, 2010). For Palaeolithic hominins, an amount of ~2.5 g ALA daily, supplied by ~140 g of mammoth subcutaneous fat, yielding ~920 kcal, would thus need to be ingested to avoid *n*-3 PUFA deficiency symptoms. This tissue would be consumed to a greater extent, as the energy needed would have forced Stone Age hominins to consume far higher amounts of fatty tissues, while the previously discussed bone marrow would provide energy figures very similar to those of subcutaneous fat.

## *Key points concerning the various n-3 PUFA groups derived from mammoths in the diet of Palaeolithic hunters*

Among the different organs of mammoths surveyed, the subcutaneous fat and similar tissues could have provided

Palaeolithic hunters with the *n*-3 LCPUFAs needed to avoid deficiency symptoms, as would have occurred when consuming this organ from other single-stomached mammals, such as horses or woolly rhinoceroses (Guil-Guerrero *et al.*, 2013, 2014, 2015; Guil-Guerrero, 2017). Such *n*-3 LCPUFA sources for hominins during the Palaeolithic in Europe could have been complemented in the warm season by selected organs of wild plants (Madella *et al.*, 2002; Hardy, 2010).

As seen above, the hunting of mammoths could have had clear nutritional advantages for Palaeolithic hunters, in contrast to ruminant prey. In this regard, it has been previously argued that Upper Palaeolithic societies devoted to reindeer as a food resource had a very restricted range (Morin, 2008).

From ALA intake, more than 75% of the ingested ALA is shunted to β-oxidation, while the total conversion to *n*-3 VLCPUFAs is less clear, probably 0–4% (Egert *et al.*, 2009). Thus, given the above-mentioned limit for meat intake, it is likely that the need for such *n*-3 VLCPUFAs, i.e. EPA and DHA, would have been fulfilled by complementing the diet with other PUFA sources, especially in older individuals, for whom the bioconversion of ALA to EPA and DHA is very low (Burdge and Calder, 2005; Bradbury, 2011). These alternative sources of *n*-3 VLCPUFAs could have included fish in the summer and some DHA-rich human organs throughout the year (Guil-Guerrero, 2017). In addition, late Upper Palaeolithic hominins, in some areas, made intensive use of marine foods, and particularly marine mammals (Richards *et al.*, 2005), which provided sufficient amounts of EPA + DHA.

Other organs not yet discussed, such as the liver, yield a FA profile similar to that of meat, although with somewhat more fat, while the intestines contain very low amounts of fat, and these organs are not discussed separately.

## Conclusions

Evidence taken from the sites reported in the literature summarized in Table S1 indicate that mammoths constituted a more or less constant prey source for both scavenger and hunter hominin societies from the early Middle Palaeolithic until recent times, and also that mammoth capture provided construction materials, useful tools and decorative objects. Moreover, for Palaeolithic hominins, the most nutritionally valuable tissues were the fatty tissues, which are quite resistant to quick degradation processes and spoiling, while providing nitrogen-free energy as well as adequate *n*-3 LCPUFAs. In addition, the mammoth brain occasionally contributed to meeting the needs for *n*-3 VLCPUFAs. Thus, adequate *n*-3 LCPUFA intake and cultural development can be linked, given that effective hunting strategies for mammoth require good mental and physical activity, while consumption of mammoth tissues provided high-quality nutrients for such activities.

## Supporting Information

Table S1. Selected sites containing mammoth remains and associated human activity.

*Abbreviations.* ALA, α-linolenic acid; DHA, docosahexaenoic acid; FA, fatty acid; GLC, gas–liquid chromatography; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acid; OA, oleic acid; PA, palmitic acid; PUFA, polyunsaturated fatty acid; VLCPUFA, very-long-chain polyunsaturated fatty acid

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Original research article

# Fatty acid profiles and *sn*-2 fatty acid distribution of γ-linolenic acid-rich *Borago* species

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ABSTRACT

The aim of this work was to assess the seed oils of different *Borago* species as sources of bioavailable γ-linolenic acid (GLA) by establishing its positional distribution within the triacylglycerol (TAG) structure. To this end, seeds of wild and farmed *Borago* species were collected and analysed. The highest GLA percentage of total fatty acids (FA) was found in *B. morisiana* and *B. pygmaea* (23.1 and 22.9% GLA of total FA, respectively). Other endemic *Borago* species (*B. trabutii* and *B. longifolia*) show a lower GLA content (18.9 and 19.0% of total FA, respectively). Oil content in seeds of *B. officinalis* ranged from 14.4 to 20.3%, while the GLA percentage of total FA was 15.7–19.4%, which leads to a 2.3–3.7% GLA range in the whole seeds. For endemic *Borago*, such amounts were 5.6, 5.0, 3.9, and 3.0%, for *B. morisiana*, *B. pygmaea*, *B. trabutii*, and *B. longifolia*, respectively. The distribution of FA in the *sn*-2 position of TAG was assessed by enzymatic hydrolysis reaction. The best regiospecific index was found in *B. longifolia* (0.8) and *B. officinalis* (1.0). This study provides useful data on novel GLA-rich *Borago* seed oils, which have potential use in the food and pharmaceutical industries.

## 1. Introduction

Higher plants contain mainly fatty acids (FA) of C16 and C18 chains, which are end products of FA biosynthesis. The main saturated FA (SFA) in plants is palmitic acid (PA, 16:0) while the main unsaturated ones are oleic acid (OA, 18:1*n*–9), linoleic acid (LA, 18:2*n*–6) and α-linolenic acid (ALA, 18:3*n*–3) (Horrobin, 1992; Guil-Guerrero, 2007). However, some unusual polyunsaturated FA (PUFA) occur in a few plant families, such as γ-linolenic acid (GLA, 18:3*n*–6) and stearidonic acid (SDA, 18:4*n*–3), which have high nutritional and industrial interest.

GLA is a PUFA belonging to the *n*–6 series, which is produced in the body from LA, an essential FA (EFA), by the enzyme Δ6-desaturase, which catalyses the introduction of a double bond at Δ6 position of the substrate (Zhou et al., 2006). This enzyme also desaturates ALA, which is also an EFA, to SDA (Horrobin, 1992; Guil-Guerrero, 2007). Most of the Boraginaceae species usually yield both GLA- and SDA-rich oils, the latter FA also having interesting healthy properties, although different affinities of the enzyme for each precursor EFA have been found, as deduced by the unequal concentrations of both desaturated FA in the various oils of the plant species expressing this enzyme (García-Maroto et al., 2006). GLA oils are scarce in common foods, and they are usually obtained from three plants: borage (*Borago officinalis* L., 21–23% GLA of total FA); evening primrose (*Oenothera biennis* L.), 9–12% GLA of total FA; and blackcurrant (*Ribes nigrum* L.), 15–20% GLA of total FA (Gunstone, 1992; Guil-Guerrero, 2007). Among the botanical families, Boraginaceae is the most feasible option for searching Δ6-desaturated FA, (Guil-Guerrero, 2007). This is because: in the seed oil of the majority of species of Boraginaceae species tested GLA reaches amounts higher than 10% of total FA; ii) in this plant family, the seed oil usually constitutes 20–30 g/100 g; iii) the oil of cultured species, i.e. those of *Echium*, *Borago* and *Buglossoides*, has been shown to have no toxicity at all; iv) most Boraginaceae species are herbaceous and can be easily grown, and are usually good seed producers.

*Abbreviations:* ALA, α-linolenic acid; ANOVA, one-way analysis of variance; DHA, docosahexaenoic acid; EFA, essential FA; FA, fatty acid; FAME, FA methyl ester; GLA, γ-linolenic acid; GC, gas chromatography; LA, linoleic acid; LOD, limit of detection; LOQ, limit of quantification; MAG, monoalyclycerol; MUFA, monounsaturated FA; MR, method reproducibility; OA, oleic acid; PA, palmitic acid; PUFA, polyunsaturated fatty acid; RI, regiospecific index; RS, recovery studies; SA, stearic acid; SD, standard deviation; SDA, stearidonic acid; TAG, triacylglycerol; TLC, thin-layer chromatography

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For some years, several species from this family are being typified for use as GLA and SDA sources: e.g. Eastern European species (Yunusova et al., 2012); Macaronesian *Echium* (Guil-Guerrero et al., 2000, 2001a), and several Boraginaceae species from the Mediterranean Basin (Guil-Guerrero et al., 2001b, 2017).

Suitable GLA-rich oils are needed to meet market demands, given that large health benefits have been attributed to dietary supplementation with GLA-rich oils: GLA has therapeutic value *per se* (Horrobin, 1992); GLA displays different anti-cancer actions, for instance by inhibiting p38 MAPK-dependent activator protein and mitochondria-mediated apoptosis pathway (Park et al., 2015; Zhang et al., 2015); GLA-rich oils improve the blood-lipid profile by slightly lowering glucose, triglycerides, total protein, albumin, and cholesterol concentrations (Tso et al., 2012); and this FA ameliorates the course of several syndromes, such as perspiration, dermatitis, skin hyperproliferation, and osteoporosis (Kawamura et al., 2011; Tasset-Cuevas et al., 2013).

On the other hand, the position in which those PUFA are esterified in natural TAG molecules is of great interest, because PUFA in *sn*-2 position are more bioavailable than those in the *sn*-1 and *sn*-3 locations (Iwasaki and Yamane, 2000; Mu and Høy, 2004). This is so because when TAG are ingested and then processed in the digestive system, the FA in *sn*-1 and *sn*-3 positions of the glycerol backbone are released by pancreatic lipase in the small intestine, whereas FA esterified in *sn*-2 position remains unreacted, being easily absorbed as *sn*-2 monoacylglycerols (2-MAG) by the intestinal brush-border membrane. This structure can take up MAG from digested fat through passive diffusion, although a protein transporter for 2-MAG has also been proposed (Yen et al., 2015) and, consistent with these findings, higher bioavailability of GLA at *sn*-2 position has been supported by data from several laboratories (Kapoor and Nair, 2005).

According to all the above, the search for species producing oils containing GLA located at *sn*-2 position is a suitable strategy to improve the nutritional benefits of GLA-rich oils, while such species could become new functional oil-producing crops. In this work, the whole and positional FA profiles of GLA-rich endemic *Borago* species compared with farmed and wild *B. officinalis* from different locations have been determined, in order to assess their feasibility as sources of highly bioavailable GLA. Moreover, the FA profiles of two endemic *Borago* species from the Maghreb (*B. trabutii* and *B. longifolia*) are reported for the first time, as well as the FA profiles of several samples of wild *B. officinalis* collected in the Mediterranean Basin.

## 2. Material and methods

### 2.1. Standards and reagents

FAME standards (PUFA No. 1, 47033; methyl γ-linolenate 98.5% purity, L6503; methyl pentadecanoate, 99.5% purity, 76560; and methyl stearidonate, 97% purity, 43959) and pancreatic lipase were purchased from Sigma-Aldrich (Madrid, Spain). All other chemicals used in this study were reagent grade and were purchased from Sigma-Aldrich, Madrid.

### 2.2. Sample collection locations

Various seeds were collected from their natural habitats or from farmed plants between May and July, and voucher specimens were deposited in the Herbarium of the University of Almería (HUAL): (Table 1).

Seeds were ground in the laboratory with the aid of a mortar; then 150–200 mg were taken for direct methylation and further gas chromatography (GC) analyses.

### 2.3. Oil extraction and transesterification

Simultaneous oil extraction and transesterification was performed according to previous works (Rincón-Cervera et al., 2012). Samples were weighed in test tubes and *n*-hexane (1 mL) was added to each one. FA methyl esters (FAME) were obtained after adding 1 mL of the methylation mixture, which was composed of methanol:acetyl chloride (20:1 *v/v*), and then heated at 100 °C for 10 min. After cooling to room temperature, 1 mL of distilled water was added in each tube, after which the tubes were centrifuged at 3500 rpm for 5 min. The upper hexane layer was removed for GC analyses.

### 2.4. Gas chromatography

FAME were analysed by using a Focus GC (Thermo Electron, Cambridge, UK) equipped with flame ionisation detector and an Omegawax 250 capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness; Supelco, Bellefonte, PA). The temperature program was: 1 min at 90 °C, heating to 200 °C at a rate of 10 °C/min, held at 200 °C (3 min), heating to 260 °C at a rate of 6 °C/min, and held at 260 °C (5 min). The injector temperature was 250 °C with a split ratio of 50:1. The injection volume was 4 μL and the detector temperature was 260 °C. Nitrogen was used as the carrier gas (1 mL/min). Peaks were identified by retention times obtained for known FAME standards, while FA contents were estimated by using methyl pentadecanoate as internal standard. The limit of detection (LOD) for all FAME was 0.004 mg, whereas the limit of quantification (LOQ) was 0.01 mg. LOD and LOQ were calculated as signal-to-noise ratios equal to 3:1 and 10:1, respectively.

### 2.5. Enzymatic hydrolysis

Lipids, either extracted in the laboratory or purchased, were enzymatically hydrolysed as previously described (López-López et al., 2001). Briefly, 50 mg of oil were hydrolysed by adding porcine pancreatic lipase (40 mg) in 4 mL of Tris solution (1 M, pH = 8.0), 1 mL of a sodium cholate solution (0.1% w/v) and 400 μL of a calcium chloride solution (22% *w/v*). Flasks were then placed in a water bath (37 °C, 120 rpm) for 40 min. After that, they were removed and cooled in a water bath at 20 °C and 2 mL of hydrochloric acid (6 M) and 1 mL of *n*-hexane were added. Flasks were shaken for 1 min and the content was transferred to test tubes prior to centrifugation (3500 rpm, 5 min). An aliquot was collected to separate the hydrolysis products by TLC as previously described (Rincón-Cervera and Guil-Guerrero, 2010).

### 2.6. Regiospecific index (RI)

*RI* was defined as the percentage of a given FA in the *sn*-2 position with respect to its percentage in the whole oil, according to the formula:

$$RI = (\text{FA\% in } sn\text{-2 position} - \text{FA\% in TAG})/\text{FA\% in TAG}.$$

The *RI* range for a given FA is between −1.0, when the FA is not esterified at all at the *sn*-2 position, and 2.0, when the esterification rate of the FA at the *sn*-2 position is 100%. Thus, $RI = 0$ means that 33.3% of the total target FA is esterified at the *sn*-2 position (Guil-Guerrero et al., 2015).

### 2.7. Quality control

The repeatability of the direct methylation was checked by analysing replicates of the same sample daily, through the assessment of the percentage of relative standard deviation. The intermediate precision was evaluated by measuring samples on different days throughout the study. Also, blank samples were analysed whenever the methylation reactions were carried out. A blank sample (hexane) was tested for

**Table 1**
Data on collection of *Borago* species.

| species | sample location | herbarium code | geographical coordinates | collection date |
|---|---|---|---|---|
| *B. longifolia* Poir. | Wild; Algeria, Skikda: Garaet Sidi Lakhdar | – | 36.910 N 7.191 E | 06/08/2016 |
| *B. morisiana* Bigazzi & Ricceri | Wild; Italy, Oristano: Laconi (Tanca de Cuccuru) | HUAL 25965 | 39.874 N 9.091 E | 07/04/2014 |
| *B. officinalis* L. | Wild; Spain, Jaén: Navas de San Juan | – | 38.181 N 3.319 W | 06/25/2016 |
| *B. officinalis* L. | Farmed; Spain, Almería | | | |
| *B. officinalis* L. | Wild; Italy, Cagliari (near Lattias mountain) | HUAL 25637 | 39.144 N 8.861 E | 05/09/2013 |
| *B. officinalis* L. | Wild; Tunisia, Béja: Ouechtata | – | 36.960 N 8.982 E | 05/17/2016 |
| *B. pygmaea* (DC.) Chater & Greuter | Wild; Italy, Ogliastra: Gairo (Montarbu di Seui, Girolamo river) | HUAL 25608 | 39.839 N 9.455 E | 07/04/2014 |
| *B. trabutii* Maire | Wild; Morocco, Marrakech-Tensift-Al Hauz (Anfli, Ourika valley) | HUAL 25966 | 31.201 N 7.739 W | 06/14/2015 |

every batch in GC as quality control.

### 2.8. Statistical analysis

All analyses were carried out in triplicate and the data were expressed as means ± standard deviations (SD). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to compare the FA profiles of all samples. Hierarchical cluster analysis was performed using the values calculated for the RI of all samples by the Group Average Method (Fig. 1). Differences were considered statistically significant at $p < 0.05$. All statistical analyses were performed using Statgraphics Plus software version 5.1, Windows package (Statistical Graphics Co., Rockville, MD).

## 3. Results and discussion

For GC analyses, FAME were recovered directly from seeds through simultaneous oil extraction and transesterification. This procedure has been selected among several available methodologies because it is the one that yields more FAME from samples of various matrices, uses low toxicity solvents, and is faster than other methods based on previous stages for oil extraction and further transesterification (Cavonius et al., 2014; Ramos-Bueno et al., 2016).

All *Borago* species analysed in this work were collected from the Tyrrhenian islands, Iberian Peninsula and the Maghreb area (all sites in the Mediterranean Basin), which constitute the speciation hotspots for *Borago* genus (Médail and Quézel, 1999). Previously, *B. morisiana* and *B. pygmaea* seed oils have been characterised (Guil-Guerrero et al., 2017), while this work constitutes the first report for the FA profiles of *B. trabutii* and *B. longifolia*, as well as for regiospecificity of seed oil FA of all endemic *Borago* species. The seed-oil FA profiles for all *Borago* species and their hydrolysis products are summarised in Table 2 (endemic *Borago* species) and Table 3 (*B. officinalis*). As has been found in other endemic *Borago* taxa (Guil-Guerrero et al., 2017), the seed oils of endemic species characterised in this work are suitable alternatives to that provided by *B. officinalis*, considering the high percentages of GLA all endemic *Borago* species show (Table 2).

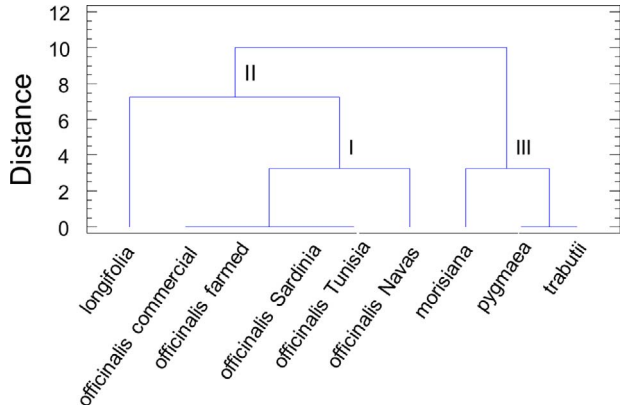


**Fig 1.** Dendrogram of cluster analysis comparing the *Borago* oils samples based on their regiospecific index. The clustering was made using the Group Average linking method ($p < 0.05$).

*B. officinalis* constitutes the only *Borago* species farmed up to now. This species is a prominent GLA source, considering both the GLA concentration in the whole oil (≈16–22% GLA of total FA) as well as the favourable GLA location within the TAG structure (Guil-Guerrero et al., 2015). However, all endemic *Borago* species could be potential sources of GLA-rich oils for the same reasons. The two novel endemic species analysed in this work, the North African *B. trabutii* and *B. longifolia*, contained high percentages of GLA in their corresponding oils (~19% GLA), comparable to levels shown by commercial *B. officinalis* seed oil (~20%), and higher than in *B. officinalis* collected in the wild (≈15–18%; Table 3). According to both the amounts of FA contained in the seeds of *B. officinalis* reported here (14.4–20.3% of total seed weight) and the GLA percentage of total FA (15.7–19.4%), a 2.3–3.7% GLA range is expected in the whole seeds of this species. For endemic species of *Borago*, these amounts are: 5.6, 5.0, 3.9, and 3.0%, for *B. morisiana*, *B. pygmaea*, *B. trabutii*, and *B. longifolia*, respectively.

The amounts of GLA shown by endemic *Borago* species analysed here are unusual among Boraginaceae seed oils. Only Macaronesian *Echium* species reached figures around 20% GLA of total FA (Guil-Guerrero et al., 2000, 2001a), while the usual figures found for GLA in most Boraginaceae species range from 5 to 12% of total FA (Guil-Guerrero et al., 2001b, 2013, 2014).

The remaining FA found in the genus *Borago* highlight the similarity of values of palmitic acid (PA, 16:0) and stearic acid (SA, 18:0) shown by all species in the whole oil: 10–14 and 4–7%, respectively); the high percentages of oleic acid (OA, 18:1*n*–9) and LA (~16–25% and 29–38%, respectively); the low amounts of ALA and SDA (less than 2% for all species); and the presence of minor amounts of C20, C22, and C24 SFA (< 1%) as well as their respective monounsaturated FA (MUFA, < 5% in all cases).

Regiospecific analyses of *B. officinalis* seed oil are already known (Gunstone, 1992; Redden et al., 1995; Guil-Guerrero et al., 2015), while the present work is the first to report on regiospecificity of FA for all endemic *Borago* species. The regiospecific distributions of FA within the glycerol backbone in the seed TAG of *Borago* species are summarised in Table 2 (endemic *Borago* species) and Table 3 (*B. officinalis*). The typical pattern for all species consists in the enrichment in the *sn*-2 position for all PUFA, which has been attributed to the weak activity on PA and SA exercised by the enzyme responsible for *sn*-2 acylation (Cahoon and Shmid, 2008). Thus, in the *sn*-2 position, all seed oils of *B. officinalis* are reported as particularly enriched in LA, ALA, GLA and SDA, while SFA are concentrated in the *sn*-1 and *sn*-3 positions, and MUFA are distributed among all available positions in similar proportions (Mattson and Volpenhein, 1963; Christie, 1992; Guil-Guerrero et al., 2015).

Consistently with these statements, *RI*, which has been used as a suitable parameter indicating the degree of enrichment in the *sn*-2 position for all FA (Guil-Guerrero et al., 2015), displays positive values for GLA in all *Borago* species analysed here. The best *RI* (1.0) was found in *B. longifolia* TAG (Table 2) and wild *B. officinalis* (Table 3), while among Tyrrhenian endemic species, *B. morisiana* registered the best

**Table 2**
Regiospecific fatty acid profiles (fatty acid% of total fatty acid area) of endemic *Borago* species [a].

| fatty acids | *B. longifolia* | | | *B. morisiana* | | | *B. pygmaea* | | | *B. trabutii* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | whole oil | *sn*-2 | *sn*-1,3 | whole oil | *sn*-2 | *sn*-1,3 | whole oil | *sn*-2 | *sn*-1,3 | whole oil | *sn*-2 | *sn*-1,3 |
| Total fatty acids (g/100 g seed) | 15.5 ± 1.3 a | – | – | 24.2 ± 2.8 b | – | – | 21.9 ± 0.5 b | – | – | 20.8 ± 1.7 b | – | – |
| 16:0 (PA) | 12.4 ± 0.2 a | 0.77 ± 1.4 b | 18.3 ± 0.1 c,e | 13.5 ± 0.1 a,d | 7.0 ± 0.4 d | 16.7 ± 0.2 c | 14.2 ± 0.3 a, d | 1.4 ± 1.2 b | 20.5 ± 0.5 e | 12.2 ± 0.5 a | 1.6 ± 1.3 b | 17.5 ± 0.2 c |
| 16:1*n*-7 (POA) | 0.3 ± 2.9 a | – | 0.5 ± 1.0 a | – | – | – | – | – | – | 0.2 ± 1.5 a | – | 0.4 ± 1.5 a |
| 18:0 (SA) | 5.5 ± 1.2 a | – | 8.3 ± 1.3 b,c | 5.6 ± 1.1 a | 5.1 ± 1.2 a | 5.8 ± 1.2 a,b | 6.7 ± 1.3 a,b | – | 10.0 ± 0.4 c | 4.2 ± 1.2 a | 1.0 ± 1.1 d | 5.9 ± 0.8 a,b |
| 18:1*n*-9 (OA) | 24.0 ± 0.1 a | 27.7 ± 0.1 b | 22.1 ± 0.1 a,d | 16.3 ± 0.2 c,h | 20.5 ± 0.2 d,h | 14.2 ± 0.2 c,f | 21.2 ± 0.3 a,d,h | 40.8 ± 0.5 e | 11.4 ± 0.6 f | 23.2 ± 0.1 a,d | 33.5 ± 0.8 g | 18.1 ± 0.2 h |
| 18:1*n*-7 (VA) | 0.3 ± 1.9 a | – | 0.5 ± 2.1 a | – | – | – | – | – | – | 0.4 ± 2.1 a | – | 0.6 ± 1.1 a |
| 18:2*n*-6 (LA) | 29.0 ± 0.1 a,c | 33.2 ± 0.1 b,c | 26.8 ± 0.2 a | 31.2 ± 0.4 c | 27.3 ± 0.1 a | 33.2 ± 0.1 b,c | 29.4 ± 0.2 a,c | 28.4 ± 0.2 a,c | 29.9 ± 0.3 a,c | 37.6 ± 0.3 d | 37.9 ± 0.3 d | 37.5 ± 0.2 d |
| 18:3*n*-6 (GLA) | 19.0 ± 0.1 a | 37.5 ± 0.4 b | 9.6 ± 0.4 c | 23.1 ± 0.8 d,f | 39.8 ± 0.3 b | 14.7 ± 0.2 e | 22.9 ± 0.1 d | 29.1 ± 0.1 f | 19.8 ± 0.7 a | 18.9 ± 0.7 a | 26.0 ± 0.2 f | 15.4 ± 0.1 e |
| 18:3*n*-3 (ALA) | 0.4 ± 3.0 a | 0.3 ± 3.2 a | 0.4 ± 2.3 a | 1.5 ± 2.4 a | – | 2.2 ± 1.7 a | 1.3 ± 1.5 a | – | 1.9 ± 2.2 a | 1.1 ± 3.0 a | – | 1.6 ± 2.6 a |
| 18:4*n*-3 (SDA) | 0.3 ± 2.1 a | 0.5 ± 3.1 a | 0.2 ± 4.3 a | 1.9 ± 2.0 a | – | 2.8 ± 1.3 a | 1.2 ± 1.1 a | – | 1.7 ± 2.4 a | 0.3 ± 2.2 a | – | 0.4 ± 2.9 a |
| 20:0 | 0.52.4 a | – | 0.8 ± 2.2 a | 1.1 ± 2.1 a | – | 1.7 ± 2.3 a | 0.4 ± 1.7 a | – | 0.5 ± 2.2 a | – | – | – |
| 20:1*n*-9 (GOA) | 3.1 ± 1.2 a | – | 4.7 ± 0.3 a | 3.2 ± 0.5 a | – | 4.8 ± 0.4 a | 2.9 ± 0.5 a | – | 4.3 ± 0.7 a | 1.7 ± 1.1 a | – | 2.6 ± 1.1 a |
| 22:0 | 0.5 ± 2.1 a | – | 0.7 ± 2.2 a | – | – | – | – | – | – | – | – | – |
| 22:1*n*-9 | 2.4 ± 1.1 a | – | 3.5 ± 0.9 a | – | – | – | – | – | – | – | – | – |
| 24:0 | 0.3 ± 2.7 a | – | 0.5 ± 2.6 a | – | – | – | – | – | – | – | – | – |
| 24:1*n*-9 | 2.1 ± 1.1 a | – | 3.2 ± 0.9 a | 1.3 ± 1.8 a | – | 2.0 ± 1.4 a | – | – | – | – | – | – |
| *Total identified fatty acids* | 100.0 | 100.0 | 100.0 | 98.6 | 99.6 | 98.1 | 99.9 | 99.7 | 100.0 | 99.9 | 100.0 | 99.8 |
| *regiospecific index* | | | | | | | | | | | | |
| 16:0 (PA) | | –0.9 | | | –0.9 | | | –0.9 | | | –0.5 | |
| 16:1*n*-7 (POA) | | – | | | – | | | – | | | – | |
| 18:0 (SA) | | – | | | – | | | –0.8 | | | –0.1 | |
| 18:1*n*-9 (OA) | | 0.2 | | | 0.9 | | | 0.4 | | | 0.3 | |
| 18:1*n*-7 (VA) | | – | | | – | | | – | | | – | |
| 18:2*n*-6 (LA) | | 0.1 | | | 0.0 | | | 0.0 | | | –0.1 | |
| 18:3*n*-6 (GLA) | | 1.0 | | | 0.3 | | | 0.4 | | | 0.7 | |
| 18:3*n*-3 (ALA) | | –0.2 | | | – | | | – | | | – | |
| 18:4*n*-3 (SDA) | | 0.6 | | | – | | | – | | | – | |
| 20:0 | | – | | | – | | | – | | | – | |
| 20:1*n*-9 (GOA) | | – | | | – | | | – | | | – | |
| 22:0 | | – | | | – | | | – | | | – | |
| 22:1*n*-9 | | – | | | – | | | – | | | – | |
| 24:0 | | – | | | – | | | – | | | – | |
| 24:1*n*-9 | | – | | | – | | | – | | | – | |

– not detected; means with the same letter in each row do not significantly differ at 0.05 level; LOD for all fatty acids was 0.004 mg.
[a] Data represent means ± standard deviation (SD) ($n$ = 3).

**Table 3**
Regiospecific fatty acid profiles (fatty acid% of total fatty acid area) of *B. officinalis* [a].

| Wild *B. officinalis* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Fatty acids | Commercial *B. officinalis* oil | | | Farmed *B. officinalis* | | | Spain | | |
| | whole oil | *sn*-2 | *sn*-1,3 | whole oil | *sn*-2 | *sn*-1,3 | whole oil | *sn*-2 | *sn*-1,3 |
| Total fatty acids (g/100 g seed) | – | – | – | 18.4 ± 0.8 a,b | – | – | 16.0 ± 0.9 a,c | – | – |
| 16:0 (PA) | 10.1 ± 0.2 a | 0.8 ± 1.1 b | 14.8 ± 0.4 c | 11.4 ± 0.1 a | 0.6 ± 1.8 b | 16.9 ± 0.2 c,d | 13.0 ± 0.1 a,c | 0.8 ± 1.0 b | 19.1 ± 0.2 d |
| 16:1*n*-7 (POA) | 0.1 ± 2.0 a | – | 0.2 ± 1.7 a | 0.2 ± 1.3 a | 0.1 ± 1.0 a | 0.2 ± 1.7 a | 0.3 ± 1.5 a | 0.1 ± 2.0 a | 0.4 ± 2.2 a |
| 18:0 (SA) | 5.1 ± 0.4 a | 0.3 ± 2.0 b | 7.4 ± 0.2 a | 4.2 ± 0.3 c | 0.2 ± 2.0 b | 6.3 ± 0.3 a,c | 6.0 ± 0.4 a,c | 0.3 ± 2.6 b | 8.8 ± 0.2 c |
| 18:1*n*-9 (OA) | 18.8 ± 0.3 a,b,c | 20.7 ± 0.3 a,c | 17.8 ± 0.4 a,b,c | 19.6 ± 0.4 a,c | 15.8 ± 0.4 b | 21.5 ± 0.3 c | 25.2 ± 0.1 d | 28.6 ± 0.5 e | 23.4 ± 0.2 c,d |
| 18:1*n*-7 (VA) | 0.2 ± 3.1 a | – | 0.3 ± 2.2 a | 0.3 ± 2.5 a | 0.2 ± 2.1 a | 0.5 ± 1.6 a | 0.5 ± 1.9 a | – | 0.6 ± 1.1 a |
| 18:2*n*-6 (LA) | 36.8 ± 0.2 a | 42.7 ± 0.3 b | 33.9 ± 0.1 c | 34.8 ± 0.2 a,c | 39.8 ± 0.3 a,b | 32.3 ± 0.2 c,d | 30.1 ± 0.2 d | 36.8 ± 0.2 a | 26.8 ± 0.6 e |
| 18:3*n*-6 (GLA) | 19.8 ± 0.3 a | 34.5 ± 0.3 b | 12.4 ± 0.2 c | 19.4 ± 0.3 a | 43.0 ± 0.2 d | 7.5 ± 0.4 e | 15.7 ± 0.2 f | 32.8 ± 0.3 b | 7.1 ± 0.4 e |
| 18:3*n*-3 (ALA) | 0.2 ± 2.4 a | 0.2 ± 2.6 a | 0.1 ± 1.3 a | 0.2 ± 2.7 a | 0.3 ± 2.1 a | 0.2 ± 2.0 a | 0.6 ± 0.1 a | 0.3 ± 2.3 a | 0.8 ± 1.0 a |
| 18:4*n*-3 (SDA) | 0.1 ± 5.6 a | 0.2 ± 1.1 a | 0.1 ± 3.1 a | 0.1 ± 2.5 a | – | 0.2 ± 1.9 a | 0.2 ± 0.1 a | – | 0.2 ± 2.0 a |
| 20:0 | 0.3 ± 0.9 a | – | 0.5 ± 2.4 a | 0.4 ± 2.0 a | – | 0.5 ± 1.9 a | 0.4 ± 2.5 a | – | 0.6 ± 2.1 a |
| 20:1*n*-9 (GOA) | 4.1 ± 0.4 a | 0.5 ± 1.9 b | 6.0 ± 0.7 a | 4.0 ± 1.2 a | 0.4 ± 2.3 b | 5.8 ± 0.3 a | 3.7 ± 0.2 a | 0.4 ± 2.1 b | 5.3 ± 0.1 a |
| 22:0 | 0.2 ± 2.4 a | – | 0.3 ± 2.1 a | 0.1 ± 1.9 a | – | 0.2 ± 2.3 a | 0.3 ± 2.5 a | – | 0.4 ± 2.1 a |
| 22:1*n*-9 | 2.5 ± 0.8 a | – | 3.8 ± 0.6 a | 3.4 ± 0.7 a | – | 5.0 ± 0.4 a | 2.5 ± 1.1 a | – | 3.7 ± 0.5 a |
| 24:0 | 0.1 ± 1.0 a | – | 0.1 ± 2.2 a | 0.1 ± 2.9 a | – | 0.2 ± 2.6 a | 0.2 ± 1.9 a | – | 0.2 ± 2.5 a |
| 24:1*n*-9 | 1.5 ± 1.3 a | – | 2.3 ± 1.1 a | 1.9 ± 1.3 a | – | 2.8 ± 0.8 a | 1.8 ± 0.7 a | – | 2.7 ± 0.8 a |
| *Total identified fatty acids* | 99.9 | 99.8 | 99.9 | 100.0 | 100.0 | 100.0 | 99.9 | 100.0 | 99.8 |
| *Regiospecific index* | | | | | | | | | |
| 16:0 (PA) | | –0.9 | | | −0.9 | | | −0.9 | |
| 16:1*n*-7 (POA) | | – | | | −0.5 | | | −0.6 | |
| 18:0 (SA) | | –0.9 | | | −1.0 | | | −0.9 | |
| 18:1*n*-9 (OA) | | 0.1 | | | −0.2 | | | 0.1 | |
| 18:1*n*-7 (VA) | | – | | | −0.7 | | | – | |
| 18:2*n*-6 (LA) | | 0.2 | | | 0.1 | | | 0.2 | |
| 18:3*n*-6 (GLA) | | 0.8 | | | 1.0 | | | 1.0 | |
| 18:3*n*-3 (ALA) | | 0.0 | | | 0.5 | | | −0.5 | |
| 18:4*n*-3 (SDA) | | 1.0 | | | – | | | – | |
| 20:0 | | – | | | – | | | – | |
| 20:1*n*-9 (GOA) | | −0.9 | | | −0.9 | | | −0.9 | |
| 22:0 | | – | | | – | | | – | |
| 22:1*n*-9 | | – | | | – | | | – | |
| 24:0 | | – | | | – | | | – | |
| 24:1*n*-9 | | – | | | – | | | – | |

| Wild *B. officinalis* | | | | | | |
|---|---|---|---|---|---|---|
| Fatty acids | Sardinia | | | Tunisia | | |
| | whole oil | *sn*-2 | *sn*-1,3 | whole oil | *sn*-2 | *sn*-1,3 |
| Total fatty acids (g/100 g seed) | 20.3 ± 1.1 b | – | – | 14.4 ± 0.9 c | – | – |
| 16:0 (PA) | 10.8 ± 0.3 a | 0.5 ± 2.0 b | 16.0 ± 0.1 c,d | 11.1 ± 0.2 a | 0.5 ± 1.0 b | 16.4 ± 0.2 c,d |
| 16:1*n*-7 (POA) | – | 0.1 ± 2.7 a | – | – | 0.1 ± 1.2 a | – |
| 18:0 (SA) | 7.4 ± 0.2 a,c | 0.3 ± 2.5 b | 11.0 ± 0.3 d | 7.5 ± 0.2 a,c | 0.3 ± 1.5 b | 11.1 ± 0.2 d |
| 18:1*n*-9 (OA) | 22.7 ± 0.3 c,d | 25.2 ± 0.3 d | 21.5 ± 0.4 c | 23.3 ± 0.3 c,d | 25.3 ± 0.1 d | 22.3 ± 0.5 c,d |
| 18:1*n*-7 (VA) | 0.4 ± 2.3 a | – | 0.6 ± 2.2 a | 0.3 ± 2.5 a | 0.2 ± 2.1 a | 0.3 ± 2.5 a |
| 18:2*n*-6 (LA) | 32.8 ± 0.4 c,d | 37.3 ± 0.5 a | 30.6 ± 0.3 d | 30.9 ± 1.0 d | 35.2 ± 0.4 a,c | 28.8 ± 0.2 d,e |

*(continued on next page)*

**Table 3** *(continued)*

| Wild *B. officinalis* | | | | | | |
|---|---|---|---|---|---|---|
| Fatty acids | Sardinia | | | Tunisia | | |
| | whole oil | *sn*-2 | *sn*-1,3 | whole oil | *sn*-2 | *sn*-1,3 |
| 18:3*n*-6 (GLA) | 18.0 ± 0.8 a,f | 35.8 ± 0.2 b,g | 9.1 ± 0.3 e | 18.2 ± 0.3 a,f | 37.7 ± 0.3 g | 8.4 ± 0.4 e |
| 18:3*n*-3 (ALA) | 0.4 ± 1.7 a | 0.3 ± 2.7 a | 0.4 ± 2.6 a | 0.4 ± 2.5 a | 0.3 ± 1.4 a | 0.4 ± 2.3 a |
| 18:4*n*-3 (SDA) | 0.2 ± 2.1 a | 0.3 ± 2.2 a | 0.2 ± 1.8 a | 0.3 ± 1.2 a | 0.4 ± 2.0 a | 0.2 ± 2.0 a |
| 20:0 | 0.4 ± 1.7 a | – | 0.7 ± 2.3 a | 0.5 ± 2.7 a | – | 0.7 ± 2.3 a |
| 20:1*n*-9 (GOA) | 3.7 ± 0.4 a | 0.4 ± 2.1 b | 5.3 ± 1.3 a | 4.1 ± 1.5 a | 0.3 ± 2.6 b | 6.0 ± 0.4 a |
| 22:0 | 0.2 ± 2.7 a | – | 0.3 ± 2.0 a | – | – | – |
| 22:1*n*-9 | 1.9 ± 0.5 a | – | 2.9 ± 1.1 a | 2.1 ± 0.9 a | – | 3.2 ± 0.4 a |
| 24:0 | – | – | – | – | – | – |
| 24:1*n*-9 | 1.2 ± 0.7 a | – | 1.8 ± 0.8 a | 1.4 ± 0.6 a | – | 2.1 ± 0.5 a |
| *Total identified fatty acids* | 100.0 | 99.9 | 100.0 | 100.0 | 99.9 | 100.0 |
| *Regiospecific index* | | | | | | |
| 16:0 (PA) | | −1.0 | | | −1.0 | |
| 16:1*n*-7 (POA) | | – | | | – | |
| 18:0 (SA) | | −1.0 | | | −1.0 | |
| 18:1*n*-9 (OA) | | 0.1 | | | 0.1 | |
| 18:1*n*-7 (VA) | | – | | | −0.5 | |
| 18:2*n*-6 (LA) | | 0.1 | | | 0.1 | |
| 18:3*n*-6 (GLA) | | 1.0 | | | 1.0 | |
| 18:3*n*-3 (ALA) | | −0.3 | | | −0.3 | |
| 18:4*n*-3 (SDA) | | 0.5 | | | 0.3 | |
| 20:0 | | – | | | – | |
| 20:1*n*-9 (GOA) | | −0.9 | | | −0.9 | |
| 22:0 | | – | | | – | |
| 22:1*n*-9 | | – | | | – | |
| 24:0 | | – | | | – | |
| 24:1*n*-9 | | – | | | – | |

– not detected; means with the same letter in each row do not significantly differ at 0.05 level; LOD for all fatty acids was 0.004 mg.

[a] Data represent means ± standard deviation (SD) ($n$ = 3).

value (0.7). Thus, in terms of GLA bioavailability, *B. longifolia* presumably has higher values than the other *Borago* species, as it contains GLA mainly in the *sn*-2 location, close in absolute values (37.5%) to that displayed by *B. morisiana* (39.8%). The *RI* values compare favourably with values previously found for GLA in other seed oils. For instance, in other Boraginaceae it ranges from 0.6 to 1.0; in Onagraceae the value is 0.1; in Scrophulariaceae 0.6; and in Saxifragaceae 0.2. Regarding ALA-rich oils, in *Linum usitatissimum* (flaxseed oil) it is 0.2, while for docosahexaenoic acid (DHA, 22:6*n*–3), the index reaches 0.6 in cod liver oil (Guil-Guerrero et al., 2015). For the remaining FA, a similar trend was observed in the *sn*-2 position; i.e., the lessening of PA, SA, and OA; the neutral action on LA, and ALA; and the increasing of SDA.

The FA profiles of Boraginaceae species have been used as a chemotaxonomic marker (Velasco and Goffman, 1999; Guil-Guerrero et al., 2001a,b). To detect similarities among the *Borago* species analysed in this study, we have performed a cluster analysis using the values calculated for the *RI*, which takes into account the percentage of a given FA in the *sn*-2 position, with respect to its percentage in the whole oil. Hierarchical cluster analysis of the *RI* results in the formation of three clusters, I, II, and III (Fig. 1). The three clusters reflect broad similarities in the *RI* of its individual species/samples. Cluster I contains all the *B. officinalis* samples collected in different locations, commercial *B. officinalis* oil and cultivated *B. officinalis*. Cluster II contains all samples of *B. officinalis* and *B. longifolia*, which are native to the Maghreb area. Cluster III contains the remaining species, i.e. the two Sardinian species (*B. morisiana* and *B. pygmaea*) as well as another North African species, *B. trabutii*. The similarities found among *Borago* species through the *RI* agree with the results of phylogenetic analyses of sequences from six chloroplast DNA (cpDNA) markers (Mansion et al., 2009). Therefore, besides helping to elucidate taxonomic relationships among species, the *RI* could be used as an appropriate tool in chemometric methods, when the aim is the classification and comparison of different vegetable oils in routine food-control analyses.

In conclusion, it can be highlighted that all endemic *Borago* taxa analysed in this work have suitable positional distribution for GLA in their seed oils, and similar or better than that showed by the hitherto cultivated *B. officinalis*. All endemic species tested in this work could be adequate candidates for culture adaptation and/or agronomic actions to increase their GLA percentages. Future lines of research could include, among others: i) the development of new GLA-producing taxa through hybridisation between *Borago* species and further culture selection; ii) application of molecular engineering tools for Δ6-desaturase gene transfer from wild endemic *Borago* species to *B. officinalis* to increase the GLA percentage in the seed oils; iii) molecular engineering techniques to transfer the gene coding of the enzyme inserting GLA in the *sn*-2 position of TAG.

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# Insects as Food: Fatty Acid Profiles, Lipid Classes, and *sn*-2 Fatty Acid Distribution of Lepidoptera Larvae

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**The aim of this work is to assess the fatty acid (FA) profiles, the lipid classes, and the positional distribution of FA within the triacylglycerol (TAG) structure of the lipids extracted from Lepidoptera larvae, compared with Diptera and Coleoptera ones. The major essential FA in most species was α-linolenic acid (ALA), at 62.5 mol% of total FA in *Caligo memnon*. Oleic acid (OA) was found in high amounts in *Galleria mellonella*, at 44.8 mol% of total FA. The *n*-6/*n*-3 ratio was below 1 in 12 of the 15 species surveyed, reaching 14.8 in *Oryctes nasicornis*. As for lipid fractions, an increase in the amounts of stearic and linoleic acids in the phospholipids fraction was observed. Distribution of FA in the *sn*-2 position of TAG is assessed through enzymatic hydrolysis reaction. *Bombyx mori* larvae contain ALA at 49.1 mol% of total FA at the *sn*-2 position of TAG, while OA is the main FA in *sn*-2 position in *G. mellonella* (88.5 mol% of total FA). Overall, Lepidoptera larvae constitute a rich source of specifically located at the *sn*-2 position OA and ALA, and similar or better than current sources. Initial data on other insect orders also points in this direction. *Practical Applications*: The present study develops knowledge about the lipid classes of Lepidoptera larvae, as well as on their regiospecific FA profiles. The data from Lepidoptera species are compared to other ones obtained from samples belonging to Diptera and Coleoptera. Insects are rich in essential amino acids; therefore if they contain omega-3 and omega-9 FA, would be healthy alternatives to other commonly consumed animal foods, which tend to be rich in saturated and omega-6 FA. Therefore, it is necessary to check different species to know their FA profile, as well as both the structure of their TAG and their lipid classes. Lepidoptera larvae constitute an alternative source of OA- and ALA-rich oils, which can be devoted for food use, as well as for using in the food and pharmaceutical industries, with agronomic implications.**

## 1. Introduction

Insect consumption is a traditional alimentary habit that comes from ancient times. In many cases, insects are a vital dietary element providing nutrients of high biological value including energy.[1] Furthermore, certain insect species, especially during the larval stage, contain high proportions of fat,[2] thereby opening interesting possibilities with alimentary and industrial purposes. Previously, it has been established that the richness of the different FA classes – *n*-3, *n*-6, *n*-9 and saturated – is species-dependent,[2–4] although it is possible to modify the FA profiles, especially of larvae, by manipulating the composition of the diet.[5,6] Among the FA groups, the *n*-3 long-chain polyunsaturated FA (LCPUFA) are especially relevant in human nutrition, given that in Western diets the *n*-6/*n*-3 ratio ranges between 15.0/1 and 16.7/1, instead the optimal dose ratio of 1/1 to 4/1, which is assumed that was in the diet of most Paleolithic peoples.[7] To improve this ratio, new food *n*-3 LCPUFA sources, as are those from insects, are desirable.

α-linolenic acid (ALA, 18:3*n*3) is especially abundant in most green organs of plants, being an essential FA (EFA). This, together with linoleic acid (LA, 18:2*n*6), which is also an EFA, are the most abundant LCPUFA in animal and vegetal foods. LA deficiency leads to poor growth, fatty liver, skin lesions, and reproductive failure. Meanwhile, ALA deficiency leads to reductions in visual acuity and to an increased cardiovascular disease risk, as well as to a decrease of docosahexaenoic acid (DHA, 22:6*n*3) in brain and retina, and thus to a poorly modulation of several signalling pathways in the brain.[8] On the other hand, oleic acid (OA, 18:1*n*9), is a monounsaturated FA (MUFA) that display modulatory effects in a wide range of physiological functions, having anti-inflammatory and anti-atherogenic properties. It develops antitumor actions, acting against autoimmune and inflammatory diseases, besides its ability to facilitate wound healing and improving the immune response associated to a more successful elimination of pathogens.[9] By the enzyme Δ6-desaturase LA yields γ-linolenic

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acid (GLA, 18:3*n*6), and by the consecutive action of other desaturase and elongase enzymes, it yields an *n*-6 very-LCPUFA (VLCPUFA), that is arachidonic acid (ARA, 20:4*n*6). Meanwhile, ALA is desaturated by the same enzyme to stearidonic acid (SDA, 18:4*n*3), and further it is metabolized to *n*-3 VLCPUFA: eicosapentaenoic acid (EPA; 20:5*n*3) and DHA.[10,11]

Whether the essentiality of ALA in the diet primarily reflects the activity of ALA itself or of VLCPUFA synthesized from ALA is a matter for debate, as well as whether DHA synthesis from ALA is sufficient to supply the need of the adult brain.[12] Today, suitable OA- and ALA-oils are needed to meet the market demand, given that large health benefits that have been attributed to their dietary supplementation. Although some controversy exists, and additional studies are needed, current evidence suggests that the position in which *n*-3 and *n*-6 PUFA are esterified in natural triacylglycerol (TAG) molecules influences their bioavailability. This is so because during digestion, TAG are hydrolyzed to *sn*-2 monoacylglycerols (2-MAG) and to free FA (FFA) in the small intestine by pancreatic lipase, being both absorbed by the enterocytes.[13,14] However, the hydrolysis of the TAG is affected by chain length and unsaturation of the FA in the *sn*-1,3 positions, with medium chain TAG (MCTAG) being hydrolyzed faster than long chain TAG (LCTAG).[14] After intestinal absorption, medium-chain FA (MCFA) are preferentially transported via the portal vein to the liver, where they are oxidized, while the 2-MAG are reesterified with FA of exogenous or endogenous origin. These form a new population of TAG, which are packed into chylomicrons and excreted into the lymph.[13,14] Overall, the structure of TAG is critical, and displacing PUFA or MUFA from the *sn*-2 position, by substitution with SFA, is hypothesized to cause lipid and lipoprotein abnormalities. Moreover, dietary FA in the *sn*-2 position could influence lipemia and platelet reactivity, as well as desaturation and elongation of PUFA.[14]

Previously, it has been reported that OA and ALA are the most abundant components in the *sn*-2 position in diacylglycerols (DAG) and TAG in the body fat of an Orthopter, *Locusta migratoria* Linnaeus, 1758.[15] Also, that in both the *sn*-1-position of the DAG and the *sn*-1,3 positions of the TAG, saturated FA (SFA), mainly palmitic (PA, 16:0) and stearic (SA, 18:0) acids are predominant.[15]

As previously exposed, the design of lipids containing bioactive LCPUFA located at *sn*-2 position, able to be structured by using specific lipases for introducing MCFA in the *sn*-1,3 positions, is a suitable strategy to avail the maximum nutritional benefits from that particular FA composition. In this work, the FA profiles, the lipid classes, and the FA distribution within the TAG structure of OA- and ALA-rich Lepidoptera species from different locations compared with Diptera and Coleoptera ones have been determined, in order to assess their feasibility as sources of *sn*-2 located OA and ALA.

## 2. Experimental Section

### 2.1. Sample Collection

Insects were collected from the wild, donated or purchased (**Table 1**). All samples were at larval stage, excepting those of *Danaus plexippus* b, which were at pupa phase. Samples were freeze-dried and ground in the lab with the aid of a mortar and stored frozen at −40 °C until analysis.

### 2.2. Lipid Analyses

Freeze-dried larvae were analyzed to determine their overall FA profile. Analyses were made by simultaneous oil extraction and transesterification, which was performed according to previous works.[16] To check lipid classes, larvae were extracted with a chloroform–methanol (2:1, v/v) mixture according to Folch et al.[17] During the extraction procedure, lipids were protected against oxidation by adding 0.05 mg mL$^{-1}$ BHT to the solvents. The lipid extracts were separated into eight fractions by preparative thin layer chromatography (TLC) on silica gel 60. After TLC development by using *n*-hexane:diethylether:acetic acid (70:30:1 v/v/v), the plate was revealed under a iodine stream and bands corresponding to the detected lipid classes were scrapped off and stored cold until checking their FA profiles. For FA derivatization, samples (150–200 mg of freeze-dried insect or ∼10 mg of each lipid class) were weighed in test tubes and *n*-hexane (1 mL) was added to each one. FA methyl esters (FAME) were obtained after adding 1 mL of the methylation mixture, which was composed of methanol:acetyl chloride (20:1 v/v), and 100 mL of a standard solution of 10 mg mL$^{-1}$ of pentadecanoic acid (15:0). The tubes were heated at 100 °C for 10 min. After cooling to room temperature, 1 mL of distilled water was added in each tube, after which the tubes were centrifuged at 3500 rpm for 5 min. The upper hexane layer was removed for GC analyses.[16,18]

### 2.3. GC Analyses

FAME were analyzed by using a Focus GC (Thermo Electron, Cambridge, UK) equipped with flame ionization detector and an Omegawax 250 capillary column (30 m × 0.25 mm id × 0.25 μm film thickness; Supelco, Bellefonte, PA, USA). The temperature program was: 1 min at 90 °C, heating to 200 °C at a rate of 10 °C min$^{-1}$, constant temperature at 200 °C (3 min), heating to 260 °C at a rate of 6 °C min$^{-1}$, and constant temperature at 260 °C (5 min). The injector temperature was 250 °C with a split ratio 50:1. The injection volume was 4 μL and the detector temperature was 260 °C. Nitrogen was used as the carrier gas (1 mL min$^{-1}$) and peaks were identified by retention times determined for known FAME standards (PUFA No. 1 from Sigma, St. Louis, USA), while the total FAME contents were estimated by using methyl pentadecanoate (15:0) as an internal standard.[16,18]

### 2.4. Enzymatic Hydrolysis of Lipids

The goal of this analysis was to obtain the regiospecific FA composition of TAG. Briefly, 50 mg of the TLC-purified TAG fraction was hydrolyzed by adding porcine pancreatic lipase (40 mg) in 4 mL of Tris solution (1M, pH = 8.0), 1 mL of a sodium cholate solution (0.1% w/v) and 400 μL of a calcium chloride solution (22% w/v). Flasks were then placed in a water bath (37 °C, 120 rpm) for 30 min. After that, they were removed

**Table 1.** Samples characteristics.

| Species | Common name | Date collection | Origin | Species distribution | Feed |
|---|---|---|---|---|---|
| Lepidoptera | | | | | |
| *Bombyx mori* Linnaeus, 1758 | Silkworm | 03/08/2016 | Reptimercado (Lorca, España) | Worldwide | *Morus alba* Linnaeus, 1753, leaves |
| *Caligo eurilochus* Cramer, 1775 | Forest giant owl | 06/04/2016 | Hostería Mariposas de Mindo (Mindo, Ecuador) | Central and South America | *Musa ×paradisiaca* Linnaeus 1753, leaves |
| *Caligo memnon* C. & R. Felder, 1867 | Giant owl | 06/04/2016 | Hostería Mariposas de Mindo (Mindo, Ecuador) | North, Central, and South America | *Musa ×paradisiaca* Linnaeus 1753, leaves |
| *Charis anius* Cramer, 1776 | Anius metalmark | 06/04/2016 | Hostería Mariposas de Mindo (Mindo, Ecuador) | Central and South America | *Mikania micrantha* Kunth 1820, leaves |
| *Danaus plexippus*[a] Linnaeus, 1758 | Monarch butterfly | 07/04/2016 | Hostería Mariposas de Mindo (Mindo, Ecuador) | North, Central, and South America | *Asclepia aurasabica* Linnaeus 1753, leaves |
| *Danaus plexippus*[b] Linnaeus, 1758 | Monarch butterfly | 02/07/2016 | Deco experience SL (Almería, España) | North, Central, and South America | *Asclepia aurasabica* leaves |
| *Dryas iulia* Fabricius, 1775 | Julia butterfly | 06/04/2016 | Hostería Mariposas de Mindo (Mindo, Ecuador) | Tropical South America | *Passiflora incarnata* Linnaeus 1753, leaves |
| *Galleria mellonella* Linnaeus, 1758 | Wax moth | 07/09/2016 | Reptimercado (Lorca, España) | Worldwide | Bran, honey and wax |
| *Methona grandior* Forbes, 1944 | Ecuadorian glasswing butterfly | 06/04/2016 | Hostería Mariposas de Mindo (Mindo, Ecuador) | South America | Grases |
| *Papilio thoas* Linnaeus, 1771 | King swallowtail | 06/04/2016 | Hostería Mariposas de Mindo (Mindo, Ecuador) | Tropical and subtropical America | *Citrus* spp. leaves |
| *Parides arcas* Cramer, 1777 | Arcas pipevine | 06/04/2016 | Hostería Mariposas de Mindo (Mindo, Ecuador) | Central- and northern South America | *Aristolochia constricta* Griseb. 1857, leaves |
| *Siproeta stelenes* Linnaeus, 1758 | Malachite | 06/04/2016 | Hostería Mariposas de Mindo (Mindo, Ecuador) | Central and South America | Acanthaceae leaves |
| *Spodoptera exigua* Hübner, 1808 | Beet armyworm | 09/13/2016 | Entomotech (Almería, Spain) | Asia in origin, worldwide | Laying hen feed |
| Coleoptera | | | | | |
| *Oryctes nasicornis* Linnaeus, 1758 | European rhinoceros beetle | 08/21/2016 | Collected in the wild | Europe and Asia | Decaying organic matter |
| Diptera | | | | | |
| *Hermetia illucens* Linnaeus, 1758 | Black soldier fly | 09/13/2016 | Entomotech (Almería, Spain) | America in origin, worldwide | Laying hen feed |

[a] Larval stage; [b] Pupa stage.

and cooled in a water bath at 20 °C and 2 mL of hydrochloric acid (6 M) and 1 mL of n-hexane were added. Flasks were shaken for 1 min and the content was transferred to test tubes prior to centrifugation (3500 rpm, 5 min). Hydrolysis reaction was performed in triplicate. An aliquot was collected to separate the hydrolysis products by TLC as previously described.[18] The FA profile by GC-FID was performed after the recovery of the 2-MAG fraction from the TLC.

The FA profile of 2-MAG and the FA profile of the unreacted fat were obtained in g/100 g and were transformed to mole percentage. Both FA profiles were used to calculate the FA profile at *sn*-1,3 by using the formula[19]:

$$\text{position } 1,3_{\text{FAi}} \frac{3x\text{TAG}_{\text{FAi}} - \text{position } 2_{\text{FAi}}}{2}$$

where position 2 is the amount of an FA at *sn*-2 and $\text{TAG}_{\text{FAi}}$ is the amount of the same FA in the unreacted TAG and *i* is one of the FA in insect fat.

### 2.5. Regiospecific Index (RI)

The RI was defined as the percentage of a given FA in the *sn*-2 position of TAG with respect to its percentage in the TAG, according to the formula:

$$\text{RI} = (\text{FA mol \% in } sn\text{-2 position} - \text{FA mol \% in TAG})/\text{FA mol \% in TAG}$$

The RI range for a given FA is between −1.0, when such FA is not esterified at all at the *sn*-2 position, and 2.0, when the esterification rate of the FA at the *sn*-2 position is 100%. Thus, RI = 0 means that 33.3% of the total target FA is esterified at the *sn*-2 position.[16]

### 2.6. Quality Control

Intermediate precision, quality control of GC analyses, detection limit, method reproducibility, and recovery studies were performed as previously described.[16]

All data for total and *sn*-2 FA shown in Tables 2–4 were obtained by GC. Results are expressed as mean values in Tables; standard deviations among means were routinely lower than 5%.

### 2.7. Statistical Analysis

All experiments and/or measurements were replicated three times. Analysis of variance (ANOVA) was carried out and the average values were compared with Fisher's Multiple Comparison Test. Differences were considered statistically significant at $P < 0.05$. All statistical analyses were performed using Statgraphics Plus software version 5.1, Windows package (Statistical Graphics Co., Rockville, MD).

## 3. Results and Discussion

This work provides novel information about the FA profiles, lipid classes, and regiospecificity of the TAG for several Lepidoptera

 

larvae, as well as for two Diptera and Coleoptera ones used for comparison, selected for this purpose because most consumed insect species belong to these two taxa. All species checked in this work are herbivorous, and therefore, toxic syndromes due to their consumption are not expected. In fact, the toxicity caused by insect consumption is exceptional.[20] Among the species analyzed, stand out by their traditional edible use *Oryctes nasicornis* Linnaeus, 1758, *Bombyx mori* Linnaeus, 1758, *Galleria mellonella* Linnaeus, 1758, and *Hermetia illucens* Linnaeus, 1758.

Previously, the FA profiles of *H. illucens* larvae have been characterized,[6] and those of *B. mori*[21] as well as its lipids classes,[22] while several years ago the regiospecific FA profiles of *L. migratoria* were reported.[15]

Given that insects are monogastric animals, their FA profiles can be partially changed by dietary manipulation[6]; however, most insect species display specific feeding habits; thus, this fact limits the possibility of modifying their FA profiles by means of specific diets.

A screening on the FA profiles of several Lepidoptera larvae is summarized in **Table 2**, while the lipid classes and the regiospecific FA profiles of Lepidoptera larvae compared to that of Coleoptera and Diptera ones are summarized in Tables 3 and 4, respectively. *H. illucens* larvae, which was one of the species used to compare the FA profiles of Lepidoptera, shows a very complex FA profile, in which highlights MCFA, as is lauric acid (LaA, 12:0), whose origin could be due to the bioconversion of carbohydrates to lipids. In this regard, the capacity of the fat body for lipogenesis from glucose is much higher than that for glycogen synthesis, which explains the higher content of lipids compared to glycogen in the insect fat.[23] Therefore, LaA seems to be a metabolic target in the metabolism of *H. illucens* larvae, although its percentage with respect to the total FA is subjected to wide changes in function of dietary manipulations.[6]

For most Lepidoptera species reported in Table 2, the FA profiles are clearly dominated by ALA, which presumably reflects the feed composition.[6] This FA was in the top of the range in the two *Caligo* species analyzed, in which ALA reached ~62 mol% of total FA (Table 2). It is also noticeable that *Danaus plexippus* Linnaeus, 1758 showed differences in oil amounts and FA profiles depending on the development stage: *D. plexippus* a, at larval stage, contained ALA at 59.0 mol% of total FA and 10.6 g FA/100 g dry wt, while *D. plexippus* b at pupa stage contained ALA at 39.1 mol% of total FA and 6.5 g FA/100 g dry wt. Such difference was clearly due to that insects at pupa phase have partially consumed their fat reservoirs for metamorphosis.[23] The only species differing from this pattern of FA distribution in Lepidoptera are *G. mellonella* and *Spodoptera exigua* Hübner (**Table 3**), which contain OA at 44.8 and 27.0 mol% of total FA, respectively. Such high content is probably due to the fed used for rearing (Table 1), although this fact needs more research. Larvae of *G. mellonella* have been recently characterized to perform fast bio-degradation of polyethylene (PE) producing ethylene glycol,[24] thus, further knowledge about its body composition is an interesting task, to discern whether the consumption of PE by larvae might alter this pattern of FA composition, this fact having economic and nutritional implications. On the other hand, *O. nasicornis*, the Coleoptera species used as control, showed also high amount of OA, at 36.7 mol% of total FA.

As to the remaining FA, it highlights the broad range of concentrations for PA and SA showed by all species in the unhydrolyzed oil (Table 2): 9.3 (*H. illucens*) to 38.3 (*Charis anius* Cramer, 1776) and 2.1 (*G. mellonella*) to 12.6 (*D. plexippus* b) mol % of total FA, respectively, and the moderate percentages of LA detected in most species: from 4.5 (*H. illucens*) to 23.2 (*S. exigua*) mol% of total FA, which reflects the differential richness of the different food sources. On the other hand, the occurrence of minor amounts of C20 and C22 SFA in some species (usually less than <1 mol% of total FA, excepting *S. exigua*); the presence or relative high amounts of gondoic acid (GOA, 20:1*n*9) in *G. mellonella* (2.9 mol% of total FA); as well as the minor amounts of DHA showed by *B. mori*, *D. plexippus* b, *Parides arcas* Cramer, 1777, *S. exigua*, and *H. illucens* (0.3, 0.3, 1.3, 0.4, and 0.9 mol% of total FA, respectively), would be due to a reduced enzymatic activity of elongation and desaturation in FA of carbon chain length >20 C.

The amount of total FA ranges from 1.7 in *Papilio thoas* Linnaeus 1771 to 31.7 g/100 g body weight (wt) in *G. mellonella*, thus, considering both the percentage of OA (46.4 g/100 g of total FA) and the FA amount found in this last species, it is expected OA at 14.7 g/100 g of the whole larva. Concerning ALA, considering the percentage of total FA (60.8 g/100 g) and the oil content (20.7 g/100 g) detected in *B. mori* larvae, the body of this last species will contain about 12.6% ALA on dry wt.

The *n*-6/*n*-3 PUFA ratio recommended by FAO for edible oils is below 10:1.[25] The insect lipids summarized in Table 2 have an appropriate ratio for 14 of the 15 species surveyed, ranging from 0.1 (*C. eurilochus*, *C. memnon*, *Dryas iulia* Fabricius, 1775 and *Methona grandior* Forbes, 1944) to 14.8 (*O. nasicornis*). However, this last value is irrelevant, since in this species *n*-3 and *n*-6 PUFA percentages are low, and in this insect highlights its high OA percentage, which is a healthy FA belonging to the *n*-9 series. Such a ratio is especially adequate for human nutrition, since excessive amounts of *n*-6 PUFA and a very high *n*-6/*n*-3 ratio, as is found in today's Western diets, promote the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases. Conversely, increased levels of *n*-3 PUFA (a low *n*-6/*n*-3 ratio) exert suppressive effects.[7]

The high amounts of ALA, which is the main *n*-3 PUFA present in these species, is responsible for the low *n*-6/*n*-3 ratio. All larvae analyzed in this work were reared with leaves (Table 1), which constitute a noteworthy source of *n*-3 FA,[26] excepting those of *O. nasicornis*, *S. exigua*, *H. illucens*, and *G. mellonella*, in which the *n*-6/*n*-3 ratio was found in the top of the range. This situation agrees with previous studies pointing out that the FA composition of the insect lipids is mainly influenced by the feed used for rearing.[6]

Table 3 presents the lipid classes of four species of Lepidoptera selected from the previous screening, compared with those of Coleoptera and Diptera. Such species were chosen according to the percentage of bioactive FA (OA and ALA) they contained. The lipids classes were separated and isolated by silica gel TLC according to their degree of polarity. They included, on a moisture-free basis, TAG (4.7–26.0%), DAG (0.3–1.8%), sterol esters (SE, 0.2–0.9%), free FA (FFA, 0.4–1.4%), and phospholipids (PL, 0.5–3.6%), while MAG were detected as traces.

**Table 2.** FA composition of several Lepidoptera larvae compared to that of Diptera and Coleoptera (mol% of total FA).[a)]

| | Lepidoptera | | | | | | | | | | | | | Diptera | Coleoptera |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acids | Bmo | Ceu | Cme | Can | Dpl a[b)] | Dpl b[c)] | Diu | Gme | Mgr | Par | Pth | Sex | Sst | Hil | Ona |
| 8:0 | 1.8 ± 0.3 | 0.2 ± 0.1 | – | – | – | 0.3 ± 0.2 | 0.4 ± 0.3 | – | – | 0.4 ± 0.2 | 0.6 ± 0.2 | – | 0.4 ± 0.2 | 0.4 ± 0.2 | 2.4 ± 0.3 |
| 10:0 | – | – | 0.2 ± 0.2 | – | 0.2 ± 0.2 | – | 0.3 ± 0.3 | – | 0.2 ± 0.2 | – | 0.5 ± 0.3 | 2.8 ± 0.5 | 0.2 ± 0.1 | 3.4 ± 0.6 | – |
| 12:0 (LaA) | – | 0.4 ± 0.1 | 0.4 ± 0.2 | 0.4 ± 0.1 | 0.4 ± 0.2 | 1.4 ± 0.7 | 0.7 ± 0.2 | – | 1.1 ± 0.3 | 0.6 ± 0.3 | 0.5 ± 0.2 | 0.1 ± 0.2 | 1.4 ± 0.3 | 57.2 ± 1.9 | – |
| 14:0 | 1.6 ± 0.4 | 0.4 ± 0.2 | 0.6 ± 0.2 | 0.2 ± 0.2 | 0.5 ± 0.1 | 0.6 ± 0.2 | 0.5 ± 0.3 | 0.8 ± 0.2 | 1.1 ± 0.2 | 0.4 ± 0.3 | 1.0 ± 0.2 | 1.3 ± 0.3 | 0.4 ± 0.1 | 8.4 ± 0.8 | 3.2 ± 0.3 |
| 15:0 | – | – | – | – | – | 0.2 ± 0.1 | – | 0.1 ± 0.2 | – | – | – | – | – | 0.2 ± 0.1 | 1.4 ± 0.3 |
| 16:0 (PA) | 17.2 ± 1.2 | 15.2 ± 0.1 | 15.9 ± 0.2 | 38.3 ± 0.3 | 14.7 ± 0.3 | 11.7 ± 0.4 | 20.9 ± 0.5 | 31.1 ± 1.6 | 30.2 ± 1.0 | 15.9 ± 0.9 | 13.1 ± 0.7 | 21.9 ± 1.1 | 21.8 ± 0.9 | 9.3 ± 0.7 | 23.4 ± 1.1 |
| 16:1*n*7 (POA) | 1.5 ± 0.3 | 0.4 ± 0.2 | 0.3 ± 0.2 | 5.6 ± 0.2 | 0.5 ± 0.2 | 0.9 ± 0.2 | 1.1 ± 0.3 | 2.8 ± 0.5 | 1.3 ± 0.2 | 1.1 ± 0.3 | 1.0 ± 0.3 | 3.3 ± 0.5 | 1.5 ± 0.3 | 1.9 ± 0.3 | 4.0 ± 0.2 |
| 17:0 | 0.5 ± 0.2 | 0.6 ± 0.4 | 0.7 ± 0.3 | – | 0.4 ± 0.3 | 1.2 ± 0.4 | 0.6 ± 0.2 | 0.6 ± 0.3 | 0.9 ± 0.2 | 0.3 ± 0.2 | 0.7 ± 0.2 | 0.1 ± 0.1 | 0.6 ± 0.2 | 0.1 ± 0.1 | 0.8 ± 0.3 |
| 18:0 (SA) | 9.8 ± 1.1 | 6.7 ± 0.2 | 6.8 ± 0.2 | 2.1 ± 0.1 | 3.0 ± 0.2 | 12.6 ± 0.9 | 4.6 ± 0.5 | 2.4 ± 0.6 | 5.1 ± 0.3 | 5.4 ± 0.8 | 6.4 ± 0.4 | 6.1 ± 0.4 | 2.9 ± 0.8 | 2.2 ± 0.4 | 6.3 ± 0.2 |
| 18:1*n*9 (OA) | 15.0 ± 1.1 | 5.6 ± 0.3 | 3.7 ± 0.3 | 17.5 ± 0.7 | 5.5 ± 0.3 | 6.9 ± 0.4 | 4.5 ± 0.7 | 44.8 ± 2.9 | 4.3 ± 0.3 | 8.3 ± 0.9 | 5.9 ± 0.4 | 27.0 ± 1.7 | 5.8 ± 0.3 | 6.5 ± 0.2 | 36.7 ± 1.1 |
| 18:1*n*7 (VA) | – | 0.2 ± 0.2 | 0.4 ± 0.2 | – | 0.5 ± 0.2 | 0.8 ± 0.2 | 0.3 ± 0.2 | 0.4 ± 0.2 | 0.3 ± 0.2 | 1.2 ± 0.3 | 0.5 ± 0.3 | 1.7 ± 0.3 | 0.2 ± 0.2 | 0.1 ± 0.2 | 2.3 ± 0.3 |
| 18:2*n*6 (LA) | 13.1 ± 1.3 | 7.1 ± 0.3 | 8.1 ± 0.3 | 6.9 ± 0.3 | 14.5 ± 0.7 | 20.7 ± 0.8 | 6.3 ± 0.4 | 12.1 ± 0.9 | 5.8 ± 0.5 | 16.3 ± 0.4 | 15.7 ± 0.8 | 23.2 ± 1.9 | 15.3 ± 0.9 | 4.5 ± 0.3 | 14.0 ± 0.8 |
| 18:3*n*6 (GLA) | 0.6 ± 0.2 | 0.2 ± 0.1 | 0.2 ± 0.2 | – | 0.2 ± 0.2 | 0.4 ± 0.2 | – | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.2 | 1.3 ± 0.3 | 0.1 ± 0.2 | – | – | 1.3 ± 0.3 |
| 18:3*n*3 (ALA) | 37.1 ± 2.0 | 62.1 ± 0.3 | 62.5 ± 0.4 | 28.9 ± 0.3 | 59.0 ± 1.1 | 39.4 ± 2.0 | 59.6 ± 1.3 | 1.7 ± 0.3 | 48.7 ± 1.3 | 45.6 ± 0.9 | 50.7 ± 0.8 | 3.7 ± 0.5 | 49.5 ± 1.2 | 3.3 ± 0.3 | 1.1 ± 0.3 |
| 18:4*n*3 (SDA) | – | – | – | – | – | 0.1 ± 0.1 | – | – | – | – | – | 0.5 ± 0.2 | 0.1 ± 0.2 | 0.5 ± 0.2 | – |
| 20:0 | 0.9 ± 0.2 | 0.4 ± 0.2 | 0.2 ± 0.1 | – | 0.3 ± 0.2 | 0.9 ± 0.3 | 0.3 ± 0.2 | 0.3 ± 0.2 | 0.4 ± 0.2 | 0.4 ± 0.2 | 0.4 ± 0.2 | 2.9 ± 0.4 | – | 0.6 ± 0.3 | 1.0 ± 0.2 |
| 20:1*n*9 (GOA) | 0.3 ± 0.2 | – | – | – | 0.3 ± 0.1 | 0.1 ± 0.3 | – | 2.9 ± 0.9 | 0.1 ± 0.1 | 0.7 ± 0.3 | 1.2 ± 0.3 | 0.9 ± 0.3 | 0.1 ± 0.2 | 0.2 ± 0.1 | – |
| 20:4*n*6 (ARA) | 0.1 ± 0.1 | – | – | – | – | – | – | – | – | 0.5 ± 0.2 | – | 0.1 ± 0.1 | – | – | 1.3 ± 0.2 |
| 20:5*n*3 (EPA) | – | – | – | – | – | 0.2 ± 0.2 | – | – | – | – | – | – | – | 0.1 ± 0.2 | – |
| 22:0 | 0.2 ± 0.2 | 0.2 ± 0.1 | – | – | – | 1.0 ± 0.3 | – | 0.1 ± 0.2 | – | 0.2 ± 0.2 | – | 2.8 ± 0.7 | – | 0.2 ± 0.2 | 0.4 ± 0.2 |
| 22:1*n*9 | – | 0.1 ± 0.1 | – | – | 0.1 ± 0.1 | – | – | – | 0.2 ± 0.1 | 1.0 ± 0.3 | 0.2 ± 0.2 | 0.9 ± 0.3 | – | – | 0.3 ± 0.2 |
| 22:4*n*6 | – | – | | – | – | – | – | – | – | – | – | – | – | – | – |
| 22:5*n*6 | 0.1 ± 0.1 | 0.2 ± 0.1 | – | – | – | 0.3 ± 0.2 | – | – | 0.2 ± 0.2 | – | – | 0.3 ± 0.2 | – | – | – |
| 22:5*n*3 | – | – | – | – | – | – | – | – | – | 0.3 ± 0.2 | 0.4 ± 0.3 | – | – | – | – |
| 22:6*n*3 (DHA) | 0.3 ± 0.2 | – | – | – | – | 0.3 ± 0.4 | – | – | – | 1.3 ± 0.3 | – | 0.4 ± 0.2 | – | 0.9 ± 0.2 | – |
| Total FA wt%[a)] | 20.7 ± 1.5 | 2.9 ± 0.4 | 2.2 ± 0.5 | 20.0 ± 0.3 | 10.6 ± 0.4 | 6.5 ± 0.6 | 12.5 ± 0.4 | 31.7 ± 0.8 | 10.7 ± 0.3 | 2.9 ± 0.5 | 1.7 ± 0.3 | 8.6 ± 0.4 | 8.5 ± 0.2 | 15.7 ± 0.8 | 10.1 ± 0.3 |
| *n*-3 PUFA | 37.3 | 62.1 | 62.5 | 28.9 | 59.0 | 40.1 | 59.6 | 1.7 | 48.7 | 47.2 | 51.1 | 4.6 | 49.5 | 4.8 | 1.1 |
| *n*-6 PUFA | 13.9 | 7.4 | 8.3 | 6.9 | 14.7 | 21.4 | 6.3 | 12.2 | 6.2 | 17.1 | 17.0 | 23.6 | 15.4 | 4.5 | 16.6 |
| *n*-6/*n*-3 PUFA | 0.4 | 0.1 | 0.1 | 0.2 | 0.2 | 0.5 | 0.1 | 7.3 | 0.1 | 0.4 | 0.3 | 5.1 | 0.3 | 0.9 | 14.8 |
| MUFA | 16.8 | 6.4 | 4.4 | 23.1 | 6.9 | 8.6 | 5.9 | 50.8 | 6.1 | 12.3 | 8.9 | 33.8 | 7.5 | 8.8 | 43.4 |
| SFA | 31.9 | 24.2 | 24.7 | 41.0 | 19.4 | 30.0 | 28.2 | 35.3 | 39.0 | 23.5 | 23.1 | 37.9 | 27.6 | 81.9 | 38.9 |

Species legend: Bmo: *Bombix mori*; Ceu: *Caligo eurilochus*; Cme: *Caligo memnon*: Can: *Charis anius*; Dpla: *Danaus plexippus* a: Diu: *Dryas iulia*; Gme: *Galleria mellonella*; Hil: *Hermetia illucens*; Mgr: *Methona grandior*; Ona: *Oryctes nasicornis*; Par: *Parides arcas*; Pth: *Papilio thoas*; Sex: *Spodoptera exigua*; Sst: *Siproeta stelenes*.

[a)] The total FA amount was determined by comparing the total peak area with that of the internal standard; [b)]Larval stage; [c)]Pupa stage.

**Table 3.** Fatty acid composition of the lipid classes of several Lepidoptera larvae compared to that of Diptera and Coleoptera (mol% of total area).[a)]

| | Lepidoptera | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Spodoptera exigua* | | | | | *Danaus plexippus* b[b)] | | | | | *Bombyx mori* | | | | | *Galleria mellonella* | | | | |
| | SE | TAG | DAG | FFA | PL | SE | TAG | DAG | FFA | PL | SE | TAG[a] | DAG | FFA | PL | SE | TAG | DAG | FFA | PL |
| Total FA wt%[a)] | 0.4 ± 0.1 | 4.8 ± 0.2 | 0.7 ± 0.1 | 0.9 ± 0.1 | 1.8 ± 0.3 | 0.2 ± 0.0 | 4.7 ± 0.1 | 0.3 ± 0.1 | 0.8 ± 0.1 | 0.5 ± 0.1 | 0.9 ± 0.1 | 13.0 ± 0.2 | 1.1 ± 0.1 | 1.3 ± 0.2 | 4.4 ± 0.1 | 0.4 ± 0.1 | 26.0 ± 0.3 | 0.3 ± 0.0 | 1.4 ± 0.2 | 3.6 ± 0.1 |
| 8:0 | – | – | – | – | – | – | 0.4 ± 0.1 | – | – | – | 2.4 ± 0.2 | 0.8 ± 0.1 | 1.0 ± 0.1 | 0.2 ± 0.1 | 5.0 ± 0.2 | – | – | – | – | – |
| 10:0 | 2.8 ± 0.3 | 3.8 ± 0.5 | 3.1 ± 0.3 | 1.3 ± 0.3 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 12:0 (LaA) | – | – | – | – | 0.5 ± 0.2 | 4.2 ± 0.2 | 1.1 ± 0.4 | 1.5 ± 0.6 | 0.5 ± 0.2 | 2.5 ± 0.3 | – | – | – | – | – | – | – | – | – | – |
| 14:0 | 1.5 ± 0.2 | 1.2 ± 0.1 | – | 0.8 ± 0.2 | 2.1 ± 0.1 | 3.6 ± 0.3 | 0.4 ± 0.1 | – | 0.1 ± 0.1 | 1.8 ± 0.2 | 1.2 ± 0.3 | 0.4 ± 0.1 | – | – | 2.1 ± 0.3 | 1.1 ± 0.1 | 0.1 ± 0.1 | – | – | 4.6 ± 0.3 |
| 15:0 | – | – | – | – | – | – | 0.2 ± 0.1 | – | 0.2 ± 0.1 | – | – | – | – | – | – | 0.1 ± 0.2 | 0.1 ± 0.1 | – | – | – |
| 16:0 (PA) | 27.2 ± 1.1 | 24.3 ± 0.9 | 24.1 ± 1.0 | 20.5 ± 1.3 | 13.5 ± 0.8 | 17.3 ± 1.1 | 12.0 ± 0.7 | 13.9 ± 1.1 | 11.2 ± 0.2 | 6.6 ± 0.5 | 22.4 ± 1.6 | 18.1 ± 0.6 | 22.0 ± 2.1 | 17.1 ± 0.9 | 15.0 ± 0.4 | 37.8 ± 1.7 | 32.7 ± 2.0 | 31.7 ± 0.9 | 33.1 ± 1.9 | 17.4 ± 0.5 |
| 16:1*n*7 (POA) | 7.3 ± 0.6 | 3.1 ± 0.2 | 5.0 ± 0.1 | 8.4 ± 0.3 | 2.3 ± 0.4 | 5.2 ± 0.2 | 0.7 ± 0.2 | 0.5 ± 0.3 | 1.1 ± 0.3 | 1.0 ± 0.1 | 9.1 ± 0.2 | 1.4 ± 0.3 | 1.0 ± 0.2 | 1.3 ± 0.3 | 0.3 ± 0.2 | 7.8 ± 0.3 | 2.6 ± 0.4 | 1.7 ± 0.4 | 1.9 ± 0.3 | 1.4 ± 0.3 |
| 17:0 | – | – | – | – | 0.5 ± 0.2 | – | 1.3 ± 0.2 | 0.2 ± 0.2 | 0.8 ± 0.2 | – | 0.9 ± 0.2 | 0.5 ± 0.2 | 0.2 ± 0.2 | – | 0.3 ± 0.1 | – | 0.7 ± 0.2 | – | 0.4 ± 0.1 | 0.6 ± 0.2 |
| 18:0 (SA) | 8.5 ± 0.3 | 3.7 ± 0.3 | 3.4 ± 0.2 | 6.4 ± 0.4 | 13.1 ± 0.3 | 14.2 ± 0.3 | 11.6 ± 0.3 | 13.2 ± 0.6 | 15.4 ± 1.1 | 19.2 ± 0.8 | 12.2 ± 0.8 | 8.5 ± 0.4 | 9.4 ± 0.3 | 11.7 ± 0.4 | 13.5 ± 0.8 | 1.6 ± 0.5 | 1.4 ± 0.4 | 2.4 ± 0.3 | 2.1 ± 0.3 | 9.3 ± 0.4 |
| 18:1*n*9 (OA) | 38.8 ± 1.8 | 29.2 ± 2.0 | 30.5 ± 1.8 | 26.6 ± 1.4 | 17.4 ± 0.9 | 13.9 ± 1.3 | 7.0 ± 0.8 | 6.7 ± 0.8 | 4.3 ± 0.5 | 2.7 ± 0.4 | 24.4 ± 0.5 | 14.5 ± 0.9 | 32.4 ± 1.7 | 18.2 ± 0.9 | 10.4 ± 0.6 | 38.1 ± 2.0 | 45.8 ± 1.8 | 45.1 ± 2.7 | 46.7 ± 1.6 | 41.8 ± 0.9 |
| 18:1*n*7 (VA) | 0.5 ± 0.4 | 2.3 ± 0.4 | 1.9 ± 0.5 | – | 0.3 ± 0.1 | 3.6 ± 0.8 | 0.6 ± 0.2 | 1.8 ± 0.5 | 0.5 ± 0.3 | 0.3 ± 0.2 | – | – | – | – | – | 1.2 ± 0.4 | 0.1 ± 0.1 | 1.2 ± 0.3 | 0.4 ± 0.2 | 1.4 ± 0.4 |
| 18:2*n*6 (LA) | 7.3 ± 0.5 | 20.4 ± 1.1 | 18.2 ± 0.8 | 27.7 ± 1.0 | 36.0 ± 1.7 | 7.1 ± 0.8 | 20.2 ± 1.4 | 21.5 ± 1.7 | 20.4 ± 1.3 | 29.0 ± 1.0 | 6.8 ± 0.8 | 12.8 ± 0.8 | 10.3 ± 0.6 | 13.0 ± 1.0 | 16.6 ± 0.8 | 9.1 ± 0.4 | 11.2 ± 0.2 | 13.8 ± 0.6 | 9.1 ± 0.9 | 18.8 ± 1.1 |
| 18:3*n*6 (GLA) | – | – | – | – | 0.4 ± 0.4 | – | 0.5 ± 0.2 | 0.4 ± 0.3 | 0.3 ± 0.2 | – | 0.1 ± 0.1 | 0.5 ± 0.2 | 0.9 ± 0.2 | – | 0.7 ± 0.2 | – | 0.1 ± 0.1 | – | 0.3 ± 0.1 | – |
| 18:3*n*3 (ALA) | 1.1 ± 0.3 | 3.6 ± 0.4 | 4.9 ± 0.1 | 3.7 ± 0.3 | 3.8 ± 0.7 | 23.9 ± 1.0 | 41.1 ± 1.8 | 38.7 ± 0.9 | 42.2 ± 1.6 | 33.7 ± 1.5 | 20.0 ± 1.3 | 41.2 ± 0.9 | 22.8 ± 1.0 | 38.1 ± 1.4 | 31.6 ± 1.5 | 1.0 ± 0.2 | 1.8 ± 0.3 | – | 1.5 ± 0.3 | 1.4 ± 0.3 |
| 18:4*n*3 (SDA) | – | 0.4 ± 0.2 | 0.3 ± 0.1 | – | 1.1 ± 0.3 | – | 0.1 ± 0.1 | – | – | – | – | – | – | – | 0.2 ± 0.1 | – | – | – | – | – |
| 20:0 | 2.3 ± 0.3 | 3.1 ± 0.3 | 3.6 ± 0.5 | 2.5 ± 0.3 | 2.1 ± 0.2 | 4.2 ± 0.4 | 0.9 ± 0.2 | 0.4 ± 0.2 | 0.3 ± 0.2 | – | – | 0.6 ± 0.1 | – | – | 2.0 ± 0.3 | – | 0.1 ± 0.1 | – | – | 1.6 ± 0.2 |
| 20:1*n*9 (GOA) | – | 1.0 ± 0.2 | 0.7 ± 0.1 | 1.2 ± 0.3 | 0.8 ± 0.2 | – | – | – | – | 0.7 ± 0.2 | 0.4 ± 0.2 | 0.4 ± 0.1 | – | – | – | 2.2 ± 0.2 | 3.2 ± 0.2 | 2.3 ± 0.2 | 4.4 ± 0.4 | 1.8 ± 0.1 |
| 20:4*n*6 (ARA) | – | – | – | – | 0.4 ± 0.1 | – | – | – | – | – | – | – | – | – | 0.5 ± 0.2 | – | – | 1.6 ± 0.3 | – | – |
| 20:5*n*3 (EPA) | – | – | – | – | – | – | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.2 | – | – | – | – | – | – | – | – | – | – | – |
| 22:0 | 1.8 ± 0.2 | 2.9 ± 0.2 | 3.3 ± 0.4 | – | 2.4 ± 0.2 | 3.0 ± 0.1 | 1.0 ± 0.0 | 0.7 ± 0.1 | 1.3 ± 0.3 | 0.6 ± 0.2 | – | – | – | – | 1.1 ± 0.2 | – | 0.1 ± 0.1 | – | – | – |
| 22:1*n*9 | 0.9 ± 0.2 | 0.8 ± 0.2 | 0.7 ± 0.2 | – | 1.2 ± 0.3 | – | – | – | – | – | 0.1 ± 0.1 | – | – | – | – | – | – | – | – | – |
| 22:4*n*6 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 22:5*n*6 | – | – | – | – | 1.2 ± 0.2 | – | 0.2 ± 0.1 | 0.4 ± 0.2 | 0.2 | 1.2 ± 0.1 | – | – | – | – | 0.6 ± 0.1 | – | – | – | – | – |
| 22:5*n*3 (DPA) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 22:6*n*3 (DHA) | – | 0.3 ± 0.1 | 0.3 ± 0.2 | 0.9 ± 0.2 | 0.8 ± 0.1 | – | 0.3 ± 0.1 | – | 1.2 ± 0.3 | 0.8 ± 0.1 | – | 0.3 ± 0.1 | – | 0.4 ± 0.2 | 0.1 ± 0.1 | – | – | – | – | – |
| *n*-3 PUFA | 1.1 | 4.4 | 5.5 | 4.6 | 5.7 | 23.9 | 41.8 | 38.9 | 43.6 | 34.4 | 20.0 | 41.5 | 22.8 | 38.5 | 31.8 | 1.0 | 1.8 | – | 1.5 | 1.4 |
| *n*-6 PUFA | 7.3 | 20.4 | 18.2 | 27.7 | 38.1 | 7.1 | 20.9 | 22.3 | 20.7 | 30.2 | 4.9 | 13.3 | 11.2 | 13.0 | 18.4 | 9.1 | 11.3 | 15.5 | 9.4 | 18.8 |
| *n*-6/*n*-3 PUFA | 6.8 | 4.7 | 3.3 | 6.0 | 6.6 | 0.3 | 0.5 | 0.6 | 0.5 | 0.9 | 0.2 | 0.3 | 0.5 | 0.3 | 0.6 | 9.1 | 6.4 | 0.0 | 6.2 | 13.0 |
| MUFA | 47.5 | 36.4 | 38.8 | 36.2 | 22.0 | 22.7 | 8.3 | 9.0 | 5.9 | 4.7 | 34.0 | 16.3 | 33.4 | 19.5 | 10.7 | 49.4 | 51.7 | 50.4 | 53.4 | 46.3 |
| SFA | 44.2 | 38.9 | 37.5 | 31.5 | 34.2 | 46.4 | 29.0 | 29.8 | 29.8 | 30.7 | 41.1 | 28.9 | 32.6 | 29.0 | 39.0 | 40.6 | 35.2 | 34.1 | 35.6 | 33.5 |

| | Diptera | | | | | Coleoptera | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Hermetia illucens* | | | | | *Oryctes nasicornis* | | | | |
| | SL | TAG | DAG | FFA | PL | SL | TAG | DAG | FFA | PL |
| Total FA wt%[a)] | 0.2 ± 0.1 | 11.0 ± 0.2 | 1.8 ± 0.1 | 1.1 ± 0.2 | 1.7 ± 0.1 | 0.5 ± 0.0 | 6.9 ± 0.0 | 0.5 ± 0.0 | 0.4 ± 0.1 | 1.8 ± 0.1 |
| 8:00 | 0.6 ± 0.1 | 0.5 ± 0.2 | 0.3 ± 0.1 | – | – | 0.5 ± 0.2 | 1.1 ± 0.1 | 2.8 ± 0.2 | 0.3 ± 0.2 | 5.7 ± 0.3 |
| 10:00 | 3.8 ± 0.4 | 3.1 ± 0.3 | 3.8 ± 0.2 | 2.0 ± 0.3 | 4.1 ± 0.4 | – | – | – | – | – |
| 12:0 (LaA) | 52.9 ± 2.1 | 57.8 ± 3.0 | 53.3 ± 1.7 | 48.6 ± 1.6 | 58.8 ± 2.0 | – | – | – | – | – |
| 14:00 | 12.8 ± 0.3 | 8.3 ± 0.4 | 7.3 ± 0.4 | 5.7 ± 0.6 | 9.8 ± 0.5 | 4.8 ± 0.2 | 1.7 ± 0.3 | 2.2 ± 0.3 | 0.7 ± 0.2 | 6.5 ± 0.4 |
| 15:00 | 1.3 ± 0.3 | 0.1 ± 0.2 | 0.2 ± 0.1 | – | 0.3 ± 0.0 | 3.0 ± 0.3 | 0.9 ± 0.5 | 1.2 ± 0.6 | 0.6 ± 0.1 | 2.3 ± 0.4 |
| 16:0 (PA) | 12.9 ± 0.5 | 9.8 ± 0.3 | 13.5 ± 0.7 | 13.3 ± 0.7 | 2.3 ± 0.2 | 24.8 ± 1.8 | 25.4 ± 2.6 | 25.3 ± 0.8 | 22.2 ± 0.2 | 17.9 ± 1.4 |
| 16:1*n*7 (POA) | 2.9 ± 0.5 | 2.2 ± 0.3 | 1.9 ± 0.1 | 2.5 ± 0.4 | 0.8 ± 0.4 | 7.8 ± 0.2 | 3.9 ± 0.3 | 4.3 ± 0.4 | 3.4 ± 0.3 | 3.1 ± 0.2 |
| 17:00 | 0.3 ± 0.2 | 0.2 ± 0.1 | – | 0.3 ± 0.1 | – | 1.4 ± 0.2 | 0.7 ± 0.3 | 0.9 ± 0.2 | 0.5 ± 0.2 | 1.0 ± 0.2 |
| 18:0 (EA) | 3.2 ± 0.5 | 1.5 ± 0.3 | 1.7 ± 0.3 | 3.1 ± 0.3 | 5.7 ± 0.4 | 12.0 ± 0.8 | 3.8 ± 0.5 | 4.3 ± 0.6 | 2.8 ± 0.2 | 10.8 ± 0.6 |
| 18:1*n*9 (OA) | 4.6 ± 0.9 | 6.3 ± 0.6 | 8.9 ± 0.4 | 14.0 ± 0.9 | 5.3 ± 0.4 | 34.5 ± 1.4 | 43.2 ± 2.1 | 39.0 ± 1.9 | 56.2 ± 1.9 | 21.9 ± 1.4 |
| 18:1*n*7 (VA) | – | 0.2 ± 0.1 | – | 0.3 ± 0.2 | – | 4.6 ± 0.5 | 2.4 ± 0.3 | 1.7 ± 0.5 | 0.6 ± 0.2 | 1.8 ± 0.3 |
| 18:2*n*6 (LA) | 1.4 ± 0.4 | 4.7 ± 0.7 | 3.5 ± 0.5 | 7.2 ± 1.0 | 5.0 ± 0.3 | 4.3 ± 0.4 | 13.5 ± 0.8 | 12.9 ± 1.1 | 10.7 ± 0.9 | 18.0 ± 0.8 |
| 18:3*n*6 (GLA) | – | – | – | – | – | – | 0.6 ± 0.2 | 0.8 ± 0.2 | – | 3.3 ± 0.2 |
| 18:3*n*3 (ALA) | 2.0 ± 0.3 | 3.3 ± 0.5 | 4.2 ± 0.7 | 1.6 ± 0.3 | 2.9 ± 0.3 | 0.4 ± 0.1 | 1.4 ± 0.2 | 1.8 ± 0.1 | 1.6 ± 0.5 | 0.6 ± 0.2 |
| 18:4*n*3 (SDA) | – | 0.4 ± 0.1 | 0.3 ± 0.2 | 0.3 ± 0.2 | 1.3 ± 0.2 | – | – | – | – | – |
| 20:00 | – | 0.7 ± 0.2 | – | 0.8 ± 0.2 | 0.8 ± 0.2 | – | 0.3 ± 0.2 | 1.4 ± 0.1 | – | 2.6 ± 0.3 |
| 20:1*n*9 (GOA) | – | 0.3 ± 0.1 | – | – | – | – | – | – | – | – |
| 20:4*n*6 (ARA) | – | – | – | – | – | – | 0.4 ± 0.1 | 0.6 ± 0.2 | 0.5 ± 0.2 | 4.2 ± 0.3 |
| 20:5*n*3 (EPA) | – | – | – | – | 0.5 ± 0.2 | – | – | – | – | – |
| 22:00 | 1.1 ± 0.3 | 0.1 ± 0.1 | 0.2 ± 0.1 | – | 0.5 ± 0.1 | 1.2 ± 0.2 | 0.4 ± 0.2 | 0.3 ± 0.1 | – | 0.3 ± 0.1 |
| 22:1*n*9 | – | – | – | – | – | 0.6 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.2 | – | 0.1 ± 0.1 |
| 22:4*n*6 | – | – | – | – | – | – | – | – | – | – |
| 22:5*n*6 | – | – | – | – | – | – | – | – | – | – |
| 22:5*n*3 (DPA) | – | – | – | – | – | – | – | – | – | – |
| 22:6*n*3 (DHA) | – | 0.6 ± 0.2 | 1.0 ± 0.1 | 0.7 ± 0.2 | 1.9 ± 0.2 | – | – | – | – | – |
| *n*-3 PUFA | 2.0 | 4.3 | 5.5 | 2.3 | 6.7 | 0.4 | 1.4 | 1.8 | 1.6 | 0.6 |
| *n*-6 PUFA | 1.4 | 4.7 | 3.5 | 7.2 | 5.0 | 4.3 | 14.5 | 14.3 | 11.1 | 25.4 |
| *n*-6/*n*-3 PUFA | 0.7 | 1.1 | 0.6 | 3.1 | 0.7 | 11.0 | 10.6 | 7.8 | 7.0 | 43.8 |
| MUFA | 7.6 | 8.9 | 10.7 | 16.7 | 6.1 | 47.6 | 49.8 | 45.4 | 60.2 | 26.8 |
| SFA | 89.0 | 82.1 | 80.3 | 73.7 | 82.3 | 47.8 | 34.3 | 38.4 | 27.1 | 47.1 |

[a)] The total FA amount of each lipid class was determined by comparing the total peak area with that of the internal standard; [b)]Pupa stage.

**Table 4.** Regiospecific fatty acid composition of several Lepidoptera larvae compared to that of Diptera and Coleoptera (mol% of total fatty acids).

| | Lepidoptera | | | | | | | | | Diptera | | | Coleoptera | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Danaus plexippus* b[a)] | | | *Bombyx mori* | | | *Galleria mellonella* | | | *Hermetia illucens* | | | *Oryctes nasicornis* | | |
| Fatty acids | TAG | *sn*-2 | *sn*-1,3 | TAG | *sn*-2 | *sn*-1,3 | TAG | *sn*-2 | *sn*-1,3 | TAG | *sn*-2 | *sn*-1,3 | TAG | *sn*-2 | *sn*-1,3 |
| 8:0 | 0.4 ± 0.2 | 0.6 ± 0.3 | 0.3 ± 0.2 | 0.4 ± 0.3 | – | 0.6 ± 0.2 | – | – | – | 0.3 ± 0.2 | 0.1 ± 0.2 | 0.4 ± 0.3 | 0.4 ± 0.3 | 0.3 ± 0.2 | 0.4 ± 0.2 |
| 10:0 | – | – | – | – | – | – | – | – | – | 2.1 ± 0.4 | 0.2 ± 0.3 | 3.1 ± 0.6 | – | – | – |
| 12:0 (LaA) | 1.1 ± 0.3 | 0.1 ± 0.1 | 1.6 ± 0.2 | – | – | – | – | – | – | 51.6 ± 2.0 | 44.5 ± 1.3 | 55.1 ± 0.9 | – | – | – |
| 14:0 | 0.4 ± 0.2 | 0.2 ± 0.1 | 0.4 ± 0.3 | 0.2 ± 0.2 | – | 0.4 ± 0.2 | 0.1 ± 0.2 | – | 0.2 ± 0.2 | 8.4 ± 0.4 | 14.7 ± 1.1 | 5.3 ± 0.8 | 1.2 ± 0.3 | 0.2 ± 0.2 | 1.7 ± 0.4 |
| 15:0 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.2 | – | – | – | 0.2 ± 0.2 | – | 0.3 ± 0.1 | 0.1 ± 0.2 | 0.3 ± 0.2 | – | 0.6 ± 0.2 | 0.4 ± 0.2 | 0.6 ± 0.3 |
| 16:0 (PA) | 12.0 ± 0.9 | 10.4 ± 0.8 | 12.8 ± 0.4 | 17.6 ± 1.0 | 2.1 ± 0.4 | 25.3 ± 0.8 | 30.4 ± 0.8 | 2.4 ± 0.5 | 44.5 ± 1.7 | 11.3 ± 0.3 | 8.3 ± 0.4 | 12.9 ± 0.7 | 23.5 ± 1.0 | 3.4 ± 0.5 | 33.6 ± 1.4 |
| 16:1*n*7 (POA) | 0.7 ± 0.2 | 1.1 ± 0.3 | 0.5 ± 0.2 | 0.8 ± 0.2 | – | 1.1 ± 0.3 | 2.4 ± 0.3 | 1.0 ± 0.3 | 3.1 ± 0.4 | 2.5 ± 0.3 | 3.8 ± 0.3 | 1.9 ± 0.3 | 3.7 ± 0.4 | 1.1 ± 0.2 | 5.0 ± 0.3 |
| 17:0 | 1.5 ± 0.3 | 3.3 ± 0.4 | 0.7 ± 0.3 | 0.5 ± 0.2 | – | 0.8 ± 0.3 | 0.7 ± 0.4 | 0.8 ± 0.3 | 0.7 ± 0.2 | 0.2 ± 0.1 | 0.1 ± 0.2 | 0.2 ± 0.1 | 0.6 ± 0.3 | – | 0.9 ± 0.3 |
| 18:0 (EA) | 11.6 ± 0.2 | 17.2 ± 0.4 | 8.7 ± 0.6 | 8.8 ± 0.6 | 1.7 ± 0.4 | 12.4 ± 0.4 | 1.2 ± 0.3 | – | 1.7 ± 0.2 | 1.8 ± 0.2 | 0.4 ± 0.2 | 2.4 ± 0.3 | 4.0 ± 0.2 | 0.5 ± 0.2 | 5.7 ± 0.3 |
| 18:1*n*9 (OA) | 7.1 ± 0.6 | 7.8 ± 0.4 | 6.7 ± 0.4 | 14.9 ± 0.4 | 33.7 ± 0.8 | 5.5 ± 0.4 | 47.0 ± 0.8 | 88.5 ± 2.2 | 26.2 ± 1.0 | 7.5 ± 0.4 | 9.7 ± 0.4 | 6.5 ± 0.4 | 44.7 ± 2.1 | 87.7 ± 1.5 | 23.2 ± 0.9 |
| 18:1*n*7 (VA) | 0.6 ± 0.2 | 0.7 ± 0.2 | 0.5 ± 0.2 | – | – | – | 0.1 ± 0.2 | – | 0.2 ± 0.1 | 0.2 ± 0.2 | 0.1 ± 0.2 | 0.3 ± 0.2 | 2.5 ± 0.3 | – | 3.8 ± 0.4 |
| 18:2*n*6 (LA) | 20.2 ± 0.6 | 11.1 ± 0.4 | 24.8 ± 0.9 | 12.9 ± 0.3 | 13.1 ± 0.3 | 12.9 ± 0.4 | 12.2 ± 0.7 | 5.7 ± 0.5 | 15.4 ± 0.9 | 6.7 ± 0.3 | 10.2 ± 0.3 | 4.9 ± 0.2 | 15.0 ± 0.4 | 5.4 ± 0.2 | 19.7 ± 0.8 |
| 18:3*n*6 (GLA) | 0.5 ± 0.2 | 0.6 ± 0.2 | 0.4 ± 0.2 | – | – | – | 0.1 ± 0.2 | 0.3 ± 0.2 | – | – | – | – | 0.6 ± 0.2 | 0.2 ± 0.1 | 0.8 ± 0.3 |
| 18:3*n*3 (ALA) | 41.1 ± 0.7 | 43.5 ± 1.2 | 39.9 ± 1.6 | 42.3 ± 1.5 | 49.1 ± 0.9 | 38.9 ± 1.2 | 1.8 ± 0.4 | 0.8 ± 0.3 | 2.3 ± 0.4 | 4.4 ± 0.3 | 1.2 ± 0.3 | 6.0 ± 0.4 | 1.4 ± 0.3 | 0.5 ± 0.2 | 1.8 ± 0.4 |
| 18:4*n*3 (SDA) | 0.1 ± 0.2 | 0.1 ± 0.1 | 0.1 ± 0.1 | – | – | – | – | – | – | 0.5 ± 0.3 | 0.5 ± 0.3 | 0.5 ± 0.3 | – | – | – |
| 20:0 | 0.9 ± 0.3 | 1.2 ± 0.4 | 0.7 ± 0.2 | 0.7 ± 0.2 | 0.3 ± 0.4 | 0.9 ± 0.3 | 0.1 ± 0.2 | – | 0.1 ± 0.2 | 1.0 ± 0.3 | 2.9 ± 0.3 | – | 0.4 ± 0.2 | 0.1 ± 0.1 | 0.5 ± 0.2 |
| 20:1*n*9 (GOA) | – | – | – | 0.4 ± 0.3 | – | 0.5 ± 0.2 | 3.6 ± 0.5 | 0.4 ± 0.2 | 5.3 ± 0.8 | 0.4 ± 0.2 | 1.0 ± 0.4 | – | – | – | – |
| 20:4*n*6 (ARA) | – | – | – | – | – | – | – | – | – | – | – | – | 0.8 ± 0.3 | 0.2 ± 0.1 | 1.1 ± 0.3 |
| 20:5*n*3 (EPA) | 0.3 ± 0.3 | 0.1 ± 0.2 | 0.4 ± 0.2 | – | – | – | – | – | – | – | – | – | | | – |
| 22:0 | 1.0 ± 0.2 | 1.3 ± 0.4 | 0.8 ± 0.2 | 0.3 ± 0.2 | 0.2 ± 0.2 | 0.4 ± 0.2 | 0.1 ± 0.2 | – | 0.1 ± 0.2 | 0.1 ± 0.2 | 0.3 ± 0.2 | 0.1 ± 0.1 | 0.2 ± 0.2 | – | 0.4 ± 0.2 |
| 22:1*n*9 | – | – | – | – | – | – | – | – | – | – | – | – | 0.3 ± 0.2 | – | 0.5 ± 0.1 |
| 22:4*n*6 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 22:5*n*6 | 0.2 ± 0.2 | 0.1 ± 0.2 | 0.2 ± 0.1 | – | – | – | – | – | – | – | – | – | – | – | – |
| 22:5*n*3 (DPA) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 22:6*n*3 (DHA) | 0.3 ± 0.2 | 0.3 ± 0.2 | 0.4 ± 0.3 | 0.3 ± 0.3 | – | 0.4 ± 0.2 | – | – | – | 0.9 ± 0.3 | 1.5 ± 0.3 | 0.6 ± 0.2 | 0.3 ± 0.2 | – | 0.4 ± 0.1 |
| *n*-3 PUFA | 41.8 | 44.0 | 40.8 | 42.5 | 49.1 | 39.3 | 1.8 | 0.8 | 2.3 | 5.8 | 3.2 | 7.1 | 1.6 | 0.5 | 2.2 |
| *n*-6 PUFA | 20.9 | 11.8 | 25.4 | 12.9 | 13.1 | 12.9 | 12.3 | 6.2 | 15.3 | 6.7 | 10.2 | 4.9 | 16.4 | 5.8 | 21.7 |
| *n*-6/*n*-3 PUFA | 0.5 | 0.3 | 0.6 | 0.3 | 0.3 | 0.3 | 6.9 | 7.8 | 6.8 | 1.1 | 3.1 | 0.7 | 9.9 | 11.6 | 12.4 |
| MUFA | 8.3 | 9.6 | 7.6 | 16.0 | 33.7 | 7.2 | 53.1 | 89.8 | 34.6 | 10.6 | 14.6 | 8.6 | 51.2 | 88.7 | 28.6 |
| SFA | 29.0 | 34.6 | 26.2 | 28.5 | 4.2 | 40.7 | 32.8 | 3.2 | 45.9 | 76.9 | 72.0 | 79.3 | 30.8 | 5.0 | 42.9 |
| RI | | | | | | | | | | | | | | | |

(*Continued*)

**Table 4.** (*Continued*)

| | Lepidoptera | | | | | | | | | Diptera | | | Coleoptera | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Danaus plexippus* b[a)] | | | *Bombyx mori* | | | *Galleria mellonella* | | | *Hermetia illucens* | | | *Oryctes nasicornis* | | |
| Fatty acids | TAG | *sn*-2 | *sn*-1,3 | TAG | *sn*-2 | *sn*-1,3 | TAG | *sn*-2 | *sn*-1,3 | TAG | *sn*-2 | *sn*-1,3 | TAG | *sn*-2 | *sn*-1,3 |
| 8:0 | | 0.6 | | | −1.0 | | | – | | | −0.6 | | | −0.2 | |
| 10:0 | | – | | | – | | | – | | | −0.9 | | | – | |
| 12:0 (LaA) | | −0.9 | | | – | | | – | | | −0.1 | | | – | |
| 14:0 | | −0.4 | | | −1.0 | | | −1.0 | | | 0.7 | | | −0.8 | |
| 15:0 | | 0.4 | | | | | | −1.0 | | | 2.0 | | | −0.2 | |
| 16:0 (PA) | | −0.1 | | | −0.9 | | | −0.9 | | | −0.3 | | | −0.9 | |
| 16:1*n*7 (POA) | | 0.6 | | | −1.0 | | | −0.6 | | | 0.5 | | | −0.7 | |
| 17:0 | | 1.1 | | | −1.0 | | | 0.2 | | | −0.5 | | | −1.0 | |
| 18:0 (EA) | | 0.5 | | | −0.8 | | | −1.0 | | | −0.8 | | | −0.9 | |
| 18:1*n*9 (OA) | | 0.1 | | | 1.3 | | | 0.9 | | | 0.3 | | | 1.0 | |
| 18:1*n*7 (VA) | | 0.2 | | | – | | | −1.0 | | | −0.6 | | | −1.0 | |
| 18:2*n*6 (LA) | | −0.4 | | | 0.0 | | | −0.5 | | | 0.5 | | | −0.6 | |
| 18:3*n*6 (GLA) | | 0.2 | | | – | | | 2.0 | | | – | | | −0.7 | |
| 18:3*n*3 (ALA) | | 0.1 | | | 0.2 | | | −0.5 | | | 0.1 | | | −0.6 | |
| 18:4*n*3 (SDA) | | 0.0 | | | – | | | – | | | −0.7 | | | – | |
| 20:0 | | 0.4 | | | −0.6 | | | −1.0 | | | 2.0 | | | −0.7 | |
| 20:1*n*9 (GOA) | | – | | | −1.0 | | | −0.9 | | | 1.5 | | | – | |
| 20:4*n*6 (ARA) | | – | | | – | | | – | | | – | | | −0.8 | |
| 20:5*n*3 (EPA) | | −0.7 | | | – | | | – | | | – | | | – | |
| 22:0 | | 0.3 | | | −0.5 | | | −1.0 | | | 1.3 | | | −1.0 | |
| 22:1*n*9 | | – | | | – | | | – | | | – | | | −1.0 | |
| 22:4*n*6 | | – | | | – | | | – | | | – | | | – | |
| 22:5*n*6 | | −0.5 | | | – | | | – | | | – | | | – | |
| 22:5*n*3 | | −0.2 | | | – | | | – | | | – | | | −1.0 | |
| 22:6*n*3 (DHA) | | – | | | −1.0 | | | – | | | 0.7 | | | – | |

[a)] Pupa stage.

As expected, TAG were present in large amounts in all samples, since they represent the largest store of metabolic energy in insects.[23] An explanation for the presence of DAG and FFA in samples is that they can be formed by lipolysis, which is presumably to occur during sample preparation.[27] The presence of greater or lesser amounts of such acyl-lipids can be due to the temperature used for sample homogenization, given that the solvent used for extraction inactivate the enzymes. However, although this phenomenon could increase such contents, DAG are expected to occur in insects, as these are the form in which lipids are transported in their body fluids.[28] We must also consider that insects can contain cold active lipases, which can be reactive even at low water concentrations.[29] Thus, although the samples were lyophilized, the time elapsed from such operation until the analysis of the lipids was effected could have been decisive in increasing to a greater or lesser extent the DAG and FFA detected in samples.

Concerning PL and SE, they have a structured role in the membrane systems of the cell. The ratio of PUFA to SFA in PL has a significant effect on the biophysical properties of biological membranes.[30] Therefore, it is expected that all insect species contain variable, albeit minority, quantities of these lipid classes. A constant trend for all species summarized in Table 3 was, in comparison with TAG, the increase in PA, SA and OA, as well as a decrease in all types of PUFA in the SE, in parallel with an increase in the amounts of SA and LA in the PL fraction.

To the best of our knowledge, this work constitutes the first report from a nutritional point of view on the positional distribution of the FA on the TAG for insects. Before performing the enzyme reaction analysis, the acyl migration phenomenon was studied. The TAG fractions of several larvae lipids were checked during the hydrolysis with pancreatic lipase to establish the degree of acyl migration over time. The bands recovered from the TLC plates, corresponding to TAG, DAG, MAG, and FFA, were analyzed for the FA profiles every 5 min. After 30 min of reaction it was found that the hydrolysis reached the highest values, while the FA profiles of each lipid class did not show significant differences among them over time ($P < 0.05$). Therefore, we decided to perform the highest degree of hydrolysis to obtain the FA profile of the more complete TAG fraction. The regiospecific distributions of the FA within the TAG structure for three species of Lepidoptera compared with that of *H. illucens* (Diptera) and *O. nasicornis* (Coleoptera) are summarized in **Table 4**. For such analysis *G. mellonella* was chosen against *S. exigua*, considering that the previous showed higher percentages of both OA and TAG, although both had similar FA profiles.

The typical pattern for all species listed in Table 4 consists in the enrichment in the *sn*-2 position for MUFA and PUFA, which has been attributed to the weak activity on PA and SA exercised by the enzyme responsible of *sn*-2 acylation.[31] It was noted an enrichment in ALA from 42.3 in the TAG to 49.1 mol% of total FA in the 2-MAG in *B. mori*, while, unexpectedly, OA was notably enriched from 44.7 and 47.0 in the TAG to 87.7 and 88.5 mol% of total FA in the 2-MAG in *O. nasicornis* and *G. mellonella* larvae, respectively. Such pattern of FA distribution was different to that observed in samples of *H. illucens*, which was weakly enriched in the 2-MAG with these FA.

Consistently with these results, the RI, which has been used as a suitable parameter indicating the level of any FA in the *sn*-2 position for the different FA,[16] displays positive values for OA in all Lepidoptera species analyzed here (1.3 and 0.9 for *B. mori* and *G. mellonella*, respectively) and Coleoptera (1.0 for *O. nasicornis*). The amounts of OA in the *sn*-2 position of *O. nasicornis* and *G. mellonella* (88.0 and 88.6 g/100 g of total FA, respectively) is similar to that shown by other typical OA sources such as olive oil, in which this FA ranges in the *sn*-2 location between 85.0 and 93.4 g/100 g of total FA, depending of the olive variety.[32] Concerning ALA, current sources such as are rosehip (*Rosa rubiginosa* Linnaeus 1771), soy (*Glycine max* Merril 1917) and flax (*Linum usitatissimum* Linnaeus, 1753) shows this EFA at 24.4, 5.7, and 44.2 g/100 g of total FA in the *sn*-2 location, respectively[16]; thus, *B. mori* larval oil, which contains ALA at 48.4 g/100 g of total FA in the *sn*-2 position (49.1 mol% of total FA), and a RI of 0.2, seems to be among the best sources of this LCPUFA found in nature. Although promising results have been obtained, the present investigation presents some limitations; for instance, the fact that 7 out of 15 species analyzed contain <10% lipids on dry wt, which is extremely low for commercial harvesting of lipids. Therefore, further research is required on the formulation of feeding pattern (and feed) or genetic selection that may result in elevated lipid content of these insects.

## 4. Conclusions

On the whole, this work provides a significant advance in knowledge on the occurrence of OA- and ALA-rich oils. Lepidoptera species constitute a promising source of *sn*-2 located OA- and ALA-rich oils, and similar or better than current sources. In addition, some data about other insect orders such as Coleoptera also points in this direction. Therefore, regardless of their nutritional interest *per se*, these insects could be reared to provide raw oils for using in the food and pharmaceutical industries. All species tested in this work could constitute adequate candidates for genetic selection to increase their oil percentages and/or targeted bioactive FA.

## Abbreviations

ARA: arachidonic acid; ALA: α-linolenic acid; DHA: docosahexaenoic acid; DL: detection limit; EFA: Essential FA; EPA: eicosapentaenoic acid; FA: fatty acid; FAME: FA methyl ester; GLA: γ-linolenic acid; GLC: Gas-Liquid Chromatography; GOA: gondoic acid; LA: linoleic acid; LaA: lauric acid; LCPUFA: long-chain polyunsaturated FA; MAG: monoacylglycerol; MR: method reproducibility; MUFA: monounsatured FA; OA: oleic acid; PA: palmitic acid; PE: polyethylene; PUFA: Polyunsaturated fatty acid; RI: Regiospecific index; RS: recovery studies; SA: stearic acid; SDA: stearidonic acid; SFA: saturated FA; TAG: triacylglycerol; TLC: thin layer chromatography; VLCPUFA: very-LCPUFA; wt: weight.

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## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

α-linolenic acid, *Bombyx mori*, lepidoptera, oleic acid, regiospecifity, triacylglycerol

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# A whole-food approach to the *in vitro* assessment of the antitumor activity of *gazpacho*

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ABSTRACT

*Gazpacho* is a traditional cold soup of the Mediterranean diet consisting of a main base of fresh pureed tomato and other vegetables. Tomato and tomato products have demonstrated chemopreventive activity against several types of cancer through *in vitro* studies, and in animal and clinical research. Here we have applied a whole-food approach for the preclinical assessment of the antitumor potential of *gazpacho*. Colon cancer cells (HT-29) were exposed to growing concentrations of *gazpacho* previously digested *in vitro* to simulate the delivery of bioactive molecules to colon cells after food consumption. The cytotoxicity of gazpacho ingredients was also tested in independent experiments. Programmed cell death by apoptosis was detected by using a multiparametric analysis that combines image-based bright-field and fluorescence cytometry, intracellular ATP level determination and enzymatic activity of caspase-3/7. Modulation of gene expression of key regulatory genes (p53, Bcl-2, BAX, and cyclin D1) was also investigated. Our cytotoxicity data showed that *in vitro* digestion of samples allowed the delivery of bioactive levels of antitumor phytochemicals to cultured cells. Controlled experiments showed significant repetitive dose and time-response cytotoxicity of *gazpacho*. Gazpacho digestates caused net cell death of cultures suggesting synergic activity among phytochemicals from its vegetable ingredients. Multiparametric and genetic analyses showed that *gazpacho* digestates can trigger colon cancer cells death by apoptosis through the activation of caspase cascade. Our results show that coupled *in vitro* methodology employed can be applied to investigate the antitumor potential of complex food matrixes or combinations of foods in the diet.

## 1. Introduction

Cancer chemoprevention is the use of natural or synthetic compounds to block, reverse or prevent the development of invasive cancer. A variety of dietary compounds have been largely investigated for their potential role in chemoprevention through diet. Bioactive phytochemicals from fresh fruits and vegetables, such as such as carotenoids, polyphenols, sulfur compounds, terpenoids and tocopherols, have been suggested as chemo-preventive agents (Béliveau & Gingras, 2007; Cilla, Alegría, Barberá, & Lagarda, 2013; Langner & Rzeski, 2012; Moosavi et al., 2018). Carotenoids, polyphenols, sulfur compounds and tocopherols).

*Gazpacho* is a traditional fresh soup of the Mediterranean diet consisting of a main base of tomato, olive oil, and small quantities of other raw vegetables like sweet pepper and cucumber. Consumption of *gazpacho* has been associated to a decreased cardiovascular risk in epidemiological studies (Lee & Foo, 2013; Medina-Remón et al., 2013). Its main component, tomato (and tomato based products), have shown anti-proliferative activity against some types of cancer in epidemiological, clinical and *in vitro* studies (Boivin et al., 2009; Giovannucci, 2002). Furthermore, different antitumor activities have been characterized in other components of the *gazpacho* mixture, such as olive oil (Pampaloni et al., 2014), cucumber (Abdelwahab et al., 2012), and garlic (Karasaki, Tsukamoto, Mizusaki, Sugiura, & Gotoh, 2001; Milner, 2006). Most of these works link antitumor activity to either different families of micronutrients, such as phenolic compounds, carotenoids, B vitamins, sulfur compounds, or to specific molecules like beta-carotene, lycopene, vitamin D or folate. Carotenoids are the main pigments found in tomato and have been associated with biological activities of this vegetable. Lycopene is the main carotenoid in tomato, and has been linked with the antitumor potential of tomato consumption (Hadley, Miller, Schwartz, & Clinton, 2002). Another bioactive compound,

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vitamin E, is a phenolic antioxidant encountered in vegetables, and includes several compounds, whose main molecular species is α-tocopherol, the most bioactive form in humans. The term "vitamin E" refers to a family of eight naturally occurring tocopherol homologues that are synthesized by plants. Vitamin E is the major lipid-soluble antioxidant in the cell antioxidant defense system and is exclusively obtained from the diet.. However, an increasing number of evidences suggest that these bioactive molecules might not act just alone, but that their activity could be enhanced in the context of the natural food matrix, and needs to be characterized in such context (Jacobs & Tapsell, 2007). Fresh vegetables are composed of a complex mixture of thousands of micronutrients. Their effective tissues concentration after consumption, and the interactions among them and with the cell metabolism that cooperate in the control of cancerous processes, are in great extent unknown. For instance, in the case of lycopene, a carotenoid suggested as responsible for the antitumor activity of tomato, plasma levels are dependent on many variables (Mayne et al., 1999). *In vitro* models often test lycopene levels above the attainable physiological range (Burgess et al., 2008). Moreover, clinical trials have generally shown limited evidence of the preventive efficacy of lycopene when administered isolated from its original food matrix (Basu & Imrhan, 2007; Talvas et al., 2010; Wang, Yang, Zhou, & Chen, 2016). Given the complex nature of the nutrition-health interface, further research is needed to characterize the bioactivity of complex food matrixes considered as a whole unit, shifting the focus from single biomolecules to a food-based approach that considers the food as a whole, and not just the nutrients, as the fundamental unit in nutrition (Jacobs & Tapsell, 2007). Based on available evidences, the U.S. Food and Drug Administration by now has not allowed claims that lycopene as nutritional complement exhibits antitumor activity in humans (Cavanaugh et al., 2007). The main rationale is that lycopene-containing foods contain other nutrients that also may interact with the metabolism of lycopene and the etiology of certain cancers, making it difficult to study isolated lycopene action (Sempos, Liu, & Ernst, 1999). For the time being, the most reliable advice for cancer chemo-prevention through food phytochemicals is still the recommendation of a frequent consumption of bioactive micronutrients-dense foods, such as fresh vegetables and fruits (Baena Ruiz & Salinas Hernández, 2016).

*In vitro* models, although with well-characterized limitations to reach conclusions at the *in vivo* level (Verhagen et al., 2003), however offer the possibility to make quick and repeatable pre-clinical assessments of the potential chemo-preventive activity of complex mixtures of micro-nutrients present in foods. One of these limitations is the need to simulate as best as possible the *in vivo* levels of supply of active micronutrients to cancer cells as best as possible. Here we have used an *in vitro* gastric and pancreatic digestion of the *gazpacho* dish and its ingredients to simulate the processes of digestion, absorption and transport of bioactive molecules to colon cancer cells after their consumption in fresh state. Even though most studies focus on the relation between tomato phytochemicals and prostate cancer (Campbell, JK., Canene-Adams, K., Lindshield, BL., Boileau, TWM., Clinton, SK., & Erdman, 2004), tomato activity against colon cancer has also been reported in both cellular (Cenariu et al., 2015; Palozza et al., 2007; Shi, Yang, Feng, Li, & Zhu, 2010), and animal models (Kim, Nam, & Friedman, 2015). Another advantage of *in vitro* model is that it allows for the study of mechanisms of control of cancer cells proliferation by apoptosis. Apoptosis is a programmed cell death activated in normal cells to control excess proliferation and embrionary development. Inhibition of apoptotic pathways plays a key role in cancer progression. Lycopene and other food phytochemicals have been linked to the activation of apoptosis in cancer cell lines *in vitro* (Arathi et al., 2016; Vijayarathna et al., 2015). Apoptosis is one key mechanism detected in cancer cell lines death when cultures are exposed to tomato digestates (Palozza et al., 2009). In this case, the caspase cascade pathway has been identified as the mechanism of apoptosis, activated by the decreased expression of tumor suppressor genes from the Bcl-2 family and the increased expression of pro-apoptotic genes such as BAX, and down-regulation of the expression of cell cycle genes such as cyclin D1.

Here we have used a whole-food approach to test the antitumor activity of gazpacho *in vitro* (Jacobs & Tapsell, 2007). Our main assumption is that cytotoxicity of our samples does not relay on the isolated activity of single phytochemicals, but is the result of the combination of all molecular species present in the complex vegetable food matrix tested.

Thus, the main goals in this work were two:

- check the performance of a whole food approach for the *in vitro* testing of antitumor potential of food samples, by using a coupled *in vitro* digestion of samples and testing the cytotoxicity of these digested samples against cancer cells cultured *in vitro*.

Testing empirically three hypotheses:

A. There is a significant antiproliferative action of *gazpacho* digestates against colon cancer cells
B. This activity is significantly higher than that of gazpacho ingredients tested separately
C. The cytotoxicity of gazpacho digestates occurs through the activation of apoptosis

Here we have assessed the growth inhibition and cytotoxic activity of digested samples of *gazpacho*, and its vegetable ingredients. Levels of gazpacho digestates tested were within the range that could be expected to be achieved in plasma when consumed *in vivo* (Mayne et al., 1999). Enzymatic and gene expression analysis have been carried to gain insight on the mechanism of action of cytotoxicity.

## 2. Materials and methods

### 2.1. Samples of vegetables and gazpacho

Samples of round tomato var. *racimo* (*Solanum lycopersicum* L.), sweet pepper var. *palermo* (*Capsicum annuum* L.), short cucumber, var. *corto* (*Cucumis sativus* L.), garlic (*Allium sativum* L.), salt, extra-virgin olive oil, Modena vinegar, and water were mixed in *gazpacho* recipe. All vegetables were purchased in local markets at ripe stage. At this stage (maximum maturity of shell life), the surfaces of the tomatoes get red in color and fruits begin to soften. Prior to exposure to cell cultures, all samples of gazpacho and its ingredients underwent *in vitro* digestion as explained later.

### 2.2. Gazpacho recipe preparation

A standardized home traditional recipe of *gazpacho* was used for all analyses and characterization of cells viability. The proportions in *gazpacho* recipe were: 385 g/l tomatoes, 58 g/l cucumber, 3 g/l sweet pepper, 2 g/l garlic, 3.3% (*v*/v) extra virgin olive oil (*Carbonell*™, Spain), 3.3% water (*v*/v), and 0.16% (v/v) vinegar (*Borges*™, Spain). Olive oil composition was characterized by 88% oleic acid and 0.2° acidity, and wine vinegar acidity was 6°. These ingredients were milled for 60 s in ambient temperature to become a puree, with a household blender (Type BLP30, *Kenwood*™, China).

### 2.3. Maturity parameters of gazpacho, tomatoes and sweet pepper

All vegetables were tested for bioactivity at their higher maturity stage. Tomato, sweet pepper and *gazpacho* were characterized using commercial standards for ripening stage assessment (Table 1). Color evaluation of tomatoes and sweet peppers was determined using a colorimeter (Spectrophotometer CM-3500D; Konika Minolta, Madrid, Spain). A CIELAB color space coordinates with L*, a*, b* values was characterized. The luminosity is represented by L* (0, black to 100,

**Table 1**
Maturity parameters of tomatoes, sweet pepper and *gazpacho*. Results are expressed as the mean values of the analysis of 3 independent samples ($n = 3$).

| | Moisture (g kg$^{-1}$) | Color parameters | | | | Brix (% sucrose) | Acidity (g/l) | Firmness (kg cm$^{-2}$) |
|---|---|---|---|---|---|---|---|---|
| | | L* | a* | b* | a*/b* | | | |
| Tomato | 935 | 33.7 | 17.3 | 18.9 | 0.91 | 5.0 | 2.18 | 1.6 |
| Sw. Pepper | – | 30.8 | 24.7 | 11.3 | 2.18 | 6.5 | 0.6 | – |
| *Gazpacho* | 899 | 54.1 | 22.9 | 25.3 | 0.90 | 4.8 | 2.12 | – |

white), a* represents the variation of greenness to redness (−60 to 60) and b* indicates the variation of blueness to yellowness (−60 to 60). The a*/b* ratio indicates the maturity stage of tomatoes. Each record was an average of three measurements on every tomato sample. One measurement was taken in the equatorial zone and the other two at the distal area. The maturity parameters of tomato and *gazpacho* are summarized in Table 1. According to a*/b* ratio, the commercial maturity stage of all tomatoes was *red*. Three samples of tomato, sweet pepper and *gazpacho* were then homogenized for analysis of moisture, color, total soluble solids content (Brix), titratable acidity and firmness.

### 2.4. In vitro digestion of food samples

Food samples were milled and underwent *in vitro* digestion without previous pulp filtering, to avoid losses of undetermined active compounds. We used an *in vitro* digestion procedure developed for assessing the bioavailability of carotenoids from meals (Garrett, Failla, & Sarama, 1999), with modifications. Namely, 4 g of *gazpacho* or milled vegetables were homogenized with 32 ml of saline solution. Gastric digestion was carried after acidification to pH 2 with 1 M HCl, and adding 2 ml of porcine pepsin (Sigma-Aldrich, St Louis, USA) (40 mg/ml in 0.1 M de HCl), incubating at 37 °C in a water bath, shaking at 95 rpm for 1 h. Prior to duodenal digestion, pH was risen to 5.3 adding 1 ml to 1.3 ml $NaHCO_3$ 0.9 M. Then 9 ml of a digestion juice were added, containing 2 g/l pancreatine and 12 g/l of bile extract (Sigma-Aldrich, St. Louis, MO, USA) in 100 mmol/l from a solution of $NaHCO_3$ at 0.1 M. pH was then raised to 7.5 adding NaOH 1 N (200 mg of NaOH in 5 ml water). The digestion mixture was then incubated at a water bath 37 °C, shaking at 95 rpm for 2 h. Finally, digestates were sterilized prior to exposure to cell cultures by filtering through 0.2 µm filters (Millipore Corp., USA). *Gazpacho s*amples were digested immediately after prepared, and all cell viability assays were done immediately after digestion of all samples to avoid possible degradation of phytochemicals under storage.

### 2.5. Analysis of key phytochemicals

*Gazpacho* samples were characterized through their content in three key bioactive compounds, lycopene, tocopherols and total flavonoids. Raw and digested samples were characterized before and after digestion. However, flavonoids content in *Gazpacho* digestate was below the detection limits of the method.. Phytochemical composition of tomato var. *racimo* employed in our *gazpacho* recipe, has been reported elsewhere (Ramos-Bueno, Romero-González, González-Fernández, & Guil-Guerrero, 2017). The composition of three key bioactive phytochemicals in *gazpacho* samples was determined before and after digestion. We selected three different biomolecular species that have been related to the antitumor activity of tomatoes *in vitro:* one carotenoid (lycopene) one tocopherol (α-tocopherol) and flavonoids (given as quercetin equivalent). Carotenoids were extracted and lycopene content was analyzed by HPLC. Briefly, the extraction method involved blending 3 times (1 h) the 50 g food samples in diethyl ether (100 ml). Later saponification was done with methanolic potassium hydroxide at 10% (*w/v*) for 1 h at 4 °C. The organic layer with saponifiable fraction was washed with water and etheric phase was collected. Then the remaining organic fraction was evaporated under vacuum at room temperature. The carotenoid residue was dissolved in methyl tert-butylhhh ether, filtered and analyzed by HPLC using the same equipment and method described elsewhere (Ramos-Bueno et al., 2017). A binary solvent system was used in isocratic elution (acetonitrile: methanol, 85: 15 (*v/v*)), with 5 µL injection and a flow rate of 1 ml/min. Carotenoids were identified by comparison with retention times of external standards (Sima-Aldrich)Tocopherols were extracted by alkaline saponification of the samples followed by extraction with organic solvent. 2–5 g of sample were saponified under nitrogen using a mixture of 50 ml ethanol, an antioxidant (ascorbic acid) (0.25 g) and aqueous potassium hydroxide (5 ml). This mixture was placed into a water bath (100 °C) for 45 min. Then, the tocopherols were extracted with n-hexane, 3 to 4 times with volumes ranging from 50 to 150 ml. The combined extracts were washed at neutral pH with water (2–4 times, 50–150 ml). Finally, the n-hexane phase containing the tocopherols was separated and the solvent removed *in vacuo* with 2 mg of BHT. The residue was dissolved in methanol, filtered and immediately analyzed by HPLC. Peak identification was based on the comparison of HPLC retention times with chemical standards (α- tocopherol), which were purchased from Sigma-Aldrich (St. Louis, MO, USA). A binary solvent system was used in isocratic elution (methanol: water, 98: 2 (*v/v*)), with 5 µL injection and a flow rate of 1 ml/min. All HPLC analyses were carried out with a Finningan Surveyor Chromatograph (Thermo Electron, Cambridge, UK) equipped with a UV detector and a reverse phase column (Hypersil Gold, 250 × 4.6 mm id, 5 µm, Thermo Electron, Cambridge, UK).

The concentration of total flavonoids was measured by spectrophotometric method (Riahi & Hdider, 2013), with modifications. Briefly, 3 g of lyophilized *gazpacho* sample were diluted in 20 ml of 80% ethanol and placed in a sonicator bath for 6 cycles of 6 min each at 60 °C. After filtration (Whatman filter, n° 5), 100 µl were diluted with ethanol 80%, and mixed with 200 µL of 1 M potassium acetate and 200 µL of 10% aluminium nitrate solutions, up to 10 ml final volume. After 40 min at room temperature, absorbance was read at 415 nm. Flavonoid content was determined using a calibration curve constructed with quercetin (Sigma-Aldrich, Q4951), and expressed as mg quercetin-equivalent (QE) per gram of dry weight.

### 2.6. Cancer cells culture

Colorectal cancer cell line HT-29 (ATCC) was used as cancer model *in vitro*, supplied by the CIC Center from the University of Granada, Spain. Additionally, Caco2 cancer cells were cultured to test for reproducibility of cytotoxicity of *gazpacho* digestates in another colon cancer line. Cell lines were grown in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.125 g/l amphotericin and 100 mg/ml penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cultures were plated in 25 cm$^2$ plastic tissue culture flasks (Sarstedt, USA) and incubated at 37 °C and 5% $CO_2$.

### 2.7. Cell viability and proliferation assays

Cell viability was determined after exposure to increasing concentrations of digested samples, and activity parameters were estimated within the range of concentrations tested. MTT assay (Mosmann, 1983) was used as quantitative colorimetric assay for cells survival and

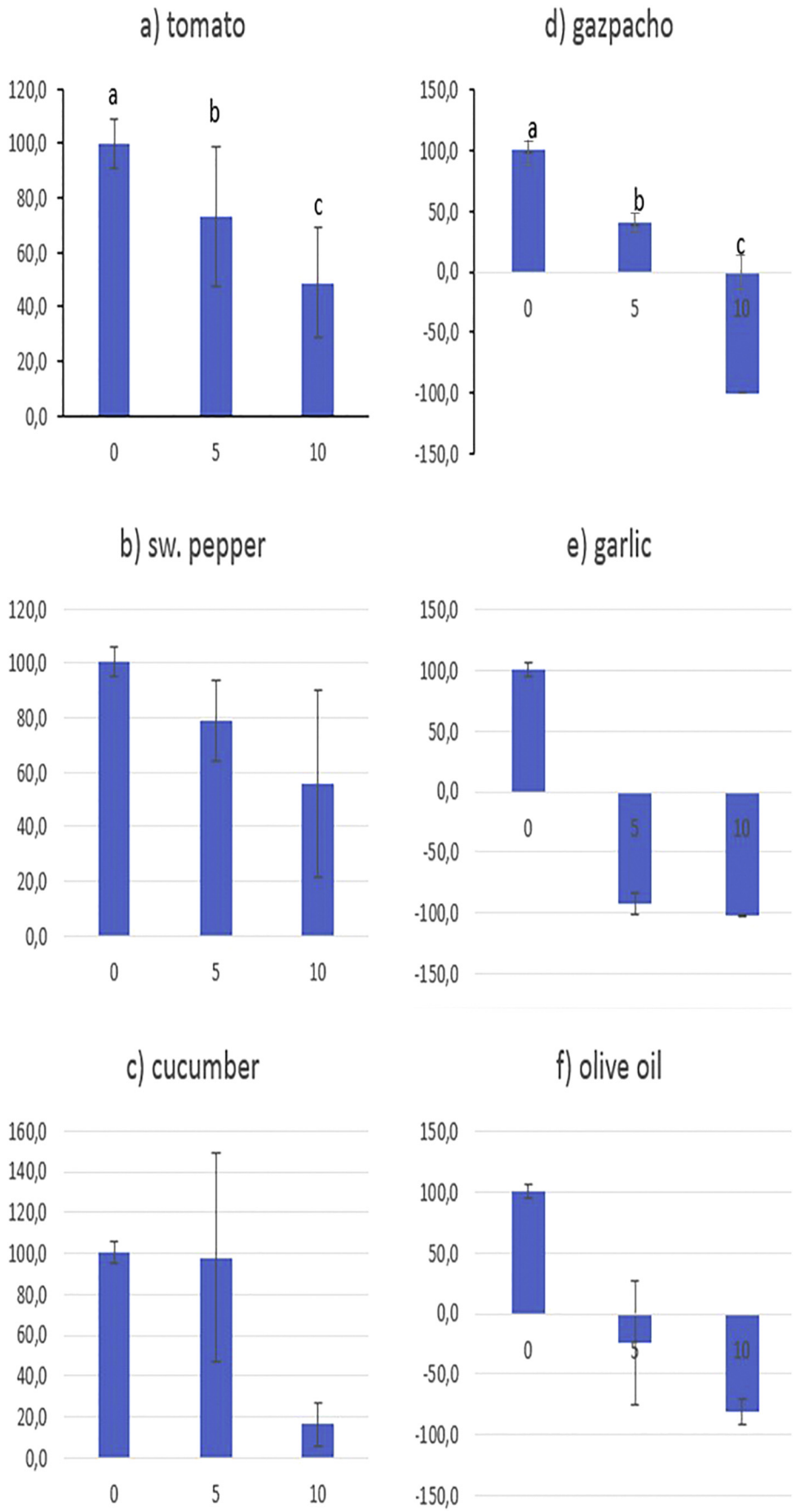


**Fig. 1.** Growth of HT-29 cells after 48 h of exposure to digested samples ("0" represent a negative control growth (=100). Concentrations of digestates is given in % v/v of culture medium. Values with unlike letters are significantly different ($P < 0{\cdot}05$), as determined by one-way ANOVA followed by Tukey's test (a,b,e), or Bonferroni test (c,d,f). Values are mean ± SD of six repeated samples.

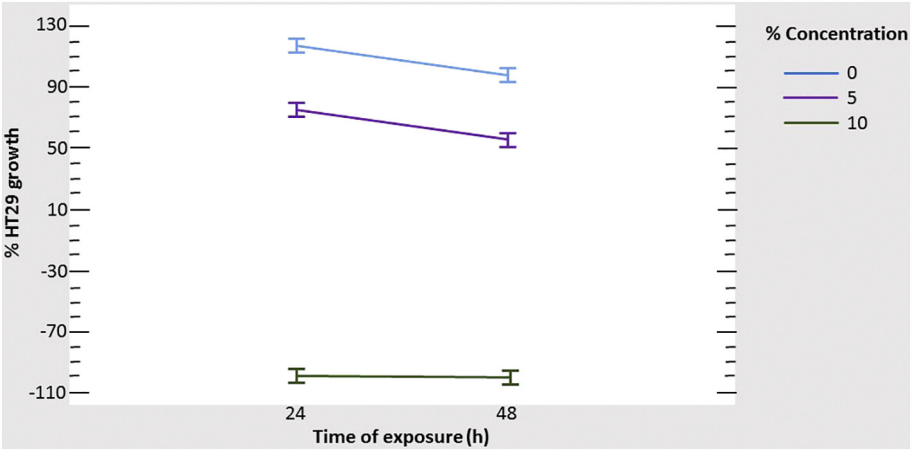


**Fig. 2.** Interactions plot of HT29 growth after two times of exposure to different concentrations of gazpacho digestate. Two-way ANOVA followed by Tukey test ($p < .05$).

proliferation. Exponentially growing cells (10,000) were seeded in 200 µl/well at 96-well plates (Corning™ 96 Well Clear Tc-treated Microplates), and exposed to digestates after allowing 24 h for culture establishment. Viable cells were determined measuring absorbance after 48 h of exposure to increasing concentrations of samples. Effect of gazpacho digestates was also determined after 24 h exposure. ELISA plate reader was used for MTT essays. Digestates concentrations are given as percent over total well volume (*v*/v), and are intended to lie below the maximum threshold expected *in vivo*, from 5 to 10% (v/v). Every assay included three different controls: a negative control (untreated, no sample added), positive control of a known HT29 cytotoxic compound (indomethacin, 0.2 mM), and a control for the activity of a mixture of digestion enzymes at the same concentrations was tested in experiments with digested samples.

Cytotoxicity parameters and cell growth data reported in Figs. 1 and 2 were calculated according to the US NCI *in vitro* screening program (Boyd, 1997). Cell viability data are expressed as percent growth inhibition relative to negative control growth. Using the MTT absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of digestate at the three concentration levels (Ti)], the percentage growth is calculated at each of the concentrations levels. Percentage growth inhibition is calculated as:

$$[(Ti - Tz)/(C - Tz)] \times 100 \text{for concentrations forwhich } Ti > /= Tz$$

$$[(Ti - Tz)/Tz] \times 100 \text{ for concentrations for which } Ti < Tz$$

When values are negative, they indicate a net reduction of viable cells compared to initial control. Values given are the mean values and standard deviations of six repetitions for each level. Every experiment was repeated three times (data not shown) with three standardized and digested vegetable samples. Negative controls and digestion enzymes controls showed no cell growth inhibition in all essays reported here. Positive control of indomethacin (C+) was tested as 0.2 mM as reference of a known cytotoxic dosage for HT29 cells. Our dosage-response data are given in (Fig. S1), showing comparable activity to previous reports (Kapitanović et al., 2006). Three dose response parameters were calculated for each sample: growth inhibition 50 (GI50) is the sample concentration (*v*/v) resulting in 50% reduction from control cells population; total growth inhibition (TGI) is the concentration resulting in 0% cells growth, and lethal concentration 50 (LC50) is the concentration resulting in a 50% reduction of culture, as compared to control growth after 24 h of establishment of culture in test plates, indicating a net cells death. Values of these three parameters are given when the level of activity is reached. If the effect is not reached or is exceeded, the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested.

### 2.8. Multiparametric analysis of cytotoxicity

To gain insight into the mechanism of the antitumor action of gazpacho digestates, we have applied a multiparametric analysis of cytotoxicity of gazpacho digestates against HT29 cells growth, by means of a combined well-imaging technology and luminescence measurement, carried out at an EnSight™ Multimode Plate Reader (PerkinElmer, Inc.). This methodology allows orthogonal assays in the same well, reducing inter-plate variation and increasing overall assay reliability, reducing artifacts caused by inter-well variability in MTT assays. Bright-field images were combined with nucleus staining and caspase enzyme activity analysis using fluorescent images. Additionally, a standard luminescence ATP detection assay was employed to determine cells viability. Similar experiment design to MTT viability tests were run, but cells were seeded in opaque walls plates to prevent well-to-well crosstalk (Corning® 96 Well Flat Clear Bottom Black Polystyrene TC-Treated Microplates). Namely, cells were seeded at 10,000 cells per well onto 96-well CellCarrier™ microplates (clear bottom, Black). Plates were incubated at 37 °C in the presence of 5% $CO_2$. After 24 h from seeding, cells were exposed to two concentrations of digestates (1, 5 and 10% *v*/v) in sextuplicate. After 24 h exposure to samples, culture medium was removed from the wells, and 100 ul/well of fluorescent dyes diluted in culture medium were added for cell staining. Assay plate was imaged on the EnSight system at 24 h and 48 h exposure. Plates were kept under controlled conditions (5% $CO_2$, 37 °C and 90% humidity) between each read and imaging took place at 37 °C using the temperature control facility of the EnSight reader, which eliminates potential water condensation effects. Cytotoxicity was characterized in this instrument using predefined image analysis algorithms provided by the EnSight system's Kaleido™ Data Acquisition and Analysis Software. Positive (indomethacin) and negative controls and all levels of concentrations were done in sextuplicate. As 1% digestates showed no significant differences in cell viability in previous MTT assays, only control, 5% and 10% concentrations were tested in multiparametric essay.

As additional information, gazpacho digestates were tested additionally with Caco2 cells to investigate anti-proliferative activity against another colon cancer line, using this multiparametric essay (Fig. S2). In this essay, cell viability was estimated as percent confluency measured in bright field. Also, HT29 cells were additionally exposed to tomato digestates and confluency, nuclei count and caspase were analyzed (Fig. S3).

#### 2.8.1. Bright field imaging

Prior to fluorescent essays, bright-field channel images were automatically analyzed by Kaleido Software, based on detection masks for

different well regions to identify relevant morphological and texture based parameters. Two image descriptors were identified as being strongly associated with the underlying cytotoxicity responses of the cells. Confluency analysis, which determines the percentage of the image area covered by cells, is a well-known indirect readout for the status of cells.

#### 2.8.2. Nucleus characteristics fluorescent image analysis

Stain solution Hoechst 33342 (Sigma, St. Louis, USA) was used as nucleic acid dye for nuclei count and nuclei characteristics assay. This dye is a membrane-permeable molecule with low cytotoxicity that stains both live and dead cells, and emits blue fluorescence when bound to DNA. The Hoechst stain was prepared at 3X, with a 50 µL addition to 100 µL of cells stained with CellEvent™. Final dye concentration was 3.3 µg/ml. Cells were incubated for at least 15 min at 37 °C prior to the first imaging. The channel was set at UV excitation 385 nm. Number of total nuclei and median nucleus area ($\mu m^2$) were determined by this essay.

#### 2.8.3. DEVD colorimetric assay of cytoplasmic caspase 3/7 activity

Activation of caspases enzymatic activity is one of the more definitive markers of apoptosis. To detect and characterize cytoplasmic differential activities we have employed the CellEvent™ Caspase-3/7 Green detection reagent (ThermoFisher Scientific), following the manufacturer's protocol. This test uses a fluorogenic substrate, the DEVD peptide sequence, (AspGlue-Val-Asp), conjugated to a nucleic acid–-binding dye. The DEVD peptide inhibits the ability of the dye to bind to DNA and thus the substrate is intrinsically non-fluorescent. The dye is cleaved from the DEVD peptide in the presence of activated caspase 3/7, and binds to DNA, producing a fluorogenic response indicative of apoptosis. In our essays we removed the media from the well and then added 100 ul/well of reagent diluted in culture medium, with a final concentration of 5 µM in each well. Fluorogenic response was measured after 30 minutes incubation at 37 °C. The channel was set at true green excitation 525 nm. DEVD area (%) and cells with DEVD staining (%) were the outputs of by this essay.

#### 2.8.4. Intracellular ATP levels

A luminescence ATP 1-step detection assay was used as a marker of cell viability (ATPlite, PerkinElmer, Inc), to confirm well-imaging results. Intracellular ATP is a marker of cell viability as it is present in all metabolically active cells and declines rapidly when the cells undergo necrosis or apoptosis. After well-imaging and fluorescence essays, cells were lysed and detection reagent containing luciferase and luciferin were added according to the kit instructions. Namely, 40 µL of solution were removed from each well and 40 µL of ATPlite one step luminescence assay solution were added (PerkinElmer, USA). All subsequent steps were performed according to the kit instructions. Luminescence was measured using the default settings on the EnSight system. ATP-dependent luciferin conversion is given by this essay. ATPlite essay is destructive and caused total cells death. This test was run only after 48 h exposure.

### 2.9. Gene expression analysis

The differential expressions of key genes involved in the regulation cell cycle (cyclin D1) and apoptosis (Bcl-2, p53 and BAX) were determined by real-time RT-PCR. After 48 h exposure of HT29 cells to gazpacho digestates, total RNA was extracted using PureLink RNA Mini Kit (Invitrogen) according to the manufacturer's instructions, after processing the samples on the FastPrep-24 5G (MP Biomedical) for 45 s at a speed of 6 m/s. RNA yield and quality were determined by a Qubit 2.0 Fluorometer (Invitrogen, by Life Technologies) and the automated electrophoresis equipment, Agilent 2100 Bioanalyzer system. Equal amounts (1 µg) of RNA from cells were reverse transcribed with High-capacity cDNA Reverse Transcription Kits (Applied Biosystems, by ThermoFisher Scientific). The cDNA was used as a template for subsequent real-time polymerase chain reaction (RT-PCR). Quantitative RT-PCR was done on a MyGo Pro® RealTime PCR System using TaqMan™ assays (Applied Biosystems, USA) following the manufacturer's instructions. The TaqMan™ Assay IDs of the genes investigated are: p53 (TP53) - Hs01034249_m1; cyclin D1 (CCND1) -Hs00765553_m1; BCL2 - Hs00608023_m1; Bax -Hs00180269_m1. The expression level of Bcl2, Bax, p53, and cyclin D1 mRNAs were all normalized with Human PGK1 (Phosphoglycerate Kinase 1) expression level. Relative expression was determined using the ΔΔCT method with PGK1 rRNA as the reference genes. Indomethacin was used as positive control of apoptosis induction in HT-29 cells.

### 2.10. Statistical analysis

For every experiment mean values of six replicates are given for every concentration of samples tested, and their standard deviations are represented as error bars in figures. When the influence of one factor (sample) was assessed in independent experiments (Fig. 1), one-way ANOVA test was used to detect any differences among levels. Multifactorial two-way ANOVA was used to assess any differences among treatments when two factors were assessed (concentration and time) (Figs. 3 and 5). When significant values were found ($P < .05$), *post hoc* comparisons of means were made using the Tukey's HSD test. Normality of residues was tested by Shapiro-Wilk test and homocedasticity by and Levene's test. When data did not comply with assumptions for ANOVA, non-parametric Kruskal-Wallis test was used to detect any significant differences, and Bonferroni procedure ($P < .05$) for comparisons pairs (Fig. 1c,d; Fig. 5c). Statgraphics Centurion XVII was used for analyses (Standpoint Technologies Inc.).

## 3. Results

### 3.1. Contents in key bioactive molecules of food samples

Maturity state of tomato, sweet pepper and gazpacho is given in Table 1. As the composition in biomolecules is closely related to the maturity stage of tomatoes (Opara, Al-Ani, & Al-Rahbi, 2012), contents of key phytochemicals (Table 2), and biological activity reported in this work correspond to that ripening stage. Values of lycopene of tomatoes var. *racimo* used in *gazpacho* recipe were in the range of those previously reported (Guil-Guerrero & Rebolloso-Fuentes, 2009). After digestion of *gazpacho*, lycopene decreased from raw *gazpacho* sample, while α-tocopherol decreased only 29% below raw sample.

### 3.2. Cell viability assays

HT29 cultures were exposed for 48 h to increasing concentrations of digested samples of gazpacho and its ingredients, in separated experiments (Fig. 1). All samples showed anti-proliferative activity in a dosage dependent manner. Digestates from the three main vegetables in the recipe (tomato, sweet pepper and cucumber) exerted growth inhibition activity, but no net culture death was caused at the highest concentration tested (TGI > 10%) (Fig. 1a–c). On the contrary, garlic and olive oil digestates caused net cell death at 10% *v*/v (TGI < 10%) (Fig. 1e–f). Gazpacho digestates caused growth inhibition response at 5%, but TGI level was between 5 and 10%. Total cell death occurred at 10% (LC50 between 5 and 10%). In all samples, lowest concentrations tested (1% *v*/v) showed no significant differences with controls (Tukey's test $p < .05$) (data not shown).

In Fig. 2, the effect of gazpacho digestate on HT29 cells growth is given at the interaction plot between concentration and time of exposure. Antiproliferative action of digestate was time and concentration dependent (Tukey test, $p < .05$). At both times of exposure, lowest concentration tested (1% v/v) showed no activity, medium response was given by 5% (*v*/v) and total response occurred with the highest

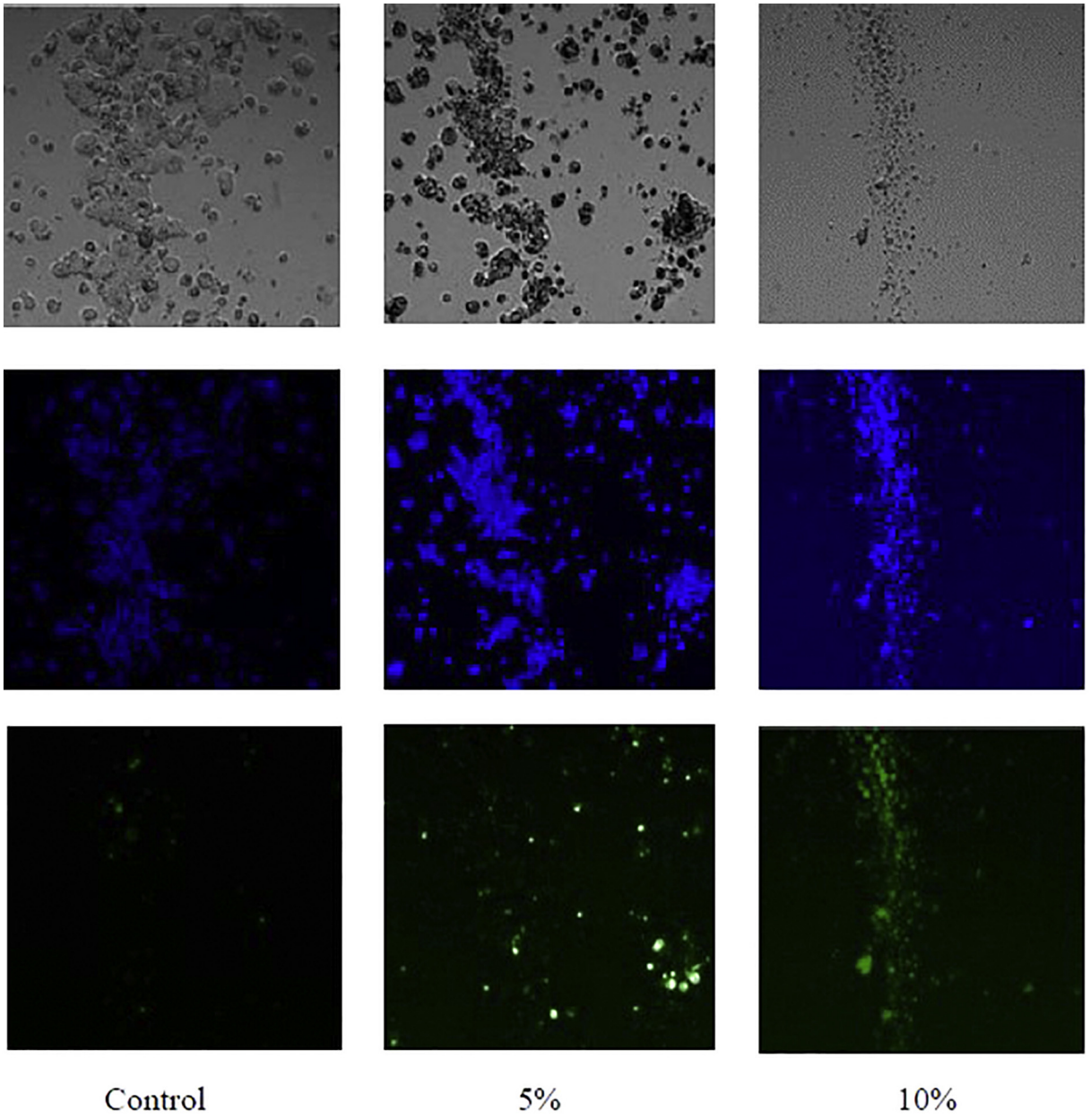


**Fig. 3.** Multiparametric analysis of cytotoxicity of *gazpacho* digestates against colon cancer HT29 cells, incubated for 48 h with two concentrations of digestates (5–10% *v*/v), and untreated control cells. Images were taken in bright-field imaging (up, black/white), blue Hoechst 3342 channel (middle) and green DEVD channels (low). 4× amplification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

level tested (10%), at which most cultured cells died at both times.

### 3.3. Multimodal analysis

Further insight on the mechanism of action of the anti-proliferative activity of *gazpacho* digestates was revealed by multiparametric

**Table 2**
Contents in key phytochemicals in *gazpacho* samples, before (raw) and after digestion (digested). Results are expressed as the mean values and standard deviations (SD) of three different samples (n = 3).

| | Lycopene ($\mu g\ g^{-1}$)* | SD | Lycopene (μM) | SD | α-tocopherol ($\mu g\ g^{-1}$)* | SD | α-tocopherol (μM) | SD | Flavonoids (mg QE $g^{-1}$)* | SD | Flavonoids (mg QE $l^{-1}$)† | SD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw *gazpacho* | 609 | 20 | 11.3 | 0.36 | 27.6 | 1.9 | 0.06 | 0.00 | 4.38 | 0.40 | 43.8 | 1.3 |
| Digested *gazpacho* | 97.1 | 3.3 | 1.57 | 0.05 | 19.8 | 3.6 | 0.40 | 0.07 | N/A+ | N/A | N/A | N/A |

N/A, non-available.
* dw, dry weight.
† Fresh weight.

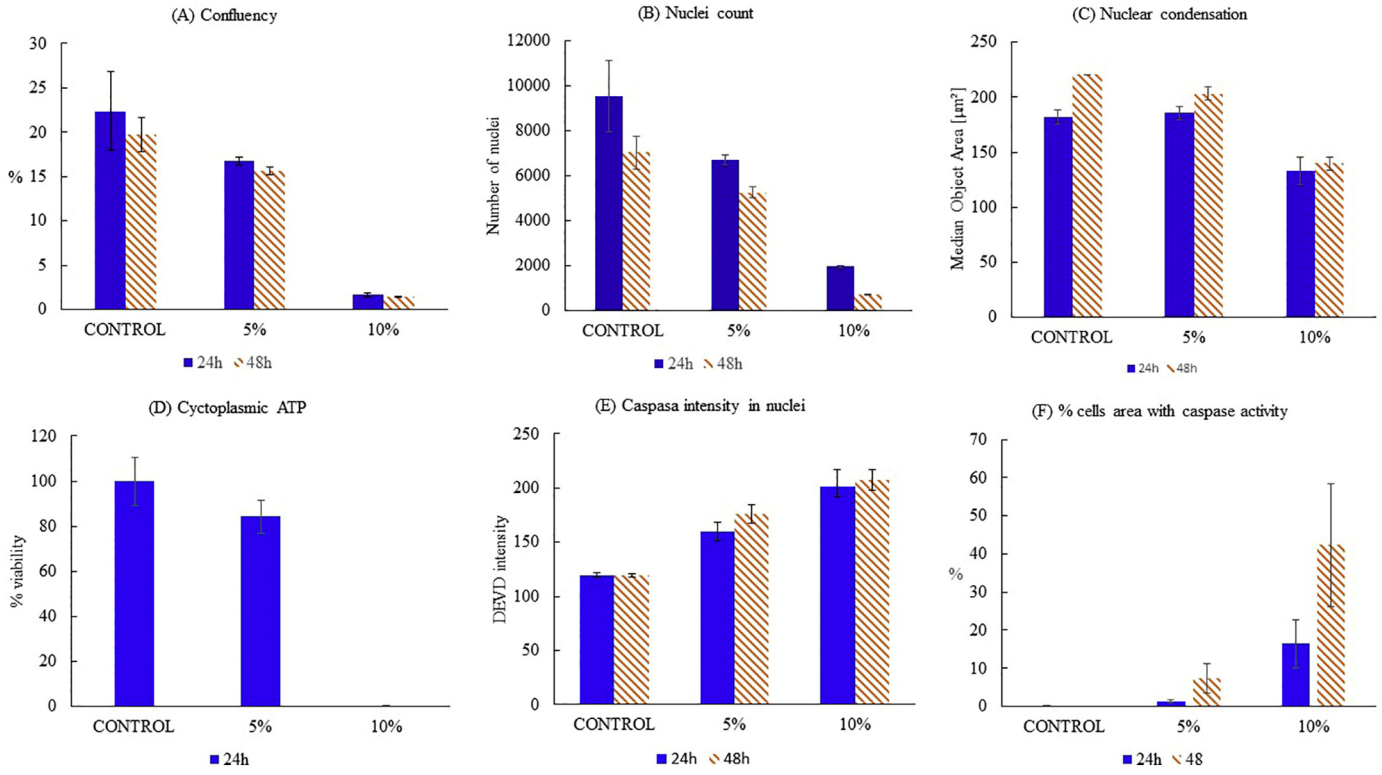


**Fig. 4.** Multiparametric analysis of cytotoxicity after exposure of HT29 cells to growing concentrations of *gazpacho* digestates for 24 h and 48 h. (A) Confluency by bright-field imaging; Nuclei count (B), and nuclear condensation (C) by Hoechst staining; (D) Luminescence cytoplasmic ATP test after 24 h; caspase 3/7 activity measured as (E) % cells stained with DEVD, and (F) % DEVD staining area; Values between 24 and 48 h are not comparable when control growth was significantly different between experiments ($p < .05$) (Fig. 3A–C). Values not sharing common letters are significantly different, as determined by two-way ANOVA (a,b,c,e,f)), or one-way ANOVA (d), both followed by Tukey's test at $p < .05$. Values represented are mean ± SD of six replicated samples. C– = Negative control; C+ = positive control (Indomethacin at 0.2 mM).

analysis of cytotoxicity against HT29 cells growth (Figs. 3 and 4). Differences among treated and non-treated cells were clearly visible through bright-field channel imaging with morphological changes associated with the onset of apoptosis, with increased roughness and darkening of cells after exposure to 5% digestate (Fig. 3, first row). Generalized cell death was observed with 10% concentration, in accordance with our previous MTT assays (Fig. 3, third column). Another apoptosis indicator, nuclear condensation, was detected by Hoechst blue staining (Fig. 3, mid row). Activation of caspase cascade, a key step in the apoptotic pathway, was revealed by DEVD green staining, showing an intense activation of caspases 3/7 in a dosage-dependent manner (Fig. 3, low row).

Numerical analysis of these images is summarized in Fig. 4. Effects of 1% (*v*/v) concentration were not significantly different form control in any of the parameters analyzed ($p < .05$) (data not shown). Significant differences between control and both 5% and 10% digestates were detected (Tukey test, $p < .05$). Interestingly, in most parameters, activity of 5% digestates was not significantly different than the level of positive control used (indomethacin, 0.2 mM). In accordance with our previous MTT assays (Fig. 3), cell viability decreased with increasing doses of *gazpacho* digestates, as shown by a decrease in % culture confluence detected by bright-field imaging (Fig. 4(A)). This anti-proliferative activity was confirmed by cytoplasmic ATP decrease after 48 h exposure (Fig. 4(D), as an indicator of impaired mitochondrial activity, with total cell death after exposure to 10% (v/v) digestate. Nuclei count by Hoechst staining (Fig. 4(B)) further illustrates this pattern of dosage-dependent activity. Nuclear condensation analysis (Fig. 4(C)), allowed the detection apoptosis, as the decrease in mean nuclear area is a key morphological change of programmed cell death. Fluorescence DEVD intensity and % area increase (Fig. 4(E–F)) showed an intensification of caspase 3/7 activity, with a clear dosage- and time-dependent pattern of caspase activation, and a sharp increase of % cells with DEVD staining after exposure to 10% digestates (Fig. 4(F)). By this parameter, we were able to estimate caspase activity related to total cell number, even if cell population was sharply reduced by cytotoxicity of the digestates. Thus, when 10% digestate was used, even if few cells survived, a very high ratio of fluorescence was detected, with > 40% active area after 48 h (Fig. 4 (E)). Anti-proliferative activity of gazpacho digestates was further tested in another colon cancer line (Caco2) by dosage- and time-dependent response of cells growth after exposition to the same digestates, as shown by decreases in confluency and nuclei count measured by using the Ensight instrument multiparametric essay (Fig. S2).

In conclusion, multimodal analysis of cytotoxicity allowed to detect and characterize the dosage and time dependent anti-proliferative activity of *gazpacho* digestates, giving strong evidence that activation of apoptosis by the caspase cascade is a key mechanism of action of *gazpacho* digestates against HT29 cells growth.

### 3.4. Genetic expression

In Fig. 5 we present the analysis of the differential genetic expression of key apoptosis-regulating genes (Bcl2, BAX, p53 and cyclin D1), after exposure of HT-29 cells to levels 5–10% *v*/v of *gazpacho* digestate for 24 h. Our analysis of the changes in expression of two genes of the BCL2 family, anti-apoptotic Bcl2 and pro-apoptotic BAX, highlight their important role in the modulation of apoptosis after exposure to our samples. A significant and dosage-dependent downregulation of the expression of Bcl2 was observed (Fig. 5(A)), falling to around 50% from normal levels of expression in untreated control HT-29 cells. Though BAX also showed downregulation, down to 65% level (Fig. 5(B)), no significant differences were observed between sample concentrations,

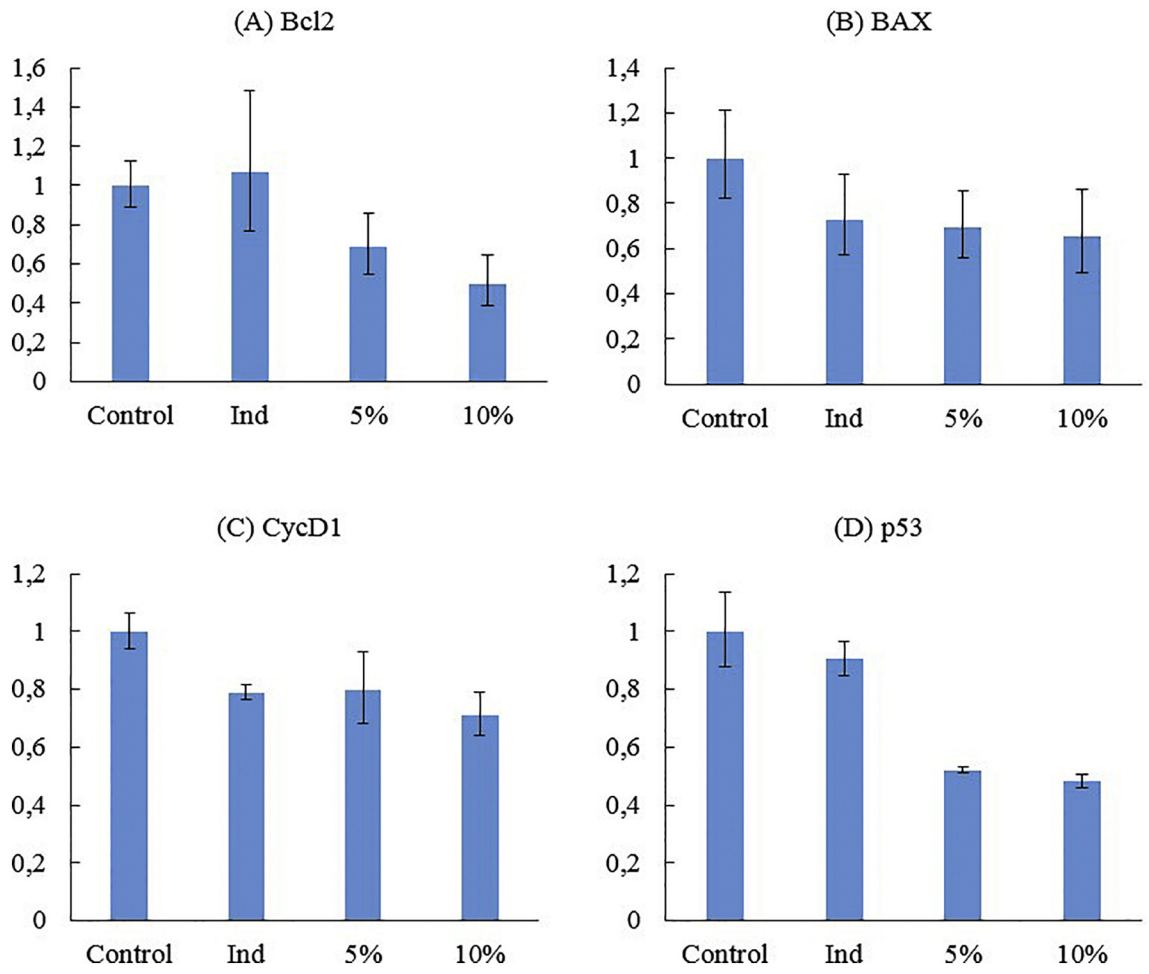


**Fig. 5.** mRNA expression in HT-29 cells of tumor suppressor genes (Bcl2, Bax and p53), and cell cycle regulator cyclin D1, after 24 h exposure to two concentrations of *gazpacho* digestates, 5% and 10% (*v*/v). Ind, indomethacin at 0.2 mM (positive control). Values are means of three independent experiments and bars denote standard deviations. Values with unlike letters are significantly different ($P < 0{\cdot}05$), as determined by one-way ANOVA followed by Tukey's test (a,b,d), or by Kruskal-Wallis test followed by Bonferroni test (c). Values are mean ± SD of six repeated samples.

and the levels of expression of this gene alone did not seem to be relevant in the activation of apoptosis. This is in accordance with findings with tomato digestates (Palozza et al., 2007). However, the reduction in the ratio Bcl2/BAX has been suggested as a mechanism of growth-inhibition by lycopene in cancer cells (Palozza et al., 2009) the determinant parameter to activate apoptosis. Our data show a clear relation between the increase in the concentration of digestates, and the increase in this ratio. When exposed to 10% (*v*/v) sample, the ratio fell to 76% from control, evidence that the change in Bcl2 and Bax, more than their separated expression, plays a key role in the activation of apoptosis by *gazpacho* digestates. Another key oncogene expression studied was cyclin D1, responsible for promoting cell cycle progression at G1/G0 phase in cancer cells. Cyclin D1 acts as a growth factor sensor in G phase and is overexpressed in HT-29 cancer tumors (Mayo & Mayol, 2009). After 24 h of exposure to *gazpacho* digestates, we detected a significant and dose-dependent downregulation of cyclin D1 expression, down to 71% from control, after exposure to 10% v/v of digestate (Fig. 5(C)). As downregulation of this gene has been reported after exposure of HT29 cells to tomato digestates (Palozza et al., 2007), our data this suggests that *gazpacho* digestate might also probably interfere with cell cycle progression at this level. Unexpectedly, gene p53 expression decreased in a dosage-dependent manner after 24 h exposure to *gazpacho* digestates, falling to 45% of control expression after 10% v/v digestate exposure ((Fig. 5(D)) Positive control indomethacin caused no significant change in expression of p53 and Bcl2, with downregulation observed in BAX and Cyclin D1.

## 4. Discussion

We present the first experimental evidence of the anti-proliferative and cytotoxic activity of *gazpacho* against cancer cell lines growth. Using a coupled *in vitro* digestion/cell culture viability assay, we have detected dose and time-dependent growth inhibition activity of tomato and *gazpacho* digestates at low concentration levels attainable *in vivo*. We have selected lycopene content as bioactive molecules reference to compare the levels of exposure tested *in vitro* with those expected *in vivo*. However, by our whole food approach we do not intend to associate the antiproliferative activity detected to any particular compound or family of phytochemicals present in the digestates. The physiological levels of lycopene in human blood and tissues vary with intake and tissue type. It has been established from clinical trials (Mayne et al., 1999) an average lycopene content of 0.5 μM in human blood (ranging 0.07–1.79). Lycopene in human tissue levels vary from 0.001 μM wet weight in adipose tissue to 0.02 μM wet weight in adrenals and testes (Stahl & Sies, 1996). In our gazpacho samples, an initial concentration in raw *gazpacho* of 11.3 μM (Table 1), was reduced in digestates to 1.57 μM, resulting final concentrations of 0.06 μM for 5% samples, and 0.15 μM for 10% samples, around 5-fold below average plasma levels of 0.5 μM, and within the range of *in vivo* attainable levels.

A methodological contribution of this research is the assessment of the performance of an *in vitro* cellular model, coupled with a standardized static *in vitro* digestion method, and a multiparametric analysis of

cytotoxicity, to characterize the chemopreventive potential of food samples against the proliferation of cancer cell cultures. The method of *in vitro* digestion of vegetable samples (Garrett et al., 1999) has proved to be a valid method to deliver complex food matrixes to cultured cell. Despite the dramatic decrease in phytochemicals concentrations after digestion (Table 2), the anti-proliferative activity of digestates was significant, and confirms that bioaccessibility and bioavailability of active biomolecules is enhanced by the digestion procedure (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009). Our data of growth inhibition of cancer cells by tomato digestates was comparable to previous values reported. Using the same digestion method, previous works (Garrett et al., 1999; Palozza et al., 2009) reported values of HT29 growth of 45% from control after 24 h of exposure to 10% (*v*/v) of tomato digestate, which was not significantly modified prolonging the incubation time until 72 h. We obtained a very similar value of 48% after 72 h exposure. This similarity is quite remarkable, as differences in tomato samples and experimental conditions may cause significant variability among studies. Another novelty of our investigation is that our data show an enhancement of activity of phytochemicals from tomato and other fresh vegetables when mixed in the context of the *gazpacho* mixture. Two factors might co-operate to reach this enhanced bioactivity. First, our data support prior observations that the higher the variety of molecular species in the food matrix, the higher the anti-proliferative action. The complex cocktail of phytochemicals present in *gazpacho* is much richer than in a simple tomato juice, including several families of biomolecules with known antitumor activity from other food components. It is possible that, when consumed regularly, such dietary mixture might act in a similar way as a metronomic chemotherapy (Scharovsky, Mainetti, & Rozados, 2009). This way, dietary food biomolecules would act in similar way to the administration of generally low doses of various chemotherapeutic drugs without extended rest periods, acting on a variety of molecular targets in cancer cells, and thus, enhancing their anti-proliferative activity. Second, the presence of olive oil in the *gazpacho* mixture enhances micellization process during the *gazpacho* puree milling process, increasing the bioaccessibility and bioavailability of hydrophobic phytochemicals, such as carotenoids (van Het Hof, West, Weststrate, & Hautvast, 2000) and polyphenols (Failla & Chitchumroonchokchai, 2005; Palmero, Lemmens, Hendrickx, & Van Loey, 2014). Such processing destroys plant tissues, increasing surface area and enhancing the interactions of digestion enzymes and emulsifiers with food particles. During *in vivo* digestion, olive oil helps transfer of lipophilic micronutrients from the food matrix to oil droplets and micelles, and stimulates the secretion of bile salts and pancreatic lipases required for micelle formation and inducing chylomicron synthesis (Borel, 2003). Olive oil is a key ingredient in *gazpacho* recipes, ranging between 3 and 5% (*v*/v) in the final mixture. The presence of oleate from olive oil in the micelles has also shown a positive effect in the absorption of carotenoids (Hollander & Ruble, 1978). In addition to this action, olive oil has some specific bioactive compounds, such as triterpenes, that have shown their own biological activities (Sánchez-Quesada et al., 2013) and direct antitumor and proapoptotic activities against cancer cells (Allouche et al., 2011; Cárdeno, Sánchez-Hidalgo, Cortes-Delgado, & Alarcón De La Lastra, 2013; Reyes-Zurita, Rufino-Palomares, Lupiáñez, & Cascante, 2009) that might also play a role in *gazpacho* activity. Also, our data suggest that garlic phytochemicals with known antitumor activity, such as sulfur compounds (Milner, 2006), though present in minor amounts in the final recipe, might also cooperate in the overall cytotoxicity of gazpacho digestate. Only gazpacho, garlic and olive oil digestates caused net cell death at 10% v/v. However, according to usual levels of dietary exposure of garlic and olive oil, we believe that such plasmatic concentration should only be attainable for gazpacho digestate. This highlights the antitumor potential of gazpacho, compared to its separated ingredients.

Our results from caspase enzymatic activity and differential gene expression analyses suggest that *gazpacho* matrix contains a rich combination of natural bioactive species able to activate the apoptotic pathway in colon cancer cells. Several components of *gazpacho*, such as carotenoids in tomato (Cenariu et al., 2015), and phenolic compounds in olive oil (Gill et al., 2005; Llor et al., 2003), have shown *in vitro* anti-proliferative activity against colon cancer (Rotelli et al., 2015). Here we have detected the activation of Caspase 3/7 route by *gazpacho* digestates. *Gazpacho* can be considered as a fresh tomato juice, enriched by other vegetables and olive oil. The activation of apoptosis by tomato digestates had been already reported in previous studies. Tomato digestates activate apoptotic targets by inducing caspase-3, and inhibit the G0/G1 progression of the cell cycle. Downregulation of cyclin D1, Bcl-2 and Bcl-xL has been also reported (Palozza et al., 2007, 2009). Our data show that apoptosis was triggered by *gazpacho* digestates mainly by the downregulation of anti-apoptotic gene Bcl-2. The inhibition of Bcl-2 seems to be determinant in the induction of apoptosis in our case. As downregulation of this gene has been reported after exposure of HT29 cells to tomato digestates (Palozza et al., 2007), our data this suggests that *gazpacho* digestate might also probably downregulate cell cycle progression at this level. Cyclin D1 is one of the main modulators of cell cycle, *via* cyclin dependent kinases, and its inhibition by tomato digestates (Palozza et al., 2007, 2009) and lycopene (Nahum et al., 2006) has been described. Further cell cycle analysis should confirm this mechanism."

One of the most interesting future lines of research is the undetermined role of p53 downregulation in the antitumor activity observed. The p53 gene has now clearly been shown to be the most commonly mutated oncogene in a wide variety of human cancers, including the most frequent adenocarcinomas. Wild-type p53 integrates a complex network of anti-proliferative responses, including apoptosis, cell cycle arrest and senescence (Fridman & Lowe, 2003). However, although loss of normal functionality is almost universal, p53 might not be really a tumor suppressor gene but a dominant negative effect is involved in most mutations (Liu, Russell, & Wang, 2006). In our case, since HT-29 cells do not contain wild-type p53 protein (Rodrigues et al., 1990), the apoptosis mechanism induced by *gazpacho* digestate detected here, through caspase activation *via* Bcl2/BAX ratio decrease, is likely independent on wild-type p53 protein in this cell line. This agrees with similar absence of significant p53 expression change in HT-29 after indomethacin exposure in our positive control test of cytotoxicity. It also agrees with the unchanged regulation of p53 in HT-29 reported after exposure to tomato digestates (Palozza et al., 2007, 2009). The p53 anti-proliferative network is controlled by different signals, including environmental factors, such as dietary active biomolecules and their metabolites. As far as we know, from tomato micronutrients only lycopene has been cited as activator of p53 to induce the intrinsic pathway of apoptosis in cancer cells, but different lines than HT-29 (Palozza et al., 2010; Pereira Soares et al., 2014). Little is known about the role of p53 mutant in HT29 carcinogenic phenotype, and thus by now the link between the p53 down-regulation reported here, and growth inhibition by *gazpacho* digestates remains to be investigated.

The main handicap of the whole food approach employed here is the weak establishment of causal links between the overall activity of the matrix characterized here and specific biomolecules of known isolated activity (Jacobs & Tapsell, 2007).

The detailed identification of these molecules, their relative role and the interactions and synergies responsible for the antitumoral activity described here remains to be investigated, and was out of the scope of our assessment. Much research has been done to assess the antitumor activity of isolated biomolecules, but few works describe paired synergies between mixed components such as those present in *gazpacho* recipe, such as tocopherols and carotenoids of tomato and pepper (Le Grandois, Guffond, Hamon, Marchioni, & Werner, 2017), garlic and tomato (Bhuvaneswari, Rao, & Nagini, 2004), and olive oil and tomato (Ramos-Bueno et al., 2017). We believe that our whole food approach provides a necessary view, complementary to these studies, for the evaluation of the overall chemopreventive potential of these molecular species when mixed in the context of their natural food matrixes.

The conclusions related to the anticancer potential of our study are limited to the model system used, and to the specified food digestates tested, and needs further support by clinical trials. Our model can only simulate the complete sequence of digestive transformation of food in part, and the final development of biologic actions inside cancer cells. Further developments should insert an intestinal absorption interface for a more realistic simulation of bioavailability of key micronutrients. Despite this limitation, we think that coupled digestion-cell viability tests such as the methodology used here, is a valuable tool for the pre-clinical assessment of the potential anti-proliferative activity of complex food matrixes rich in active biomolecules, and for the design of controlled experiments to elucidate their molecular mechanisms of action.

## Conflicts of interest

None.

## Funding sources

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## Contributors

PC designed the experiment, coordinated the group, and wrote and edited the manuscript. JAGC carried out the analyses related to the gene expression, and reviewed and corrected the manuscript. MJAG, RPRB and MJGR carried out all of the cell cytotoxicity tests. HK performed the multiparametric activity test and the caspase activity analysis.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.11.058.

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Original Research Article

# Borage oil: Tocopherols, sterols and squalene in farmed and endemic-wild *Borago* species

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ABSTRACT

Endemic *Borago* species contain notably high amounts of γ-linolenic acid (GLA, 18:3*n*6), the main bioactive fatty acid of borage oil. However, little is known about the occurrence of nutritionally relevant phytochemicals in seed oil of such plants. This work was designed to test the occurrence of important phytochemicals (tocopherols, sterols and squalene) in the seed oil of *Borago* species. To this end, seeds of endemic-wild and farmed *Borago* species were collected and analyzed. All compounds were analyzed by HPLC-diode array detector, while sterol and squalene peaks were confirmed using a LC–MS device and a library software. The highest amounts of tocopherols were found in two endemic *Borago* species: *B. pygmaea* and *B. morisiana*, with 514 and 296 mg/100 g oil, respectively. Squalene was predominant in *B. officinalis* collected in Tunisia and *B. morisiana* (3.9 mg/100 g seeds). The main sterols were $\Delta^5$-avenasterol, campesterol, and β-sitosterol, which were especially high in *B. morisiana* and *B. pygmaea*, with 107 and 102 mg of total sterols by100 g of seeds, respectively. The latter species are noteworthy GLA producers. The study suggested that oils from the endemic species are more nutritionally advantageous than those from the common cultivated borage (*B. officinalis*) and other current sources.

## 1. Introduction

Squalene (Sq), sterols (St) and tocols (Tc) constitute a large fraction of the unsaponifiable materials of vegetable oils (Choo et al., 2007). The generic term "Tocols" refers to a group of eight lipophilic compounds of tocopherol (Tp) and tocotrienol (T3) homologues, some of which show vitamin E–like activity. Both groups are structurally similar but differ in the degree of unsaturation in their side chains. While $T_3$ have an isoprenoid chain with three double bonds with *trans* configuration at 3′, 7′ and 11′ position, the side chain of Tp is completely hydrogenated. The four natural isomers of each group are named as α-, β-, γ- and *δ*-Tp/$T_3$. These four isomers differ in the number and position of the methyl groups present on a chromanol ring (Eitenmiller and Lee, 2004). The structures of all naturally occurring Tc, together with common St and Sq, are depicted in Fig. 1.

Tc display lipophilic antioxidant properties to prevent lipid peroxidation (Traber and Atkinson, 2007), and they also act as scavengers of reactive oxygen species (Esterbauer et al., 1991). Therefore, their main role in plants is related to the protection of lipids against oxidation (Havaux et al., 2005). Furthermore, all these compounds reportedly possess antitumor activities (Campbell et al., 2006; Yu et al., 1999), prevent cardiovascular diseases (Jialal et al., 1995; Loffredo et al., 2015) and diabetes (Fang et al., 2010; Bharti et al., 2013), and display anti-inflammatory activity (Mocchegiani et al., 2014). Regarding Tc

*Abbreviations:* AIF, all-ions fragmentation; DAD, diode array detector; ESI, electrospray interface; EU, European Union; FA, fatty acids; GLA, gamma-linolenic acid; HCD, higher collisional dissociation; HPLC, high pressure liquid chromatography; MS, mass spectrometry; RP, reverse phase; Sq, squalene; St, sterols; $T_3$, tocotrienols; Tc, tocols; Tp, tocopherols

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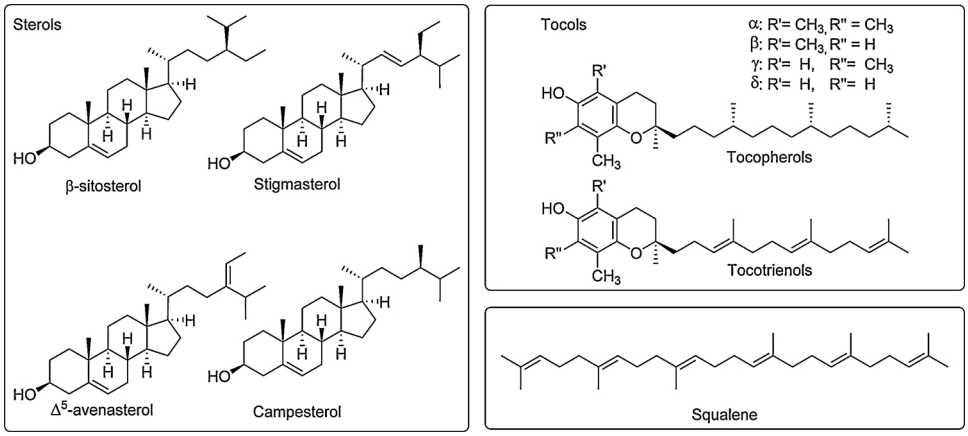


**Fig. 1.** Structure of tocols and the main sterols found in plant foods. Most vegetables contain variable amounts of α-, β-, γ-, and δ-tocopherol, while the occurrence of tocotrienols is restricted to some taxa. Among sterols, β-sitosterol, stigmasterol, avenasterol, and campesterol are the more frequent ones, and they have been identified in borage oil. Structures were drawn using Chem Draw Ultra® software (Chem Draw Ultra, Cambridge Soft Co., MA, USA).

occurrence, while Tp reach high concentrations in most seed oils, $T_3$ compounds are restricted to a few botanical families (Horvath et al., 2006). In addition, the amounts of Tc are lower in fruits and vegetables than in seed oils because of the lower concentration of lipids (Shahidi and de Camargo, 2016).

Phytosterols are bioactive isoprenoids occurring in plants. More than 150 phytosterols have been found so far. Among them, β-sitosterol, campesterol and stigmasterol are dominant compounds, representing 65, 30, and 3%, respectively, of the dietary phytosterol intake (Weihrauch and Gardner, 1978). Phytosterols have cholesterol-lowering effects. They are found in plants either as esters or as free alcohols both of which are beneficial to health as the hydrolysis of the esters occurs in the digestive system (Carden et al., 2015). The nutritional value of such compounds is connected to the inhibition of the absorption of cholesterol, and to the reabsorption of cholesterol excreted to the bile during the enterohepatic cycle, leading to lower values of total serum cholesterol and LDL-cholesterol (Piironen et al., 2002). St (campesterol, β-sitosterol and stigmasterol) show antitumor actions, especially on those of colon, breast, and prostate. Their mode of action is connected to cancer cell growth inhibition, and to the promotion of cancer cell apoptosis (Bradford and Awad, 2007).

Sq is a 30-carbon isoprenoid and the biosynthetic precursor of steroids both in plants and animals, which is metabolized to St in plant cells (Goodwin, 1980). Although no report was found about its antitumor actions in humans, in rodents Sq inhibits induced tumorigenesis, and can reduce free radical oxidative damage to the skin (Huang et al., 2009; Auffray, 2007). The primary application of Sq in cancer therapy seems to be as potentiating agent for anticancer drugs. Promising results were obtained when Sq was tested in combination with antitumor agents (Popa et al., 2015). Moreover, animal experimentation suggested the protective effect of Sq against cardiovascular heart disease, explained by its ability to inhibit the isoprenaline-induced lipid peroxidation (Popa et al., 2015).

The *Borago* genus belongs to the Boraginaceae family. It comprises only five species. *B. officinalis, B. trabutii, B. longifolia, B. pygmaea,* and *B. morisiana*, all them native to the Mediterranean Basin. *B. officinalis* constitutes the only farmed taxon in this genus and can be found outside this region. The remaining species are endemics and threatened for extinction (Selvi et al., 2006). The seed oil of *B. officinalis* is considered one of the richest natural sources of γ-linolenic acid (GLA, 18:3*n*6), ranging from 20 to 23% of the total fatty acid (FA) composition (Deng et al., 2001). GLA displays interesting medicinal properties, such as anti-inflammatory, anti-cancer, emollient of the skin and mucous membranes, among others, and for this reason, this oil is widely marketed (Guil-Guerrero, 2007; Horrobin, 1992).

The nutritional composition of *B. officinalis* seed oil has been widely studied (Czaplicki et al., 2011; Eskin, 2008; Nogala-Kalucka et al., 2010). However, for the remaining *Borago* species, only analyses of the FA profiles and *sn*-2 FA distribution within the triacylglycerol (TAG) structure have been accomplished (Guil-Guerrero et al., 2017, 2018). The results of these studies showed that both *B. morisiana* and *B. pygmaea* are better sources of GLA than *B. officinalis*, and that all *Borago* species are remarkable GLA-producers. However, the unsaponifiable fraction of such new oils remains unstudied and should be checked in order to know their phytochemical and nutrient profiles as well as their potential activity against oxidative damage. To this end, this work was conducted to stablish the Tc, St, and Sq composition of the whole *Borago* genus and other GLA-rich Boraginaceae species, *i.e. Symphytum caucasicum* (Guil-Guerrero et al. (2014)) and *Echium gentianoides* (Guil-Guerrero et al., 2001).

## 2. Materials and methods

### 2.1. Sample collection

Seeds were gathered either from their natural habitats or farms (Table 1). Each wild species was collected from five well-differentiated subpopulations in each collection location. Given that such endemic species are highly adapted to environmental conditions, the environments for each subpopulation have not differences among them. Upon

**Table 1**
Data on collection of *Borago* and other Boraginaceae species.

| Species | Sample location | Herbarium code | Geographical coordinates | Collection date |
|---|---|---|---|---|
| *Borago morisiana* Bigazzi et Ricceri | Wild; Italy, Oristano: Laconi (Tanca de Cuccuru) | HUAL 25965 | 39.874 N 9.091 E | 07/04/2014 |
| *Borago officinalis* L. | Farmed; Spain, Almería | – | | |
| *Borago officinalis* L. | Wild; Tunisia, Béja: Ouechtata | – | 36.960 N 8.982 E | 05/17/2016 |
| *Borago pygmaea* (DC.) Chater & Greuter | Wild; Italy, Ogliastra: Gairo (Montarbu di Seui, Girolamo river) | HUAL 25608 | 39.839 N 9.455 E | 07/04/2014 |
| *Borago longifolia* Poir. | Wild; Algeria, Skikda: Garaet Sidi Lakhdar | – | 36.910 N 7.191 E | 06/08/2016 |
| *Borago trabutii Maire* | Wild; Morocco, Marrakech-Tensift-Al Hauz (Anfli, Ourika valley) | HUAL 25966 | 31.201 N 7.739 W | 06/14/2015 |
| Borage oil | Purchased | – | – | – |
| *Symphytum caucasicum* Bieb. | Purchased | | | |
| *Echium gentianoides* Webb ex Coincy | La Palma island | – | 28.755 N 17.884 W | 07/09/2017 |

receipt, the seeds were cleaned, labeled, and then placed in a glass desiccator until analysis. Commercial oil from *B. officinalis* was purchased from local markets. Seeds of *Echium gentianoides* were collected in La Palma island (Canary archipelago) while seeds from *Symphytum caucasicum* were acquired from B & T World Seeds (Olonzac, France).

### 2.2. Reagents

Solvents and reagents were purchased from Merck (Darmstadt, Germany). Stigmasterol, β-sitosterol and squalene were purchased from Sigma-Aldrich (Steinheim, Germany). Tp and $T_3$ homologues (α, β, γ, and *δ*) and α-Tp acetate were obtained from Sigma-Aldrich (Steinheim, Germany).

### 2.3. Total lipids

Approximately 1 g of ground seed were used for lipid extraction with 10 mL of chloroform:methanol (2:1, v/v) containing 5 mg/L of butylated hydroxytoluene (BHT) (Folch et al., 1957). Total lipids data were used to quantify the compounds in mg/100 g seed and mg/100 g oil.

### 2.4. Saponification and extraction of Tc, st, and sq

Saponification was carried out according to the methodology described by López-Ortiz et al. (2006). Approximately 0.2–0.3 g of seeds or 30 mg of *Borago* oil were weighed in a 100 mL screw cap flask. Solutions of ascorbic acid (0.1 M, 5 mL) and potassium hydroxide (2 M, 20 mL) were added. The mixture was heated for 45 min at 60 °C. Extraction of unsaponifiable components was carried out by filtration of the initial mixture, followed by addition of 10 mL of saturated NaCl solution and 10 mL of *n*-hexane with BHT (5 mg/L). The sample was stirred for 1 min in Vortex and the organic layer was collected in a 50 mL round bottom flask. The aqueous layer was re-extracted with 5 mL of *n*-hexane and then the hexane layer obtained was combined with the first organic phase. The *n*-hexane solution obtained was evaporated in a rotavapor at 60 °C to dryness. The resulting residue was dissolved in 0.98 mL of 2-propanol and then combined with 0.02 mL of *n*-hexane solution containing α-Tp acetate (5 mg/mL) as an internal standard. The final concentration of the internal standard was 0.1 mg/mL. Finally, an inert atmosphere was created with the aid of a stream of nitrogen. The resultant aliquots were stored in darkness at -10 °C until HPLC analysis.

### 2.5. HPLC analysis of tp and T3

#### 2.5.1. Column and mobile phase

Tp and $T_3$ homologs were determined using RP-HPLC/DAD (Agilent 1100 series, Palo Alto, CA, USA) equipped with a ProntoSIL $C_{30}$ column (4.6 × 250 mm, 3 µm; Bischoff Chromatography, Leonberg, Germany) cooled at 15 °C according to Pérez-Fernández et al. (2017). Mixtures of methanol:acetonitrile (95:5, v/v, phase A) and 2-propanol:*n*hexane (50:50, v/v, phase B) were used as mobile phase at a flow rate of 0.8 mL/min. The following sequence was used to elute each sample: 25 min of phase A (100%) followed by 20 min of phase B (100%). Additional 15 min of phase A (100%) were used to re-equilibrate the column. Phase B was used as a washing solution. The wavelength selected for DAD was 290 nm.

#### 2.5.2. Standards, internal and external quantification of Tp and $T_3$

Stock standard solutions of α-, β-, γ-, and *δ*-$T_P$ and -$T_3$ isomers were prepared in ethanol (1 mg/mL) and were stored at − 20 °C in the dark.

An external calibration was performed prior to analyses of *Borago* seeds. All previously detailed standards were injected individually to estimate retention times. Mixtures of the standards at nine different concentrations, ranging from 0.5 to 20 mg/L, were then injected to establish the correlation between peak area and Tc concentration.

A solution of α-Tp acetate (60 mg in 100 mL of ethanol) was used for internal calibration. A calibrated quantity (5 µg) of the working solution was added to all Tc final extracts as an internal calibrator for determination of mass and retention time. In the final extracts, all Tc originated from seeds were in their alcohol forms due to saponification step.

#### 2.5.3. Linearity and sensitivity of Tc analysis

The linearity and the limits of detection (LOD) and quantification (LOQ) were determined. For each compound, a 6-level calibration curve was constructed using the peak-area ratio between the different Tc versus concentration of the standard (mg/L). The average of triplicate determinations for each level was used. LOD were calculated as the concentration corresponding to three times the standard deviation of the baseline noise, and LOQ were investigated by sample dilution. Only values are reported for the detected compounds, that is, Tp. **HPLC analysis for St and Sq**

St and Sq were determined by RP-HPLC/DAD using Luna C18 column (250 × 4.6 mm, 5 µm; Phenomenex) at a fixed temperature of 30 °C. The mobile phase was programmed in isocratic mode, containing methanol:acetonitrile (70:30, v/v), and the flow rate was 0.8 mL/min for 55 min. The wavelength selected for DAD was 210 nm. St were quantified using stigmasterol as an external standard, while Sq was quantified by means of a calibration curve made with pure Sq.

For St and Sq identification, samples were run on a HPLC-mass spectrometry (HPLC-MS) (Thermo Fisher Scientific Transcend 600 LC, San Jose, CA, USA). Mass spectra were acquired using Exactive-Orbitrap MS analyzer (-Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA) in positive and negative ion modes. ESI parameters were as follows: spray voltage, 4 kV; sheath gas ($N_2$, N95%), 35 (non dimensional); auxiliary gas ($N_2$, N95%), 10 (non dimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 °C; and capillary temperature, 300 °C. The mass spectra were acquired employing four alternating acquisition functions: (1) full MS, ESI+, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25,000 full width at half maximum (FWHM); scan time = 0.25 s, (2) full MS, ESI-, using the above mentioned settings, (3) all-ions fragmentation (AIF), ESI+, with fragmentation (HCD on, collision energy 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s, and (4) AIF, ESI−, using the settings explained for (3). The mass range in the full scan experiments was set to *m/z* 50–1500. The database used for compounds identification was Compound Discoverer software (version 2.1, Thermo Scientific).

### 2.6. Accuracy and validity of the analysis protocols

To evaluate the accuracy and validity of the developed analysis protocols, a food-based Certified Reference Material (CRM) for St and Tp was analyzed. Among the available CRM, NIST-3251- *Serenoa repens* extract was selected, which contains phytosterols, fatty acids, ß-carotene, and γ-Tp, purchased from Sigma-Aldrich (Steinheim, Germany). This material was analyzed in triplicate.

The accuracy of the method was also evaluated by the standard addition procedure (% of recovery) with three addition levels (15%, 30% and 60% of the expected values, each one in duplicate). Standard mixtures were added to the samples, and all the extraction procedures were carried out. Recovery rates for α-Tp, α-Tp acetate, β-Tp, *δ*-Tp, γ-Tp, stigmasterol and Sq were calculated by the following equation:

$$R\% = [(Cs\text{-}Cp)/Ca]*10$$

where R (%) is the percent recovery of added standard; Cs the compound content in spiked sample; Cp the compound content in sample;

and Ca the compound standard added.

### 2.7. Statistical analysis

All data in tables were analyzed using one-way ANOVA (Statgraphics Centurion XVI.I, Warrenton, VA, USA) and expressed as the average ± SD of seeds from five different species populations, each of which was analyzed in triplicate. Borage oil data correspond to the average ± SD obtained from three different commercial packages from the same manufacturer, each of them analyzed in triplicate. Differences among mean values were tested using Duncan's test at $P < 0.05$.

## 3. Results and discussion

### 3.1. Methods validation

Concentrations of Tc, St and Sq in the seeds of five *Borago* species, as well as the commercial oil of *B. officinalis*, were checked and compared with those in *Echium gentianoides* and *Symphytum causcasicum* seed oils. To accomplish this objective, prior actions were performed to validate our analytical procedures. In order to improve the accuracy, precision and robustness, the quantification of both Tp and $T_3$ by an internal standard, *i.e.* α-Tp acetate, was accomplished. This compound exhibited different retention times when compared to the compounds under analysis. The identification of eight Tc in a RP-HPLC/DAD system equipped with a $C_{30}$ column using non-aqueous mobile phase at similar conditions that described by Pérez-Fernández et al. (2017) was conducted. An HPLC UV-chromatogram of Tp from *B. pygmaea* seed oil is presented in Fig. 2. It is noteworthy that under the above-mentioned conditions, separation of β- and γ-Tc homologs was successfully achieved.

Unlike the Tc family, which has a limited number of components (only 8), St are a group of a large terpenoids in which a large number of compounds can be present in vegetal samples. This means that while Tc can be identified by comparison of their HPLC retention times with pure standards, the analyses of St need a different approach. For instance, the identity of critical peaks of any sample can be solved through the comparison of their mass spectra with library standards. Therefore, a HPLC/MS was used for St and Sq identification while the quantification was achieved using HPLC/DAD with internal standards. The mass spectrum of each peak in the sample was compared with that in the data base (Fig. 4). The identifications of the St reported were based on a probability match equal or higher than 95%. Fig. 4 also showed an expansion window of the molecular ion zone in which the fragmentation patterns of the measured spectra were compared with those of the library's standards. After peaks identification, nutritionally relevant St ($\Delta^5$-avenasterol, campesterol, and β-sitosterol) and Sq (Table 3) were simultaneously determined using a RP-HPLC/DAD system equipped with a C18 column (Fig. 3).

The linearity and sensitivity of the analyses was satisfactory. For α-Tp, α-Tp acetate, β-Tp, δ-Tp, and γ-Tp, LOD were 3, 1, 2, 3, and 2 mg/L, respectively; LOQ were 5, 2, 4, 5, and 4 mg/L, respectively; linearity ranges – ($R^2$) were 2 – 20 (0.995), 3 – 18 (0.987), 5–20 (0.986), 3 – 18 (0.980), and 6 – 20 (0.979) mg/L, respectively. For

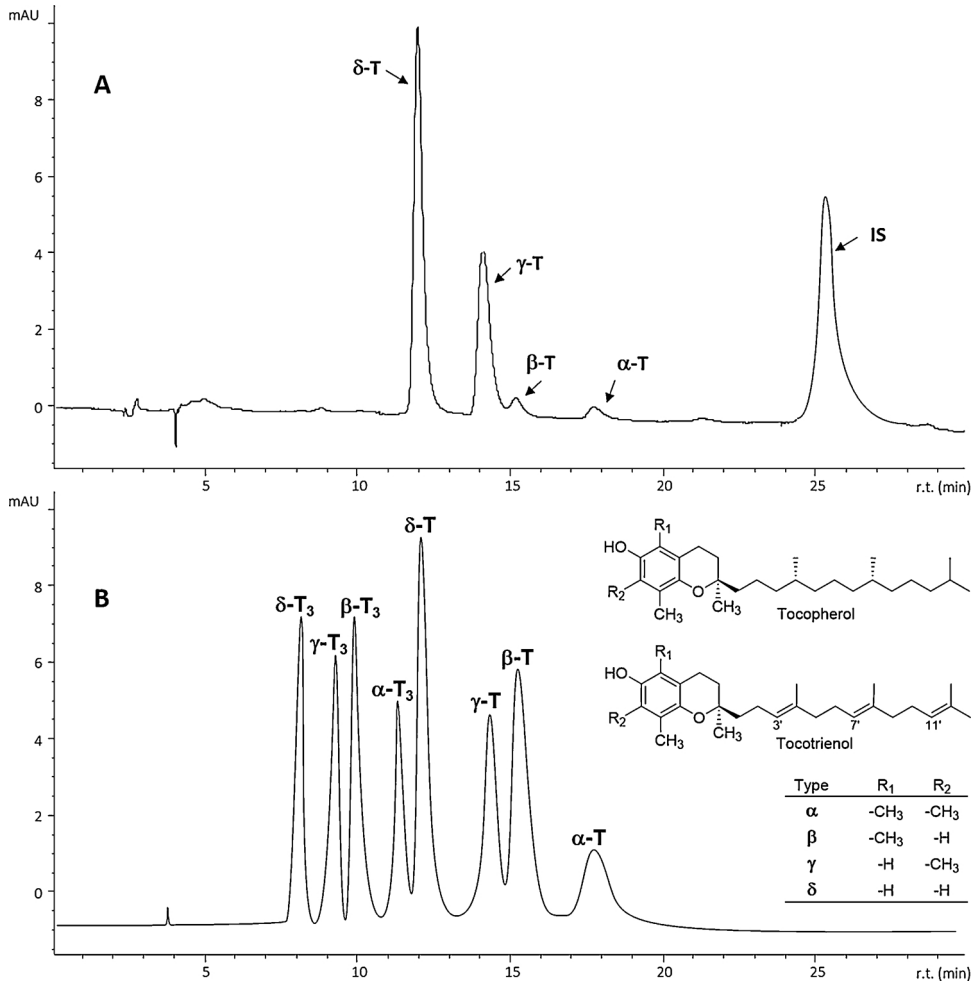


**Fig. 2.** A) RP 290 nm HPLC-DAD chromatogram of the tocopherols (Tp) containing fraction of *B. pygmaea* (α-Tp acetate as Internal Standard (IS)). B) Chromatogram of a standard mixture of all tocols including Tp and tocotrienol ($T_3$) homologues.

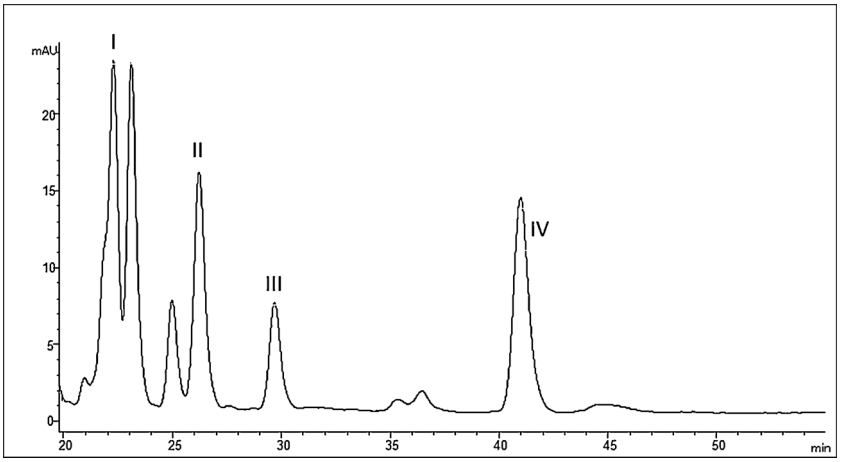


**Fig. 3.** RP 210 nm HPLC-DAD of sterols and squalene of *B. pygmaea*. I ($\Delta^5$-avenasterol), II (campesterol), III (β-sitosterol), and IV (squalene).

stigmasterol, LOD and LOQ were 1.5 and 3 mg/L, respectively; linearity range – ($R^2$) was 4 − 220 mg/L (0.959). For Sq, LOD and LOQ were 0.3 and 1 mg/L, respectively; and the linearity range – ($R^2$) was 2 − 90 mg/L (0.989).

The accuracy and validity of the analysis protocols were checked. For CRM, the values obtained for γ-Tp, β-sitosterol, campesterol, and stigmasterol were all within the 95% level of confidence of the NIST certified concentrations. The value observed in this study for γ-Tp was slightly higher at 3.68 ± 0.08 mg/100 g CRM compared to the NIST assigned values of 3.53 ± 0.05 mg/100 g. The value for β-sitosterol was slightly lower at 156.6 ± 8.4 mg/100 g compared to the NIST assigned values of 166.6 ± 6.4 mg /100 g. The value for campesterol was at 52.8 ± 6.5 mg /100 g, similar to the NIST assigned values of 53.3 ± 3.1 mg /100 g. The value for stigmasterol was slightly higher at 26.0 ± 5.1 compared to the NIST assigned values of 24.7 ± 4.0 mg/100 g. The accuracy of the method was also evaluated by the standard addition procedure. For α-Tp, α-Tp acetate, β-Tp, *δ*-Tp, γ-Tp, stigmasterol and Sq, recoveries were 88.6 ± 6.6, 92 ± 2.9, 93.6 ± 1.7, 87.8 ± 4.0, 101.2 ± 0.7%, 88.7 ± 1.5, and 98.3 ± 4.0, respectively.

### 3.2. Tc content in Borago species

Four Tp (α, β, γ and *δ*) were identified (Table 2), although the $T_3$ homologs were not detected. *δ*-Tp was the predominant Tp in all *Borago* and *E. gentianoides* sampled seeds, to 88.1 and 86.2% of total Tp for *B. morisiana* and farmed *B. officinalis*, respectively. For *B. officinalis* collected in Tunisia and Almería, the Tp profiles were consistent with previously published results (Eskin, 2008; Nogala-Kalucka et al., 2010). α-Tp was the main Tp found in commercial borage oil, and γ-Tp in the case of *S. caucasicum*. Among the remaining samples, α-Tp was detected at 1.1 and 1.4% of total Tp in only *B. morisiana* and *B. pygmaea* seeds, respectively. The high concentration of α-Tp detected in commercial borage oil was because this compound was added as a natural antioxidant to protect the oil against oxidation, as specified on the package label.

*B. morisiana* and *B. pygmaea* are the only species where the four Tp homologs have been detected, even including β-Tp. It is remarkable that the last species has the largest amount of total Tp, 112 mg/100 g seed, and 514 mg/100 g oil. Previously, Tp content of *B. officinalis* was found higher than that of other seeds, as are viper's bugloss, sea buckthorn, black seed, rose mosqueta, evening primrose, quince and safflower (Nogala-Kalucka et al., 2010). The analyses conducted in this work confirmed the high amounts of Tp in *B. officinalis* seeds, although *B. morisiana*, *B. pygmaea*, and *B. trabutii* contained exceptionally high amounts, much more than those present in the seeds commonly used for functional oils extraction (Nogala-Kalucka et al., 2010).

### 3.3. St and sq content in Borago species

Among *Borago* species, *B. officinalis* collected in Tunisia had the largest amount of total St, with 499 mg/100 g oil, which was significantly higher than other quantities detected in the remaining samples, except for *B. pygmaea*, with 466 mg/100 oil. However, considering the whole seed, *B. morisiana* and *B. pygmaea* reached higher concentrations than the remaining species, with 107 and 102 mg/100 g seeds, respectively. Total St contents in oils extracted from *B. officinalis*, *B. morisiana* and *B. pygmaea* were higher than those reported in oils from walnut (307 mg/100 g oil), peanut (284 mg/100 g oil), pecan (283 mg/100 g oil) and almond (271 mg/100 g oil), but lower than some other nut oils such as sweet chestnut oil (800 mg/100 g) (Alasalvar and Bolling, 2015).

The amounts of total St in *B. officinalis* seeds were slightly higher than those reported by Szterk et al. (2010), whose study also reported small amounts of other minor St, such as 25-hydroxy-24-methylcholesterol. Moreover, they showed similar concentrations of $\Delta^5$-avenasterol, campesterol, and β-sitosterol, while in this work β-sitosterol reached lower percentages of total St than the remaining St in all *B. officinalis* seeds/oil analyzed.

Campesterol was the most abundant St in all *Borago* endemic species, accounting for more than a third of the total St. Conversely, in *B. officinalis*, both from Almeria and Tunisia, the predominant St was $\Delta^5$-avenasterol, reaching to almost half of the total St. β-sitosterol was found in all *Borago* species, and specially accounted for more than a third of total St in *B. longifolia*. Borage oil purchased contained lower amounts of total St compared to the oils from *Borago* species, and its predominant St was campesterol, followed by $\Delta^5$-avenasterol. In *S. caucasicum*, all St showed similar percentages whereas β-sitosterol was the predominant St in *E. gentianoides*.

Sq content in *B. officinalis* was previously reported by Czaplicki et al. (2011). Results for Sq in *Borago* seeds ranged from 4.4 in *B. trabutii* to 27.4 mg/100 g oil in *B. officinalis* collected in Tunisia. However, this last sample and *B. morisiana* showed the same level of concentration in the whole seed (3.9 mg/100 g). Commercial borage oil, *S. caucasicum* and *E. gentianoides* contained 8.7, 28.1, and 2.6 mg Sq/100 g oil, respectively. Sq concentrations detected in all *Borago* species are higher than other amounts detected in another GLA source, as is Evening primrose (*Oenothera biennis*) oil, which contains 0.04 mg Sq/100 g oil (Timoszuk et al., 2018). However, these amounts are far from the Sq concentrations achieved in some commercial seed oils, such as *Amaranthus* spp. oil, which contains 4.2 g Sq/100 g oil (He and Corke, 2003).

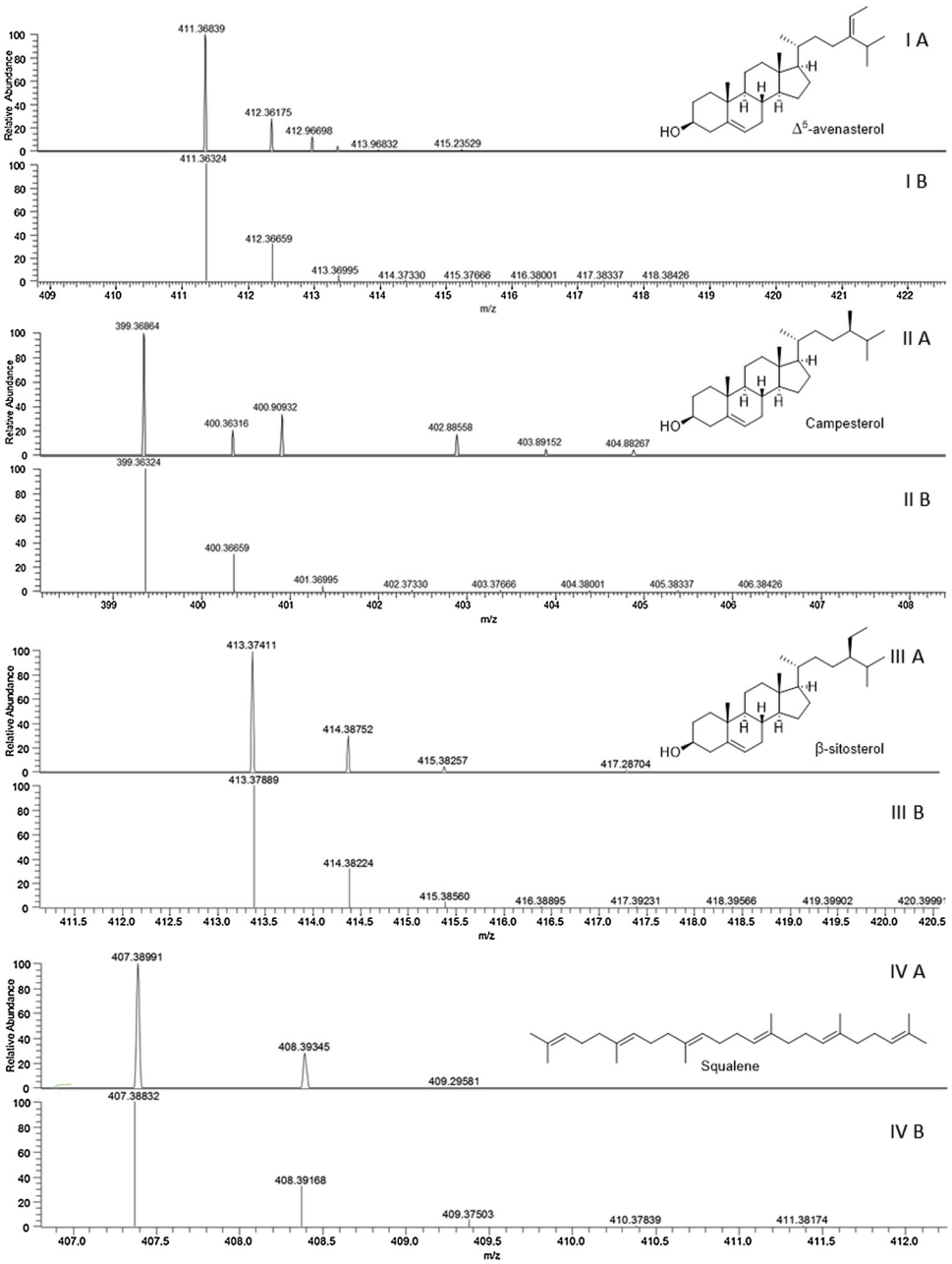


**Fig. 4.** ESI (ion mode + ve or –ve) mass spectrum of sterols and squalene: I ($\Delta^5$-avenasterol), II (campesterol), III (β-sitosterol) and IV (squalene). A: Spectra of analyzed samples. B: spectra of data base standards. Only a zoom window comparing the fragmentation pattern in the molecular ion zone is shown for clarity reasons.

**Table 2**
Tocopherols (Tp) content of Borago species in comparison with other Boraginaceae taxa[a].

| | Tp% of total Tp area | | | | Total Tp (α-Tp acetate equivalents) | |
|---|---|---|---|---|---|---|
| Species | α-Tp | β-Tp | γ-Tp | δ-Tp | (mg/100 g seeds) | (mg/100 g oil) |
| *B. morisiana* | 1.1 ± 1.5 [d] | 2.7 ± 0.7 [a] | 8.1 ± 3.1[f] | 88.1 ± 5.3 [a] | 71.7 ± 4.0 [b] | 296 ± 16 [c] |
| *B. officinalis* L. (Almería, farmed) | n.d. | n.d. | 13.8 ± 2.2 [e] | 86.2 ± 4.6 [a] | 44.1 ± 4.3 [d] | 240 ± 15 [d] |
| *B. officinalis* L. (Tunisia, wild) | n.d. | n.d. | 24.7 ± 3.8 [c] | 75.3 ± 6.7 [c, d] | 15.1 ± 3.3 [f] | 105 ± 12 [e] |
| *B. pygmaea* | 1.4 ± 1.4 [d] | 2.8 ± 0.6 [a] | 19.2 ± 4.7 [d] | 76.6 ± 4.7 [c] | 112 ± 17 [a] | 514 ± 28 [b] |
| *B. longifolia* | n.d. | n.d. | 29.2 ± 3.2 [b] | 70.8 ± 8.0 [d] | 16.2 ± 3.4 [f] | 105 ± 10 [e] |
| *B. trabutii* | n.d. | n.d. | 18.1 ± 1.8 [d] | 81.9 ± 1.9 [b] | 51.3 ± 1.7 [c] | 247 ± 6 [d] |
| Borage oil, commercial | 98.5 ± 0.4 [a] | n.d. | 0.2 ± 0.1[g] | 1.3 ± 0.4 [f] | n.d. | 2150 ± 39 [a] |
| *Symphytum caucasicum* | 3.6 ± 0.8 [c] | n.d. | 92.0 ± 0.8 [a] | 4.3 ± 0.8 [e] | 30.9 ± 1.1 [e] | 90.2 ± 2.6 [f] |
| *Echium gentianoides* | 9.7 ± 0.9 [b] | n.d. | n.d. | 90.3 ± 3.3 [a] | 17.7 ± 0.8 [f] | 59.0 ± 1.3 [g] |

Data represent means ± standard deviation of seeds from 5 different species populations, each of them analyzed in triplicate; n.d. not detected.
Differences in Tp percentages and Tp amounts were tested according to one-way ANOVA followed by Duncan's test.
In a column, means followed by different letter are significantly different at $P < 0.05$.

**Table 3**
Sterols (St) and squalene (Sq) content of *Borago* species in comparison with other Boraginaceae taxa.

| | St% of total St area | | | Total St (Stigmasterol equivalents) | | Sq | |
|---|---|---|---|---|---|---|---|
| **Species** | $\Delta^5$-Avenasterol | Campesterol | β-sitosterol | mg/100 g seed | mg/100 g oil | mg/100 g seed | mg/100 g oil |
| *B. morisiana* | 22.4 ± 1.7 $^{e}$ | 42.7 ± 2.4 $^{a}$ | 34.9 ± 1.1 $^{c}$ | 107 ± 3.1 $^{a}$ | 444 ± 21.7 $^{b}$ | 3.9 ± 0.1 $^{b}$ | 16.0 ± 0.1 $^{c}$ |
| *B. officinalis* (Almería, farmed) | 42.8 ± 1.8 $^{b}$ | 39.6 ± 1.0 $^{b}$ | 17.6 ± 0.7 $^{e}$ | 65.1 ± 2.3 $^{d}$ | 354 ± 10.8 $^{c}$ | 3.6 ± 0.7 $^{b}$ | 19.7 ± 0.3 $^{b}$ |
| *B. officinalis* (Tunisia, wild) | 48.6 ± 2.1 $^{a}$ | 35.7 ± 0.7 $^{c}$ | 15.8 ± 0.9 $^{f}$ | 71.8 ± 3.3 $^{c}$ | 499 ± 20.8 $^{a}$ | 3.9 ± 0.4 $^{b}$ | 27.4 ± 0.6 $^{a}$ |
| *B. pygmaea* | 29.9 ± 3.1 $^{c, d}$ | 37.9 ± 1.0 $^{c}$ | 32.2 ± 2.9 $^{c}$ | 102 ± 3.9 $^{a}$ | 466 ± 31.5 $^{a, b}$ | 2.7 ± 0.4 $^{c}$ | 12.1 ± 0.7 $^{d}$ |
| *B. longifolia* | 18.9 ± 1.3 $^{e}$ | 40.6 ± 2.9 $^{a, b}$ | 40.5 ± 2.3 $^{b}$ | 48.9 ± 3.3 $^{e}$ | 315 ± 10.6 $^{d}$ | 1.8 ± 0.3 $^{d}$ | 11.5 ± 0.4 $^{d}$ |
| *B. trabutii* | 27.6 ± 0.9 $^{d}$ | 43.7 ± 1.8 $^{a}$ | 28.7 ± 1.6 $^{d}$ | 61.6 ± 4.2 $^{d}$ | 296 ± 10.9 $^{d, e}$ | 0.9 ± 0.1 $^{e}$ | 4.4 ± 0.2 $^{f}$ |
| Borage oil | 41.4 ± 2.0 $^{b}$ | 42.8 ± 3.4 $^{a}$ | 15.8 ± 1.0 $^{f}$ | n.d. | 196 ± 14.0 $^{f}$ | n.d. | 8.7 ± 0.1 $^{e}$ |
| *Symphytum caucasicum* | 33.8 ± 2.7 $^{c}$ | 33.4 ± 1.3 $^{d}$ | 32.9 ± 1.7 $^{c}$ | 48.4 ± 2. 7 $^{e}$ | 141 ± 10.7 $^{g}$ | 9.6 ± 0.2 $^{a}$ | 28.1 ± 0.8 $^{a}$ |
| *Echium gentianoides* | 9.2 ± 0.4 $^{f}$ | 37.9 ± 2.2 $^{c}$ | 52.9 ± 4.1 $^{a}$ | 83.2 ± 5.0 $^{b}$ | 277 ± 14.8 $^{e}$ | 0.8 ± 0.1 $^{e}$ | 2.6 ± 0.2 $^{g}$ |

Data represent means ± standard deviation of seeds from 5 different species populations, each of them analyzed in triplicate; n.d. not detected.
Differences in St percentages, and St and Sq amounts were tested according to one-way ANOVA followed by Duncan's test.
In a column, means followed by different letter are significantly different at $P < 0.05$.

### 3.4. Significance of the results and potential use of the species analyzed

Overall, the highest concentrations of Tp, St and Sq appeared in *B. morisiana* and *B. pygmaea* seeds, while these species have been reported as notable GLA-producers (Guil-Guerrero et al., 2017, 2018); thus, oils from these species can be interesting alternatives to the widely cultivated *B. officinalis*. The compounds detected in the seed oils of *Borago* species are widely recognized for their health-promoting actions, especially for their role in reducing CVD risk, while increasing antioxidant defenses and reducing inflammation. Furthermore, it has been suggested that oily nuts consumption reduces cancer incidence and benefit cognitive function, as well as ameliorates the risks of asthma and inflammatory bowel disease, among others (Alasalvar and Bradley, 2015). Thus, given their favorable Tp, St, and Sq content, *B. morisiana* and *B. pygmaea* oils consumption may shows better health beneficial actions than traditional *B. officinalis* oil consumption.

Among the analyzed endemic species, *B. morisiana* seems to be a plant with good potential for farming: although it is a perennial hemicryptophyte, it develops every year flowering branches easily harvestable. As a hydrophyte, it might be adapted without difficulty to a hydroponic culture system.

Before approval for human consumption, the oils analyzed in this work should be given the consideration of novel foods. The "Novel Foods Regulation" (Regulation (EC) No 258/97) defines novel food as a food that does not have a significant history of consumption within the European Union (EU) before the 15th of May 1997. Such foods are subject to a pre-market safety assessment, before a decision is made on EU-wide authorization. However, the Novel Foods Regulation includes a simplified procedure for marketing certain types of products, if they are considered "substantially equivalent" to an existing food that is already marketed within the EU. Such equivalency is mainly based on both their compositional and nutritional values and their levels of undesirable substances (Coppens et al., 2006). In this regard, the oils from endemic *Borago* species analyzed in this work can benefit from such procedure to be easily and promptly introduced in markets.

## 4. Conclusions

The results of this study showed that all *Borago* species were excellent sources of Tp, St, and Sq. Both *B. pygmaea* and *B. morisiana* contained all Tp types, while *B. pygmaea* were the Tp-richest *Borago* species, exceeding more than twice the concentration of such compounds found in *B. officinalis*. All *Borago* species showed similar concentrations of total St, and all of them contained $\Delta^5$-avenasterol, campesterol, and β-sitosterol. In endemic *Borago* species, campesterol was the more abundant St, while in both wild and farmed *B. officinalis*, $\Delta^5$-avenasterol had the greatest values. Concerning Sq, wild *B. officinalis* from Tunisia and *B. morisiana* showed the highest concentrations in the whole seed. Overall, the highest concentration of Tp, St, and Sq appeared in *B. morisiana* and *B. pygmaea* seeds. In order to know the whole phytochemical profile of *Borago* oils, further studies to assess their phenolic profile and antioxidant activity are needed.

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# *Ribes* taxa: A promising source of γ-linolenic acid-rich functional oils

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ABSTRACT

Fifty *Ribes* species and *R. nigrum*-based cultivars from eight *Ribes* sections were surveyed for γ-linolenic acid (GLA, 18:3, n-6)- and stearidonic acid (SDA, 18:4, n-3)-rich oils. *R. pallidiflorum*, *R. glabellum* and *R. pubescens* seed oils contain noticeable GLA amounts: 13.3, 11.8, and 11.9% of total fatty acids (FA), respectively. However, the highest GLA contents were found in the seed oils of several blackcurrant cultivars, highlining *Ribes* 'Myuryucheene' with 20.2% GLA of total FA. Principal Component Analysis showed that similarities in FA profiles allow grouping species as botanical criteria for *Ribes* sections do. The main GLA-taxa detected in this study correspond to blackcurrant cultivars, all of them native to Siberia. Considering that such cultivars are notable fruit-producers, its cultivation in Siberia besides producing fruits in very difficult agronomic areas, could produce a valuable by-product, i.e. the seeds, which will add economic value to agricultural systems if devoted to GLA-rich oils extraction.

## 1. Introduction

The genus *Ribes* (Grossulariaceae), which includes the cultivated currants and gooseberries, contains 120–150 species distributed in the temperate and Mediterranean regions of the Northern Hemisphere and South America. Approximately thirty species are recognized in Andean South America. One or two species occur in Central America at high elevations giving the genus a continuous range from Alaska to Tierra del Fuego. Also, some European species are found in Western North Africa (Wes, 1994).

*R. nigrum* (blackcurrant) is the most widely used species among *Ribes* genus, which is used at industrial scales. This bush is grown primarily for processing into juices, cordials and jams, while the seeds and pomaces of this species constitute some raw by-products commonly used for functional oils extraction, to be used in the food and health sectors (Dobson et al., 2012). The interest in blackcurrant in the areas of pharmacy and medicine is largely due to the variety of potentially health beneficial bioactive compounds such as flavonols, pectins, organic acids, invert sugars, polysaccharides and some phenolic acids, which act as natural antioxidants. The fatty acids (FA) that can be found mainly in fruit and seeds, but also in leaves and buds. The main antioxidants present in blackcurrant fruit are phenolic acids (gallic, caffeic, p-coumaric and ferulic acids) and ascorbic acid. In addition, blackcurrant fruit, like other purple colored fruits, is a rich source of anthocyanins (specifically delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside) (Flores & Ruiz del Castillo, 2016; Gopalan et al., 2012).

Previous studies have demonstrated several biological activities of *Ribes* seed oil, highlighting immunomodulating and anti-inflammatory ones: a blackcurrant seed oil rich-diet suppressed significantly the monosodium urate crystal-induced inflammation in rats (Jurgoński, Fotschki, & Juśkiewicz, 2015). Moreover, from a few decades ago, the seeds of blackcurrants have been attracting attention for use in cosmetics and dermatology for skin regeneration and neurodermitis (Messing, Niehues, Shevtsova, Borén, & Hensel, 2014).

The seeds of *Ribes* species contains C-18 polyunsaturated FA (PUFA) and monounsaturated FA (MUFA), such as oleic acid (OA, (*Z*)-octadec-9-enoic acid, 18:1, n-9). Among PUFA, γ-linolenic acid (GLA, (6*Z*,9*Z*,12*Z*)-octadeca-6,9,12-trienoic acid, 18:3, n-6) has pharmaceutical applications, and for that reason, *R. nigrum* seed oil is marketed as functional oil (Guil-Guerrero, 2007). Besides Grossulariaceae, this FA occurs also in some botanical taxa, such as Boraginaceae and Onagraceae (Guil-Guerrero, 2007). GLA is the metabolite of linoleic acid (LA, (9*Z*,12*Z*)-octadeca-9,12-dienoic acid, 18:2, n-6), as result of the

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Δ6-desaturase enzyme activity. Further, GLA is metabolized to dihomo-γ-linolenic acid (DGLA, (8*Z*,11*Z*,14*Z*)-icosa-8,11,14-trienoic acid, 20:3, n-6), which in the downstream metabolic pathway produces anti-inflammatory eicosanoids (series-1 prostaglandins and series-3 leukotrienes). These are hormone-like bioactive compounds, which contributes to the regulation of several physiological mechanisms in animals and humans (Calder, 2015; Guil-Guerrero, 2007). GLA and derived molecules also affect the expression of various genes involved in immune functions and apoptosis (Kapoor & Huang, 2006), as well as inhibit tumor cell cycle progression and angiogenesis in cancer cells (Xu & Qian, 2014). In this regard, the dietary supplementation with GLA achieves clear health benefits: it improves blood lipid profile and skin perspiration, while ameliorates the symptoms of dermatitis, skin hyperproliferation and osteoporosis, among several other syndromes (Tasset-Cuevas et al., 2013; Tso, Caldwell, Lee, Boivin, & DeMichele, 2012). Thus, GLA has several applications in the field of cosmetic and food industries (Bruheim & Griinari, 2013).

Besides GLA, other PUFA occurs in the seed oil of *Ribes* species. These are LA, α-linolenic acid (ALA, (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoic acid, 18:3, n-3), and stearidonic acid (SDA, (6*Z*,9*Z*,12*Z*,15*Z*)-octadeca-6,9,12,15-tetraenoic acid, 18:4, n-3) (Šavikin et al., 2013). The healthy properties of all these PUFA have been reviewed (Abedi & Sahari, 2014; Ristić-Medić, Vučić, Takić, Karadžić, & Glibetić, 2013).

Among *Ribes* species, blackcurrant (*R. nigrum*) seed oil is marketed as a health supplement due to its high GLA. However, little is known about the FA composition of the seed oils of other *Ribes* species. In this regard, only few works have been focused to study wild northern *Ribes* (Finland): *R. alpinum* and *R. spicatum* (Johansson, Laakso, & Kallio, 1997); *R. rubrum* seed oil from the region of Asturias (Spain) (Bada, León-Camacho, Copovi, & Alonso, 2014); and different cultivars of redcurrant (*R. rubrum* L.), gooseberry (*R. uva-crispa* L.) and jostaberry (*R. nidrigolaria* Bauer) (Piskernik et al., 2018).

Furthermore, in addition to an interesting FA profile, blackcurrant seed oil has also been reported to be a good source of tocopherols and phytosterols, which constitute a large fraction of the unsaponifiable material of vegetable oils (Jurgoński et al., 2015).

*Ribes* fruits are appreciated due to their multiple use, both for fresh consumption and for industrial preparations in the form of juices, jams and related products. On the other hand, a small number of *Ribes* species are used to obtain GLA-rich functional oils from industrial by-products; i.e., the seeds. However, there is a great number of *Ribes* species not yet checked for their GLA content. In this context, we hypothesize that many high-quality fruit-producers *Ribes* taxa all over the world, besides for fruits consumption, could be also used for GLA-rich functional oils extraction. Therefore, the aim of this study was to determine the FA profiles of the seed oils of several *Ribes* taxa. For this action, several seeds were taken from crops or purchased, while some others were collected from their natural habitats.

## 2. Material and methods

### 2.1. Samples

All seed sources are detailed in Table 1 where all species are taxonomically grouped by subgenus and sections following Schultheis and Donoghue (2004). Several *Ribes* seeds were supplied by botanical gardens from the Russian Federation; other seeds were taken from very-ripe fruits collected at their natural habitats in Spain; and several others were acquired from B & T World Seeds (Olonzac, France). Fruits collected in the wild were transported to the laboratory in paper bags (one day at most), and then seeds were removed from fruits and dried in a forced-air oven at 40 °C up to constant weight. However, most of the seeds were received at the laboratory from suppliers by mail, contained in small plastic or paper bags. All seeds were cleaned if necessary, labeled and placed in a glass desiccator until analysis, which was delayed one week maximum. Just prior to analysis, seed size was determined with a Vernier caliper. When there was enough sample available, moisture of seeds was gravimetrically determined by drying in a forced-air oven at 105 °C for 24 h, and this was always within the 6.5–7.0% range. Thereafter, seeds were ground to powder with a mortar, and then they were immediately analyzed.

### 2.2. FA analyses

FA determination in seeds was carried out after direct derivatization of the oil to FA methyl esters (FAME) (Rodríguez-Ruiz, Belarbi, Sánchez, & Alonso, 1998). For this, accurately weighed 50 mg of seeds were placed in test tubes and then 50 μL of the internal standard (heptadecanoic acid (17:0) 98% purity, H3500 from Sigma, St. Louis, USA) in ethanol (10 mg/mL) were added. After that, 2 mL of a methylating mixture (methanol:acetyl chloride, 20:1, v/v) and 1 mL *n*-hexane were carefully poured over the described material. Tubes were then capped and heated at 100 °C for 30 min. After tubes were cooled to room temperature, 1 mL distilled water was added in each one, then they were centrifuged (2000*g*, 5 min) and the hexane layer was collected for GC-FID analysis. FAME were analyzed in a Focus GC (Thermo Electron. Cambridge. UK), equipped with a flame ionization detector (FID) and an Omegawax 250 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Supelco. Bellefonte, USA), as previously described (Guil-Guerrero, Gómez-Mercado, Ramos-Bueno, Rincón-Cervera, & Venegas-Venegas, 2014). The peak area of the internal standard was used as reference to calculate the mass of each FA in the resulting chromatograms, and results were computed as FA percentages of total FA (Table 2). Peaks were identified by retention times obtained for known FAME standards (PUFA No. 1, 47033; methyl γ-linolenate 98.5% purity, L6503; and methyl stearidonate 97% purity, 43959 FLUKA) from Sigma, (St. Louis, USA).

### 2.3. Quality control

The quality control of FA analyses was performed following previous protocols (Griffiths, Van Hille, & Harrison, 2010; Guil-Guerrero et al., 2014). To determine the limits of detection (LOD) and quantification (LOQ) of the assays, pure LA and ALA were diluted in the 0.001–20 mg/mL range in toluene in triplicate, transmethylated and quantified by GC-FID. Negative controls made without OA or LA addition were also analyzed. The LOD was defined as the minimum concentration at which distinct peaks could be detected above the baseline noise. The LOQ was defined as the lowest concentration of FA that could be quantified with an accuracy and precision within 15%. The estimated LODs for LA and ALA were in the 0.8–0.9 μg/mL range, while LOQs for such molecules were in the 2.4–3.8 μg/mL range. The recovery was calculated by the formula [C(Exp)/C(Theo) × 100]. Recoveries for LA were in the 94.4–100.3% range, while for ALA were in the 97.3–102.0% range. As quality control of GC, a blank sample (hexane) was run together with the samples in every batch to check the GC performance. Control oil samples were analyzed prior and after running samples. Canola oil (46961 SUPELCO, from SIGMA) was used for the control tests. As quality control of GC, a blank sample (hexane) was run together with the samples in every batch to check the GC performance.

### 2.4. Statistical analysis

Experimental results were expressed as means ± SD for three different determinations. Data were submitted to Principal Component Analysis using Statgraphics© centurion XVI (StatPoint Technologies, Warrenton-Virginia, USA).

## 3. Results

Basic data on analyzed samples are detailed in Table 1, while the

**Table 1**
Data collection and taxonomic position of samples (*Ribes* L.).

| Label | Samples | Sample location | Collection year | 100 seed weight (g) | Seed length mm | Width mm |
|---|---|---|---|---|---|---|
| **Subgenus *Ribes* (Currants)** | | | | | | |
| *Sect. Berisia Spach (Alpine Currants)* | | | | | | |
| 1 | *R. alpinum* L. A | Sukachev Institute of Forest of the Siberian Branch of the RAS (SIF SB RAS), Krasnoyarsk, Russia | 2017 | 0.39 | 2.31 | 1.78 |
| 2 | *R. alpinum* L. B | Sierra de Baza, Spain | 2011 | 0.42 | 2.65 | 1.82 |
| 3 | *R. alpinum* L. C | Botanical Garden of North-Eastern Federal University, Yakutsk, Russia | 2014 | 0.46 | 2.77 | 1.83 |
| 4 | | | 2015 | 0.45 | 2.74 | 1.79 |
| 5 | | | 2016 | 0.43 | 2.75 | 1.80 |
| 6 | *R. alpinum* L. D | South-Ural Botanical Garden-Institute – Subdivision of the Ufa Federal Research Centre of the Russian Academy of Sciences. Ufa, Republic of Bashkortostan, Russia | 2018 | 0.32 | 2.68 | 1.77 |
| 7 | *R. alpinum* L. E | Federal Budget Institution “Northern research institute of forestry”. Arkhangelsk, Russia | 2017 | 0.33 | 2.68 | 1.74 |
| 8 | | | 2018 | 0.33 | 2.70 | 1.74 |
| 9 | *R. alpinum* L. F | Amur Branch of Botanical Garden Institute, Far East Branch of the Russian Academy of Sciences, Blagoveshchensk, Russia | 2018 | 0.31 | 2.31 | 1.43 |
| 10 | *R. diacanthum* Pall. | Amur Branch of Botanical Garden Institute, Far East Branch of the Russian Academy of Sciences, Blagoveshchensk, Russia | 2018 | 0.31 | 1.59 | 0.48 |
| 11 | *R. orientale* Desf. | B & T World Seeds | 2012 | 0.29 | 2.13 | 1.49 |
| 12 | *R. pulchellum* Turcz. | Sukachev Institute of Forest of the Siberian Branch of the RAS (SIF SB RAS), Krasnoyarsk, Russia | 2017 | 0.48 | 2.39 | 1.62 |
| 13 | *R. saxatile* Pall. A | Amur Branch of Botanical Garden Institute, Far East Branch of the Russian Academy of Sciences, Blagoveshchensk, Russia | 2018 | 0.23 | 1.91 | 1.48 |
| 14 | *R. saxatile* Pall. B | Federal Foresty Agency Northern Research Institute of Foresty, Arkhangelsk, Russia | 2017 | 0.24 | 1.92 | 1.48 |
| *Sect. Calobotrya (Spach) Jancz. (Ornamental Currants)* | | | | | | |
| 15 | *R. cereum* Douglas | B & T World Seeds | 2014 | 0.18 | 2.40 | 1.33 |
| 16 | *R. sanguineum* var. *glutinosum* (Benth.) Loudon | B & T World Seeds | 2011 | 0.21 | 2.16 | 1.52 |
| *Sect. Coreosma (Spach) Jancz. (Black Currants)* | | | | | | |
| 17 | *R. dikuscha* Fisch. ex Turcz. | Botanical Garden of North-Eastern Federal University, Yakutsk, Russia | 2015 | 0.11 | 2.27 | 0.75 |
| 18 | *R. hudsonianum* Richardson | Botanical Garden of North-Eastern Federal University, Yakutsk, Russia | 2014 | 0.07 | 1.29 | 0.83 |
| 19 | | | 2015 | 0.06 | 1.29 | 0.78 |
| 20 | | | 2016 | 0.06 | 1.39 | 1.04 |
| 21 | *R. nigrum* L. | State Dendrological Park «Alexandria» of the National Academy of Sciences of Ukraine, Bila Tserkva City, Ukraine | 2018 | 0.17 | 2.08 | 1.24 |
| 22 | *R. nigrum* L. 'Hara katarlik' (=*R. pauciflorum* Turcz. ex Pojark) | Botanical Garden of North-Eastern Federal University, Yakutsk, Russia | 2015 | 0.17 | 2.29 | 1.16 |
| 23 | *R. nigrum* L. 'Koksa' | Botanical Garden of North-Eastern Federal University, Yakutsk, Russia | 2014 | 0.17 | 2.56 | 1.13 |
| 24 | | | 2015 | 0.18 | 2.69 | 1.43 |
| 25 | | | 2016 | 0.17 | 2.57 | 1.13 |
| 26 | *R. viburnifolium* A. Gray | Botanical Garden of Cagliari, Sardinia, Italy | 2014 | 0.12 | 1.28 | 1.14 |
| 27 | *Ribes* "Yakutskaya' (=*R. dikuscha* Fisch. ex Turcz. × *R. procumbens* Pall.) | Botanical Garden of North-Eastern Federal University, Yakutsk, Russia | 2014 | 0.14 | 2.65 | 1.10 |
| 28 | | | 2015 | 0.13 | 2.64 | 1.11 |
| 29 | | | 2016 | 0.13 | 2.63 | 1.07 |
| 30 | *Ribes* 'Erkeeni' (=*R. nigrum* subsp. *europaeum* (Jancz.) Pavl. × *R. nigrum* subsp. *sibiricum* E. Wolf. × *R. dikuscha* Fisch. ex Turcz. × *R. pauciflorum* Turcz. ex Pojark) | Botanical Garden of North-Eastern Federal University, Yakutsk, Russia | 2016 | 0.17 | 2.30 | 1.06 |
| 31 | *Ribes* 'Myuryucheene' (=*R. procumbens* Pall. × *R. nigrum* subsp. *europaeum* | Botanical Garden of North-Eastern Federal University, Yakutsk, Russia | 2014 | 0.18 | 1.72 | 0.80 |
| 32 | (Jancz.) Pavl.) | | 2015 | 0.18 | 1.74 | 0.77 |

(*continued on next page*)

**Table 1** (*continued*)

| Label | Samples | Sample location | Collection year | 100 seed weight (g) | Seed length mm | Width mm |
|---|---|---|---|---|---|---|
| *Sect. Parilla Jancz. (Andine Currants)* | | | | | | |
| 33 | *R. cucullatum* Hook. & Arn. | B & T World Seeds | 2017 | 0.28 | 2.57 | 1.59 |
| 34 | *R. gayanum* (Spach) Steud. | B & T World Seeds | 2017 | 0.20 | 1.79 | 1.11 |
| 35 | *R. magellanicum* Poir. | B & T World Seeds | 2011 | 0.15 | 2.07 | 1.13 |
| *Sect. Ribes (Red Currants)* | | | | | | |
| 36 | *R. glabellum* (Trautv. & C.A. Mey.) Hedl. | Botanical Garden of North-Eastern Federal University, Yakutsk, Russia | 2014 | 0.37 | 2.32 | 1.56 |
| 37 | | | 2015 | 0.38 | 2.40 | 1.291.57 |
| 38 | | | 2016 | 0.41 | 2.76 | |
| 39 | *R. manshuricum* Kom. | Amur Branch of Botanical Garden Institute, Far East Branch of the Russian Academy of Sciences, Blagoveshchensk, Russia | 2018 | 1.14 | 3.26 | 2.16 |
| 40 | *R. multiflorum* subsp. *sandalioticum* Arrigoni | Monte Novo San Giovanni, Sardinia, Italy | 2014 | 0.23 | 2.71 | 1.39 |
| 41 | *R. pallidiflorum* Pojark. | Amur Branch of Botanical Garden Institute, Far East Branch of the Russian Academy of Sciences, Blagoveshchensk, Russia | 2018 | 0.34 | 2.15 | 1.39 |
| 42 | *R. pubescens* (Sw. ex C. Hartm.) Hedl. | Federal Foresty Agency Northern Research Institute of Foresty, Arkhangelsk, Russia | 2018 | 0.33 | 2.33 | 1.43 |
| 43 | *R. rubrum* L. | State Dendrological Park «Alexandria» of the National Academy of Sciences of Ukraine, Bila Tserkva City, Ukraine | 2018 | 0.55 | 1.93 | 0.68 |
| 44 | *R. triste* Pall. | Botanical Garden of North-Eastern Federal University, Yakutsk, Russia | 2015 | 0.30 | 2.11 | 1.38 |
| *Sect. Symphocalyx Berland. (Golden Currants)* | | | | | | |
| 45 | *R. aureum* Pursh A | State Dendrological Park «Alexandria» of the National Academy of Sciences of Ukraine, Bila Tserkva City, Ukraine | 2018 | 0.24 | 2.45 | 0.89 |
| 46 | *R. aureum* Pursh B | Amur Branch of Botanical Garden-Institute, Far East Branch of the Russian Academy of Sciences, Blagoveshchensk, Russia | 2018 | 0.23 | 2.35 | 1.79 |
| 47 | *R. odoratum* J.C. Wendl. | B & T World Seeds | 2017 | 0.34 | 2.66 | 1.62 |
| **Subgenus *Grossularia* (Mill.) Pers. (Gooseberries)** | | | | | | |
| *Sect. Grossularia* | | | | | | |
| 48 | *R. divaricatum* Douglas | B & T World Seeds | 2011 | 0.26 | 2.58 | 1.32 |
| 49 | *R. uva-crispa* L. | Sierra Nevada, Spain | 2011 | 0.24 | 2.62 | 1.56 |
| **Subgenus *Hesperia* A. Berger (Gooseberries)** | | | | | | |
| *Sect Hesperia* | | | | | | |
| 50 | *R. menziesii* Pursh var. *menziesii* | B & T World Seeds | 2014 | 0.26 | 2.84 | 2.22 |

**Table 2**
Seed oil content and fatty acid profiles of *Ribes* samples.

| | Species | Fatty Acids (FA% of total FA)[a] | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10:0 | 12:0 | 14:0 | 16:0 | 17:0 | 18:0 | 18:1, n-9 OA | 18:1, n-7 | 18:2, n-6 LA | 18:3, n-6 GLA | 18:3, n-3 ALA | 18:4, n-3 SDA | n-3 PUFA | n-6 PUFA | n-3/n-6 | MUFA | SFA | FA seed g/100 g |
| **Subgenus *Ribes* (Currants)** | | | | | | | | | | | | | | | | | | | |
| | Sect. *Berisia* (Alpine Currants) | | | | | | | | | | | | | | | | | | |
| 1 | *R. alpinum* A | – | – | – | 8.4 ± 0.3 | – | – | 8.7 ± 0.7 | – | 40.7 ± 2.1 | 11.8 ± 0.4 | 20.3 ± 0.9 | 3.2 ± 0.1 | 23.5 | 72.8 | 0.3 | 8.7 | 8.4 | 15.7 |
| 2 | *R. alpinum* B | – | – | – | 7.4 ± 0.1 | – | 1.9 ± 0.0 | 19.4 ± 0.8 | – | 39.3 ± 0.3 | 4.5 ± 0.0 | 25.1 ± 1.7 | 2.5 ± 0.2 | 27.5 | 43.8 | 0.6 | 19.4 | 9.3 | 11.7 |
| 3 | *R. alpinum* 2014 C | – | – | – | 8.9 ± 0.2 | – | 1.0 ± 0.2 | 8.5 ± 0.2 | 2.1 ± 0.6 | 42.1 ± 1.9 | 8.4 ± 0.5 | 25.7 ± 0.5 | 3.2 ± 0.9 | 28.9 | 50.6 | 0.6 | 10.6 | 9.9 | 15.1 |
| 4 | *R. alpinum* 2015 C | 0.6 ± 0.0 | – | 0.7 ± 0.2 | 7.3 ± 0.8 | – | 1.3 ± 0.4 | 10.0 ± 0.4 | 1.5 ± 0.2 | 45.1 ± 2.5 | 5.4 ± 0.6 | 26.3 ± 1.1 | 1.9 ± 0.1 | 28.2 | 50.5 | 0.6 | 11.6 | 9.9 | 15.9 |
| 5 | *R. alpinum* 2016 C | 0.4 ± 0.1 | – | 0.2 ± 0.0 | 7.6 ± 1.1 | – | 1.2 ± 0.0 | 9.1 ± 0.3 | 1.6 ± 0.0 | 43.6 ± 1.8 | 6.9 ± 0.5 | 26.6 ± 0.9 | 2.8 ± 0.6 | 29.4 | 50.5 | 0.6 | 10.6 | 9.4 | 16.2 |
| 6 | *R. alpinum* D | – | – | – | 7.2 ± 0.5 | – | 1.8 ± 0.1 | 15.6 ± 0.5 | 1.4 ± 0.9 | 39.1 ± 0.4 | 9.2 ± 0.1 | 21.6 ± 0.9 | 4.2 ± 0.3 | 25.8 | 48.2 | 0.5 | 17.0 | 9.0 | 11.8 |
| 7 | *R. alpinum* E 2017 | – | – | – | 6.3 ± 0.3 | – | 1.4 ± 0.4 | 10.5 ± 0.0 | 1.2 ± 0.1 | 40.8 ± 1.5 | 9.3 ± 0.1 | 26.3 ± 0.6 | 4.1 ± 0.5 | 30.5 | 50.1 | 0.6 | 11.8 | 7.7 | 12.1 |
| 8 | *R. alpinum* E 2018 | – | – | – | 7.9 ± 0.1 | – | 1.6 ± 0.3 | 13.5 ± 0.3 | 1.5 ± 0.3 | 36.7 ± 0.5 | 7.1 ± 0.8 | 27.7 ± 0.6 | 4.1 ± 0.4 | 31.8 | 43.8 | 0.7 | 14.9 | 9.5 | 13.1 |
| 9 | *R. alpinum* F | – | 0.7 ± 0.1 | 0.2 ± 0.0 | 6.7 ± 0.6 | – | 1.3 ± 0.1 | 8.9 ± 0.8 | 1.2 ± 0.5 | 43.0 ± 0.6 | 7.2 ± 0.6 | 27.5 ± 0.9 | 3.4 ± 0.8 | 30.9 | 50.2 | 0.6 | 10.1 | 8.9 | 11.9 |
| 10 | *R. diacanthum* | – | | 0.4 ± 0.1 | 7.0 ± 0.2 | – | 1.3 ± 0.6 | 7.7 ± 0.1 | 1.2 ± 0.3 | 39.0 ± 1.8 | 14.1 ± 0.8 | 24.6 ± 0.8 | 4.8 ± 0.6 | 29.3 | 53.1 | 0.6 | 8.9 | 8.7 | 11.6 |
| 11 | *R. orientale* | – | – | – | 9.1 ± 1.1 | – | 1.7 ± 0.0 | 17.6 ± 0.4 | – | 35.9 ± 0.1 | 0.9 ± 0.0 | 31.4 ± 1.7 | 0.5 ± 0.0 | 31.6 | 36.3 | 0.1 | 17.6 | 10.7 | 6.0 |
| 12 | *R. pulchellum* | – | – | – | 6.6 ± 0.7 | – | 1.4 ± 0.7 | 9.6 ± 0.7 | 1.0 ± 0.9 | 44.0 ± 0.6 | 11.4 ± 2.1 | 21.9 ± 3.1 | 3.3 ± 0.6 | 25.2 | 55.4 | 0.5 | 10.6 | 7.9 | 19.0 |
| 13 | *R. saxatile* A | – | – | – | 6.5 ± 0.2 | – | 1.3 ± 0.1 | 6.4 ± 0.5 | 1.1 ± 0.4 | 46.0 ± 1.9 | 11.9 ± 1.1 | 22.6 ± 2.1 | 4.1 ± 0.1 | 26.7 | 57.9 | 0.5 | 7.6 | 7.8 | 12.8 |
| 14 | *R. saxatile* B | – | – | – | 6.3 ± 0.1 | – | 1.2 ± 0.0 | 9.1 ± 0.4 | 1.1 ± 0.4 | 44.2 ± 3.1 | 6.5 ± 0.9 | 28.5 ± 0.8 | 3.2 ± 0.1 | 31.6 | 50.7 | 0.6 | 10.3 | 7.5 | 11.0 |
| | Sect. *Calobotrya* (Ornamental Currants) | | | | | | | | | | | | | | | | | | |
| 15 | *R. cereum* | – | – | – | 10.2 ± 0.1 | – | 2.3 ± 0.8 | 15.7 ± 0.7 | – | 36.9 ± 1.3 | 1.7 ± 0.1 | 31.9 ± 0.7 | 1.4 ± 0.1 | 33.3 | 38.6 | 0.9 | 15.7 | 12.5 | 2.2 |
| 16 | *R. sanguineum* var. *glutinosum* | – | – | – | 6.7 ± 0.1 | – | 1.4 ± 0.0 | 21.4 ± 0.4 | – | 39.7 ± 0.1 | 0.9 ± 0.0 | 29.4 ± 0.3 | 0.6 ± 0.0 | 30.0 | 40.6 | 0.7 | 21.3 | 8.0 | 13.0 |
| | Sect. *Coreosma* (Black Currants) | | | | | | | | | | | | | | | | | | |
| 17 | *R. dikuscha* | – | – | – | 7.8 ± 0.7 | – | 1.3 ± 0.9 | 10.3 ± 1.1 | 0.3 ± 0.2 | 37.2 ± 0.6 | 12.5 ± 1.2 | 23.9 ± 0.6 | 5.0 ± 0.1 | 28.9 | 49.6 | 0.6 | 10.7 | 9.1 | 14.7 |
| 18 | *R. hudsonianum* 2014 | – | – | – | 7.9 ± 0.5 | – | 1.4 ± 0.5 | 11.8 ± 0.0 | 0.9 ± 0.1 | 41.2 ± 0.6 | 8.3 ± 0.8 | 23.9 ± 0.7 | 3.3 ± 0.1 | 27.1 | 49.5 | 0.6 | 12.7 | 9.3 | 19.3 |
| 19 | *R. hudsonianum* 2015 | – | – | – | 7.3 ± 0.1 | – | 1.9 ± 0.9 | 17.8 ± 0.7 | 0.3 ± 0.6 | 36.0 ± 0.2 | 6.1 ± 0.7 | 26.3 ± 3.1 | 2.9 ± 0.9 | 29.2 | 42.1 | 0.7 | 18.1 | 9.2 | 17.9 |
| 20 | *R. hudsonianum* 2016 | – | – | – | 7.0 ± 0.6 | – | 1.7 ± 0.0 | 15.3 ± 1.1 | 0.3 ± 0.4 | 39.4 ± 3.1 | 7.5 ± 0.1 | 24.7 ± 2.1 | 3.2 ± 0.8 | 27.9 | 46.8 | 0.6 | 15.7 | 8.6 | 23.0 |
| 21 | *R. nigrum* | 1.0 ± 0.8 | 1.3 ± 0.3 | 0.7 ± 0.0 | 8.8 ± 0.6 | 0.9 ± 0.1 | 1.7 ± 0.2 | 10.4 ± 0.9 | – | 39.2 ± 3.1 | 10.4 ± 0.6 | 20.0 ± 2.3 | 3.0 ± 0.5 | 23.7 | 49.6 | 0.5 | 11.4 | 14.4 | 16.4 |
| 22 | *R. nigrum* 'Hara katarlik' | – | – | – | 7.2 ± 0.1 | – | 1.4 ± 0.5 | 13.4 ± 0.7 | 0.4 ± 0.8 | 44.5 ± 1.2 | 15.7 ± 3.1 | 12.8 ± 0.6 | 2.4 ± 0.4 | 15.2 | 60.2 | 0.3 | 13.8 | 8.5 | 15.1 |
| 23 | *R. nigrum* 'Koksa' 2014 | – | – | – | 7.5 ± 0.2 | – | 1.3 ± 0.4 | 11.1 ± 0.6 | 0.9 ± 0.1 | 38.9 ± 4.1 | 15.7 ± 0.7 | 17.7 ± 0.3 | 3.8 ± 0.9 | 21.5 | 54.6 | 0.4 | 12.0 | 8.8 | 14.1 |
| 24 | *R. nigrum* 'Koksa' 2015 | – | – | – | 7.9 ± 0.1 | – | 1.6 ± 0.0 | 12.5 ± 0.3 | 0.8 ± 0.6 | 38.6 ± 1.1 | 14.6 ± 0.7 | 17.3 ± 0.3 | 3.9 ± 0.2 | 21.3 | 53.1 | 0.4 | 13.3 | 9.5 | 12.0 |
| 25 | *R. nigrum* 'Koksa' 2016 | – | – | – | 8.2 ± 0.4 | – | 1.7 ± 0.4 | 10.9 ± 0.3 | 1.2 ± 0.3 | 38.8 ± 3.7 | 17.0 ± 1.1 | 15.1 ± 0.9 | 3.8 ± 0.1 | 18.9 | 55.7 | 0.3 | 12.1 | 9.9 | 12.7 |
| 26 | *R. viburnifolium* | – | – | – | 19.1 ± 0.8 | – | 3.4 ± 0.1 | 7.7 ± 0.6 | – | 35.6 ± 2.1 | 1.5 ± 0.8 | 28.8 ± 0.8 | 2.0 ± 0.1 | 30.8 | 37.1 | 0.8 | 7.7 | 22.5 | 2.0 |
| 27 | *Ribes* 'Yakutskaya' 2014 | – | – | – | 9.0 ± 2.1 | 0.3 ± 0.5 | 1.5 ± 0.4 | 9.7 ± 1.4 | 1.0 ± 0.0 | 38.7 ± 0.6 | 14.8 ± 2.7 | 19.9 ± 0.3 | 4.5 ± 1.1 | 24.4 | 53.5 | 0.5 | 10.8 | 10.7 | 13.9 |
| 28 | *Ribes* 'Yakutskaya' 2015 | 0.2 ± 0.0 | – | – | 8.6 ± 0.3 | – | 1.6 ± 0.4 | 11.1 ± 0.9 | 0.5 ± 0.5 | 38.8 ± 0.4 | 12.7 ± 0.3 | 21.4 ± 0.7 | 4.1 ± 1.1 | 27.4 | 50.8 | 0.5 | 11.5 | 10.2 | 14.9 |
| 29 | *Ribes* 'Yakutskaya' 2016 | 0.2 ± 0.6 | – | – | 8.7 ± 0.4 | 0.6 ± 0.9 | 1.7 ± 0.1 | 11.5 ± 0.2 | 0.4 ± 0.3 | 37.1 ± 0.1 | 13.8 ± 0.8 | 20.2 ± 4.1 | 4.6 ± 0.6 | 24.7 | 50.9 | 0.5 | 12.0 | 10.4 | 17.0 |
| 30 | *Ribes* 'Erkeeni' | – | – | – | 7.4 ± 0.4 | – | 1.6 ± 0.6 | 11.7 ± 0.1 | 0.5 ± 0.5 | 40.9 ± 0.4 | 15.0 ± 0.9 | 17.2 ± 1.7 | 3.7 ± 0.4 | 20.9 | 55.9 | 0.4 | 12.1 | 9.0 | 13.9 |
| 31 | *Ribes* 'Myuryucheene' 2014 | – | – | – | 10.0 ± 0.2 | – | 1.4 ± 0.0 | 8.4 ± 0.1 | 0.9 ± 0.5 | 36.1 ± 2.1 | 20.2 ± 1.1 | 15.8 ± 3.1 | 4.8 ± 0.9 | 20.5 | 56.3 | 0.4 | 9.3 | 11.4 | 12.8 |

(*continued on next page*)

**Table 2** (*continued*)

| | Species | Fatty Acids (FA% of total FA)[a] | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10:0 | 12:0 | 14:0 | 16:0 | 17:0 | 18:0 | 18:1, n-9 OA | 18:1, n-7 | 18:2, n-6 LA | 18:3, n-6 GLA | 18:3, n-3 ALA | 18:4, n-3 SDA | n-3 PUFA | n-6 PUFA | n-3/n-6 | MUFA | SFA | FA seed g/100 g |
| 32 | *Ribes* 'Myuryucheene' 2015 | – | – | – | 9.6 ± 1.1 | – | 1.9 ± 0.1 | 11.8 ± 0.5 | 0.8 ± 0.3 | 35.4 ± 0.7 | 16.2 ± 0.0 | 17.4 ± 4.1 | 4.7 ± 0.3 | 22.1 | 51.6 | 0.4 | 12.6 | 11.5 | 13.0 |
| | Sect. *Parilla* (Andine Currants) | | | | | | | | | | | | | | | | | | |
| 33 | *R. cucullatum* | – | – | – | 8.2 ± 0.0 | – | 2.5 ± 0.0 | 24.6 ± 0.2 | – | 36.9 ± 0.0 | 0.8 ± 0.1 | 26.4 ± 0.2 | 0.6 ± 0.1 | 27.0 | 37.7 | 0.7 | 24.6 | 10.7 | 13.6 |
| 34 | *R. gayanum* | – | – | – | 8.5 ± 0.4 | – | 2.1 ± 0.1 | 30.7 ± 0.6 | – | 35.0 ± 0.1 | – | 23.6 ± 0.3 | – | 23.6 | 35.0 | 0.7 | 30.7 | 10.4 | 11.9 |
| 35 | *R. magellanicum* | – | – | – | 7.8 ± 0.3 | – | 1.5 ± 0.1 | 21.1 ± 0.2 | – | 38.2 ± 0.1 | 0.6 ± 0.0 | 30.1 ± 0.4 | 0.5 ± 0.0 | 30.6 | 38.8 | 0.9 | 21.0 | 9.2 | 14.0 |
| | Sect. *Ribes* (Red Currants) | | | | | | | | | | | | | | | | | | |
| 36 | *R. glabellum* 2014 | – | – | – | 8.4 ± 0.8 | – | 2.9 ± 0.5 | 12.0 ± 0.1 | – | 30.4 ± 1.7 | 7.8 ± 0.1 | 28.6 ± 0.9 | 5.9 ± 0.9 | 34.5 | 38.2 | 0.9 | 12.0 | 11.3 | 10.9 |
| 37 | *R. glabellum* 2015 | – | – | – | 6.4 ± 0.5 | – | 2.3 ± 0.4 | 11.0 ± 0.6 | – | 32.6 ± 0.9 | 10.9 ± 1.1 | 28.9 ± 3.1 | 7.9 ± 0.1 | 36.9 | 43.5 | 0.9 | 11.0 | 8.7 | 10.9 |
| 38 | *R. glabellum* 2016 | – | – | – | 6.0 ± 0.2 | – | 2.0 ± 0.1 | 10.4 ± 0.0 | 0.1 ± 0.0 | 32.9 ± 3.6 | 11.8 ± 0.9 | 28.9 ± 0.7 | 7.9 ± 0.5 | 36.7 | 44.7 | 0.8 | 10.5 | 8.0 | 11.3 |
| 39 | *R. manshuricum* | 0.7 ± 0.0 | – | – | 6.9 ± 0.2 | – | 1.4 ± 0.6 | 11.8 ± 0.9 | 0.5 ± 0.0 | 39.9 ± 1.9 | 7.1 ± 0.5 | 26.5 ± 0.2 | 3.5 ± 0.4 | 30.1 | 48.7 | 0.6 | 12.3 | 8.9 | 7.5 |
| 40 | *R. multiflorum* subsp. *sandalioticum* | – | – | – | 8.2 ± 0.7 | – | 1.8 ± 0.9 | 13.6 ± 0.0 | – | 42.3 ± 4.1 | 1.0 ± 0.1 | 31.0 ± 0.8 | 0.6 ± 0.7 | 31.6 | 43.3 | 0.7 | 13.6 | 10.0 | 3.4 |
| 41 | *R. pallidiflorum* | – | – | – | 5.7 ± 0.1 | – | 1.9 ± 0.3 | 11.0 ± 0.4 | 0.4 ± 0.5 | 36.9 ± 0.1 | 13.3 ± 0.6 | 23.6 ± 0.7 | 7.2 ± 0.8 | 30.8 | 50.2 | 0.6 | 11.4 | 7.5 | 10.9 |
| 42 | *R. pubescens* | – | – | – | 5.0 ± 0.1 | 0.6 ± 0.1 | 2.0 ± 0.1 | 11.3 ± 0.8 | – | 34.8 ± 1.8 | 11.9 ± 0.1 | 27.5 ± 1.1 | 6.9 ± 0.6 | 34.5 | 46.7 | 0.7 | 11.3 | 7.7 | 8.0 |
| 43 | *R. rubrum*[b] | – | – | – | 9.7 ± 1.1 | – | 2.4 ± 0.6 | 15.7 ± 0.1 | 0.7 ± 0.0 | 29.0 ± 1.3 | 3.4 ± 0.3 | 29.9 ± 1.6 | 3.3 ± 0.8 | 33.1 | 32.4 | 1.0 | 21.9 | 12.1 | 4.2 |
| 44 | *R. triste* | – | – | – | 6.1 ± 0.1 | – | 1.8 ± 0.3 | 13.1 ± 0.1 | 0.3 ± 0.0 | 41.8 ± 0.2 | 11.5 ± 0.7 | 20.8 ± 0.5 | 4.3 ± 0.9 | 25.2 | 53.2 | 0.5 | 13.4 | 7.8 | 18.0 |
| | Sect. *Symphocalyx* (Golden Currants) | | | | | | | | | | | | | | | | | | |
| 45 | *R. aureum* A | – | – | – | 12.4 ± 0.4 | – | 2.7 ± 0.9 | 9.8 ± 0.1 | 0.5 ± 0.6 | 32.8 ± 0.6 | 4.9 ± 0.3 | 30.8 ± 0.6 | 4.6 ± 0.0 | 35.4 | 37.6 | 0.9 | 10.2 | 15.1 | 9.7 |
| 46 | *R. aureum* B | – | – | – | 6.8 ± 0.2 | – | 1.2 ± 0.6 | 9.2 ± 0.6 | 1.3 ± 0.1 | 40.5 ± 2.4 | 8.6 ± 0.7 | 28.2 ± 0.6 | 4.1 ± 0.7 | 32.4 | 49.1 | 0.7 | 10.5 | 8.0 | 8.3 |
| 47 | *R. odoratum* | – | – | – | 9.3 ± 0.4 | – | 1.3 ± 0.1 | 9.8 ± 0.9 | – | 38.2 ± 3.3 | 6.9 ± 0.4 | 29.6 ± 3.4 | 5.1 ± 0.8 | 34.6 | 45.0 | 0.8 | 10.0 | 10.5 | 12.0 |
| | **Subgenus *Grossularia* (Gooseberries)** | | | | | | | | | | | | | | | | | | |
| | Sect. *Grossularia* | | | | | | | | | | | | | | | | | | |
| 48 | *R. divaricatum* | – | – | – | 6.9 ± 0.1 | – | 1.5 ± 0.1 | 13.9 ± 0.4 | – | 45.6 ± 0.4 | 6.1 ± 0.3 | 23.2 ± 0.4 | 3.0 ± 0.1 | 26.2 | 51.7 | 0.5 | 13.9 | 8.2 | 8.0 |
| 49 | *R. uva-crispa* | – | – | – | 7.4 ± 0.3 | – | 2.3 ± 0.1 | 26.7 ± 0.2 | – | 35.3 ± 0.1 | 4.1 ± 0.3 | 21.6 ± 0.1 | 2.7 ± 0.1 | 24.3 | 39.4 | 0.6 | 26.6 | 9.6 | 4.0 |
| | **Subgenus *Hesperia* (Gooseberries)** | | | | | | | | | | | | | | | | | | |
| | Sect. *Hesperia* | | | | | | | | | | | | | | | | | | |
| 50 | *R. menziesii* var. *menziesii*[c] | – | – | 0.8 ± 0.1 | 11.5 ± 0.6 | – | 1.7 ± 0.7 | 10.2 ± 0.8 | – | 38.3 ± 1.1 | 2.3 ± 0.6 | 16.6 ± 0.6 | 0.8 ± 0.1 | 17.4 | 40.6 | 0.4 | 10.2 | 14.0 | 2.2 |

[a] Other FA of undetermined structure accounted for 100% of the total FA.
[b] 24:1n9 (4.3%) in addition to those above reported.
[c] 24:0 (15.3%) in addition to those above reported.

seed oil content and FA profiles of *Ribes* species are reported in Table 2.

The total FA content in the *Ribes* seeds analyzed here ranged from 2.0 in *R. viburnifolium* to 23.0 g/100 g seeds in *R. hudsonianum*, collected in 2016. The SFA fraction ranges between 7.6 and 22.5% of the total FA. The main SFA in all *Ribes* species investigated was palmitic acid (PA, hexadecanoic acid, 16:0), which is the most common SFA found in animals and plants. The richest PA-species was *R. viburnifolium* (19.1% of total FA), while *R. pubescens* had the lowest percentage (5.0% of total FA). Such percentage was followed by those of stearic acid (SA, octadecanoic acid, 18:0), which ranged from 1.0 in *R. alpinum* 2014 to 2.9% of total FA% in *R. glabellum* 2014. The remaining SFA were found in amounts below 1% of total FA. LA was the main PUFA present in all *Ribes* species surveyed, ranging between 29.0 in *R. rubrum* and 56.5% of total FA in *R. diacanthum*. ALA content ranged from 12.8 (*R. diacanthum*) to 31.9% (*R. cereum*).

GLA, the main FA targeted in this work, was detected in all seeds excepting in *R. gayanum*, while in *R. orientale* reached the lowest amounts, with 0.5% total FA. Four *Ribes* species had GLA at amounts below 1.0% of total FA. These were *R. diacanthum*, *R. sanguineum* var. *glutinosum*, *R. magellanicum* and *R. cucullatum*. Among different cultivars of blackcurrant, *Ribes* 'Myuryucheene' 2014 was at the top of the range, with 20.2% GLA of total FA, followed closely by another blackcurrant, *R. nigrum* 'Koksa' 2016 (17.0% of total FA). Other noticeable GLA amounts were detected in *R. diacanthum*, *R. pallidiflorum*, *R. pubescens*, and *R. glabellum*, with 14.1, 13.3, 11.8, and 11.9% of total FA, respectively. SDA, another bioactive n-3 PUFA, was not in such high percentages as GLA, and it ranged from 0.5 in *R. magellanicum* to 7.9% of total FA in *R. glabellum*. This PUFA was absent in *R. gayanum*. Finally, ALA percentages were about 1/2 to 2/3 of the LA ones, ranging from 12.8% in *R. nigrum* 'Hara katarlik' to 31.9% of total FA in *R. cereum*.

The dimensions and weight of the *Ribes* seeds here analyzed are summarized in Table 1. The weight of 100 seeds ranged from 60 mg in *R. hudsonianum* 2015 and 2016 to 550 mg in *R. rubrum*. The seed length varies between 1.29 mm in *R. hudsonianum* 2014 and 2015, and 2.84 mm in *R. menziesii*, while the seed width ranged from 0.48 mm in *R. diacanthum* 2.22 mm in *R. menziesii*.

## 4. Discussion

The results of the present study revealed that several oils extracted from *Ribes* seeds here analyzed were exceptionally high in different potentially healthy PUFA, especially in GLA. Previously, very few GLA-rich *Ribes* species have been reported. Goffman and Galletti (2001) indicated GLA% for *R. nigrum*, *R. grossularia*, and *R. rubrum* at 15.8, 8.0 and 6.2% of total FA, respectively. Wolf, Kleiman, and England (1983) analyzed *R. alpinum*, *R. inebrians*, *R. montigenum*, and *R. orientale*, for which GLA% were 8.9, 3.4, 3.7, and 1.9% of total FA, respectively. Johansson et al. (1997) checked *R. nigrum*, *R. uva-crispa,* and *R. alpinum*, summarizing 18.0, 11.0, and 9.6 GLA% of total FA, respectively. In all these works, *R. nigrum* was always the more prominent GLA-species. A later study on the FA content and juice characteristics in black currant genotypes was conducted, which was focused to select GLA contents without negatively affecting juice quality, to combine both aspect in a single cultivar. Authors reported values ~20% GLA of total FA in four of the checked genotypes (Ruiz del Castillo, Dobson, Brennan, & Gordon, 2004). Such data indicated a high variability for GLA content in *R. nigrum*. In this regard, we found for this species 10.4% GLA of total FA, lower figure than those previously reported (between 14 and 19%) (Dobson et al., 2012). However, our value for SDA in such species (3.0% of total FA) is comparable to those reported in the literature (2.9% of total FA) (Piskernik et al., 2018). Such findings connect with previous observation on the high variability in GLA content in the seed oil of *R. nigrum*.

Regarding GLA percentage of total seeds, most cultivars of blackcurrant widely surpassed 2 g GLA/100 g seeds, with *Ribes* 'Myuryucheene' 2014 in the top of the range, with 2.6 g GLA/100 g seeds. *R. nigrum* 'Hara katarlik' 2014 and *Ribes* 'Yakutskaya' 2016 had figures very close to this, with 2.4 and 2.3 g GLA/100 g seeds, respectively.

The percentage of GLA in *Ribes* seed oils is characteristic of each taxon, since it depends on the Δ6-desaturase enzyme activity, as it occurs in the seed oils of Boraginaceae (Guil-Guerrero, 2007). Such percentages in some of the species analyzed in this work are comparable to those reported for borage (*Borago officinalis*) oil, which contains 20–23% GLA of total FA (Deng, Hua, Li, & Lapinskas, 2001), while GLA% for most analyzed cultivars of blackcurrants surpass most values previously reported for *R. nigrum*. Also, several species have higher values than those previously reported for *Echium plantagineum*, which contains 10–14% GLA of total FA (Guil-Guerrero, 2007), and also surpass the values reported for evening primrose (*Oenothera biennis*) oil, which contains ~9% GLA of total FA (Deng et al., 2001).

However, it was detected some intrataxon variability regarding GLA% of total FA. For example, for *R. alpinum*, which was collected in areas as diverse as Siberia and the mountains of Southern Spain, GLA ranges from 4.5 to 11.8% of total FA (Table 2), which could indicate the existence of different chemotypes. Interestingly, for seeds collected from plants located in the same area, the margin of variation is narrower. In this regard, for *R. alpinum* C, which was collected in North-Eastern Yakutsk, Russian Federation, in the 2014–2016 period (Table 1), GLA ranged between 5.4 and 8.4% of total FA The same can be noted in the case of *R. hudsonianum*, since for the same period GLA ranged between 6.1 and 8.3% of total FA. This is also true for *R. glabellum* in the same period, which contains 7.8–11.8 GLA% of total FA, and this observation is also true for the blackcurrant cultivars. Thereby, for the same period 2014–2016, *Ribes* 'Yakutskaya', contains GLA between 12.7 and 13.8% of total FA, while *R. nigrum* 'Koksa' contains GLA at 14.6–17.0% of total FA. In short, all evidences indicate that the percentage of GLA of total FA is an improved characteristic of *R. nigrum* achieved by genetic selection and breeding techniques, which can be moderately affected by weather conditions (Leskinen, Suomela, & Kallio, 2009).

Blackcurrant cultivars analyzed here come from the Republic of Sakha (Yakutia). This is the coldest region of Russia, located in the north-east. Sharply continental climate, a combination of a long cold period and extremely low temperatures in winter create the uniqueness of the nature of Yakutia. In the harsh conditions of Yakutia, the evolutionary processes formed a unique gene pool plants with complex resistance. Wild relatives of cultivated plants of natural flora of Yakutia have a wide range of necessary qualities, e.g.: frost resistance and high levels of biologically active substances and main food components. In this region, the combination of genomes of 3–4 local species of *Ribes*, or interspecific local forms, served as material for the creation of cultivars (Korobkova, 2011). These are characterized by a rich diversity of traits and pass beyond this species area of distribution. According to various sources of past years, Yakutia's flora includes a rich variety of wild forms of *R. nigrum* group (Popov, 1957). All currants are characterized by large polymorphism, which allows to select among wild-growing plants. The ancestor of almost black currant cultivars is easily crossed with other relative *Ribes* species, which originated new cultivars: *R. nigrum* × *R. pauciflorum* × *R. dikuscha*; *R. nigrum* × *R. procumbens*, and several others. Considering that species from one section easily interbreed with each other and forming similar hybrids, it is difficult to systematize them. Whereas Korobkova (2011) recognizes a rich variability, Pikunova, Martirosian, Kniazeva, and Ryzhova (2012) reported low genetic differences for black currants group.

*Ribes* 'Hara katarlik', 'Erkeeni', 'Yakutskaya' and 'Myuryuchana', which are ready-made varieties (cultivars) of black currant, are included in the «State register of breeding achievements permitted for utilization. Plant cultivars» of the Russian Federation (Danilova & Korobkova, 2014).

In order to investigate a possible correlation between FA profiles and phylogenetic relationships among *Ribes* taxa, we have performed

correlation and multivariable analyses using the UFA profiles reported in Table 2. A similar analysis was performed for *Echium* species from Macaronesia (Guil-Guerrero, Gómez-Mercado, García-Maroto, & Campra-Madrid, 2000), and several European GLA-producer species (Guil-Guerrero, García Maroto, & Giménez Giménez, 2001). In both works, good correlation was found between the taxonomic sections defined by morphological or genetic data and PUFA profiles.

Principal Components Analysis (PCA) was performed using all UFA percentages of total FA; that is, those of OA, LA, ALA, GLA and SDA, for all reported taxa. The purpose of the analysis is to obtain a reduced number of linear combinations of the 5 variables that explain the greater variability in the data. In this case, 5 components have been extracted as requested. Together they explain 100.0% of the variability in the original data. In the present analysis, the first two PC explained 45.5 and 28.2 of the total variance, respectively, which represent the 73.7% of the total variance. The PCA scores and loading plots were derived with respect to the species level correlation and FA correlation accordingly. Among the different graphics offered by the PCA tools, we selected the biplot one. This is a graphical representation which allows rapid visualization of the structure of the matrix, while providing a conceptual overview of the samples (Gower & Hand, 1996). This graphic superimposed score and load factors and allows to the simultaneous interpretation between variables and observations (Gower, 2003). The horizontal axis represents PC1 and vertical axis represents PC2. Load, Score and Biplot represents variance of PC thus highlighting dominant or hidden patterns in dataset. The visualization of the compounds through geometrical representation results in a better understanding of the source of variance in the matrix, and in this case, the representation indicates that species can be grouped according to their UFA contents. Fig. 1 shows the projection of the PCA biplots on the plane formed by the first two principal components: PC1 and PC2.

In this analysis, all UFA have a high influence on the model. The major UFA variables along the PC1 were GLA and SDA (negative loads), and ALA and OA (positive loads); for PC2, SDA (negative loads) and LA (positive loads). This graph clearly differentiated scores for sections *Parilla, Calobotrya*, and *Grossularia*, which have positive scores for PC1, as a result of their relative high ALA and OA content. The biplot also shows that all blackcurrant cultivars, which belong to *Coreosma* section, have negative scores for PC1, which is mainly due to their high GLA content. Negative scores for PC2 allows grouping the species of *Symphocalyx* and *Ribes* sections. Concerning the later section, two samples are outside this grouping, which have positive values for PC2, these are *R. multiflorum* subsp. *sandalioticum* and *R. triste*. In this plot it can be noted that similarities between species were coincident in most cases within *Ribes* sections. Influence on group formation can be assigned to a particular UFA; for instance, OA has a great influence in the grouping of sections *Colobotrya* and *Parilla*; GLA on section *Coreosma* (which includes *Ribes nigrum* and its cultivars); and SDA on section *Ribes*. As can be noted, the variables GLA and SDA have similar and negative high loads for PC1, which allows to both variables to be relatively close in the plot. Such closeness was much better detected in the 3D biplot, in which 92% of the total variance was accumulated (graph not shown). In such graph, a nearly complete overlap of both PUFAs was plotted. This situation can be interpreted considering that both PUFA constitute the metabolic result of the Δ6-desaturase enzyme activity, thus PCA could be useful for detecting metabolic reaction rates among FA in some plant taxa.

According to Fig. 1, the groups with greater distance would be the circumboreal section *Coreosma* and South American section *Parilla*. The gooseberries (subgenus *Grossularia* and *Hesperia*) are well grouped but not so well separated from the currants (subgenus *Ribes*) as to break the genus into two groups and even into two different genus.

In addition to using the biplots for taxonomical purposes, the PCA showed here could be useful in order to give an approximate prediction of the FA profile for an unknown *Ribes* species belonging to any taxonomic group considered in this study.

Our data on the composition of FA suggest the possibility of using *Ribes* seed oils as functional food. According to Regulation (EU) 2015/2283 on novel foods (the new Regulation), products if they are considered "substantially equivalent" (in their composition and nutritional value and their levels of undesirable substances) to an existing food that is already marketed within the EU, have a simplified procedure for marketing (Coppens, Da Silva, & Pettman, 2006). This implies that the seed oils of these species could reach a rapid presence in the market.

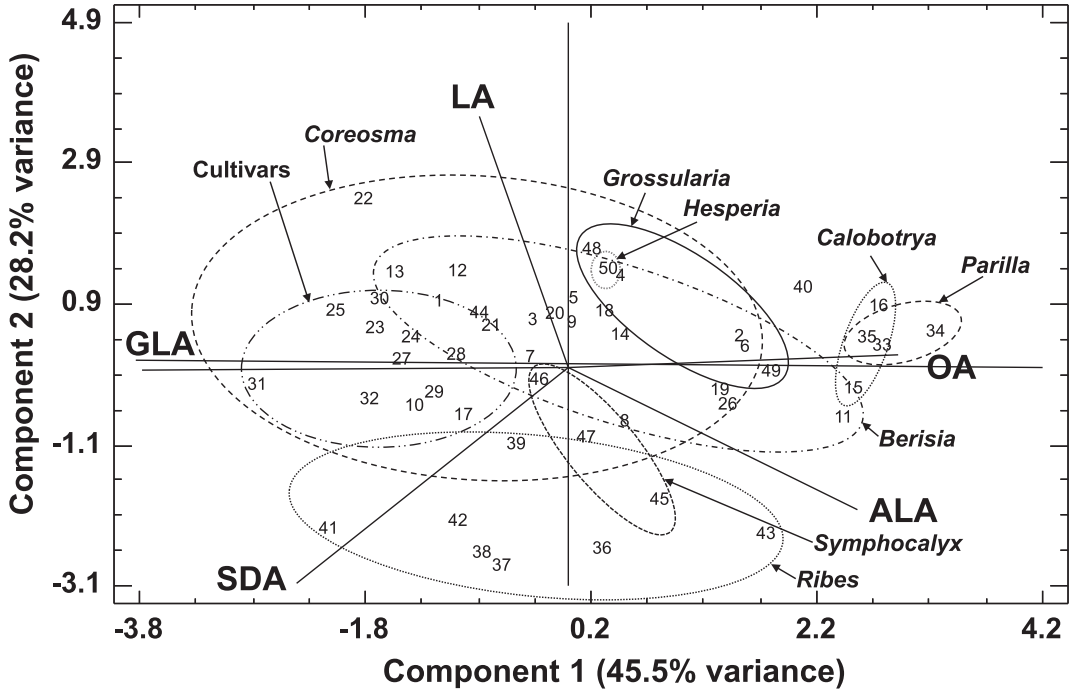


**Fig. 1.** Principal Component Analysis (PCA) of *Ribes* species based on percentages of unsaturated fatty acid of total FA. The numbers represent the species as listed in Table 1.

On the other hand, the size of the seeds is an important characteristic to establish the quality of the by-product resulting from currants juice extraction. That is why reports on the weight and size of the seeds here analyzed (Table 1). The by-product resulting from currant juice extraction is made up mainly by seeds, pulp and skin of the fruits. Logically, at industrial scale, to a larger seed size, the separation of these from the remaining by-product components become easier. In this regard, the taxa having the best GLA percentages were the blackcurrant cultivars. Although blackcurrants (*Coreosma*) show (Table 1) the smallest and lightest seeds, it is interesting to note that hybridization does not produce a significant decrease in the size of seeds with respect to conventional *R. nigrum*, which is the main currant used for juice extraction. Therefore, the techniques applied to the industrial processing of currant by-products to extract GLA-rich oils would be applicable in all cases to the cultivars described in this work.

After having analyzed the UFA content of a large number of *Ribes* species, this taxon confirms its potential as GLA-rich seed oil producer. It is noteworthy to consider that the main GLA-taxa detected in this study correspond to cultivars derived from blackcurrant and related species, all them native to Siberia. Such hybrids produce edible fruits that can be devoted to direct consuming or for industrial juice production. Therefore, the cultivation of such shrubs besides producing fruits in very difficult agronomic areas, could produce a valuable by-product, i.e., the seeds, which could add a notable economic value to agronomic processes if devoted to GLA-rich oils obtainment.

## 5. Conclusions

In short, some unexplored taxa belonging to *Ribes* genus constitute raw sources of potentially healthy GLA-rich seed oils, while the content in ALA and SDA is much lower. Among the different species analyzed, highlights *R. nigrum* cultivars. These are potentially valuable resources to be utilized for fruits production while simultaneously yielding a valuable functional oil-rich seed by-product. Moreover, these could constitute advantageous cultures in hard climatic environments, as is Northern Siberia. Additionally, we demonstrate that the UFA profiles of *Ribes* seed oils have utility as taxonomical markers. In this regard, PCA showed that GLA and OA were the more discriminant UFA for species clustering. This procedure has utility to predict the FA profiles of the seed oils of any *Ribes* species before being analyzed and indicates the presumably best GLA-producers when exploring the FA content within this genus.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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