Comparison of the instrumental response of different constituents of specific pesticides

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1. Aim and scope

This document reports the comparison of the instrumental response produced by the different isomers or constituents in various pesticides whose residue definition involves more than one compound, such as spinosad (sum of constituents spinosyns A and spinosyn D) or metaflumizone (sum of (*E*) and (Z) isomers).

2. Introduction and short description

Until not long ago, the commercial availability of individual constituents of certain pesticide mixtures was scarce, although the situation has improved in recent years and individual constituents are readily available through plenty of commercial vendors. However, many laboratories still purchase the analytical standards as mixtures of the constituents. In some cases, laboratories acquire the mixtures due to routine; in other cases, some of the laboratories may not be willing to acquire the often more expensive individual standards.

When a pesticide is defined as a sum of constituents and/or isomers, the laboratories have various options regarding the acquisition of the analytical standards and their quantitation. In some cases, the individual constituents and/or isomers can be purchased, optimized, and analysed but, usually, technical mixtures are employed for quantitation purposes.

However, this approach is only applicable to those compounds whose components provide the same instrumental response. Pesticides with a different instrumental signal for each constituent will result in incorrect quantitation (as has been observed during previous EUPTs), due to under or overestimations derived from the different relative intensities of these components. Therefore, an assessment of which pesticides can be quantitated as a sum of their constituents and which ones must be independently analysed is essential to ensure a correct performance of the laboratories.

The present study includes the compounds chlordane, cyfluthrin, cypermethrin, fenpyroxymate, metaflumizone, spinetoram and spinosad. The instrumental responses of the isomers or constituents of these compounds, together with an evaluation of their commercial availability (including their CAS number and relative costs) are also detailed.

3. Apparatus and consumables

- Automatic pipettes, suitable for handling volumes from 1 µL to 5 mL
- Graduated 10 mL pipette
- 50 mL and 15 mL PTFE centrifuge tubes
- Vortex Shaker IKATM 4 Basic
- Axial shaker Agytax SR1 CP57
- Centrifuge Orto Alresa Consul 21, suitable for the centrifuge tubes employed in the procedure and capable of achieving 4000 rpm
- Concentration workstation
- Injection vials, 2 mL, suitable for LC and GC auto-sampler
- Amber vials, 4 mL

4. Chemicals

- Acetonitrile ultra-gradient grade
- Trisodium citrate dihydrate
- Disodium hydrogenocitrate sesquihydrate
- Sodium chloride
- Anhydrous magnesium sulphate
- Primary secondary amine (PSA)
- Supel™ QuE Z-Sep
- Ammonium formate
- Ultra-pure water
- Methanol HPLC grade
- Formic acid
- Ethyl acetate
- Pesticide analytical standards

5. Procedure

5.1. Extraction of blank samples of representative matrices

The evaluation of the aforementioned pesticides and their constituents was performed in three vegetable matrices representative of high-water content (tomato), high acid content (orange) and high fat content (avocado). Blank samples were extracted using the QuEChERS method, with a modification in the clean-up step for the avocado matrix.

1. Weigh 10 g of homogenate sample (after cryogenic milling) in a 50-mL PTFE centrifuge tube.
2. Add 10 mL acetonitrile.
3. Shake the samples in an Agytax axial extractor for 4 min.
4. Add 4 g anhydrous magnesium sulphate, 1 g sodium chloride, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogencitrate sesquihydrate.
5. Shake the samples in an Agytax axial extractor for 4 min.
6. Centrifuge at 4000 rpm for 5 min.
7. Transfer a 5 mL aliquot of the supernatant to a 15 mL PTFE tube containing 750 mg anhydrous magnesium sulphate and 125 mg PSA (tomato, orange) or 750 mg anhydrous magnesium sulphate and 175 mg of Z-Sep (avocado).
8. Vortex the tubes for 30 sec.
9. Centrifuge at 4000 rpm for 5 min.
10. Acidify with 10 µL 5 % formic acid in acetonitrile (V/V) per mL of extract.

5.2. Spiking of blank extracts

Individual pesticide stock solutions (1000–2000 mg/L) were prepared in acetonitrile or ethyl acetate and were stored in screw-capped glass vials in the dark at -20 °C. These individual solutions were used to spike three replicates of each blank extract at a final concentration of 100 µg/L or 50 µg/L, depending on the sensitivity of each compound (but always the same for components of the same compound). With that purpose, 50 µL of each matrix were evaporated and reconstituted with the same volume of a solution containing one individual isomer/constituent -in acetonitrile for liquid chromatography coupled to triple quadrupole tandem mass spectrometry (LC-MS/MS) analysis and ethyl acetate for gas chromatography coupled to triple quadrupole tandem mass spectrometry (GC-MS/MS) analysis- at the corresponding concentration. The LC-MS/MS aliquots were afterwards diluted with 250 µL of ultrapure water. Dimethoate-D_6 (LC-MS/MS) or lindane-D_6 (GC-MS/MS) were added as internal standards (50 µg/L) prior to injection.

5.3. Methodology

The GC and LC systems were operated in selected reaction monitoring (SRM). First, full scan (FS) analyses were carried out to select the most sensitive precursor ions. Then, product ion scans (PIS) were performed to select the most abundant product ions. Finally, two SRM transitions and the correct ratio between the abundances of the two optimised SRM transitions (SRM1/SRM2) were used, alongside retention time matching. to obtain the maximum sensitivity for the

detection of the target molecules. The instrumental responses produced by the different constituents and/or isomers of a single pesticide were assessed and compared in terms of area and ratio between two transitions (quantitation and confirmation transitions). The mass transitions used are presented in the **Appendix** (**Table A1** for LC-MS/MS parameters and **Table A2** for GC-MS/MS parameters).

5.4. Instrumentation and analytical conditions for the LC-MS/MS system

5.4.1. 1290 UHPLC (Agilent)

- Column: Zorbax Eclipse Plus C8 2.1x100 mm and 1.8 µm particle size (Agilent)
- Mobile phase A: Water (0.1 % formic acid, 5 mM ammonium formate, 2 % MeOH)
- Mobile phase B: Methanol (0.1 % formic acid, 5 mM ammonium formate, 2 % water)
- Column temperature: 35 °C
- Flow rate: 0.3 mL/min
- Injection volume: 5 µL

Mobile phase gradient for pesticides analysis:

Time [min]	Mobile phase A	Mobile phase B
0	100 %	0 %
2	80 %	20 %
15	0 %	100 %
18	0 %	100 %

Re-equilibration time with initial mobile phase was set to 2.5 minutes.

5.4.2. 6490A triple quadrupole system (Agilent)

- Ionisation mode: positive mode and negative mode
- Capillary (positive and negative): 3000 V
- Nebulizer: 45 psi
- Nozzle: 400 V
- Drying gas flow: 13 L/min
- Drying gas temperature: 120 °C
- Sheath gas flow: 10 L/min
- Sheath gas temperature: 375 °C

- High Pressure RF (positive): 150 V
- High Pressure RF (negative): 110 V
- Low Pressure RF (positive): 60 V
- Low Pressure RF (negative): 60 V

5.5. Instrumentation and analytical conditions for the GC- MS/MS system

5.5.1. Intuvo 9000 GC system (Agilent)

- Column: 2 planar columns HP-5MS UI (15 m long × 0.25 mm i.d. × 0.25 µm film thickness)
- Injection mode: splitless
- Ultra-inert inlet liner with a glass wool frit from Agilent
- Injection volume: 1 µl
- Injector temperature: 80 °C hold for 0.1 min, then up to 300 °C at 600 °C/min, hold for 5 min and then to 250 °C at 100 °C/min
- Carrier gas: Helium at constant flow = 1.28 mL/min column 1, 1.48 mL/min column 2
- Carrier gas purity: 99.999 %
- Oven temperature: 60 °C for 0.5 min, up to 170 °C at 80 °C/min, and up to 310 °C at 20 °C/min

5.5.2. 7410 triple quadrupole system (Agilent)

- Ionisation mode: electron impact ionisation
- Temperature of the transfer line: 280 °C
- Temperature of ion source: 280 °C
- Collision gas: nitrogen
- Collision gas purity: 99.999 %
- Solvent delay: 2.6 min

6. Results

The compounds included in the present study were chlordane, cyfluthrin, cypermethrin, fenpyroximate, metaflumizone, spinetoram and spinosad, with up to four different constituents and/or isomers commercially available. In the past, several cases of wrong or inaccurate CAS numbers were detected in the main companies selling pesticide analytical standards, which could result in wrong optimization, identification, and quantitation by the laboratories. Therefore, when purchasing any of these compounds, laboratories must take into consideration which component they intend to acquire, and make sure the one offered by the manufacturer has the same CAS number (**Table 1**). A re-check of the CAS number in the certificate of analysis (CoA) should also be performed after the arrival of the standard in the lab. This practice should be extended to other compounds that comprise a mixture of isomers and/or constituents, or to any standard in general.

Table 1. CAS number and example price of the isomers/constituents of the studied compounds.

Compound name	Components				
Chlordane	**Mixture**	***cis*-Chlordane**		***trans*-Chlordane**	
Price*	0.11 €/mg	6.93 €/mg		6.93 €/mg	
CAS number	57-74-9	5103-71-9		5103-74-2	
Cyfluthrin	**Mixture**	**beta-Cyfluthrin**			
Price*	0.16 €/mg	0.21 €/mg			
CAS number	68359-37-5	1820573-27-0			
Cypermethrin	**Mixture**	**Alpha**	**Beta**	**Zeta**	**Theta**
Price*	0.43 €/mg	0.58 €/mg	0.58 €/mg	0.76 €/mg	1.58 €/mg
CAS number	52315-07-8	67375-30-8	1224510-29-5	1315501-18-8	71697-59-1
Fenpyroximate	**Mixture**	**(*E*)-Fenpyroximate**		**(Z)-Fenpyroximate**	
Price*	0.67 €/mg	13.86 €/mg		16.38 €/mg	
CAS number	111812-58-9	134098-61-6		149054-57-9	
Metaflumizone	**Mixture**	**(*E*)-Metaflumizone**		**(Z)-Metaflumizone**	
Price*	1.04 €/mg	1.04 €/mg		1.60 €/mg	
CAS number	139968-49-3	852403-68-0		139970-56-2	
Spinetoram	**Mixture**	**Spinetoram J**		**Spinetoram L**	
Price*	2.90 €/mg	NF		NF	
CAS number	935545-74-7	187166-40-1		187166-15-0	
Spinosad	**Mixture**	**Spinosyn A**		**Spinosyn D**	
Price*	1.51 €/mg	21.3 €/mg		137.1 €/mg	
CAS number	168316-95-8	131929-60-7		131929-63-0	

**Prices shown in the table are illustrative. Inclusion of a specific vendor in the References section does not imply endorsement of said vendor on behalf of the EURL-FV.*

From an availability point of view, the individual isomers and/or constituents can be purchased from the main manufacturers in most cases (**Table 1**). The technical mixtures always have a lower price but, in most cases, the difference is not very large –the exception being spinosad, with an estimated price of 1.51 €/mg of the mixture of components and more than 150 €/mg for the individual spinosyns, mainly due to the elevated cost of spinosyn D-. However, as will be discussed below, the technical mixtures are not always amenable for a correct quantification and their purchase is not always advised.

Table 2 shows the average instrumental response obtained for each individual isomer/constituent of the pesticides included in the study, in the three matrices evaluated (three replicates per matrix). Moreover, the ratio of the two SRM transitions illustrates whether a difference in the fragmentation process might take place. The results of the individual pesticides are discussed below.

Table 2. Average instrumental responses and ion ratios for the components of the evaluated compounds in three representative matrixes.

Compound		Parameters	Matrix			Technique
			Tomato	Orange	Avocado	
Chlordane	*Cis*-isomer	Avg. response	5.6E+05	6.8E+05	6.3E+05	GC-MS/MS
		Avg. ion ratio	30	31	29	
	Trans-isomer	Avg. response	5.6E+05	4.4E+05	7.0E+05	
		Avg. ion ratio	29	31	30	
	Ratio *cis/trans*	Response	100 %	153 %	91 %	
		Ion ratio	102 %	100 %	96 %	
Cyfluthrin	β-cyfluthrin	Avg. response	3.6E+05	5.8E+05	7.1E+05	GC-MS/MS
		Avg. ion ratio	198	204	215	
	Technical mixture	Avg. response	3.1E+05	5.5E+05	7.3E+05	
		Avg. ion ratio	213	205	232	
	Ratio β/technical	Response	115 %	105 %	97 %	
		Ion ratio	93 %	99 %	92 %	
Cypermethrin	α-cypermethrin	Avg. response	4.1E+05	5.4E+05	7.9E+05	GC--MS/MS
		Avg. ion ratio	13	11	9	
	β-cypermethrin	Avg. response	3.8E+05	3.6E+05	7.9E+05	
		Avg. ion ratio	14	12	9	
	θ-cypermethrin	Avg. response	3.3E+05	5.9E+05	8.3E+05	
		Avg. ion ratio	15	11	8	
	ζ-cypermethrin	Avg. response	2.8E+05	4.9E+05	7.7E+05	
		Avg. ion ratio	19	13	9	
	Ratio	Response				
		Ion ratio		*See text*		
Fenpyroximate	(*E*)-isomer	Avg. response	8.2E+05	1.0E+06	1.5E+06	LC-MS/MS
		Avg. ion ratio	32	30	29	
	(*Z*)-isomer	Avg. response	1.7E+06	1.8E+06	2.1E+06	
		Avg. ion ratio	5	5	5	
	Ratio (*E*)/(*Z*)	Response	47 %	56 %	70 %	
		Ion ratio*	635 %	570 %	557 %	
Metaflumizone	(*E*)-isomer	Avg. response	3.5E+04	6.8E+04	1.6E+05	LC-MS/MS
		Avg. ion ratio	10	9	9	
	(*Z*)-isomer	Avg. response	2.1E+04	2.6E+04	7.8E+04	
		Avg. ion ratio	6	5	6	
	Ratio (*E*)/(*Z*)	Response	167 %	261 %	201 %	
		Ion ratio*	163 %	178 %	158 %	
Spinetoram	Spinetoram J	Avg. response	5.0E+05	4.6E+05	4.8E+05	LC-MS/MS
		Avg. ion ratio	3	3	4	
	Spinetoram L	Avg. response	5.7E+05	5.1E+05	4.5E+05	
		Avg. ion ratio	7	7	8	
	Ratio (*J*)/(*L*)	Response	87 %	89 %	106 %	
		Ion ratio*	49 %	48 %	47 %	
Spinosad	Spinosyn A	Avg. response	1.6E+06	1.5E+06	1.5E+06	LC-MS/MS
		Avg. ion ratio	3	3	3	
	Spinosyn D	Avg. response	6.4E+05	6.1E+05	6.0E+05	
		Avg. ion ratio	5	5	5	
	Ratio (*A*)/(*D*)	Response	243 %	247 %	255 %	
		Ion ratio*	64 %	67 %	61 %	

**Ion ratios outside of the 70-130 % acceptable ion ratio range.*

6.1. Chlordane

Chlordane is an organochlorine insecticide with two geometric isomers, *cis*- and *trans*-chlordane, included in its residue definition (**Figure 1**). Although obsolete and not approved in the European Union since 1981, its high persistence in soil and water results in possible detections in current vegetable and/or environmental samples. As can be observed in **Table 2**, the instrumental response of both isomers is virtually the same in tomato and avocado. Conversely, in orange, the response of *cis*-chlordane is 1.53 times more intense than the one of the *trans* isomer. Therefore, the technical mixture should not be employed for quantification in this type of matrices. The ratio between the isomers remains constant in all cases studied. These isomers can be easily separated by chromatography, with a significant difference in the retention time (0.15 min difference out of a total 12.3 min run time, as can be seen in **Figure 1**). Therefore, the individual optimization and quantitation of the isomers is highly advised.

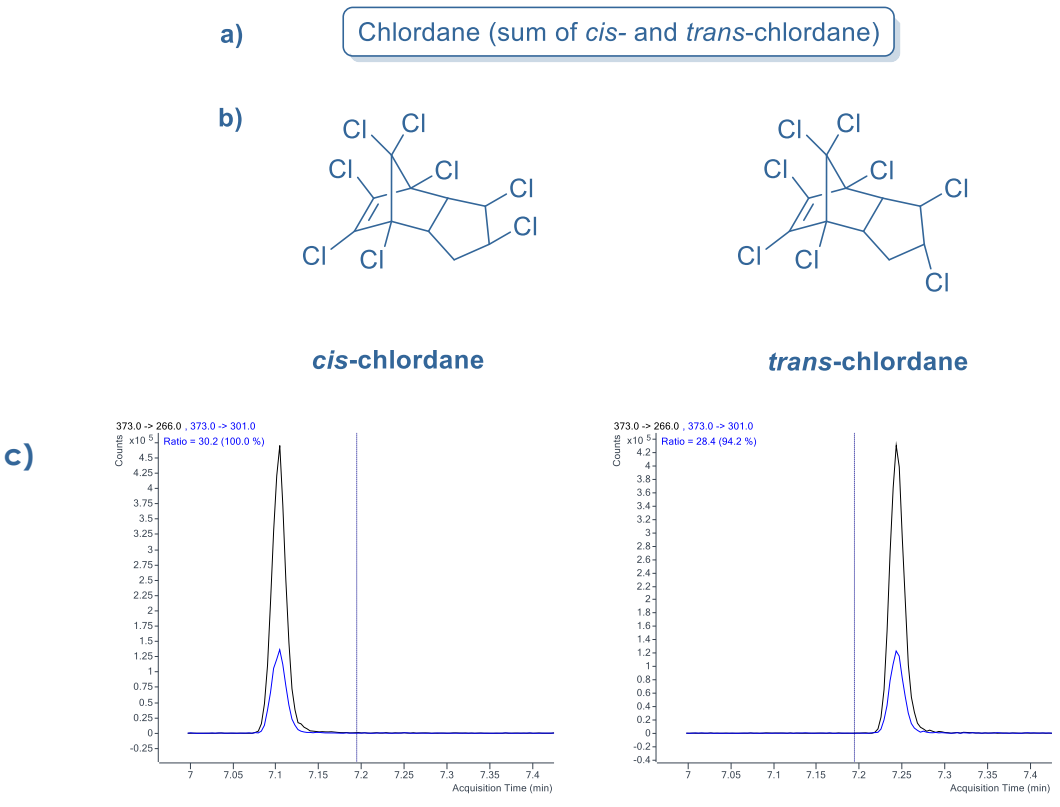


Figure 1. **a)** Residue definition, **b)** chemical structure and **c)** instrumental response of *cis*-chlordane and *trans*-chlordane.

6.2. Cyfluthrin

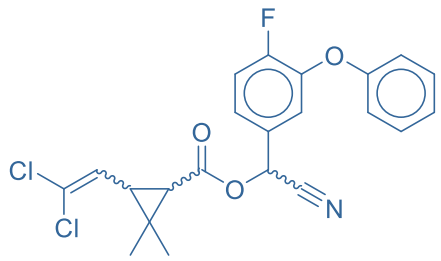
The pyrethroid cyfluthrin is an insecticide not approved in the European Union to control Lepidoptera, Coleoptera, migratory locusts, and grasshoppers (among others) in cereals, cotton, fruits, and vegetables. It contains three chiral centres that result in eight stereoisomers (four pairs of enantiomers, **Figure 2** and **Table 3**). Therefore, four cyfluthrin signals can be obtained with non-chiral chromatography although, in some cases, two or more of these signals are not separated and a lower number is obtained. The first chromatogram showed in **Figure 2** shows only three signals, the last one comprising two peaks which are not separated in the total 12.3 min run time. The technical mixture of cyfluthrin comprises the eight isomers, whereas beta-cyfluthrin is an enriched mixture of the two biologically active diastereomers (II and IV, which coelute in the second chromatogram of **Figure 2**).

Table 3. Distribution of the diastereomers of cyfluthrin

Diastereomer (comprising two enantiomers each)	Composition (in %)	
	Cypermethrin	β-Cypermethrin
I (1*R*,3*R*, α*R* + 1*S*,3*S*, α*S* = 1:1; *cis*)	23-27	< 2
II (1*R*,3*R*, α*S* + 1*S*,3*S*, α*R* = 1:1; *cis*)	17-21	30-40
III (1*R*,3*S*, α*R* + 1*S*,3*R*, α*S* = 1:1; *trans*)	32-36	< 3
IV (1*R*,3*S*, α*S* + 1*S*,3*R*, α*R* = 1:1; *trans*)	21-25	57-67

a) Cyfluthrin (cyfluthrin including other mixtures of constituent isomers (sum of isomers))

b)



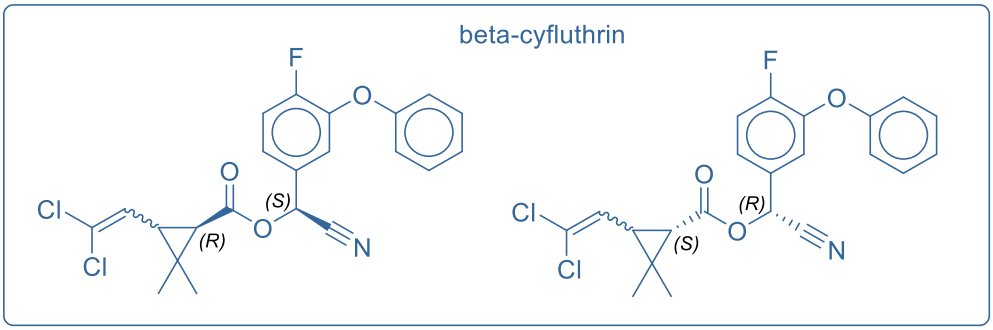


Figure 2. **a)** Residue definition, **b)** chemical structure and **c)** instrumental response of cyfluthrin (technical mixture) and beta-cyfluthrin (*continues*).

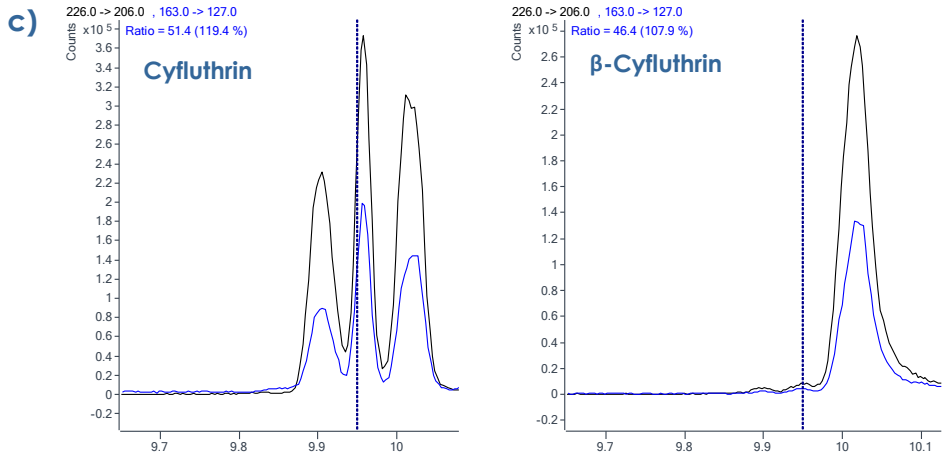


Figure 2. **a)** Residue definition, **b)** chemical structure and **c)** instrumental response of cyfluthrin (technical mixture) and beta-cyfluthrin (*continuation*).

The signal intensity of beta-cyfluthrin and the technical mixture of isomers is equivalent in all matrices studied, and the same happens with the ratio between both transitions employed for identification. Therefore, the technical mixture of cyfluthrin seems amenable for the quantitation of this compound.

6.3. Cypermethrin

Cypermethrin is another pyrethroid insecticide used for the control of Lepidoptera, Coleoptera, Diptera, Hemiptera, and other insects in citrus, vines, lettuce, Solanaceae, cocoa, and cereals (among others). The cypermethrin molecule contains three chiral centres, resulting in eight stereoisomers: up to four chromatographic signals in non-chiral chromatography, although some might not be completely resolved (**Figure 3**). The only structural difference between cyfluthrin and cypermethrin is the absence of the fluorine atom the benzene ring closer to the ester functional group of the latter. However, in this case, five combinations of isomers are commercially available:

- alpha-cypermethrin (α): 2 enantiomers, 1 peak (approved in the EU).[1]
- beta-cypermethrin (β): 2 pairs of enantiomers, up to 2 peaks (not approved).
- theta-cypermethrin (θ): 2 enantiomers, 1 peak (no data).
- zeta-cypermethrin (ζ): 4 isomers (not enantiomers), ≤ 4 peaks (not approved)
- Technical mixture: 8 isomers, up to 4 peaks (approved).

[1] The approval expired on 7th June 2021.

a) Cypermethrin (cypermethrin including other mixtures of constituent isomers (sum of isomers))

b)

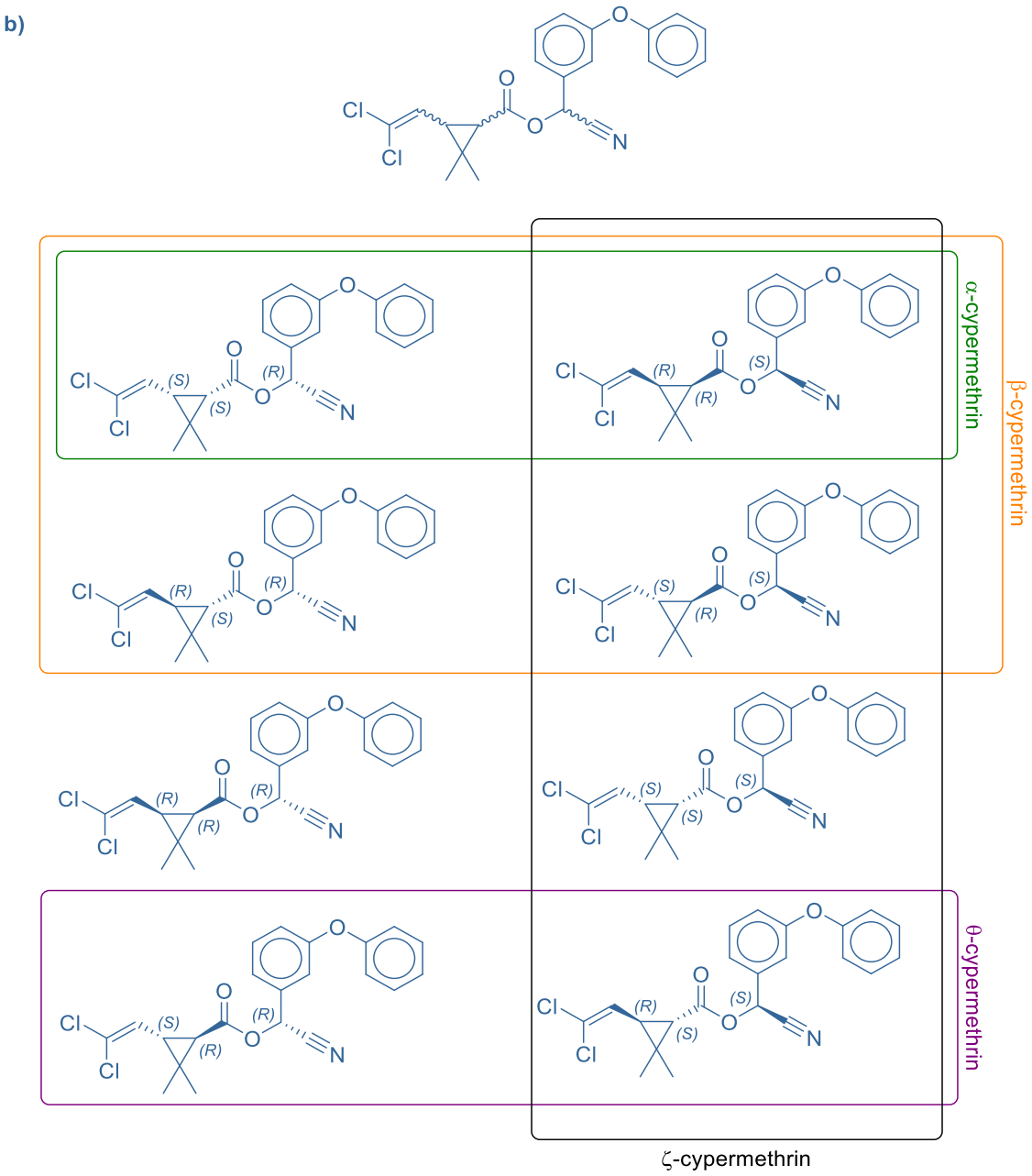


Figure 3. a) Residue definition, **b)** chemical structure and **c)** instrumental response of cypermethrin (α, β, θ and ζ isomers) (*continues*).

c)

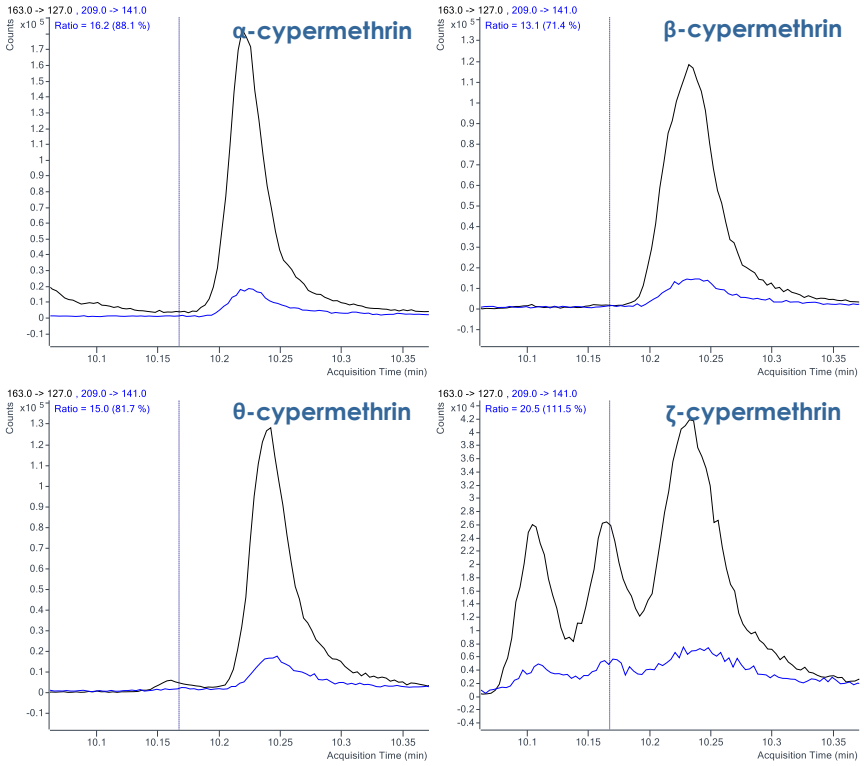


Figure 3. a) Residue definition, **b)** chemical structure and **c)** instrumental response of cypermethrin (α, β, θ and ζ isomers) (*continuation*).

The relative instrumental response of the isomers of cypermethrin was found to vary significantly among the matrices studied. In tomato, the order of signal intensity was α > β > θ > ζ, whereas in orange matrix the relative intensities were θ > α > ζ >> β. The difference between the most intense and the least intense isomers was 1.5 and 1.7 for tomato and orange, respectively. Conversely, in avocado matrix, the relative intensities of the different isomers were found to be practically identical.

The ratio of the two transitions employed for the identification of cypermethrin were consistent in all isomers for each matrix. However, these ratios changed slightly among matrices (13-19 in tomato, 11-14 in orange, 8-9 in avocado).

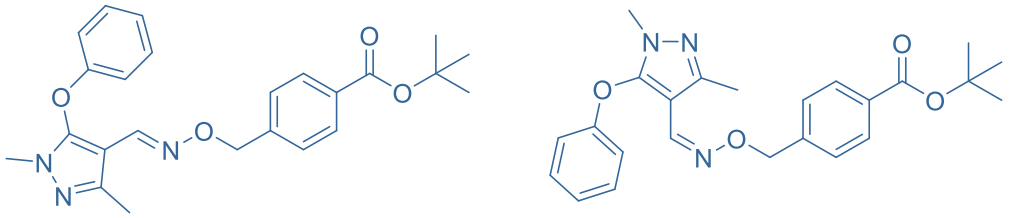
However, due to (*i*) the multiple chromatographic signals generated by this compound; (*ii*) the coelution of some of them; and (*iii*) the relatively small differences in the relative intensities, in this specific case, the use of the technical mixture for quantitation purposes is justified.

6.4. Fenpyroximate

Fenpyroximate is pirazol acaricide employed to control phytophagous mites in citrus, apples, pears, peaches, and grapes, among others. The structure of fenpyroximate contains a double bond that gives rise to (*E*) and (*Z*) geometric isomers (**Figure 4**).

a) Fenpyroximate

b)



(*E*)-Fenpyroximate (*Z*)-Fenpyroximate

c)

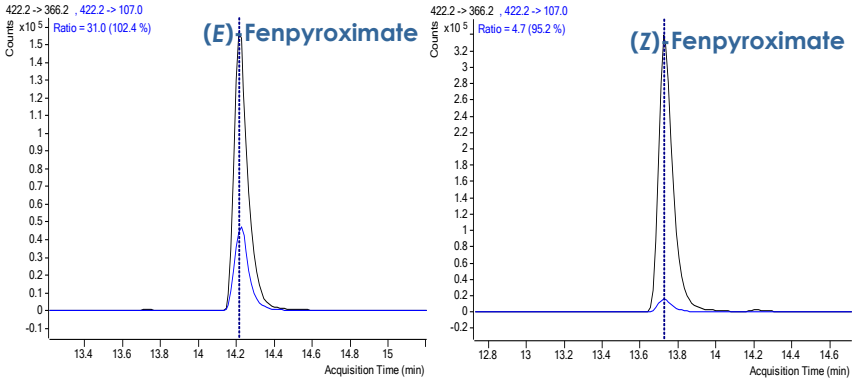


Figure 4. **a)** Residue definition, **b)** chemical structure and **c)** instrumental response of fenpyroximate ((*E*) and (*Z*) isomers).

The response of the later eluting (*E*)-isomer ranges between 47 % and 70 % compared to the (*Z*)-isomer, depending on the matrix. The ion ratio of (*E*)-fenpyroximate is also different to that of (*Z*)-fenpyroximate, ranging between 557 % and 635 % higher in the later.

The acquisition of the individual geometric isomers of fenpyroximate is significantly more expensive than the purchase of a technical mixture. When evaluating several vendors, many include "fenpyroximate" without specifying neither (*E*)- nor (*Z*)-fenpyroximate. However, all the cases evaluated, this corresponds to (*E*)-fenpyroximate (CAS no. 134098-61-6). The (*E*)-fenpyroximate

analytical standard usually contains ≤ 5 % of the (*Z*)-isomer. In fact, the fenpyroximate technical mixture (CAS no. 111812-58-9) is not readily available through many vendors. The price difference between the technical mixture and the individual (*E*)-isomer (the former being approximately 20 times less expensive) is a good indicator of whether an analytical standard in question corresponds to either the technical mixture or to the (*E*)-isomer.

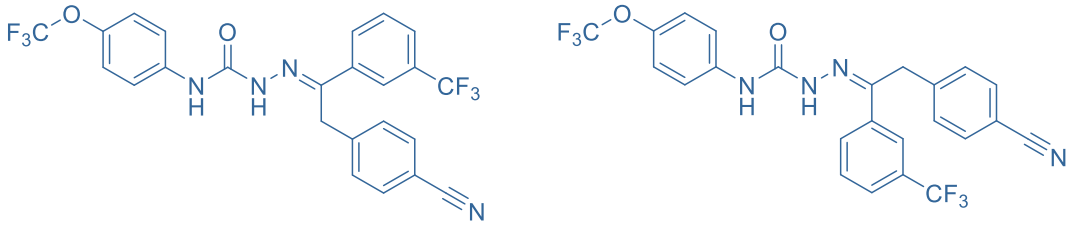
Since the residue definition for fenpyroximate does not include any reference, citation, requirements for inclusion, nor evaluation whatsoever for (*Z*)-fenpyroximate, its inclusion in standard work solution mixes is left to the discretion of the laboratories. Nevertheless, special care must be taken not to report (*Z*)-fenpyroximate as fenpyroximate if it were to be found during sample analysis.

6.5. Metaflumizone

Metaflumizone is a semicarbazone insecticide with veterinary applications employed to control Lepidoptera, Coleoptera, Hemiptera, Homoptera, Hymenoptera, Diptera, Isoptera, and Siphonaptera in brassicas, leafy vegetables, and fruiting vegetables, among others. The structure of metaflumizone contains a double bond that gives rise to (*E*) and (*Z*) geometric isomers with different instrumental response (**Figure 5**).

a) Metaflumizone (sum of E- and Z- isomers)

b)



(*E*)-Metaflumizone (*Z*)-Metaflumizone

Figure 5. **a)** Residue definition, **b)** chemical structure and **c)** instrumental response of metaflumizone ((*E*) and (*Z*) isomers) (*continues*).

c)

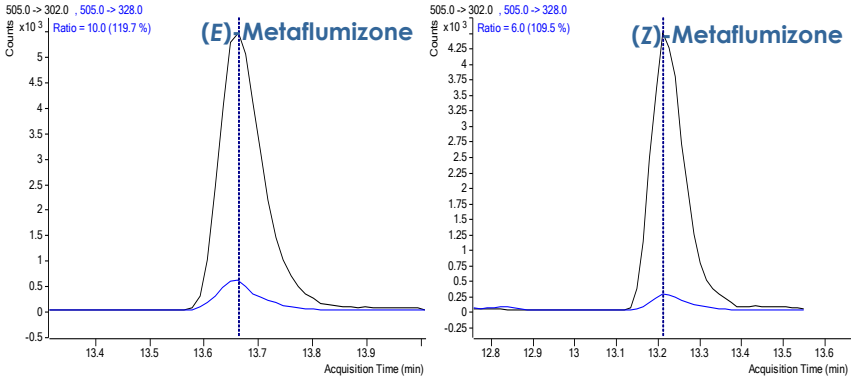


Figure 5. **a)** Residue definition, **b)** chemical structure and **c)** instrumental response of metaflumizone ((*E*) and (*Z*) isomers) (*continuation*).

The response of the later eluting (*E*)-isomer ranges between 167 % and 261 % compared to the (*Z*)-isomer, depending on the matrix. The ion ratio of (*E*)-metaflumizone is also different to that of (*Z*)-metaflumizone, ranging between 158 % and 178 % higher in the former.

The acquisition of the individual geometric isomers of metaflumizone is more expensive than the purchase of a technical mixture, however, the additional cost is justified considering their different instrumental behaviour. Furthermore, technical metaflumizone only contains ≤ 10 % of the (*Z*)-isomer, which might prove to be insufficient for routine use. Worth remarking is that metaflumizone was included in EUPT-FV-20, and bimodality was observed. One possible cause for bimodality was lack of knowledge from the laboratories regarding the (*Z*)-isomer, which may elute outside of the acquisition window for (*E*)-metaflumizone, thus, the reported concentration in this case would not match the assigned value. Another possible source of error, discussed herein, is the quantitation of one isomer with the other, as the instrumental response for both geometric isomers is not equivalent. Metaflumizone was also included in EUPT-FV-21, after participating laboratories had been made aware of this issue, and consequently, no bimodality was observed.

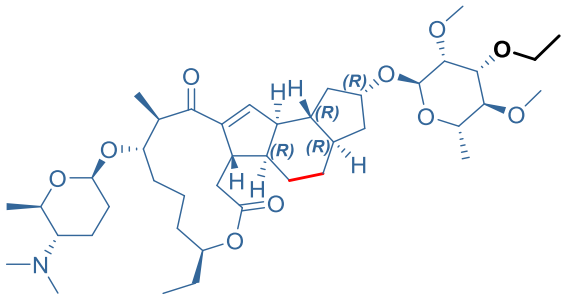
6.6. Spinosyns and spinosyn derivatives

Spinosyns are a series of naturally occurring macrocyclic lactones produced by the *Saccharopolyspora spinosa* actinomycete bacterium. Two main spinosyn-based insecticides are employed nowadays: spinosad and spinetoram. Spinosad and spinetoram are insecticides employed for the control of Lepidoptera, Diptera, Thysanoptera, Isoptera, Coleoptera, Orthoptera and certain Homoptera in pome and stone fruit, vines, tree nuts, cotton, and vegetables. Spinosad and spinetoram

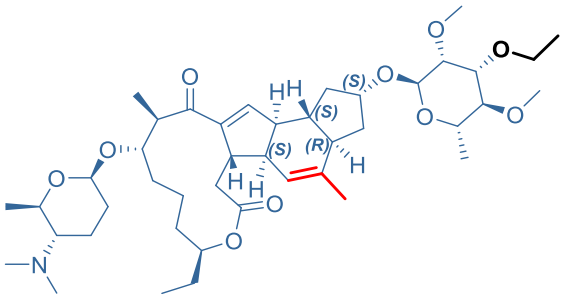
activate the nicotinic acetylcholine receptor, but at a distinct site from that of nicotine or neonicotinoids. Both spinosad and spinetoram are a mixture of two spinosyns, spinosyn A and spinosyn D in the case of spinosad, and two derivatives of spinosyn J and spinosyn L in the case of spinetoram. Thus, their molecular formulae and exact masses are very similar among these four compounds, with only minor structural differences.

The aforementioned differences are depicted in **Figure 6**: shown in black, spinosad constituents present 3'-*O*-methyl substituents in the methylated rhamnose moiety, whereas spinetoram constituents contain a 3'-*O*-ethyl group in the methylated rhamnose moiety. The remaining structural differences among the spinosyns can be found in the central tetracyclic ring system, in the six-membered ring. In red, the structural differences between spinetoram constituents are marked, whereas the differences between spinosad constituents are shown in green.

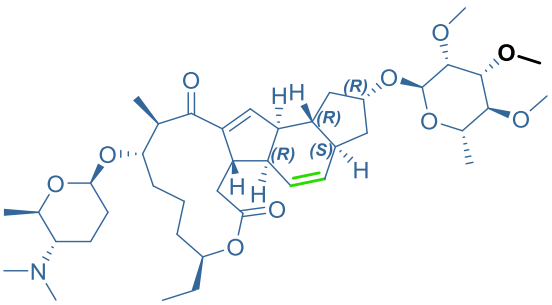
Their chemical similarities give rise to comparable chromatographic and spectrometric behaviour, with common mass fragments for all four molecules.



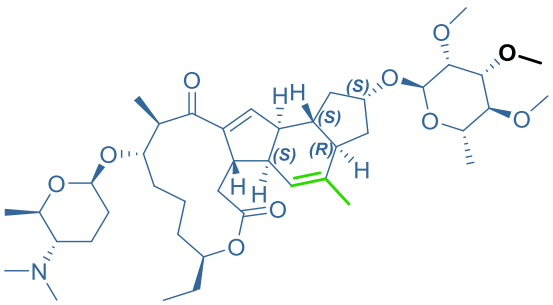
Spinetoram J
Chemical Formula: $C_{42}H_{69}NO_{10}$
Exact Mass: 747.4921 Da



Spinetoram L
Chemical Formula: $C_{43}H_{69}NO_{10}$
Exact Mass: 759.4921 Da



Spinosyn A
Chemical Formula: $C_{41}H_{65}NO_{10}$
Exact Mass: 731.4608 Da



Spinosyn D
Chemical Formula: $C_{42}H_{67}NO_{10}$
Exact Mass: 745.4765 Da

Figure 6. Structure, chemical formulae and exact masses for four spinosyns: spinosyn A and spinosyn D (spinosad constituents) and spinetoram J and spinetoram L 3'-*O*-ethylated spinosyn derivatives (spinetoram constituents).

6.6.1. Spinetoram

Spinetoram is a mixture of the 3'-*O*-ethylated naturally occurring spinosyn J and spinosyn L. The major component (spinetoram J) is further transformed by the dihydrogenation of one double bond in the tetracyclic ring system (**Figure 7**).

To date, the isolated spinetoram J and spinetoram L cannot be found as separated analytical standards to the best of our knowledge, with only mixtures of spinetoram J and spinetoram L being commercially available as spinetoram. Hence, to compare their relative responses, appropriate dilutions of the spinetoram stock solution were prepared to ensure that the same concentration of spinetoram J and spinetoram L was evaluated (according to the relative concentrations stated in the reference standard certificate of analysis).

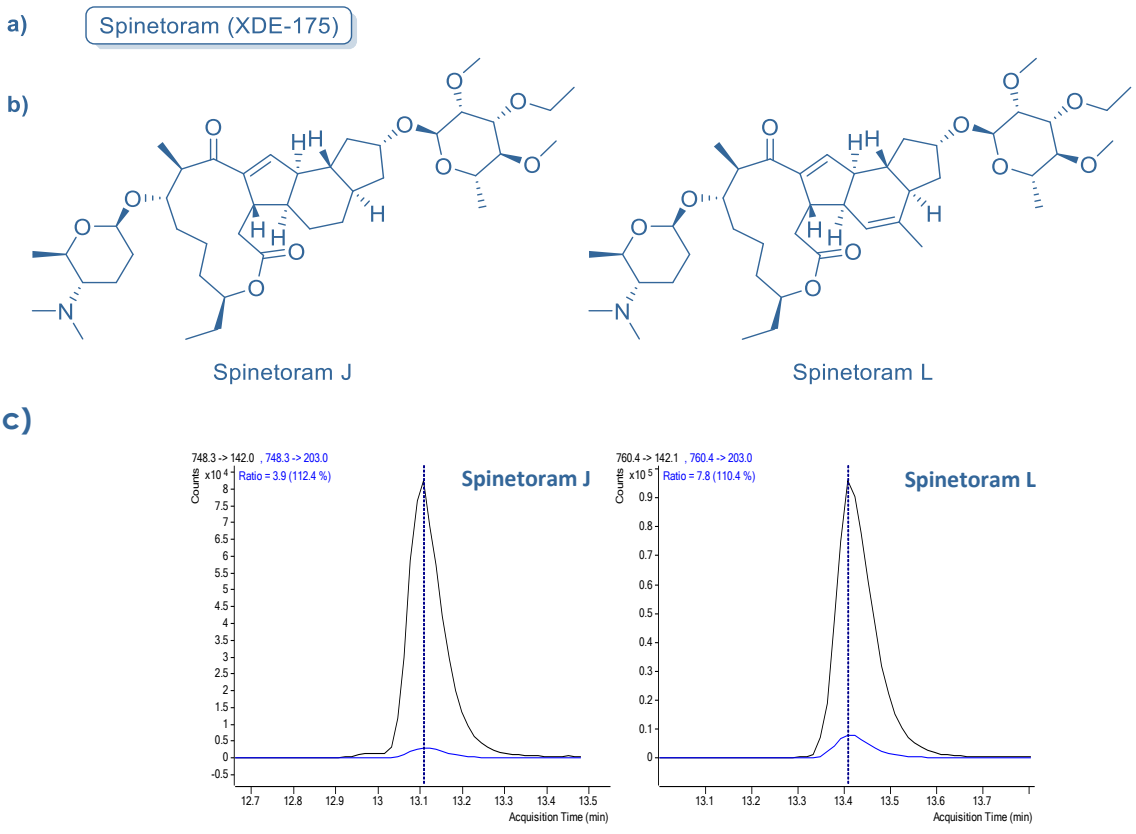


Figure 7. a) Residue definition, **b)** chemical structure and **c)** instrumental response of spinetoram (spinetoram J and spinetoram L constituents).

Spinetoram is referenced to as XDE-175 in its residue definition, a name that carries over from its original development code number by Dow AgroSciences but that possesses no analytical implications. Spinetoram J and spinetoram L can be found as XDE-175-J and XDE-175-L, respectively. For reference, the internal

development code number for spinosad was XDE-105 by DowElanco which, again, possesses no analytical relevance and may only be a source of confusion to the laboratories.

The relative instrumental responses of spinetoram J and spinetoram L are very close in the three matrixes evaluated, with a worst case of 87 % relative J/L ratio in tomato, and the best case as 106 % relative J/L ratio in avocado. Consequently, only minor divergences in the response of both spinetoram constituents are found, and quantitation employing one or the other would cause only minor deviations from the true result. However, when comparing the ion ratios for both, it was observed that the ion ratio in the case of spinetoram J was about half of the ion ratio for spinetoram L, which indicates differences in the fragmentation mechanisms.

Since both constituents are sold together and there exist differences in their instrumental behaviour (albeit minor, being the ion ratio the most affected analytical parameter), the most appropriate course of action is to quantitate and identify spinetoram J and spinetoram L separately.

6.6.2. Spinosad

Spinosad is a mixture of spinosyn A and spinosyn D. The technical mixture of Spinosad contains spinosyn A (the major component) at 50-90 % and spinosyn D at 5-50 %. As opposed to spinetoram, individual spinosyn A and spinosyn D analytical standards can be found for purchase; however, their prices are significantly higher than those for the technical mixture. The price of spinosyn D is almost one hundred times higher than the price of the spinosad mixture, which may prevent laboratories from considering including spinosyn A and spinosyn D in their pesticide mixtures instead of the commonplace technical spinosad.

Nevertheless, when evaluating the relative instrumental responses of spinosyn A and spinosyn D, the importance of quantitating and identifying each spinosad constituent separately becomes evident (**Figure 8**). The relative instrumental response of the same concentration of spinosyn A and spinosyn D ranged from 243 % relative A/D ratio in tomato to 257 % ratio in avocado, with ion ratios between 33 % and 39 % lower for spinosyn A compared to spinosyn D. These differences indicate very significant differences in their fragmentation mechanisms that are not self-evident from their molecular structures alone.

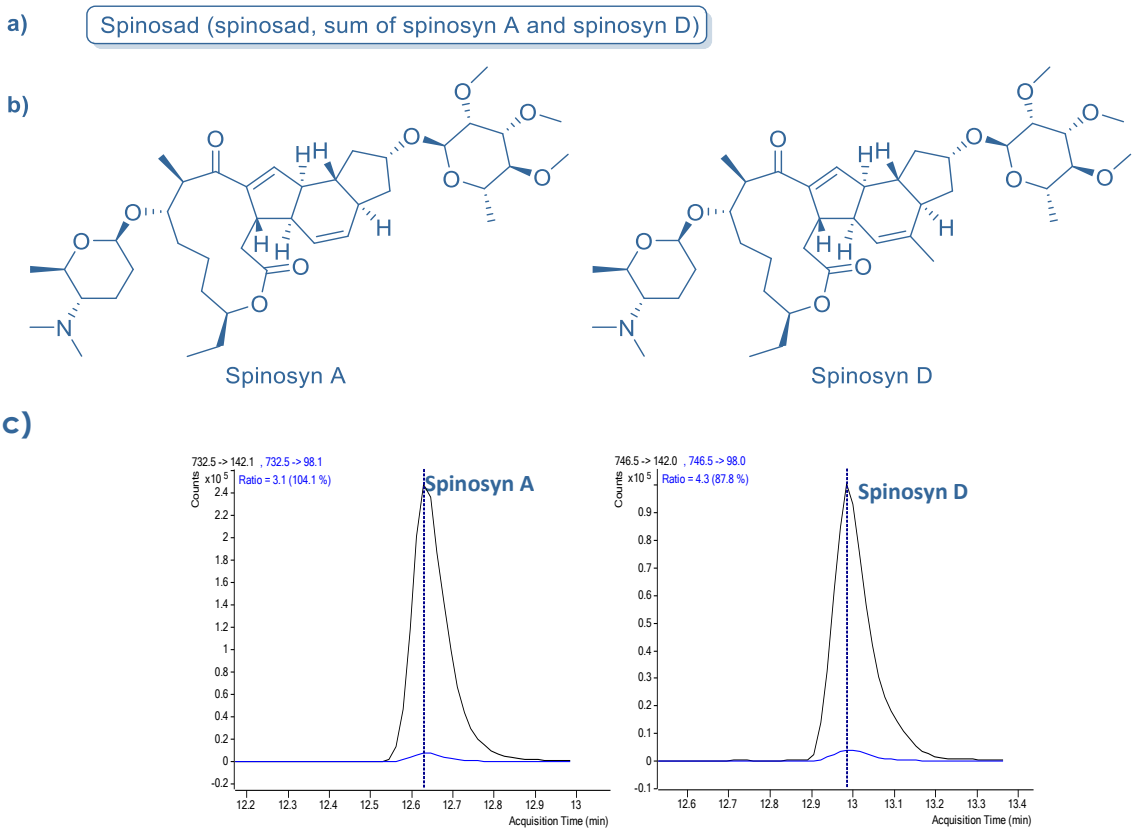


Figure 8. a) Residue definition, **b)** chemical structure and **c)** instrumental response of spinosad (spinosyn A and spinosyn D constituents).

As mentioned before, the individual analytical standards are far more expensive than the technical mixture. Thus, to compare whether the same instrumental differences were found in the technical mixture, and to check whether this mixture provides comparable results to the individual standards, the same procedure described in 6.6.1. for spinetoram was performed. To compare the relative responses of spinosyn A and spinosyn D employing the technical mixture, appropriate dilutions of a technical spinosad stock solution were prepared to ensure that the same concentration of spinosyn A and spinosyn D was evaluated (according to the relative concentrations stated in the reference standard certificate of analysis). The results were comparable to those obtained for the individual analytical standards, further reaffirming the importance of quantitating, and identifying each spinosad constituent separately, and not as the sum of the instrumental responses for both.

During the data evaluation step in EUPT-FV-23, bimodality was observed for spinosad. Bimodality may have arised from the use of either the technical mixture of the individual standards for quantitation, the quantitation of spinosyn D using spinosyn A (or vice versa), and/or using the sum of instrumental responses.

6.7. Conclusions

The instrumental responses of seven pesticide residues (chlordane, cyfluthrin, cypermethrin, fenpyroximate, metaflumizone, spinetoram, and spinosad) which are comprised of different isomers and/or constituents have been evaluated in three representative commodities for high water content, high acid content, and high oil content. The comparison has been performed in terms of relative instrumental responses and relative ion ratios.

The quantitation of different isomers and/or constituents of a specific pesticide using only one -or a narrow combination- of some of its constituents -with the assumption that the instrumental response will be equivalent- will lead to inaccuracies regarding quantitation in certain cases.

Out of the evaluated pesticides, the assumption of equivalent responses was found to be true only for chlordane, cyfluthrin, cypermethrin and, to a lesser extent (but still acceptably similar), for spinetoram.

In the case of fenpyroximate, metaflumizone, and spinosad, the error such approach will cause in the quantitative result implies that this strategy is not valid for the aforementioned pesticide residues. Thus, neither quantitating the sum of the constituents using the sum of the instrumental responses, nor calculating the concentration of one constituent with the calibration curve of the other constituent will provide accurate results. In the worst case, spinosad, the instrumental response for constituent spinosyn A was found to be six times higher than the instrumental response of spinosyn D. Ion ratios were also found to be outside the 70-130 % acceptable range for fenpyroximate, metaflumizone, and spinosad.

Hence, for these compounds, the acquisition of individual standards and individual calibration curves is mandatory for a correct quantitation of each separate constituent, except for fenpyroximate, for which only (*E*)-fenpyroximate is required for regulatory purposes. An alternative approach to quantitate each constituent, if chromatographic separation is achieved, is to prepare exact concentrations of each constituent according to their relative purities stated in the certificate of analysis.

After an evaluation of the commercial availability of every constituent from the seven studied pesticides, all of them were found to be commercially available except for spinetoram J and spinetoram L. The prices of the individual analytical standards were found to be higher than those of the technical mixtures.

In conclusion, differences in the instrumental responses have been found for some of the evaluated pesticides comprised of two or more isomers and/or constituents, whereas for others these differences have been found to be negligible. Hence, laboratories should evaluate every pesticide residue which is the combination of two or several different analytes, and check whether individual standards should be obtained and employed during routine analysis, or whether technical mixtures are sufficient for correct identification and quantitation. Training courses, such as those provided annually by the EURL-FV, are a very useful tool which the laboratories can use to gain insight on compounds with several constituents.

7. References

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APPENDIX: MASS TRANSITIONS

Table A1. Detection and chromatographic parameters for the LC-MS/MS instrument.

Name	t_R (min)	Cone voltage (V)	Precursor ion 1 (m/z)	Product ion 1 (m/z)	CE 1 (eV)	Precursor ion 2 (m/z)	Product ion 2 (m/z)	CE 2 (eV)	Polarity
Dimethoate-D_6	5.99	380	236.0	205.0	4	236.0	131.0	16	Positive
Fenpyroximate (*E*)	14.33	380	422.2	166.2	12	422.2	107.0	64	Positive
Fenpyroximate (*Z*)	13.81	380	422.2	166.2	12	422.2	107.0	64	Positive
Metaflumizone (*E*)	13.68	380	505.0	328.0	10	505.0	302.0	10	Negative
Metaflumizone (*Z*)	13.28	380	505.0	328.0	10	505.0	302.0	10	Negative
Spinetoram J	13.08	380	748.3	203.0	30	748.3	142.0	25	Positive
Spinetoram L	13.39	380	760.4	203.0	35	760.4	142.1	35	Positive
Spinosyn A	12.59	380	732.5	142.1	30	732.5	98.1	40	Positive
Spinosyn D	12.95	380	746.5	142.0	25	746.5	98°.0	40	Positive

t_R: retention time
CE: collision energy

Table A2. Detection and chromatographic parameters for the GC-MS/MS instrument.

Name	t_R (min)	Precursor ion 1 (m/z)	Product ion 1 (m/z)	CE 1 (eV)	Precursor ion 2 (m/z)	Product ion 2 (m/z)	CE 2 (eV)
Chlordane (*cis*)	7.24	373.0	266.0	20	373.0	301.0	10
Chlordane (*trans*)	7.10	373.0	266.0	20	373.0	301.0	10
Cyfluthrin	9.95	163.0	127.0	5	226.0	206.0	10
Cypermethrin	10.17	163.0	127.0	5	209.0	141.0	20
Lindane-D_6	5.61	224.0	187.0	5	224.0	150.0	20

t_R: retention time
CE: collision energy