Microchemical Journal 200 (2024) 110442

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Degradation of co-formulants and metabolites from plant protection products in courgette and tangerine using LC-HRMS: Laboratory tests

Beatriz Martín-García *, Roberto Romero-González, Francisco Javier Egea González, Antonia Garrido Frenich

Research Group "Analytical Chemistry of Contaminants", Department of Chemistry and Physics, Research Centre for Mediterranean Intensive Agrosystems and Agri-Food Biotechnology (CIAMBITAL), University of Almería, Agri-Food Campus of International Excellence, ceiA3, 04120 Almería, Spain

ARTICLE INFO

Keywords:
Degradation
Co-formulants
Plant protection products
LC-Q-Orbitrap-MS
Metabolites

ABSTRACT

Plant protection products contain co-formulants that could end up in vegetables, and they can generate transformation products that can be more toxic than their original molecule, which are a potential risk to food safety. Therefore, this study evaluated the dissipation of two co-formulants, dodecylbenzenesulfonic acid and 1-ethyl-2-pyrrolidone, in courgette and tangerine samples to determine possible harmful metabolites under laboratory conditions. The analyses of these substances were performed by liquid chromatography coupled to quadrupole-Orbitrap high-resolution mass spectrometry (LC-Q-Orbitrap-HRMS). For the degradation of both compounds, a single-phase kinetic model was fitted, with R^2 values greater than 0.99. In courgette and tangerine, half-lives (DT_{50}) for dodecylbenzene sulfonic acid were 1.83 and 1.42 days, while DT_{50} was 6.26 and 5.04 days for 1-ethyl-2-pyrrolidone, respectively. Three metabolites of dodecylbenzenesulfonic acid and five metabolites of 1-ethyl-2-pyrrolidone were found in courgette, while the same metabolites were detected in tangerine samples, except benzoic acid and 4-aminobutanoic acid. These metabolites were identified for the first time from these compounds except for benzoic acid, observing that 5-hydroxy-N-ethylpyrrolidone was the most concentrated metabolite in tangerine 14 days after application, reaching a maximum concentration of 149 μg/kg. Furthermore, some of the detected metabolites possessed a value of LD_{50} lower than their original molecules. In consequence, such metabolites, derived from these co-formulants, should be controlled to prevent negative health effects, and ensure food safety.

1. Introduction

Public authorities and researchers have established that a substantial number of co-formulants used in pesticide products are hazardous to human health and the environment. The European Union (EU) Commission issued a first list of "unacceptable co-formulants" in 2021, featuring 144 chemicals to be prohibited due to their inherent harmful qualities [1]. This list mainly includes solvents such as ethyl and methyl-2-pyrrolidone that are toxic to reproduction, naphtha and petroleum distillates, which are considered mutagenic and carcinogenic, and nonyl, octyl phenols, and their ethoxylated forms, which have endocrine disrupting properties. The acceptable limit for the presence of these substances in the finished product is 0.1 % (*w/w*) [1]. However, the content of most of these substances is not always included in the composition of the product [2], despite the fact that many studies have shown clear evidence that certain co-formulants contained in plant protection products (PPPs), such as alcohol ethoxylates, glyphosate ethoxylated adjuvants and dodecylbenzene sulfonate pose significant dangers to human health, the environment, and/or non-target organisms [3–7]. Finally, fruits and vegetables can contain these compounds, which can have a harmful effect on human health. For this reason, the amount of these compounds in vegetables needs to be controlled.

Few studies have determined the presence of co-formulants in fruits and vegetables derived from PPPs. Some of them were based on gas chromatography coupled to Q-Orbitrap high-resolution mass spectrometry (GC-Q-Orbitrap-HRMS) to analyze volatile co-formulants after applying PPPs in vegetables [8,9]. A study identified volatile co-formulants in tomato, as benzene and naphthalene, terpenoids, terpenes, and dioxolanes after the Altacor 35 WG application [9]. Other co-formulants were detected in tomato and grapes after Mytrus application, such as 1,2,4-trimethylbenzene, mesitylene, 2-ethyl-biphenyl, biphenyl, naphthalene, pentamethylbenzene and *tert*-butylbenzene [8]. Some of

* Corresponding author.
E-mail address: bea91mg@ual.es (B. Martín-García).

https://doi.org/10.1016/j.microc.2024.110442
Received 16 February 2024; Received in revised form 18 March 2024; Accepted 28 March 2024
Available online 30 March 2024

these compounds, as benzene derivative detected compounds, can cause health problems such as skin, eye, and respiratory irritation, and may have narcotic and toxic effects [8]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to analyze less volatile co-formulants in vegetable samples, which were mainly solvents as N,N-dimethyldecanamide and octylpyrrolidone and anionic surfactants as sodium dodecylsulfate, alkyl benzenesulfonates, docusate, and dibutylnaphthalene sulfonate [10–12]. However, these studies were focused on the analysis of target co-formulants that are commonly found at high concentrations in PPPs and have not evaluated their toxicity at the levels found in vegetables. Furthermore, PPPs may contain co-formulants that are not listed in their composition, and these compounds may be harmful and wind up in high proportions in vegetables. A recent study analyzed a total of 37 co-formulants in vegetables, fruits and leaves, performing a suspect screening, and 12 compounds were quantified by LC coupled to Q-Orbitrap high-resolution mass spectrometry (LC-Q-Orbitrap-HRMS) and 9 by GC-Q-Orbitrap-HRMS [13]. The fruits contained a high concentration of 1-ethyl-2-pyrrolidone ranging from 22 μg/kg in strawberries to 722 μg/kg in red grape [13]. This co-formulant has a median lethal dose (LD_{50}) equal to 1.440 g/kg [14]. Therefore, to prevent negative health effects, it is important to control these types of PPP co-formulants in fruits and vegetables.

Furthermore, monitoring the dissipation of co-formulants in vegetable samples after the application of PPPs is crucial to control food product quantities and prevent harmful effects on human health. Previous studies have evaluated the dissipation of these substances in vegetables and fruits under field conditions using LC [10–12] and GC coupled to a triple quadrupole mass spectrometer (QqQ-MS/MS) [8,12]. The study of the dissipation of tristyrylphenol ethoxylates in lettuce showed rapid degradation under greenhouse and field conditions, with half-lives of 2.18–5.39 and 1.82–5.52 days, respectively [12]. Balmer et al. studied the dissipation of co-formulants in vegetables after treatment with PPPs [11,12]. They found that most of the co-formulants were completely degraded in 14 days [11,12]. Specifically, the dissipation of three anionic surfactants (docusate, sodium dodecyl sulfate, sodium salt dibutyl naphthalene sulfonate) and organic solvent N,N-dimethyldecanamide (DMDA) in various vegetables such as leek, celery, rondini, parsley, head lettuce, oak leaf lettuce and apple samples, provided half-lives in the range $<$ 0.3–7 days [11]. These authors have also estimated that the DT_{50} for anionic surfactants, DMDA, octyl pyrrolidone, and 1- and 2- in parsley and lettuce ranged between 0.2 and 8.3 days [12]. Therefore, these compounds differ in their degradation times and the solvents DMDA and octyl-pyrrolidone declined very rapidly (half-life of a few hours) in accordance with their volatility. Another study used GC-HRMS to determine seven co-formulants, detecting 2-methyl-biphenyl and 3-methyl-biphenyl at the highest concentration (71 μg/kg) in tomato and grapes. Furthermore, all of these compounds decreased rapidly until 12 days after application [8].

In these studies, the dissipation of co-formulants in PPPs was only evaluated [8,11,12], but the metabolites that could be produced were not studied although they can be more hazardous than the original chemicals and therefore constitute a health risk. The complexity and variety of co-formulants in PPPs could make it challenging to study metabolites in vegetables derived from specific co-formulants [11]. For that reason, in the present study the individual evaluation of the dissipation of two co-formulants and the possible metabolites derived from them in vegetable samples were proposed. These co-formulants were an anionic surfactant, dodecylbenezenesulfonic acid (DBS), and the 1-ethyl-2-pyrrolidone solvent. They were chosen based on previous studies that evaluated their composition in PPPs by LC-Q-Orbitrap-HRMS, founding DBS at high concentration in many PPPs [14,15] and being 1-ethyl-2-pyrrolidone a highly toxic substance [14]. For all of that, the dissipation of DBS and 1-ethyl-2-pyrrolidone was evaluated in courgette and tangerine for the first time after the application of these co-formulants in laboratory trials by using LC-HRMS. These co-formulants have been applied individually to vegetables to evaluate the possible metabolites derived from this type of compounds. The analysis of metabolites has been carried out performing unknown analyses to search for new compounds using two software (Compound Discoverer v3.2, and MassChemSite 3.1).

2. Materials and methods

2.1. Samples, standards, reagents and equipment

Ecological courgettes were chosen to carry out this study, and they were purchased from Atochares-Pueblo Blanco, Nijar (lot: 230417–25609). Ecological tangerines (Nadorcott) were purchased from Cofrdeca, Coop. V., Av. El Salvador, sn, Belgida, 46,868 Valencia (lot: 435177066351).

Regarding analytical grade standards, DBS (purity 100 %) supplied by Sigma Aldrich (St. Louis, MO, USA), and 1-ethyl-2-pyrrolidone (purity $>$ 98 %) was acquired from TCI (Zwijndrecht, Belgium).

Magnesium sulfate and sodium acetate were purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile (both LC-MS Chromasolv™, $\geq$ 99.9), purchased from Honeywell (Charlotte, NC, USA), and water (LC-MS LiChromasolv®), obtained from Merck (Darmstadt, Germany). Ammonium acetate and ammonium hydroxide (LC-MS, 99.0 %) were acquired from Fischer Scientific (Waltham, MD, USA). Vortex provided by VWR International (Darmstadt, Germany), emulsifier cutter SK-3 from Sammic S.L. (Azkoitia, Spain), and a centrifuge FC5718R 230 V from Ohaus (Parsippany, USA) were used for sample treatment.

2.2. Sample processing

The entire tangerine and courgette samples, including their peel, were previously crushed with the emulsifier cutter according to 90/642/ECC (Council of the European Union, 1990) [16]. Then, an aliquot of the homogenized sample (10 g) was processed according to the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method. For this purpose, 10 g of sample were extracted with 10 mL of acetonitrile for 1 min [11] (tangerine) or 10 mL of acetonitrile with 1 % acetic acid (courgette) [17]. Then 4 g of magnesium sulfate and 1 g of sodium acetate were added to the sample and the mixture was agitated in a vortex for 3 min. All samples were centrifuged at 3700 rpm (3061 *g*) for 10 min. The supernatants were filtered and injected into LC-Orbitrap-MS. Three replicates of each homogenate were processed.

2.3. Laboratory trials

A laboratory trial was carried out during the Spring of 2023 (from April to May) to evaluate the dissipation of the two co-formulants in courgette and tangerine samples. Samples were kept for 21 days under regular laboratory conditions, which implies ambient temperature (15 $\pm$ 5 °C). Courgette and tangerine samples were treated with the standard dilution prepared for each target compound (100 mg/L), to obtain a final dose of 200 μg/kg of each co-formulant in the selected matrices. For that, in the case of courgette, 1 mL of each co-formulant solution at 100 mg/L was applied to each courgette (around 500 g of weight). Regarding tangerines, 0.1 mL of the standard dilution previously indicated (100 mg/L) was applied to each tangerine (approximately 50 g). Courgettes and tangerines were stored at room temperature and three replicates were analysed at 0, 1, 2, 8, 14, and 21 days for each co-formulant. One courgette and four tangerine samples were considered blank samples, and they were not treated with the standard solution. The weights of two blank courgettes and tangerines were checked to control for water loss during the period of the study, and the data was considered in the quantification of co-formulants during the dissipation process.

2.4. LC-Q-Orbitrap-MS conditions

The determination of co-formulants has been previously developed

by our research group [14]. Furthermore, the stationary polymeric phase Shodex ODP2 HP-2D (2 × 150 mm, 5 μm) (Symta, Madrid, Spain) and the LC equipment with the HRMS analyser was previously used and optimized [14].

2.5. Data treatment

The processing of the chromatograms was performed by using Xcalibur version 3.0, using Qual Browser and Quan Browser. Compound Discoverer 3.2 (Thermo Fisher Scientific) and MassChemSite 3.1. (Molecular Discovery Ltd, London, UK) were used for unknown metabolite analysis. The unknown analyses by Compound Discoverer were applied according to various filters: mass accuracy limit of 10ppm, no peak area in any blank, and good peak shape as a symmetrical or Gaussian peak. After application of these filters, detected compounds were individually checked by searching for the extracted ion in Xcalibur. The peak shape and structure of each compound were visualized. Therefore, molecules that did not match the structures derived from co-formulants, had an irregular peak shape, or had an excessively low S/N ratio were not further considered.

2.6. Data calculation

The dissipation of DBS and 1-ethyl-2-pyrrolidone was determined by plotting the concentration of these compounds versus time. A single first order (SFO) was fitted to the model. The residual concentration, or final concentration (Ct) and the half-life time (DT_{50}) were calculated using Eqs. (1) and (2) for the single first-order model [18]:

$$C(t) = C_0 e^{-kt} \quad (1)$$

$$DT_{50} = \frac{ln2}{k} \quad (2)$$

Where C_0 and C(t) are the concentrations expressed as μg/kg of the co-formulants in the sample (either courgette or tangerine) at time 0 and time t (day) respectively, while k is the dissipation kinetic rate constant ($days^{-1}$).

3. Results and discussion

3.1. Method validation

Validation of the method was carried out for the two co-formulants under study according to the parameters proposed by the SANTE guideline [19]. Table 1 shows the analytical parameters that were evaluated: linearity, intra and inter day recovery and precision, limit of quantification and matrix effect.

Linearity was tested by injecting a matrix-matched calibration, injecting blank samples extracted at the concentrations of 5, 10, 25, 50, 100 and 200 μg/kg. Regression coefficients were higher than 0.9945 for the two co-formulants in the two target matrices.

The matrix effect was determined by comparing the slope obtained by matrix-matched calibration used for linearity with a solvent calibration at the same concentrations (Eq. (3).

$$ME = \left(\frac{\text{slope of the line for the matrix}}{\text{slope of the line for the solvent}} - 1\right) x100 \quad (3)$$

The matrix effect was 2.2 % in courgette and 19.4 % in tangerine for DBS, and −12.5 % and −15.1 % for 1-ethyl-2-pyrrolidone in courgette and tangerine, respectively. Thus, soft matrix effects were observed for all compound/matrix combinations, as these values were within the range between −20 and + 20 [20]. Thus, standard calibration curves were prepared in solvent for quantification purposes.

The LOQs were set at 5 μg/kg for courgettes and tangerines for both compounds as it is the lowest concentration that provides acceptable recovery and precision. Recoveries of the two target co-formulants were evaluated by comparing the standard prepared in the extract from blank samples with that obtained by adding in the sample at a concentration of 5 and 200 μg/kg before the extraction (n = 5). Intra-day and inter-day recovery at 5 and 200 μg/kg were 90.0–105.3 % for DBS and 94.4–119.1 % in courgette and tangerine, while the intraday and interday recovery at 5 and 200 μg/kg of 1-ethyl-2-pyrrolidone was 95.5–103.6 % and 82.7–84.1 % in courgette and tangerine, respectively. Furthermore, intra-day and inter-day precision (%) was evaluated by analyzing the standards of target co-formulants at 5 and 200 μg/kg prepared in the blank samples of courgette and tangerine (n = 5). Intraday precision (% RSD) at 5 μg/kg was lower than 6.8 % and at 200 μg/kg was lower than 4.8 % in all cases, while inter-day precision at 5 μg/kg was lower than 8.4 % in all cases and at 200 μg/kg was lower than 7.8 %.

3.2. Dissipation study of co-formulants in courgette and tangerine

The dissipation profile of DBS and 1-ethyl-2-pyrrolidone was evaluated. For that purpose, co-formulant concentrations were measured at each sampling time. Three kinetic models (zero order, first order and second order) were evaluated. Table 2 shows the parameters of the kinetic models for the dissipation of each co-formulant in the samples under study. The R^2 value provided a suitable fit for all cases with the first order model, which was higher than 0.99. The R^2 in the second order also showed good adjustment values. Previous studies have also employed a single first-order (SFO) rate model kinetics to study the dissipation of co-formulant residues from PPPs in vegetables and apples under field conditions [11,12]. Therefore, the selection of the SFO model for the current study was based on its good fit, similarity to previous studies that have successfully applied it to analyse the dissipation of co-formulants. Fig. 1 shows the variation in DBS and 1-ethyl-2-pyrrolidone concentrations in courgette and tangerine samples using SFO kinetics. In courgette, DBS had an initial concentration of 182 μg/kg, gradually decreasing to 19 μg/kg eight days after applying the product. Then, the concentration of DBS was below the LOQ. In tangerine, DBS started to decrease its concentration from 235 μg/kg to 6 μg/kg after 21 days. The DT_{50} values for DBS in courgette and tangerine were similar (1.83 and 1.42 days) (Table 2). In the case of 1-ethyl-2-pyrrolidonde, the initial concentration (C_0) was 168 μg/kg in courgette, which began to degrade until it reached its final concentration of 16 μg/kg 21 days after the application of the product. Concerning tangerine, 1-ethyl-2-pyrrolidone showed a similar behaviour as observed in courgette, with a decrease from 197 μg/kg at 0 days to 11 μg/kg at 21 days.

Table 1
Validation parameters for dodecylbenzenesulfonic acid and 1-ethyl-2-pyrrolidone in courgette and tangerine.

Samples	Co-formulants	Linearity (R^2)	Matrix effect	Recovery, % (RSD, %)* at 5 μg/kg		Recovery, % (RSD, %)* at 200 μg/kg		LOQ (μg/kg)
				Intraday	Interday	Intraday	Interday	
Courgette	Dodecylbenzenesulfonic acid	0.9974	2.2	95.4 (6.8)	90.0 (8.4)	103.4 (4.8)	105.3 (5.9)	5
	1-Ethyl-2-pyrrolidone	0.9963	−12.5	95.5 (4.5)	100.4 (7.4)	102.9 (4.6)	103.6 (4.5)	5
Tangerine	Dodecylbenzenesulfonic acid	0.9945	19.4	94.4 (5.5)	96.4 (3.3)	119.1 (3.5)	117.7 (3.5)	5
	1-Ethyl-2-pyrrolidone	0.9967	−15.1	82.8 (1.5)	82.7 (3.3)	84.1 (2.1	83.0 (7.8)	5

*n = 5.

Table 2
Single phase model kinetic parameters for dodecylbenzenesulfonic acid and 1-ethyl-2-pyrrolidone dissipation in courgette and tangerine.

Kinetic parameters	Dodecylbenzenesulfonic acid						1-Ethyl-2-pyrrolidone					
	Courgette			Tangerine			Courgette			Tangerine		
	Zero	First	Second	Zero	First	Second	Zero	First	Second	Zero	First	Second
C_0 (μg/kg)	135.17	176.91	184.34	162.66	230.04	236.54	157.92	167.61	172.94	180.28	196.87	213.34
k ($days^{-1}$)	11.11	0.38	0.0038	13.24	0.48	0.0037	9.16	0.11	0.0011	11.45	0.13	0.0014
DT_{50}	6.08	1.83	1.41	6.14	1.42	1.14	8.62	6.26	5.06	7.87	5.04	3.28
R^2	0.9136	0.9952	0.9883	0.8607	0.9929	0.9943	0.9895	0.9935	0.9780	0.9778	0.9943	0.9973

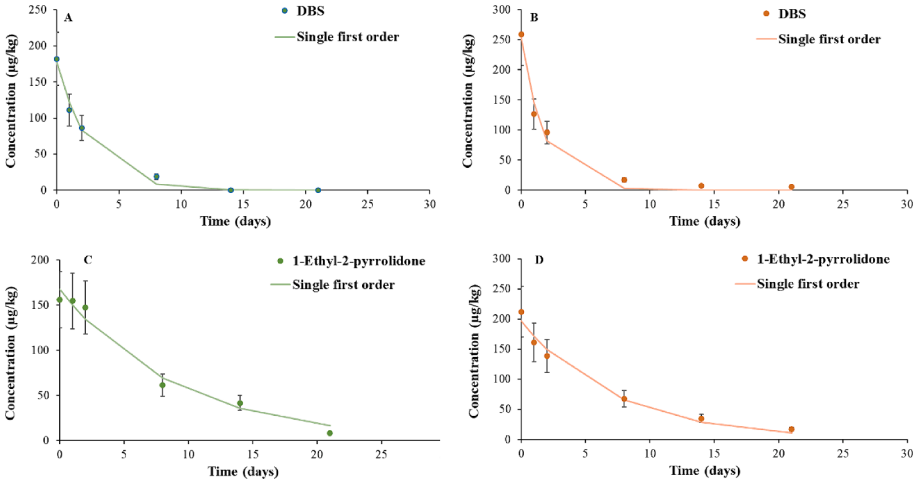

Fig. 1. First-order single kinetic model of DBS and 1-ethy-2-pyrrolidone in courgette (A,C) and tangerine (B,D). The error bars correspond to n = 3.

The 1-ethyl-2-pyrrolidone content slowly decreased from 14 to 21 days. DT_{50} values for 1-ethyl-2-pyrrolidone were also similar for the studied samples (6.26 days in courgette and 5.05 days in tangerine).

The DT_{50} results for DBS were comparable to those obtained by previous research, which found a DT_{50} value of 1.5 days for linear alkylbenzenesulfonates. This fact could be due to DBS belongs to this family of alkylbenzene sulfonate [12]. Furthermore, 1-octyl-2-pyrrolidone is a derivative of 1-ethyl-2-pyrrolidone, whose DT_{50} in an open field is 3.9 days in parsley, while in lettuce it is 0.46 days [12]. These DT_{50} values were lower than the obtained for 1-ethyl-2-pyrrolidone. However, the decline of plant residues is influenced by several factors, such as volatilization, chemical degradation, and plant metabolism. Hence, the degradation of target co-formulants has been demonstrated using simple kinetic approaches.

3.3. Co-formulant metabolites

3.3.1. Identification of metabolites

The next step was the evaluation of the dissipation of these molecules in metabolites, and the tentative identification of metabolites was carried out through an unknown analysis by MassChemSite 3.1 and Compound Discoverer. MassChemSite is a useful tool for elucidating the

Table 3
Tentative identification of metabolites derived from dodecylbenzenesulfonic acid and 1-ethyl-2-pyrrolidone in courgette and tangerine by unknown analyses.

N°	Compound name	Molecular formula	Retention time (min)	Adduct	Characteristic ions		Fragment ions			Samples	Software
					Theoretical mass (*m/z*)	Mass error (ppm)	Molecular formula	Theoretical mass (*m/z*)	Mass error (ppm)		
Dodecylbenzenesulfonic acid											
1	Benzoic acid	$C_7H_6O_2$	2.61	$[M+H]^+$	123.0441	0.520	C_6H_6 $C_7H_4O_2$	79.0542 121.0284	6.998 0.860	Both samples	Compound Discoverer
2	4-phenolsulfonic acid	$C_6H_6O_4S$	1.28	$[M-H]^-$	172.9906	1.411	C_6H_6O C_4H_4O	93.0346 67.0189	−2.487 −1.161	Both samples	Compound Discoverer
3	12-Phenyldodecanoic acid	$C_{18}H_{28}O_2$	1.59	$[M+H]^+$	277.2159	−1.828	$C_{18}H_{26}O_2$ $C_{18}H_{24}O$	275.2005 257.1899	−1.078 −2.457	Both samples	Compound Discoverer
1-Ethyl-2-pyrrolidone											
1	N-Ethylsuccinimide	$C_6H_{10}O_2N$	2.66	$[M+H]^+$	128.0705	−0.352	C_5H_9ON	100.0757	4.092	Both samples	MassChemSite
2	5-Hydroxy-N-ethylpyrrolidone	$C_6H_{11}O_2N$	2.63	$[M+H]^+$	130.0862	−0.578	C_5H_9N	84.0808	7.542	Both samples	MassChemSite
3	4-(Ethylamino) butanoic acid	$C_6H_{13}NO_2$	2.62	$[M+H]^+$	132.1019	−0.721	$C_5H_{11}N$ C_4H_5ON	86.0964 84.0444	5.854 6.183	Both samples	MassChemSite
4	4-Aminobutanoic acid	$C_4H_9NO_2$	2.66	$[M+H]^+$	104.0709	2.449	$C_4H_6O_2$ C_4H_7ON	87.0441 86.0600	7.513 6.036	Courgette	Compound Discoverer MassChemSite
5	Proline	$C_5H_9NO_2$	2.67	$[M+H]^+$	116.0708	1.076	C_4H_9N $C_4H_6O_2$	72.0808 87.0440	7.548 4.986	Both samples	Compound Discoverer

structure of organic molecules produced in chemical reactions based on HRMS [21]. This software has been used for forced degradation studies, allowed automatic identification of the products obtained from the original molecules providing their molecular structures, and the name reactions imply to obtain these metabolites [22]. Additionally, Compound Discoverer selected metabolites by searching through the list of unknown compounds and selected those whose structures could be related to the original molecules under study. Table 3 shows the identification parameters of the tentative identification of metabolites derived from the studied co-formulants. These parameters include the compound name, molecular formula, retention time, adduct, theoretical mass, and mass errors from the characteristic and fragment ions. The fragment ions found for each metabolite were confirmed by the fragments obtained by Mass Frontier. The mass error established for the characteristic ions was below 5 ppm, whereas for the fragment ions the mass error was lower than 10 ppm. This could be explained by the fact that the mass error depends on the intensity of the signal. The lower the number of ions measured, the higher the mass error; therefore, low-abundance fragments often show higher mass errors in comparison with their original molecular ion [23]. Additionally, according to a previous study that used the same LC-Q-Orbitrap-MS, the mass error had to be lower than 10 ppm for fragment ions for triazole compounds and metabolites, including triazole derivative metabolites [24]. According to the level of confidence provided by [25], the identified metabolites were classified at level 2. The structure of the molecule is shown in Fig. 2. Regarding the metabolites derived from DBS, a total of 3 metabolites were putatively identified by unknown analyses and their structures are also shown in Fig. 2. All of them were detected in the two samples under study with the exception of benzoic acid that has not been detected in tangerine. Fig. 1S shows the mass spectrum (MS) and fragmentation mass spectrum (MS^2) of each metabolite derived from DBS in courgette at 14 days. The tentatively identified metabolites were benzoic acid, 4-phenolsulfonic acid and 12-phenyldodecanoic acid, which were detected by Compound Discoverer software. Benzoic acid and 12-phenyldodecanoic acid were detected in the positive mode, while 4-phenolsulfonic acid was detected in the negative mode. The benzoic acid metabolite has been previously detected by biodegradation via new pathways in *Alcanivorax sp*. strain MBIC 4326. This metabolite could be derived from a previous desulfonation, and then the carboxylic group is formed by the oxidation of the terminal methyl group of the alkyl side chain of DBS. Subsequently, carboxylic or acetic acid derivatives are produced by a classical β-oxidation [26]. This compound was detected at *m/z* 123.0441, and a fragment ion at *m/z* 79.0542 that was obtained from the loss of the carboxylic group [27]. The compound 4-phenolsulfonic acid detected at *m/z* 172.9906 $[M-H]^-$ showed the most abundant fragment ion at *m/z* 93.0346, which was derived from the loss of sulfonate group. 12-Phenyldodecanoic acid, detected at 2.55 min, showed an abundant fragment ion at *m/z* 257.1899, which corresponded to the loss of a hydroxyl group and the formation of a double bond at the C_3-C_4 position (Fig. 2).

Additionally, five unknown metabolites were detected from 1-ethyl-2-pyrrolidone: three of them, N-ethylsuccinimide, 5-hydroxy-N-ethylpyrrolidone and 4-(ethylamino)butanoic acid, tentatively identified using MassChemSite software, while compound 4-aminobutanoic acid was detected by Compound Discoverer and MassChemSite, and compound proline was only detected by Compound Discoverer software. The MS and MS^2 of 1-ethyl-2-pyrrolidone metabolites in tangerine at 21 days shows in the **Fig. S2.** According to a previous study of novel biomarkers and age-related metabolite correlations in plasma, proline is obtained from 2-pyrrolidone [28]. Proline was detected in both samples, while 4-aminobutanoic acid was only detected in courgette samples. N-ethylsuccinimide was identified at *m/z* 128.0705 $[M+H]^+$, which possessed a fragment ion at *m/z* 100.0757 $[M+H]^+$ that was derived from the loss of the ethyl group, obtaining unsaturated 5-hydroxypyrrolidin-2-one. 5-Hydroxy-N-ethylpyrrolidone detected at 2.63 min with *m/z* 130.0862 possessed at *m/z* 84.0808 an abundant fragment ion. Fig. 3 shows the tentative identification of 4-(ethyalmino)butanoic at a retention time of 2.65 min. According to its MS^2 spectrum, the compound 4-(ethylamino) butanoic acid has a fragment ion at *m/z* 86.0964, which was obtained from the loss of carboxylic acid (Fig. 2S). 4-Aminobutanoic acid was identified at *m/z* 104.0709, which showed two fragment ions at *m/z* 87.0441 and 86.0600. Among these fragments, the most abundant was found at *m/z* 87.0441, which was due to the loss of the amine group, obtaining 4-dihydroxy-1-butenyl. Finally, the compound at 2.67 min and *m/z* 116.0708 $[M+H]^+$ was detected as proline. Its fragment ion at *m/z* 72.0808 is attributed to the rupture of pyrrolidone and the loss of carboxylic acid in this compound. Fig. 4 shows the structures of the metabolites derived from 1-ethyl-2-pyrrolidone and the corresponding mechanisms to obtain these transformation products.

A previous study reported the metabolic pathway of a similar

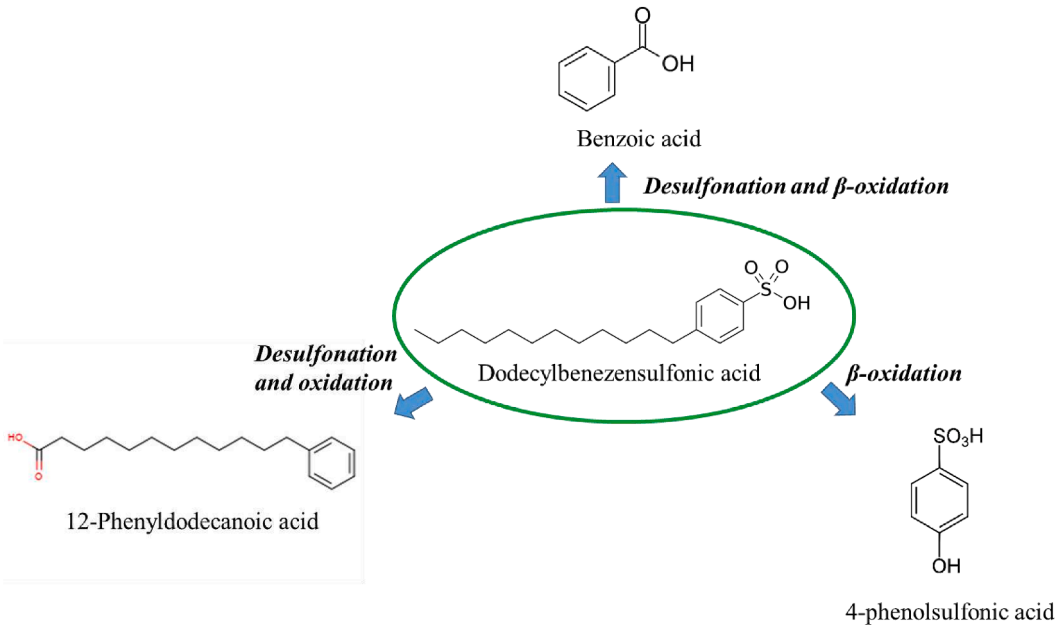

Fig. 2. Scheme of transformation mechanisms of DBS metabolites detected in courgette and tangerine.

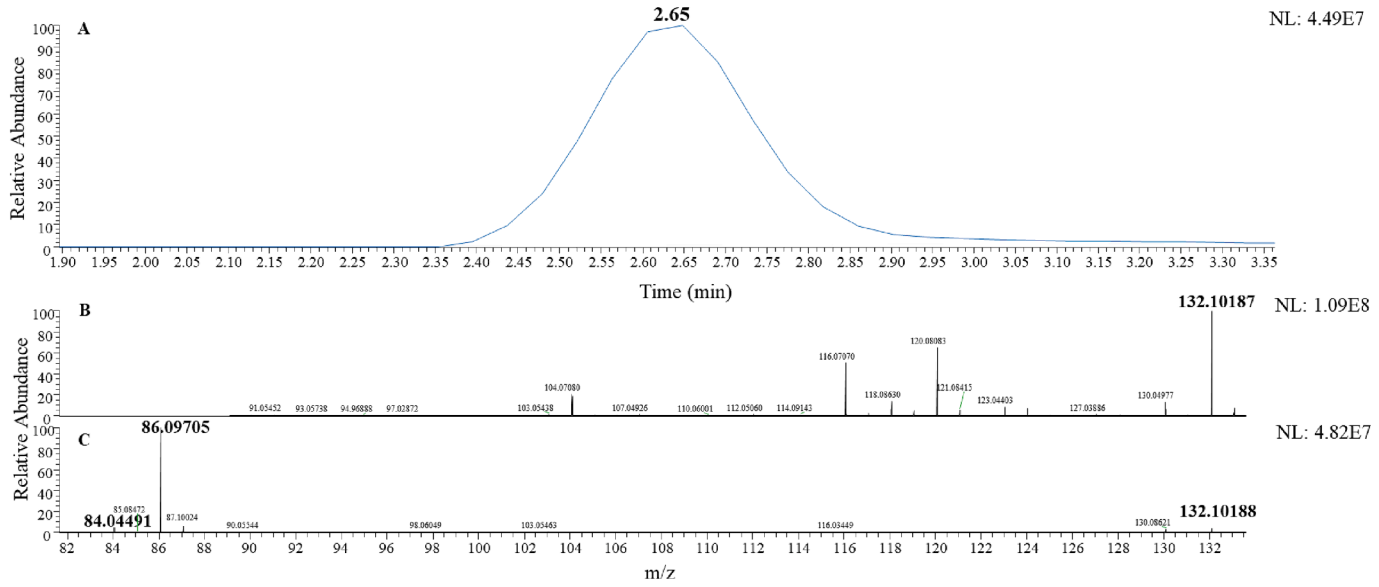

Fig. 3. Tentative identification of 4-(ethyalmino)butanoic acid: (A) extracted ion chromatogram; (B) full-scan MS spectrum showing $[M + H]^+$ adducts, and (C) $ddMS^2$ spectrum of the ion *m/z* 132.1019.

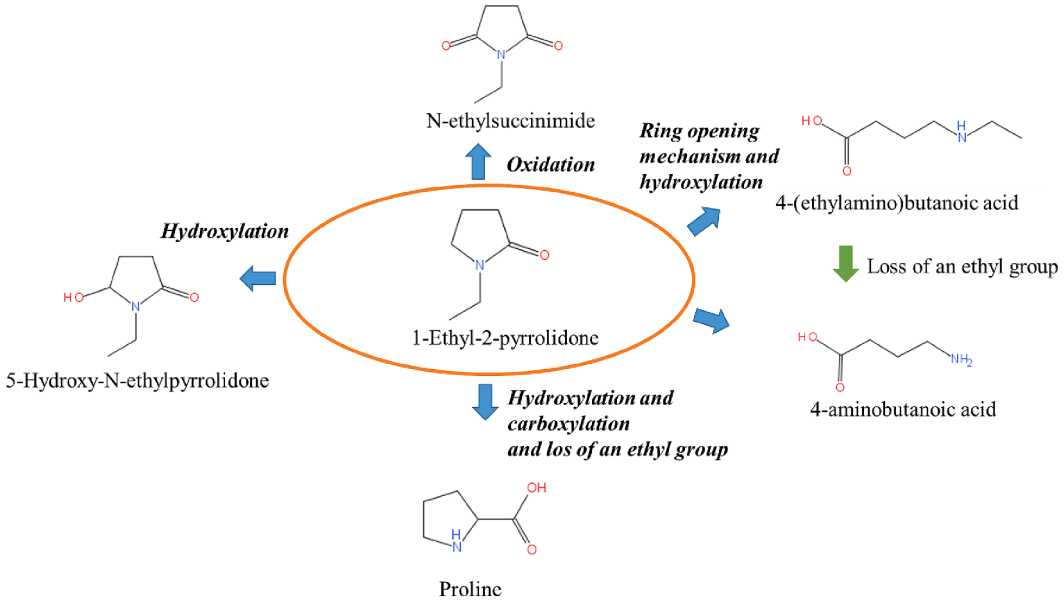

Fig. 4. Scheme of transformation mechanisms of 1-ethyl-2-pyrrolidone metabolites detected in courgette and tangerine.

compound (N-methyl-2-pyrrolidone) in humans. This study found 2 common metabolites of N-methyl-2-pyrrolidone, which were 5-hydroxy-N-methyl-2-pyrrolidone and N-methylsuccinimide in urine [29]. Therefore, in the present samples 5-hydroxy-N-ethyl-2-pyrrolidone at *m/z* 130.0862 and N-ethylsuccinimide at *m/z* 128.0705 were detected. These compounds were derived from the hydroxylation of 1-ethyl-2-pyrrolidone and the oxidation of 1-ethyl-2-pyrrolidone. Furthermore, compound 4-(ethylamino)butanoic acid can be proceeded by means of a ring opening mechanism, causing the formation of N-ethyl-4-aminobutanoic acid [30].

3.3.2. Degradation of metabolites

Table 5 shows the degradation curves of DBS and 1-ethyl-2-pyrrolidone for courgette and tangerine. As a result of the absence of standards, the metabolites were semi-quantified by using the standards of their precursor compounds.

Table 4
Toxicological information on target co-formulants and their metabolites.[a]

Compound	LD_{50} (g/kg)[a]
DBS	1.551
Benzoic acid	1.262
4-Phenolsulfonic acid	2.416
12-Phenyldodecanoic acid	6.964
1-Ethyl-2-pyrrolidone	1.440
N-Ethylsuccinimide	0.168
5-Hydroxy-N-ethylpyrrolidone	0.196
4-(ethyl amino) Butanoic acid	3.233
4-Aminobutanoic acid	2.436
Proline	1.238

[a] Abbreviation: LD_{50}: Median lethal dose estimated by the Toxicity Estimation Software Tool (T.E.S.T).

Table 5
Estimated concentration of DBS and 1-ethyl-2-pyrrolidone metabolites for courgette and tangerine. The quantities for all metabolites are expressed as μg/kg.

DBS metabolites						
Time (days)	**Courgette**			**Tangerine**		
	4-Phenolsulfonic acid	12-Phenyldodecanoic acid	Benzoic acid	4-Phenolsulfonic acid	12-Phenyldodecanoic acid	
0	N.D.	5.6	7.7	<LOQ	<LOQ	
1	N.D.	23.9	9.5	<LOQ	5.8	
2	N.D.	36.4	9.6	<LOQ	24.7	
8	N.D.	40.6	16.7	<LOQ	34.6	
14	5.1	47.7	12.2	<LOQ	40.8	
21	6.0	48.5	N.D.	5.2	59.6	

1-Ethyl-2-pyrrolidone metabolites									
Times (days)	**Courgette**					**Tangerine**			
	N-Ethyl succinimide	5-Hydroxy-N-ethyl-2-pyrrolidone	4-(Ethylamino) butanoic acid	4-Aminobutanoic acid	Proline	N-Ethyl succinimide	5-Hydroxy-N-ethyl-2-pyrrolidone	4-(Ethylamino) butanoic acid	Proline
0	N.D.	3.2	N.D.	8.0	N.D.	14.2	44.0	N.D.	21.6
1	N.D.	4.7	N.D.	10.8	N.D.	14.1	77.6	N.D.	15.3
2	N.D.	16.9	71.6	24.8	20.0	47.9	109.6	N.D.	50.6
8	13.1	16.4	66.1	29.5	23.1	48.7	131.1	16.6	54.5
14	9.3	22.1	106.9	36.8	52.3	66.1	148.6	35.7	67.1
21	N.D.	23.0	112.2	41.2	56.4	63.4	146.4	17.6	65.9

LOQ: Limit of quantification; N.D: Not detected.

In relation to DBS metabolites, two of them, including 4-phenolsulfonic acid, and 12-phenyldodecanoic acid were quantified in both samples, while benzoic acid was only found in courgette (Table 5). A clear increase in the concentrations of these metabolites can be observed in both samples. For metabolites derived from DBS, the highest content of most metabolites in courgette was obtained 14 days after the application of the co-formulants, while in tangerine it was at 21 days. Additionally, benzoic acid was only detected in courgette and its maximum concentration was 17 μg/kg, which was achieved after 8 days. It should be noted that the most concentrated degradation product of DBS was 12-phenyldodecanoic acid in both target samples, reaching concentrations of 48 and 53 μg/kg in day 21 in courgette and tangerine samples. However, the highest increase in its content was shown from 0 to 8 days. This result could be explained because the DBS was completely degraded after 8 days.

The content of 1-ethyl-2-pyrrolidone metabolites in courgette and tangerine increased between day 0 and 14, but remained constant or slightly decreased until day 21, except for N-ethylsuccinimide in courgette, which reached its highest content at 13 μg/kg after 8 days of application. The most concentrated metabolite in courgette was 4-(ethylamino)butanoic acid, whose concentration reached 107 μg/kg at day 14, and it was similar to that obtained on day 21 (112 μg/kg). However, the most concentrated metabolite derived from 1-ethyl-2-pyrrolidone in tangerine was 5-hydroxy-N-ethyl-2-pyrrolidone, reaching a content of 149 μg/kg in day 14, keeping its concentrations until the end of the study. Proline was the second most concentrated metabolite in both samples, reaching a similar level over 14 days in courgette and tangerine (52 and 67 μg/kg). Therefore, the fact that most metabolites derived from 1-ethyl-2-pyrrolidone increase their concentration 14 days after the application could be justified by the fact that the original molecule degraded almost completely 14 days after its application (Fig. 1).

3.4. Toxicity of metabolites derived from co-formulants

Toxicological information on metabolites is required to know which are a risk to health. According to the safety data sheet, the oral median lethal dose (LD_{50}) in rats for benzoic acid, phenolsulfonic acid and proline was 1700, 1900 and 5110 mg/kg and the LD_{50} in mice for aminobutanoic acid was 12680 mg/kg. However, LD_{50} is not available for the rest of the metabolites, but previous studies have used the Toxicity Estimation Software Tool (T.E.S.T) to estimate the toxicity of drugs and pesticides' metabolites [2,31]. Therefore, this tool was used to estimate and compare the LD_{50} of these metabolites derived from the co-formulants under study. LD_{50} values of the metabolites are shown in Table 4. Regarding the DBS metabolites, a similar LD_{50} of benzoic acid (1.262 g/kg) and its parent compound (1.551 g/kg) could be observed. In the literature, the reference dose for chronic oral exposure (RfD) to benzoic acid is 4 mg/kg/day. However, this metabolite was only found in courgette at a low concentration level of 16.67 μg/kg and completely degraded after 21 days. The rest of the metabolites derived from DBS could be less toxic than their original molecule because these possess a higher predictable LD_{50} in comparison with their precursor DBS.

Regarding the metabolites of 1-ethyl-2-pyrrolidone, it should be noted that the estimated LD_{50} values for N-ethylsuccinimide (0.168 g/kg), 5-hydroxy-N-ethylpyrrolidone (0.196 g/kg) and proline (1.238 g/kg) were lower than the value of the parent compound (1.440 g/kg), indicating that N-ethylsuccinimide and 5-hydroxy-N-ethylpyrrolidone could be ten times more toxic than their original molecule. Among these substances, the most concentrated metabolite in tangerine was 5-hydroxy-N-ethylpyrrolidone, whose content at 21 days after the application of the co-formulant represented around 69 % of the concentration at 0 days of its original molecule. Proline was also detected at high quantities in both samples at day 21, and its content was approximately 31 and 36 % from the original concentration of 1-ethyl-2-pyrrolidone. Proline at 50 mg/kg body weight/day is toxic and results in liver and kidney lesions [32]. For that reason, the amount of these substances in vegetables should be controlled and toxicity tests should be performed to determine their potential health-threatening levels.

4. Conclusions

An efficient method based on the use of LC-HRMS has been used for the evaluation of the dissipation, as well as the identification of potential metabolites of two co-formulants derived from PPPs in two vegetable samples for the first time. Specifically, the dissipation of DBS and 1-ethyl-2-pyrrolidone was carried out under laboratory conditions in courgette and tangerine and applying a SFO model. The half-lives show that DBS was degraded three times faster than 1-ethyl-2-pyrrolidone. Tangerine has a slightly faster degradation of both compounds (0.41 and 1.21 days lower) compared to courgette.

The proposed LC-HRMS method allowed the tentative identification of three DBS and five 1-ethyl-2-pyrrolidone metabolites in courgette and three DBS and four 1-ethyl-2-pyrrolidone metabolites in tangerine

samples. According to their LD_{50}, N-ethylsuccinimide, 5-hydroxy-N-ethylpyrrolidone and proline were even more toxic than their original molecule, 1-ethyl-2-pyrrolidone. Additionally, most metabolites are quite persistent (they are detected at 21 days with high concentrations). Therefore, this research is very important to understand the natural behaviour and dissipation process of DBS and 1-ethyl-2-pyrrolidone and their derived metabolites to ensure food safety.

CRediT authorship contribution statement

Beatriz Martín-García: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Roberto Romero-González:** Writing – review & editing, Supervision, Conceptualization. **Francisco Javier Egea González:** Supervision. **Antonia Garrido Frenich:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

Acknowledgments

The authors express their gratitude to the Spanish Ministry of Economy and Competitiveness (MINECO), Spain, and FEDER-EU (project ref. PID2019-106201RB-I00) for financial support. B.M.G. acknowledges to the Ministry of Science and Innovation (Agency State Research) MCIN/AEI/10.13039/501100011033 and the European Union "NextGenerationEU/PRTR for financial support from 'Juan de la Cierva', ref FJC2020-046307-I.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2024.110442.

References

[1] Official Journal of the European Union, Commission regulation (EU) 2021/383 of 3 March 2021 amending Annex III to Regulation (EC) No 1107/2009 of the European Parliament and of the Council listing co-formulants which are not accepted for inclusion in plant protection products, 74 (2021).
[2] M.E. Hergueta-Castillo, R. López-Ruiz, P. Marín Membrive, R. Romero-González, A. Garrido Frenich, Dissipation of penconazole formulation in horticultural crops by ultrahigh performance liquid chromatography-high resolution mass spectrometry: from the active substance to metabolites, Food Chem. 422 (2023), https://doi.org/10.1016/j.foodchem.2023.136266.
[3] E.A. Straw, M.J.F. Brown, Co-formulant in a commercial fungicide product causes lethal and sub-lethal effects in bumble bees, Sci. Rep. 11 (2021), https://doi.org/10.1038/s41598-021-00919-x.
[4] R. Mesnage, B. Bernay, G.E. Séralini, Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity, Toxicology 313 (2013) 122–128, https://doi.org/10.1016/j.tox.2012.09.006.
[5] E. Adams, V. Gerstle, T. Schmitt, C.A. Brühl, Co-formulants and adjuvants affect the acute aquatic and terrestrial toxicity of a cycloxydim herbicide formulation to european common frogs (Rana temporaria), Sci. Total Environ. 789 (2021), https://doi.org/10.1016/j.scitotenv.2021.147865.
[6] S. Klátyik, G. Simon, M. Oláh, R. Mesnage, M.N. Antoniou, J.G. Zaller, A. Székács, Terrestrial ecotoxicity of glyphosate, its formulations, and co-formulants: evidence from 2010–2023, Environ. Sci. Eur. 35 (2023), https://doi.org/10.1186/s12302-023-00758-9.
[7] M. Karaca, B.C. Fischer, C.T. Willenbockel, T. Tralau, P. Marx-Stoelting, D. Bloch, Effects of co-formulants on the absorption and secretion of active substances in plant protection products in vitro, Arch. Toxicol. 95 (2021) 3205–3221, https://doi.org/10.1007/s00204-021-03140-x.
[8] J. Marín-Sáez, R. López-Ruiz, R. Romero-Gonzalez, A. Garrido Frenich, I. Zamora Rico, Looking beyond the active substance: comprehensive dissipation study of myclobutanil-based plant protection products in tomatoes and grapes using chromatographic techniques coupled to high-resolution mass spectrometry, J. Agric. Food Chem. 70 (2022) 6385–6396, https://doi.org/10.1021/acs.jafc.2c01767.
[9] A.J. Maldonado-Reina, R. López-Ruiz, J. Marín Sáez, R. Romero-González, P. Marín-Membrive, A. Garrido-Frenich, Uncovering the dissipation of chlorantraniliprole in tomatoes: identifying transformation products (TPs) and coformulants in greenhouse and laboratory studies by UHPLC-Q-orbitrap-MS and GC-Q-orbitrap-MSJ, J. Agric. Food Chem. 71 (2023) 7230–7238, https://doi.org/10.1021/acs.jafc.3c00816.
[10] C. Li, P. Zhang, Q. He, H. Shao, L. Zheng, J. Wang, F. Jin, Dissipation profiles of tristyrylphenol ethoxylate homologs in lettuce under greenhouse and field conditions, J. Agric. Food Chem. 68 (2020) 1507–1513, https://doi.org/10.1021/acs.jafc.9b03678.
[11] M.E. Balmer, D. Janser, U. Schaller, J. Krauss, H.C. Geiserc, T. Poiger, Magnitude and decline of pesticide co-formulant residues in vegetables and fruits: results from field trials compared to estimated values, Pest Manag. Sci. 77 (2020) 1187–1196, https://doi.org/10.1002/ps.6128.
[12] M.E. Balmer, D. Janser, U. Schaller, J. Krauss, H.C. Geiser, T. Poiger, Residues of pesticide co-formulants in lettuce and parsley: identification of decline processes using field trials in different cropping systems, Pest Manag. Sci. 79 (2023) 2792–2800, https://doi.org/10.1002/ps.7455.
[13] B. Martín-García, R. Romero-González, A. Garrido Frenich, Suspect screening of pesticide co-formulants in fruits, vegetables and leaves by liquid and gas chromatography coupled to high resolution mass accuracy spectrometry: potential impact on human health, Food Chem. 434 (2024), https://doi.org/10.1016/j.foodchem.2023.137555.
[14] B. Martín-García, R. Romero-González, J.L. Martínez Vidal, A. Garrido Frenich, Unveiling coformulants in plant protection products by LC-HRMS using a polyhydroxy methacrylate stationary phase, J. Agric. Food Chem. 71 (2023) 15842–15854, https://doi.org/10.1021/acs.jafc.3c03600.
[15] A.J. Maldonado-Reina, R. López-Ruiz, R. Romero-González, J.L. Martínez Vidal, A. Garrido-Frenich, Assessment of co-formulants in marketed plant protection products by LC-Q-orbitrap-MS: application of a hybrid data treatment strategy combining suspect screening and unknown analysis, J. Agric. Food Chem. 71 (19) (2023) 7230–7238, https://doi.org/10.1021/acs.jafc.2c01152.
[16] 90/642/EEC Official Journal of the European Communities (1990), http://data.europa.eu/eli/dir/1990/642/oj. (accessed January 31, 2024).
[17] J.J. Schenck, A.N. Brown, L.V. Podhorniak, A. Parker, M. Reliford, J.W. Wong, A rapid multiresidue method for determination of pesticides in fruits and vegetables by using acetonitrile extraction/partitioning and solid-phase extraction column cleanup, J. AOAC Int. 91 (2) (2008) 422–438, https://doi.org/10.1093/JAOAC/91.2.422.
[18] P. Fantke, Variability of pesticide dissipation half-lives in plants, Environ. Sci. Tech. 47 (2013) 3548–3562, https://doi.org/10.1021/es303525x.
[19] SANTE/11312. (2021). Guidance Document on Analytical Quality Control and Method Validation for Pesticide Residues Analysis in Food and Feed Safety of the Food Chain Pesticides and Biocides. European Commission, 2021, pp. 1–57.
[20] B. Zhang, X. Chen, S.Y. Han, M. Li, T.Z. Ma, W.J. Sheng, X. Zhu, Simultaneous analysis of 20 mycotoxins in grapes and wines from hexi corridor region (China): based on a QuEChERS–UHPLC–MS/MS method, Molecules 23 (8) (2018) 1–17, https://doi.org/10.3390/molecules23081926.
[21] R. López-Ruiz, R. Romero-González, B. Serra, A. Garrido Frenich, Dissipation kinetic studies of fenamidone and propamocarb in vegetables under greenhouse conditions using liquid and gas chromatography coupled to high-resolution mass spectrometry, Chemosphere 226 (2019) 36–46, https://doi.org/10.1016/j.chemosphere.2019.03.118.
[22] S. Bonciarelli, J. Desantis, S. Cerquiglini, L. Goracci, MassChemSite for in-depth forced degradation analysis of PARP inhibitors olaparib, rucaparib, and niraparib, ACS Omega 8 (2023) 7005–7016, https://doi.org/10.1021/acsomega.2c07815.
[23] K.F. Blom, Estimating the precision of exact mass measurements on an orthogonal time-of-flight mass spectrometer, Anal. Chem. 73 (2001) 715–719, https://doi.org/10.1021/ac001064v.
[24] M.E. Hergueta-Castillo, E. López-Rodríguez, R. López-Ruiz, R. Romero-González, A. Garrido Frenich, Targeted and untargeted analysis of triazole fungicides and their metabolites in fruits and vegetables by UHPLC-orbitrap-MS2, Food Chem. 368 (2022), https://doi.org/10.1016/j.foodchem.2021.130860.
[25] E.L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H.P. Singer, J. Hollender, Identifying small molecules via high resolution mass spectrometry: Communicating confidence, Environ. Sci. Tech. 48 (2014) 2097–2098, https://doi.org/10.1021/es5002105.
[26] T.K. Dutta, S. Harayama, Biodegradation of n-Alkylcycloalkanes and n-Alkylbenzenes via New Pathways in Alcanivorax sp. Strain MBIC 4326, Appl. Environ. Microbiol. 67 (2001) 1970–1974, https://doi.org/10.1128/AEM.67.4.1970-1974.2001.
[27] N. Penner, R. Ramanathan, J. Zgoda Pols, S. Chowdhury, Quantitative determination of hippuric and benzoic acids in urine by LC-MS/MS using surrogate standards, J. Pharm. Biomed. Anal. 52 (2010) 534–543, https://doi.org/10.1016/j.jpba.2010.01.016.
[28] T. Kirby, D.C. Walters, X. Shi, C. Turgeon, P. Rinaldo, E. Arning, P. Ashcraft, T. Bottiglieri, M. Dibacco, P.L. Pearl, J.B. Roullet, K.M. Gibson, Novel biomarkers and age-related metabolite correlations in plasma and dried blood spots from patients with succinic semialdehyde dehydrogenase deficiency, Orphanet J. Rare Dis. 15 (2020), https://doi.org/10.1186/s13023-020-01522-5.

[29] M.A. Carnerup, A.M. Saillenfait, B.A.G. Jönsson, Concentrations of N-methyl-2-pyrrolidone (NMP) and its metabolites in plasma and urine following oral administration of NMP to rats, Food Chem. Toxicol. 43 (2005) 1441–1447, https://doi.org/10.1016/J.FCT.2005.04.007.

[30] G. Lennon, S. Willox, R. Ramdas, S.J. Funston, M. Klun, R. Pieh, S. Fairlie, S. Dobbin, D.F. Cobice, Assessing the Oxidative Degradation of N-methyl Pyrrolidone (NMP) in Microelectronic Fabrication Processes by using a Multi-platform Analytical Approach, J. Anal. Methods Chem. 2020 (2020) 8265054, https://doi.org/10.1155/2020/8265054.

[31] J. Trawiński, M. Wroński, M. Gawlik, R. Skibiński, Identification of the new metabolite of nebivolol using liquid chromatography coupled with high-resolution mass spectrometry and chemometrics, Molecules 27 (2022), https://doi.org/10.3390/molecules27030763.

[32] Y. Tada, N. Yano, H. Takahashi, K. Yuzawa, H. Ando, Y. Kubo, A. Nagasawa, N. Ohashi, A. Ogata, D. Nakae, Toxicological evaluation of l-proline in a 90-day feeding study with Fischer 344 rats, Regul. Toxicol. Pharm. 58 (2010) 114–120, https://doi.org/10.1016/j.yrtph.2010.04.011.