Research Article

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Characterization of the composition of plant protection products in different formulation types employing suspect screening and unknown approaches

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Abstract

BACKGROUND: Plant protection products (PPPs) are used extensively in agriculture to control crops. These PPPs, which may be found in different types of formulations, are composed of a designated pesticide (active principle) and other inactive ingredients as co-formulants. They perform specific functions in the formulation, as solvents, preservatives or antifreeze agents, among others.

RESULTS: A research technique based on ultra-high-performance liquid chromatography (UHPLC) coupled to a Quadrupole-Orbitrap mass analyzer was successfully applied to characterize the composition of six different PPPs in terms of the presence of co-formulants and types of formulations: emulsifiable concentrate (EC), emulsion in water (EW), suspension concentrate and water-dispersible granule. These PPPs (FLINT MAX, MASSOCUR 12.5 EC, IMPACT EVO, TOPAS, LATINO and IMPALA STAR) had antifungal activity, containing one triazole compound as active principle (tebuconazole, penconazole, myclobutanil, flutriafol or fenbuconazole, respectively). Non-targeted approaches, applying suspect and unknown analysis, were carried out and ten compounds were identified as potential co-formulants. Six (glyceryl monostearate, 1-monopalmitin, dimethyl sulfoxide, *N*,*N*-dimethyldecanamide, hexaethylene glycol and 1,2-benzisothiazol-3(2*H*)-one) were confirmed by injecting analytical standards. Finally, these compounds were quantified in the PPPs.

CONCLUSION: The current study allowed for detecting co-formulants in a wide range of concentrations, between 0.04 (dimethyl sulfoxide) and 19.00 g L^{-1} (glyceryl monostearate), highlighting the feasibility of the proposed analytical methodology. Moreover, notable differences among the types of formulations of PPPs were achieved, revealing that EC and EW were the formulations that contained the largest number of co-formulants (four out of six detected compounds).

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

Keywords: plant protection products; co-formulants; UHPLC-Q-Orbitrap-MS/MS; suspect screening; unknown analysis

INTRODUCTION

Globally, 4.2 million tonnes (Mt) of pesticides were used in agriculture in 2019. European countries exported about 1.4 Mt of pesticides per year during the period 1990–2019, representing more than one-third of the global market.[1] In Spain, the agricultural production relies, among other means of production, on plant protection products (PPPs), which are of great economic and environmental importance. PPPs have been widely used in agriculture over the years. In accordance with the data provided by the Spanish Ministry of Agriculture, Fisheries and Food, the total amount of marketed PPPs was estimated at 73 286 t in 2018, and 75 397 t in 2019, assuming an increase of 2.9%.[2]

According to the European Commission:

> PPPs are the products consisting of, or containing at least, intentionally one approved active substance and may contain any of the following substances or preparations: safeners, synergists or co-formulants, for any of the following

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uses: protecting plants or plant products against all harmful organisms, preventing the action of such organisms, influencing the life processes of plants, preserving plant products, destroying undesired plants or parts of plants and/or checking or preventing undesired growth of plants.[3]

Therefore, PPPs are composed of a designated pesticide (active principle) and 'other ingredients' with different functionalities.[4,5] Commercialization on the European market of PPPs, widely referred to as pesticides, is adequately regulated, and PPPs must be authorized in accordance with the updated Regulation as commercial formulations (EC) No. 1107/2009.[3,6]

Co-formulants are usually defined as substances that are added to PPPs but are neither active substances nor safeners or synergists,[3] and they provide specific properties for their application,[7] such as enhancing formulation stability or even optimizing the distribution of formulations on plant surfaces, among others. Co-formulants perform specific functions in the formulation, such as solvents, surfactants, diluents, thickeners, dispersing agents, binding agents, stabilizing agents, wetting agents, antifoaming agents, preservatives or antifreeze agents.[8-10]

In the context of PPP toxicity, Regulation (EU) 284/2013[11] mainly evaluates acute effects through tests for acute toxicity, irritation and skin sensitization. However, as generally active substances assumed to dominate toxicity, they are analyzed thoroughly for acute, chronic and subchronic effects in short- and long-term *in vivo* studies.[12] However, no further toxicological evaluation or authorization is required for co-formulants, because they are commonly subject to the REACH (Registration, Evaluation, Authorization and restriction of Chemicals) regulation and hence toxicologically tested and assessed depending on their annual production volume.[7] Nevertheless, some studies have demonstrated that some co-formulants, which might not be toxic themselves, can influence the toxicity of PPPs via toxicodynamic and toxicokinetic interactions.[8] Consequently, the Commission Regulation (EU) 2021/383 sets a list of co-formulants that are not accepted for inclusion in PPPs.[13]

Additionally, PPPs may be found in different types of formulations that contain diverse quantities of active substances and other ingredients and perform specific functions in the formulations. The most common formulation types are the following: emulsifiable concentrate (EC) that is composed of blends of pesticide, emulsifiers and adjuvants dissolved in a volatile oil; emulsion in water (EW), which is a similar mixture to EC but using water instead of oil; suspension concentrate (SC), based on suspensions of micronized active pesticide in water; and water-dispersible granule (WG), which contains active ingredient in spray form of the constituents insoluble in water.[10,14]

Published analytical methods focused on the analysis of the composition of PPPs usually use low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) analyzers. In relation to LRMS, Tush *et al.*[15] described the characterization of a non-ionic surfactant, polyoxyethylene tallow amine, in glyphosate formulations. The authors used ultra-high performance liquid chromatography (UHPLC) coupled to a triple quadrupole (QqQ) as MS analyzer. Balmer *et al.*[16] also reported a method based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). A full characterization of three anionic surfactants (docusate, sodium dodecyl sulfate and dibutylnaphthalene sulfonate) and one organic solvent (*N*,*N*-dimethyldecanamide) present in three PPPs (WG and EC formulations) was carried out. In the study performed by Lara-Martín *et al.*,[17] an analytical procedure allowing for the identification and quantification of the most frequently anionic surfactants (linear alkylbenzene sulfonates, alkyl ethoxysulfates and alkyl sulfates) in aqueous and sediment samples was developed. For that, authors applied LC-MS, using an ion trap as MS analyzer.

Few publications use HRMS for the analysis of PPPs, despite the fact that retrospective analysis can be carried out, allowing for both targeted and non-targeted studies.[18] Glaubitz *et al.*[4] performed a study to quantify sulfosuccinate surfactant in a commercial formulation, using LC coupled with time-of-flight mass spectrometry (LC-ToF-MS). Furthermore, PPPs have been investigated by LC-Exactive Orbitrap-MS[19] and nine compounds were characterized in three EC formulations. Glycol ether, and benzene and naphthalene derivatives were detected and a semi-quantitation of these compounds was performed.

Because of the importance of all the above mentioned, the aim of the present study was the characterization of the composition of six PPPs (FLINT MAX, MASSOCUR 12.5 EC, IMPACT EVO, TOPAS, LATINO and IMPALA STAR) with antifungal activity, as they contain one triazole compound as active principle: tebuconazole, penconazole, myclobutanil (two PPPs containing this compound were evaluated), flutriafol or fenbuconazole, respectively, evaluating the differences between several types of formulations (EC, EW, SC and WG).

For that, a non-targeted approach (suspect screening and unknown analysis) was carried out for the identification of co-formulants using UHPLC-Q-Orbitrap-MS. The strategy includes a tentative identification of co-formulants, which were finally confirmed injecting available analytical standards, testing the suitability of the method.

MATERIALS AND METHODS

Equipment, material and reagents

Six commercial formulations, with different composition, have been characterized in the present study: FLINT MAX (50% tebuconazole, WG); MASSOCUR 12.5 EC (12.5% myclobutanil, EC); IMPACT EVO (12.5% flutriafol, SC); TOPAS (19.4% penconazole, EW); LATINO (formerly known as MITRUS, 12.5% myclobutanil, EC); and IMPALA STAR (2.5% fenbuconazole, EW). These pesticide formulations were acquired from various vendors, and they are described in detail in Supporting Information Table S1.

Analytical standards of the compounds glyceryl monostearate, 1-monopalmitin, hexaethylene glycol and 1,2-benzisothiazol-3(2*H*)-one were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) (≥99.7% of purity) and *N*,*N*-dimethyldecanamide were acquired from Honeywell Riedel-de-Haën (Seelze, Germany).

Methanol (99.9% purity) and water, both LC-MS grade, were obtained from Honeywell Riedel-de-Haën and from JT Baker (Deventer, Netherlands), respectively. Formic acid was purchased from Fisher Scientific (Erembodegem, Belgium).

A mixture of acetic acid, caffeine, Met-Arg-Phe-Ala-acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive and negative) from Thermo-Fisher (Waltham, MA, USA) were employed for the accurate mass calibration of the Q-Orbitrap analyzer.

UHPLC-Q-ORBITRAP-MS analysis

For chromatographic analysis, a Thermo Fisher Scientific Vanquish Flex Quaternary LC (Transcend, Thermo Fisher Scientific, San Jose,

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CA, USA) was used. It was equipped with a Hypersil GOLD aQ C18 column (100 mm × 2.1 mm × 1.9 μm particle size), supplied by Thermo Fisher Scientific. The flow rate was set at 0.2 mL min^{-1}. In relation to the mobile phase, it consisted of eluent A, which was an aqueous solution of formic acid (0.1%), and eluent B, methanol.

The step gradient was as follows: 0–2 min 5% B; from 2–16 min, it was increased to 100% B and then the composition was kept constant for 10 min. Finally, it returned to the initial conditions in 1 min and remained constant for 3 min. The total running time was 30 min. The column temperature was set at 30 °C and the injection volume at 10 μL.

The chromatographic system was coupled to a hybrid mass spectrometer (Q-Exactive Orbitrap, Thermo Fisher Scientific, Bremen, Germany) using an electrospray interface (ESI; HESI-II, Thermo Fisher Scientific, USA) in positive and negative mode. ESI parameters were as follows: spray voltage, 4 kV; sheath gas (N_2, 95%), 35 (arbitrary units); auxiliary gas (N_2, 95%), 10 (arbitrary units); S-lens RF level, 50 (arbitrary units); 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 = 70 000 full width at half maximum (FWHM); AGC target = 1e6, scan time = 250 ms; (2) full MS, ESI^-, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 70 000 FWHM; AGC target = 1e6, scan time = 250 ms; (3) all-ion fragmentation (AIF), ESI^+, setting higher energy collisional dissociation (HCD) on, and collision energy = 30 eV, mass resolving power = 35 000 FWHM, scan time = 125 ms; (4) AIF, ESI^- (setting HCD on, and collision energy = 30 eV), mass resolving power = 35 000 FWHM, scan time = 125 ms. Furthermore, the following acquisition functions were tested by combination with full MS: (1) data-dependent mass spectrometry fragmentation (dd-MS^2), ESI^+ (HCD on, collision energy = 30 eV), mass resolving power = 35 000 FWHM; AGC target = 1e6; and (2) dd-MS^2, ESI^- (HCD on, collision energy = 30 eV), mass resolving power = 35 000 FWHM; AGC target = 1e6.

The mass range in the full-scan MS experiments was set to *m/z* 50–750.

Data treatment

The chromatograms were acquired using the external calibration mode and then processed using Xcalibur version 4.3.73, with Quan Browser and Qual Browser, and Mass Frontier 8.0 (Thermo Fisher Scientific, Les Ulis, France) *in silico* software.

Compound Discoverer version 3.2 (Thermo Fisher Scientific) was also employed with ChemSpider databases (EPA DSST and FDA-UNIII-NLM) when unknown approach was performed.

Data processing in suspect screening

Raw files obtained by UHPLC-Q-Orbitrap analysis were carefully studied to detect any peak belonging to a compound present in the sample. For that, raw files were processed with a homemade database (implemented in Compound Discoverer software) containing 105 compounds. This database contains information about name, molecular structure, molecular formula and exact mass (u) for each compound (Table S2). Mass error was adjusted to 5 ppm to identify co-formulants in the samples.

Furthermore, raw data obtained by LC-Q-Orbitrap analysis were manually processed with Xcalibur Qual Browser in order to monitor the spectra of the detected compounds, and confirm the fragment ions provided by Mass Frontier.

Data processing in unknown analysis

To detect other co-formulants an unknown analysis was performed, and raw files obtained from each analysis were processed with Compound Discoverer. The identification criteria were defined according to SANTE guidance.[20] These criteria were as follows: suitable peak shape signals; in case noise was absent, a signal should be present in at least five subsequent scans per peak of each ion, mass error lower than or equal to 5 ppm; and at least two fragment ions of each co-formulant were selected. Additionally, ChemSpider database, previously described in the 'Data treatment' section, was employed and a threshold filter of ≤1e5 was set for peak intensity.

Sample treatment

Individual standard solutions of each commercial formulation were initially prepared by dissolving 40 μL of each one in 40 mL methanol (in the case of MASSOCUR 12.5 EC, LATINO and IMPALA STAR) or water (in the case of FLINT MAX, IMPACT EVO and TOPAS).

The mixture of each commercial formulation was well shaken and 100 μL was transferred to an LC-MS vial and then diluted with 900 μL methanol, obtaining a final dilution of 1:100 000, v/v.

RESULTS AND DISCUSSION

For the identification of potentially expected compounds in the studied formulations, a non-targeted approach (suspect screening and unknown analysis) was performed. For that purpose, dilutions of the PPPs (described in the 'Sample treatment' section) were injected into the UHPLC-Q-Orbitrap-MS system, using positive and negative ionization mode (ESI^+ and ESI^-, respectively). Before preparing the dilution, three out of the six formulations (FLINT MAX, IMPACT EVO and TOPAS) were not completely dissolved in methanol. For that reason, these PPPs were prepared in water, obtaining a clear solution.

It should be noted that a dilution (1:100 000, v/v) was applied to the samples to prevent possible contamination of equipment, as it was observed in preliminary studies as well as to achieve optimum results related to sensitivity and peak areas.

Suspect screening

Full-scan MS was selected to acquire the total ion chromatogram (TIC) of the characteristic ions of the compounds included in the database. Fragment ions were obtained using AIF mode. Additionally, dd/MS^2 mode was tested, because it uses a very narrow quadrupole isolation window, providing much better selectivity than AIF.[21] However, some fragments of the studied compounds were not found using dd/MS^2 mode; meanwhile, when AIF mode was employed, they were detected. Therefore, dd/MS^2 was not used for suspect screening.

A homemade database, containing 105 compounds (see Supporting Information Table S2), was implemented in Compound Discoverer software, using a working node of suspect screening. This tool allowed for searching all compounds included in the database in the studied samples, selecting a mass error of ±5 ppm. In this context, three compounds were tentatively identified as potential co-formulants: glyceryl monostearate, DMSO and *N*,*N*-dimethyldecanamide, as shown in Table 1. Glyceryl monostearate was detected in all the PPPs analyzed, *N*,*N*-

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Table 1. Characteristic parameters for tentatively identified compounds by suspect screening

Compound	RT (min)	Molecular formula	Theoretical mass (*m/z*)	Mass error (ppm)	Fragment ions: Theoretical mass (*m/z*)	Fragment ions: Molecular formula	Fragment ions: Mass error (ppm)	Ionization mode	Commercial formulation
DMSO	1.33	C_2H_6OS	79.02121	3.79	—			ESI (+)	FLINT MAX
Glyceryl monostearate	20.98	$C_{21}H_{42}O_4$	359.31559	−4.70	177.11214	$C_8H_{17}O_4$	4.34	ESI (+)	FLINT MAX, MASSOCUR 12.5 EC, IMPACT EVO, TOPAS, LATINO, IMPALA STAR
					311.29446	$C_{20}H_{39}O_2$	−4.04		
					341.30502	$C_{21}H_{41}O_3$	−4.10		
N,N-Dimethyldecanamide	17.56	$C_{12}H_{25}NO$	200.20089	0.04	94.06513	C_6H_8N	3.40	ESI (+)	MASSOCUR 12.5 EC, TOPAS, IMPALA STAR
					111.11683	C_8H_{15}	2.06		
					194.15394	$C_{12}H_{20}ON$	−3.86		

DMSO, dimethyl sulfoxide; ESI (+), electrospray interface in positive mode; RT, retention time. Compounds were confirmed by acquisition of analytical standards.

dimethyldecanamide in three samples and DMSO was only identified in one of them. According to the results, the compounds showed suitable mass error (no higher than 5 ppm for characteristic ions). Because noise was absent, the characteristic ion was observed in five subsequent scans per peak. In relation to retention time, it ranged from 1.33 (DMSO) and 20.98 min (glyceryl monostearate).

For each one of these possible three co-formulants, different fragment ions, acquired by AIF, were generated in positive mode and compared with those obtained using Mass Frontier software. Therefore, a total of three fragments were monitored for glyceryl monostearate and for *N,N*-dimethyldecanamide. Suitable mass errors for the fragment ions, with values between 2.06 and 4.34 ppm, were achieved. Due to the low molecular mass of DMSO (theoretical mass *m/z* 79.02121), fragments could not be provided (Table 1), but the isotopic pattern between the experimental and theoretical spectrum was compared, bearing in mind the presence of one sulfur atom.

Unknown analysis

An unknown approach was then performed to identify other co-formulants not included in the previous suspect screening. For that purpose, the raw files obtained for each PPP were processed using Compound Discoverer, applying an 'unknown analysis mode' and employing a workflow that includes ChemSpider databases (indicated in the 'Data treatment' section). Thus the following criteria were taken into account to detect co-formulants: appropriate peak shape signals, a signal present in at least five subsequent scans per peak of each ion and mass error ≤ 5 ppm. When these settings were used, 716 features were achieved. Due to a high number of candidates, different strategies were carried out to decrease the false positives and identify potential co-formulants. First, blank solvent signals were subtracted from the samples, reducing the initial amount from 716 to 311 candidates. Second, signals from triazole compounds were filtered and subtracted, achieving 304 positives. These features were evaluated and their corresponding spectra and chromatograms were independently studied to identify the potential unknown compounds related to co-formulants. Furthermore, the analysis of the structures of each compound (provided by the Compound Discoverer software) helped to discriminate co-formulant compounds. Finally, based on the proposed structures (provided by the software) and considering the type of compounds sought (co-formulants), seven compounds were selected as potential co-formulants (see Table 2). It should be noted that the majority of the tentatively identified compounds were obtained in positive mode (1-monopalmitin, hexaethylene glycol, 1,2-benzisothiazol-3(2*H*)-one, 3,6,9,12-tetraoxapentacosan-1-ol and 3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxanonatetracontan-1-ol), and only two of them (4-decylbenzenesulfonic acid and 4-nonyl benzenesulfonic acid sodium salt) in negative mode, as displayed in Table 2. Mass error was always lower than 5 ppm. In relation to retention time, it ranged from 10.69 (1 2-benzisothiazol-3(2*H*)-one) to 20.35 min (1-monopalmitin) (Table 2). Subsequently, these co-formulants were included in the homemade database.

The spectra of characteristic ions and their fragments were monitored and studied using Qual Brwoser, in order to provide more information about the compounds. The similarity between some of the spectra of different compounds suggested relevant information. The fragmentation pattern of the octaethylene glycol and tetraethylene glycol was similar to hexaethylene glycol, detecting common fragment ions at *m/z* values 134.09375 and 177.11214. These three compounds belong to the same family, which corresponds to $C_{12}H_{26}O_7$–$(C_2H_4O^-)_n$. Likewise, the compounds 3,6,9,12,15,18,21,24,27,30-decaoxatritetracontan-1-ol and 3,6,9,12,15,18,21,24,27,30,33-undecaoxahexatetracontan-1-ol, which have common fragments at *m/z* 487.36293 ($C_{27}H_{51}O_7$) and 547.33242 ($C_{24}H_{51}O_{13}$), have similar behavior. Based on this observation, this pattern suggested the presence of compounds belonging to the 3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxanonatetracontan-1-ol family, which corresponds to $C_{37}H_{76}O_{13}$–$(C_2H_4O)_n$ (Table 2).

As observed in Table 1, 2-monopalmitin was tentatively detected in five of the analyzed samples (except in IMPALA STAR), followed by 4-decylbenzenesulfonic acid, which was found in four PPPs. Some compounds were only tentatively identified in one sample, as 3,6,9,12-tetraoxapentacosan-1-ol, 3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxanonatetracontan-1-ol (IMPACT EVO) and 4-nonyl benzenesulfonic acid sodium salt (MASSOCUR 12.5 EC).

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Table 2. Characteristic parameters for tentatively identified compounds by unknown analysis

Compound	RT (min)	Molecular formula	Theoretical mass (*m/z*)	Mass error (ppm)	Fragment ions: Theoretical mass (*m/z*)	Fragment ions: Molecular formula	Fragment ions: Mass error (ppm)	Ionization mode	Commercial formulation
1- Monopalmitin[a]	20.35	$C_{19}H_{38}O_4$	331.28429	−4.19	125.09609 177.11214 313.27372	$C_8H_{13}O$ $C_8H_{17}O_4$ $C_{19}H_{37}O_3$	1.19 3.33 −3.41	ESI (+)	FLINT MAX, MASSOCUR 12.5 EC, IMPACT EVO, TOPAS, LATINO
1,2- Benzisothiazol-3(2H)-one	10.69	C_7H_5NOS	156.01147	−3.96	105.03349	C_7H_5O	0.66	ESI (+)	IMPACT EVO, TOPAS, IMPALA STAR
3,6,9,12-Tetraoxapentacosan-1-ol	19.57	$C_{21}H_{44}O_5$	377.32615	−4.90	311.25807 319.28429	$C_{19}H_{35}O_3$ $C_{18}H_{39}O_4$	−4.44 −2.16	ESI (+)	IMPACT EVO
3,6,9,12,15,18,21,24,27,30,33,36-Dodecaoxanonatetracontan-1-ol and derivates[b]	19.49	$C_{37}H_{76}O_{13}$–$(C_2H_4O)_n$	729.53587	−0.01	487.36293 547.33242	$C_{27}H_{51}O_7$ $C_{24}H_{51}O_{13}$	−4.84 1.18	ESI (+)	IMPACT EVO
4- Decyl benzenesulfonic acid	16.76	$C_{16}H_{26}O_3S$	297.15299	−0.30	—			ESI (−)	MASSOCUR 12.5 EC, IMPACT EVO, TOPAS, LATINO
4-Nonyl benzenesulfonic acid sodium salt	16.59	$C_{15}H_{23}O_3SNa$	283.13734 $[M-Na]^-$	0.17	—			ESI (−)	MASSOCUR 12.5 EC
Hexaethylene glycol and derivates[c]	19.69	$C_{12}H_{26}O_7$–$(C_2H_4O–)_n$	283.17513	−3.31	134.09375 177.11214	$C_6H_{13}O_3$ $C_8H_{17}O_4$	−0.75 −1.29	ESI (−)	MASSOCUR 12.5 EC, LATINO, IMPALA STAR

[a] Compounds confirmed by acquisition of analytical standards are in bold.
[b] Including the compounds. 3,6,9,12,15,18,21,24,27,30-decaoxatritetracontan-1-ol and 3,6,9,12,15,18,21,24,27,30,33-undecaoxahexatetracontan-1-ol.
[c] Including the compounds: octaethylene glycol and tetraethylene glycol.
ESI (+) or (−): electrospray interface in positive or negative mode; RT, retention time.

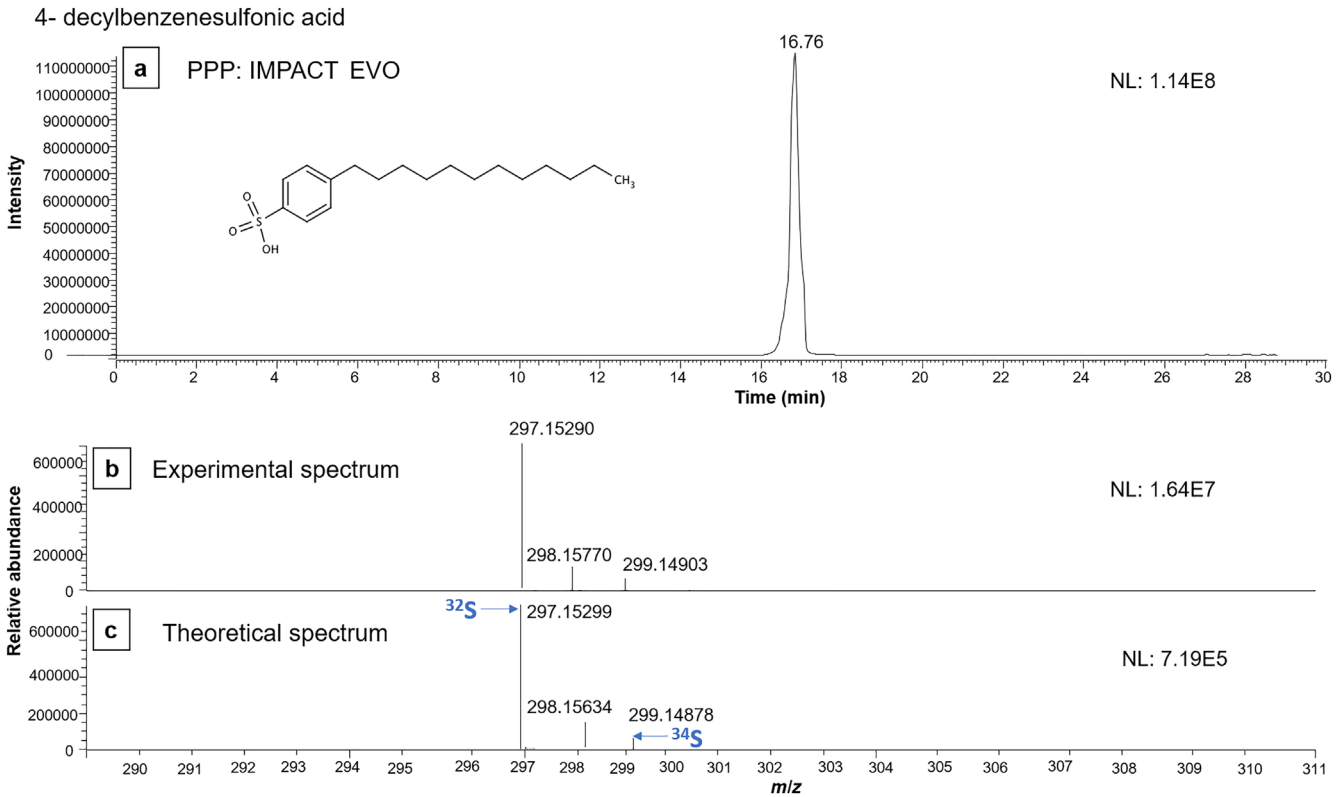

Figure 1. (a) Extracted ion chromatogram of 4-decylbenzenesulfonic acid in IMPACT EVO commercial product; (b) full-scan experimental mass spectrum at 16.76 min; and (c) full-scan theoretical mass spectrum. Abbreviation: NL: normalized level.

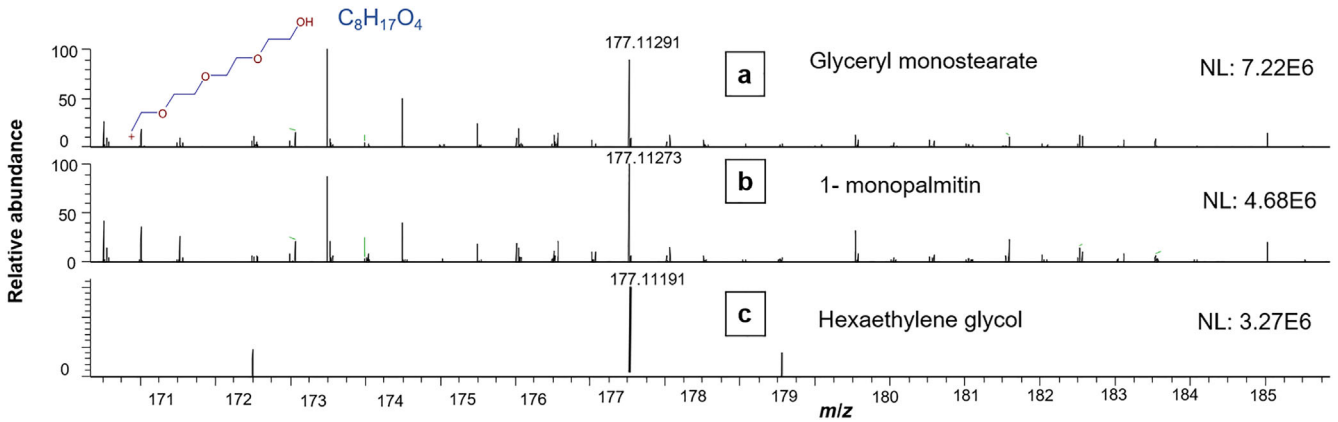

Figure 2. Full-scan experimental mass spectra of co-formulants with the common fragment 177.11214 *m/z* ($C_8H_{17}O_4$): (a) glyceryl monostearate; (b) 1-monopalmitin; and (c) hexaethylene glycol. Abbreviation: NL: normalized level.

Once the possible potential co-formulants have been detected in the PPPs, the theoretical exact mass of each one and plausible structure were combined with Mass Frontier to search fragments of each compound. The fragments were sorted in accordance with the following criteria: most abundant ion; retention time, which has to be equal to the corresponding precursor ion; and mass error (lower than 5 ppm). Table 2 shows the characteristic parameters obtained for unknown compounds, where it can be observed that at least one fragment was monitored for each possible co-formulant, except for 4-decylbenzenesulfonic acid and 4-nonyl benzenesulfonic acid sodium salt (Table 2). For instance, the extracted ion chromatogram (EIC) of 4-decylbenzenesulfonic acid in IMPACT EVO and experimental and theoretical full MS scan spectra are shown in Fig. 1. It can be observed that, although no fragments were detected, the isotopic patterns of the experimental and theoretical spectra were similar, observing the characteristic one when there was a sulfur atom in the molecule.

Regarding the fragments of the characteristic ions, a common fragment, *m/z* 177.11214 ($C_8H_{17}O_4$), was found between three co-formulants: glyceryl monostearate (detected by suspect screening), 1-monopalmitin and hexaethylene glycol (both detected by unknown analysis). This fragment corresponded to a carbon chain composed by ethoxy and methylene groups. The full MS scan experimental spectra of each one of these

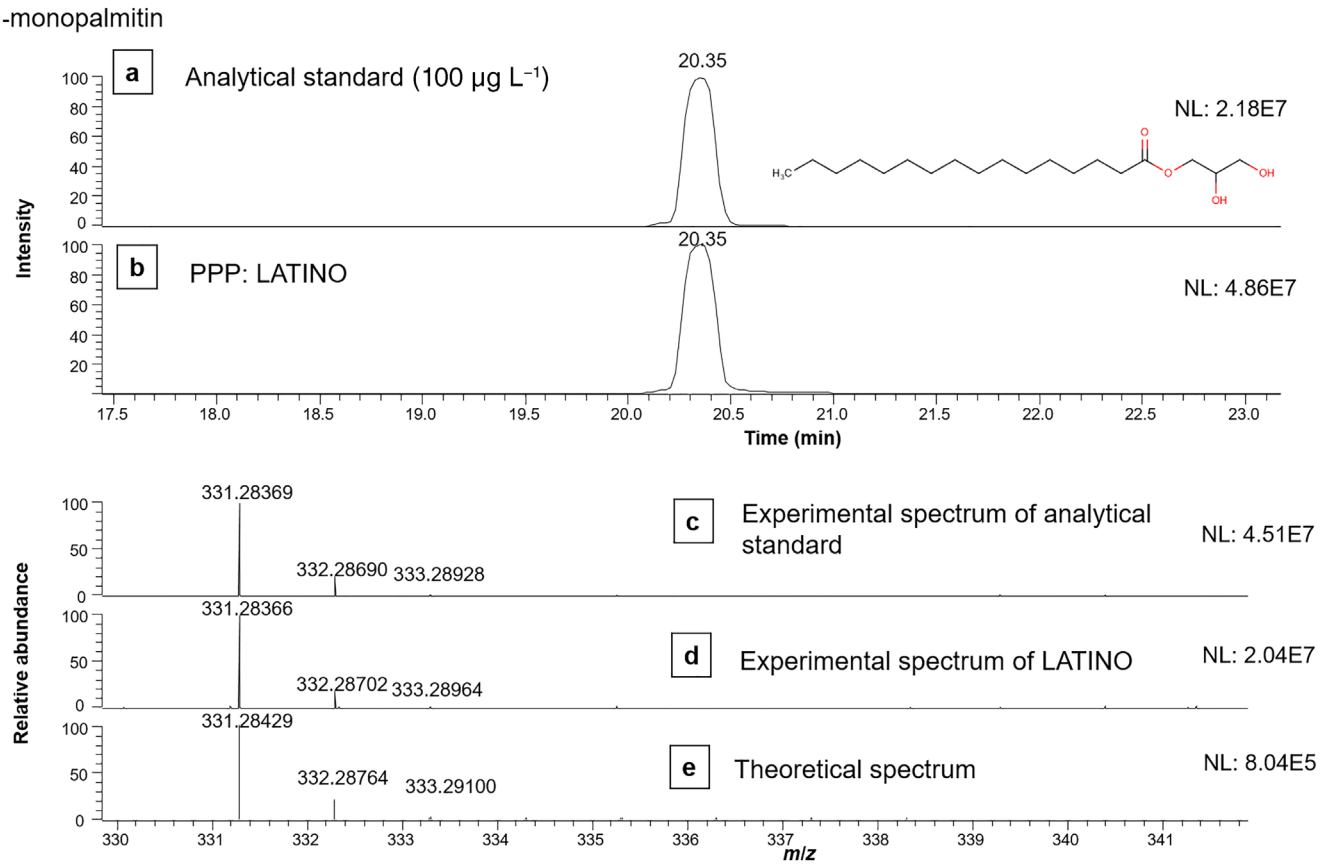

Figure 3. Extracted ion chromatograms of 1-monopalmitin: (a) analytical standard at 100 µg L^{-1} and (b) LATINO commercial product; (c) full-scan experimental mass spectrum at 20.35 min of analytical standard; (d) full-scan experimental mass spectrum of LATINO commercial product; and (e) full-scan theoretical mass spectrum. Abbreviation: NL: normalized level.

Table 3. Concentration of co-formulants in the tested plant protection products (g compound L^{-1} formulation)

	FLINT MAX (WG)	MASSOCUR 12.5 (EC)	LATINO (MITRUS, EC)	IMPACT EVO (SC)	TOPAS (EW)	IMPALA STAR (EW)
1-Monopalmitin	3.41	5.66	13.69	2.16	5.06	ND
1,2-Benzisothiazol-3(2*H*)-one	ND	ND	ND	0.16	0.92	0.10
DMSO	0.04	ND	ND	ND	ND	ND
Glyceryl monostearate	2.99	5.98	6.61	1.78	5.63	19.0
Hexaethylene glycol	ND	0.25	0.29	ND	ND	0.14
N,*N*-Dimethyldecanamide	ND	1.84	ND	ND	0.29	0.31

DMSO, dimethyl sulfoxide; EC, emulsifiable concentrate; EW, emulsion in water; ND, non-detected compound; SC, suspension concentrate; WG, water-dispersible granule.

compounds can be observed in Fig. 2, with a mass error lower than 5 ppm: 4.34 ppm for glyceryl monostearate (Table 1), and 3.33 and −1.29 ppm for 1-monopalmitin and hexaethylene glycol, respectively (Table 2).

Considering the formulation type, one EC (MASSOCUR 12.5 EC) and two EW (TOPAS and IMPALA STAR) were the PPPs that contained a major number of co-formulants, a total of four compounds. According to these results, no correlation associated with a higher number of compounds was observed between the samples diluted in water (FLINT MAX, IMPACT EVO and TOPAS) in comparison with those dissolved in methanol (MASSOCUR 12,5, LATINO and IMPALA STAR). The PPPs that contained fewer compounds were FLINT MAX and IMPACT EVO, with three compounds each one.

Confirmation of the identified co-formulants

After the tentative identification of possible ten co-formulants in the studied samples, commercially available analytical standards (Tables 1 and 2) were acquired to confirm their presence in the PPPs. Due to low availability of commercial standards, only six analytical standards were purchased: glyceryl monostearate, 1-monopalmitin, hexaethylene glycol, *N*,*N*-dimethyldecanamide,

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1,2-benzisothiazol-3(2*H*)-one and DMSO, which is a typical solvent in conventional laboratories. The other compounds (3,6,9,12-tetraoxapentacosan-1-ol, 3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxanonatetracontan-1-ol and family, 4-decylbenzenesulfonic acid and 4-nonyl benzenesulfonic acid sodium salt), whose standards were not available (Table 2), were only tentatively identified (level 2) in the analyzed samples.[22]

Consequently, the six standards were injected in UHPLC-Q-Orbitrap-MS. Once the spectrum of each analytical standard was compared with the experimental one (obtained in the step of tentative identification), it was concluded that all proposed compounds were satisfactorily confirmed in the samples. For example, Fig. 3(a,b) shows the EIC of 1-monopalmitin analytical standard (100 μg L^{-1}) and LATINO, respectively, observing that in both cases a peak was observed at 20.35 min. Additionally, the full-scan mass spectra of an analytical standard (Fig. 3c) and LATINO (Fig. 3d), were compared with the theoretical one (Fig. 3e), obtaining similar behavior. Therefore, the methodology applied in this study has been successful, due to 100% of the acquired compounds being confirmed in the studied samples.

Estimation of the concentration of co-formulants in the commercial samples

Finally, the concentration of the confirmed compounds was estimated. For that purpose, calibration curves of each co-formulant were prepared in methanol, ranging from 10 to 100 μg L^{-1}.

As displayed in Table 3, the concentrations of co-formulants found in the studied PPPs ranged from 0.04 g L^{-1} (DMSO) in FLINT MAX to 19.00 g L^{-1} (glyceryl monostearate) in IMPALA STAR. It was found up to four different co-formulants in three samples (MASSOCUR 12,5, TOPAS and IMPALA STAR).

The highest concentration was for glyceryl monostearate (IMPALA STAR) at 19.00 g L^{-1} (Table 3), and the second highest value was observed for 1-monopalmitin (LATINO) at 13.69 g L^{-1}. It should be noted that both compounds were the most recurrent co-formulants observed, hence finding them in all analyzed PPPs, except in IMPALA STAR, where 1-monopalmitin was not detected. Therefore, glyceryl monostearate achieved concentration levels between 1.78 (IMPACT EVO) to 19.00 g L^{-1} (IMPALA STAR), and 1-monopalmitin, from 2.16 (IMPACT EVO) to 13.69 g L^{-1} (IMPALA STAR). In contrast, hexaethylene glycol, 1,2-benzisothiazol-3(2*H*)-one and DMSO were found below 1 g L^{-1}. DMSO was only detected in FLINT MAX at 0.04 g L^{-1} (Table 3).

In the investigation carried out by Balmer *et al.*,[16] three anionic surfactants (docusate, sodium dodecyl sulfate and dibutylnaphthalene sulfonate) and one organic solvent (*N*,*N*-dimethyldecanamide) were studied in three PPPs, one of them being FLINT MAX. The results revealed that dibutylnaphthalene sulfonate (HPLC–ultraviolet) was the only compound found in this PPP. Consequently *N*,*N*-dimethyldecanamide was not presented in FLINT MAX. No differences could be observed in the present study, due to *N*,*N*-dimethyldecanamide not being found in FLINT MAX either.

The labels of IMPACT EVO and TOPAS revealed that these PPPs contained 1,2-benzisothiazol-3(2*H*)-one in their composition. According to these results, the compound was confirmed in both PPPs, at concentration levels of 0.16 and 0.92 g L^{-1}, respectively. Nonetheless, it was observed that 1,2-benzisothiazol-3(2*H*)-one was also present in IMPALA STAR, at 0.10 g L^{-1}, but it was not declared. In general, co-formulants are not labeled but in case of doing so, the whole composition of the PPP is not described. This information reveals that further revisions might be performed, due to the importance of knowing the integral composition of commercial pesticide for the environment and human health.

In relation to other studies, glyceryl monostearate and 4-decylbenzenesulfonic acid were also detected in the research carried out by López-Ruiz *et al.*,[19] where glyceryl monostearate had been confirmed and quantified at concentrations between 1.40 and 1.64 g L^{-1}, in EC PPPs. However, 4-decylbenzenesulfonic acid could only be tentatively identified. Similar information was achieved in the current research, and with regard to glyceryl monostearate, this was detected in the EC formulations, achieving concentrations up to 5.98 g L^{-1} (in MASSOCUR 12.5) and 6.61 g L^{-1} (in LATINO). Based on these results, concentrations up to five times higher than in the study carried out by López-Ruiz *et al.*[19] were achieved. Differences between both studies could be explained because the suppliers used in each study were different.

Study of type of formulations

In the present study four formulation types were analyzed: WG (in one PPP), EC (in two), SC (in one) and EW (in two), as displayed in Table 3, and differences among them were observed.

As shown in Table 4, EC and EW formulations accounted for the largest number of compounds: four for each one. By contrast, WG and SC were considered as the formulations with the lowest number of co-formulants, detecting only three in each one.

Furthermore, several compounds have been reported at higher concentrations in one type of formulation and at lower concentrations in others. This happens with glyceryl monostearate, which was detected at 19.00 g L^{-1} in EW formulation (IMPALA STAR), whereas its concentration was 1.78 g L^{-1} in SC (IMPACT EVO). Therefore, considering these results, it can be assumed that the concentration of the co-formulants is different, but there is not a clear correlation. The same conclusion was made by López-Ruiz *et al.*,[19] observing that, depending on the brand of PPP, diverse ranges of co-formulant concentrations are used.

The composition of two EC formulations (MASSOCUR 12.5 and LATINO) containing the same pesticide (myclobutanil) was compared. Four co-formulants were detected in MASSOCUR 12.5 and three in LATINO, *N*,*N*-dimethyldecanamide being found only in MASSOCUR 12.5. The highest values were achieved at 5.98 and 13.69 g L^{-1} for glyceryl monostearate and 1-monopalmitin, respectively (Table 4). Despite the fact that it was the same type

Table 4. Co-formulants detected in plant protection products

Compound	Function	Formulation found
1-Monopalmitin	Surfactant	WG, EC, SC and EW
1,2-Benzisothiazol-3(2*H*)-one	Preservation	SC, EW and EC
DMSO	Solvent	WG
Glyceryl monostearate	Surfactant	WG, EC, SC and EW
Hexaethylene glycol	Frost protectant	EC
N,*N*-Dimethyldecanamide	Solvent	EC and EW

DMSO, dimethyl sulfoxide; EC, emulsifiable concentrate; EW, emulsion in water; ND, non-detected compound; SC, suspension concentrate; WG, water-dispersible granule.

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of formulation containing the same active substance, notable differences were observed, which might be due to the supplier of each PPP.

Moreover, EW formulation type was studied in two PPPs, and they contained different active substances but from the same family. In this context, different solvents had to be employed by dissolving each individual solution, because TOPAS could not be dissolved in water, unlike IMPALA STAR. Four co-formulants were detected in both PPPs: 1-monopalmitin, 1,2- benzisothiazol-3(2*H*)-one, glyceryl monostearate and *N*,*N*-dimethyldecanamide in TOPAS, and 1,2-benzisothiazol-3(2*H*)-one, glyceryl monostearate, hexaethylene glycol and *N*,*N*-dimethyldecanamide in IMPALA STAR. Glyceryl monostearate was the compound that achieved the highest concentration in both formulations: 5.63 and 19.0 g L^{-1}, respectively.

Considering the function of each detected co-formulant contained in the samples, the compounds could be classified as follows: 1-monopalmitin and glyceryl monostearate as surfactants, 1,2-benzisothiazol-3(2*H*)-one as preservative, DMSO and *N*,*N*-dimethyldecanamide as solvents and hexaethylene glycol as frost protectant, as displayed in Table 4.[10,23]

Toxicity of co-formulants

The increasing use of PPPs has led to widespread concerns about their adverse effect on the environment and especially on human health, particularly those surfactants with non-ionic properties.[7] According to the harmonized classification and labeling approved by the European Union, 1,2-benzisothiazol-3(2*H*)-one is very toxic to aquatic life and harmful if swallowed. Moreover, this compound causes serious eye damage, skin irritation and might even cause an allergic skin reaction.[24] Glyceryl monostearate is a non-ionic surfactant,[23] whose toxicity is considered medium, i.e. 200 mg kg^{-1} body weight in rats.[19] Even though *N*,*N*-dimethyldecanamide has been classified as a developmental toxicant in rodents, there are no data related to its toxicity.[25] In general, DMSO has low acute and chronic toxicity for animal, plant and aquatic life. It is rapidly absorbed, reaching a peak in serum at 4–8 h after oral or transcutaneous administrations, and it is cleared from the blood within 120 h after ingestion of a single dose.[26] A DMSO dose of 15.50 g kg^{-1} borders the single intraperitoneal dose LD_{50}, although lower LD_{50} doses of ~7.00 g kg^{-1} have also been reported.[27] Regarding the family of hexaethylene glycol, the range of LD_{50} of ethylene glycol is established as 5.00–15.30 g kg^{-1}.[28] No toxic effects have been reported for 1-monopalmitin. Therefore, further studies are required to determine the toxicity values for this compound.

CONCLUSIONS

Due to the relevance of the development and application of PPPs in agriculture, in the present research six commercial formulations with antifungal activity were characterized in terms of the presence of co-formulants and formulation type. UHPLC-Q-Orbitrap-MS was applied to identify the potential co-formulants contained in the studied samples using two non-targeted approaches (suspect screening and unknown analysis). A total of ten compounds were tentatively identified, and six of them were confirmed when available standards were acquired. Thus the robustness of the proposed methodology can be highlighted, confirming 100% of the compounds, for which there were commercially available standards. Furthermore, the compounds were quantified, in a wide range of concentrations, even at very low concentrations (<1 g L^{-1}), with values between 0.04 (DMSO) and 19.00 g L^{-1} (glyceryl monostearate). Of all the detected co-formulants, 1,2-benzisothiazol-3(2*H*)-one was considered the most toxic.

The results revealed that EC and EW were the formulations that contained the largest number of co-formulants: four. Some differences regarding the active substances and amount of co-formulants were reported among formulation types. Therefore, it can be concluded that similar formulations may have different co-formulants, which could be due to the suppliers.

Finally, it is demonstrated that the proposed methodology is feasible for identifying, confirming and quantifying co-formulants in different commercial products with different types of formulations. Despite the fact that this information is relevant to consumer health, few databases and analytical standards are available.

Future investigations could focus on estimating co-formulant residues from PPPs applied on different crops. Therefore, attempts could be made to avoid possible adverse effects to consumers and the environment.

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SUPPORTING INFORMATION

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

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