Development and validation of analytical methods:

Guidance for overcoming difficulties in the quantification of complex residue definitions containing isomers

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1. Aim and scope

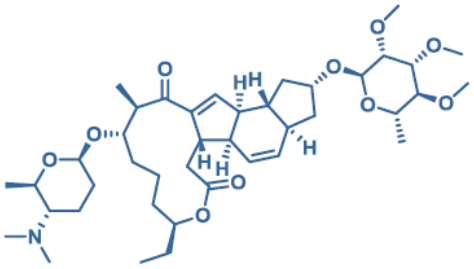
This document reports an evaluation of the quantification of spinosad taking into account the isomer proportion provided by different commercial vendors by liquid chromatography coupled to mass spectrometry.

2. Short description

The method most commonly used employed by laboratories to quantify spinosad is to use the mixture of isomers instead of the individual components. It has been found that, in some cases -as in the case of spinosad-, it is necessary to quantitfy spinosyn A and spinosyn D separately, either by using individual standards, or by the correct application of their respective purities within the spinosad standard mixture.

During the EUPT-FV23 proficiency test, spinosad was applied to the test item. The evaluation of the results demonstrated bimodality for this compound because of the different approaches laboratories followed for its quantification. A survey answered by the participants showed that 46 % of the respondents did not adequately quantify spinosad. Furthermore, after the complete evaluation of the results with the newfound information from the survey, the high dispersion of spinosyn D results might be explained by its low proportion in some of the technical mixtures of spinosad. An alternative, more worrisome cause for this dispersion might be the inaccuracy of the certified ratio of spinosyn A and spinosyn D by the standard suppliers. The goal of the EURL-FV is to investigate the latter possibility by purchasing spinosad analytical standards (mixture of spinosyn A and D (**Figure 1**)) from different certified standard providers, after which they will be employed to quantitate PT samples.

In the present work, six different commercial vendors (V1, V2, V3, V4, V5, V6) have been compared with standard of the separate isomers (spinosyn A and spinosyn D) in terms of quantification in tomato and orange matrices.



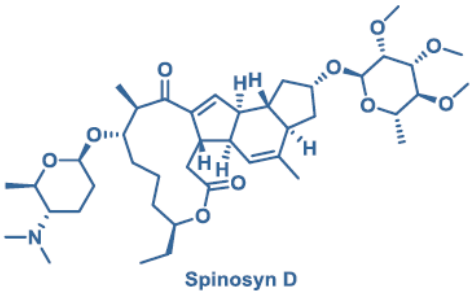


Figure 1: Chemical structure spinosyn A and spinosyn D

3. Experimental

3.1. Sample treatment

The evaluation of spinosad was performed in tomato as representative matrix of high-water content commodity group and in orange as representative of high acid content commodity group. Blank samples were extracted using the QuEChERS:

1. Weigh 10 g of sample in a 50-mL PTFE centrifuge tube.
2. Add 10 mL acetonitrile.
3. Shake the sample in an axial agitator (Agitax) for 4 minutes.
4. Add 4 g anhydrous magnesium sulphate, 1 g sodium chloride, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogencitrate sesquihydrate and shake manually (3 sec).
5. Shake the sample in an axial agitator (Agitax) for 4 minutes.
6. Centrifuge the tubes at 4000 rpm for 5 min.
7. Transfer 5 mL of the supernatant to a 15-mL PTFE centrifuge tube containing 750 mg anhydrous magnesium sulphate and 125 mg PSA (primary secondary amine) and vortex for 30 sec.
8. Centrifuge the tubes at 4000 rpm for 5 min.
9. Transfer the supernatant to a 4-mL vial and add 10 µL of a formic acid solution in acetonitrile (5 % volume) per mL of extract.

3.2. Analysis by LC-QqQ-MS/MS

All samples were analyzed by LC system operating in multiple reaction monitoring mode (MRM). Selected reaction monitoring (SRM) experiments were carried out to obtain the maximum sensitivity for the detection of the target molecules. For confirmation of the studied compounds, two SRM transitions and a correct ratio between the abundances of the two optimised SRM transitions (SRM2/SRM1) were used, along with retention time matching. The mass transitions used are presented in **Table 1**

Table 1. Detection and chromatographic parameters for the compounds analyzed by LC-MS/MS.

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Ret Time (min)	Collision Energy (eV)	Polarity
Spinosyn A	732.6	142.2	7.291	29	Positive
Spinosyn A	732.6	98.1	7.291	53	Positive
Spinosyn D	746.6	142.1	7.550	30	Positive
Spinosyn D	746.6	98.1	7.550	54	Positive
Dimethoate-d6	235.8	205.0	5.135	10	Positive
Dimethoate-d6	235.8	177.0	5.135	15	Positive
Dichlorvos-d6	227.1	133.1	5.895	17	Positive
Dichlorvos-d6	227.1	115.1	5.895	20	Positive
Malathion-d10	340.9	132.1	7.425	14	Positive
Malathion-d10	340.9	290.1	7.425	10	Positive

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

- Column: Shim-pack UC-X 2.1x150 mm and 3 µm particle size
- 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: 40 °C
- Flow rate: 0.3 ml/min
- Injection volume: 5 µL
- Autosampler temperature: 15 °C

Mobile phase gradient for pesticides analysis (**Figure 2**):

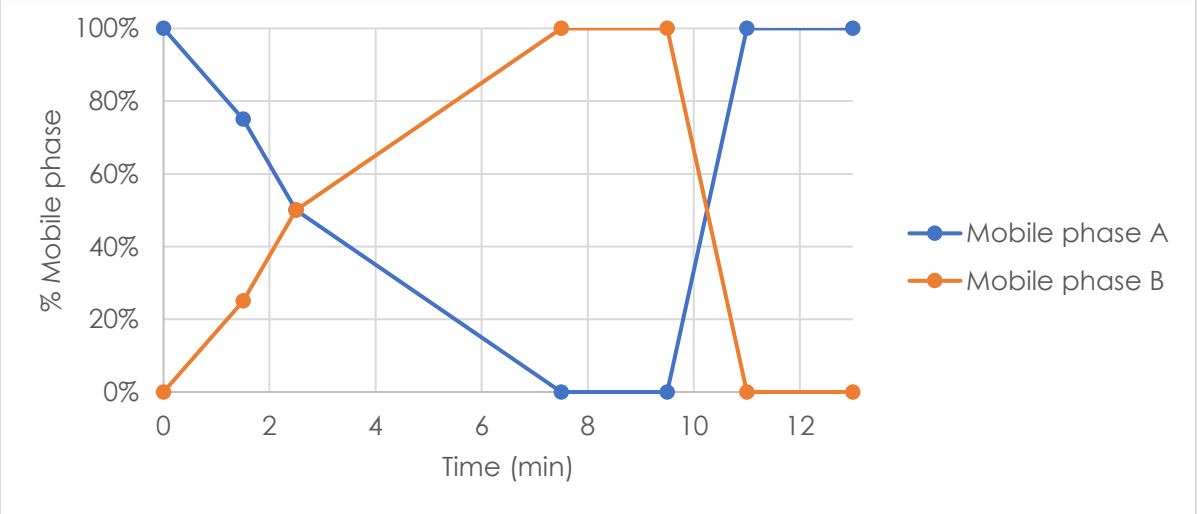


Figure 2: Mobile phase gradient used. A (Water (0.1 % formic acid, 5 mM ammonium formate, 2 % MeOH)) and B (Methanol (0.1 % formic acid, 5 mM ammonium formate, 2 % water))

Triple quadrupole system

- Ionisation mode: Positive mode
- Capillary (positive and negative): 4 kV
- Switching polarity: 5 ms
- Intereface temperature: 300 °C
- Desolvation line temperature: 526 °C
- Heat block temperature: 400°C
- Nebulizer gas flow: 3 L/min
- Heating gas flow: 10 L/min
- Drying gas flow: 10 L/min

4. Results and discussion

Five replicates of blank extracts (tomato and orange) spiked at 10 µg/L with the individual standards of spinosyn A and spinosyn D (spinosad sum concentration: 20 µg/L) were analysed to evaluate the accuracy of the certified ratio of spinosyn A and spinosyn D provided by different commercial suppliers. The five replicates were quantified with the calibration curves prepared from the mixtures provided by the suppliers at concentrations of 5, 10, 50, 100 and 200 µg/L **(Figure**

3). For the quantification of spinosyn A and D in the replicates, the ratio of the isomers of each mixture provided by the suppliers were considered (**Table 2**)

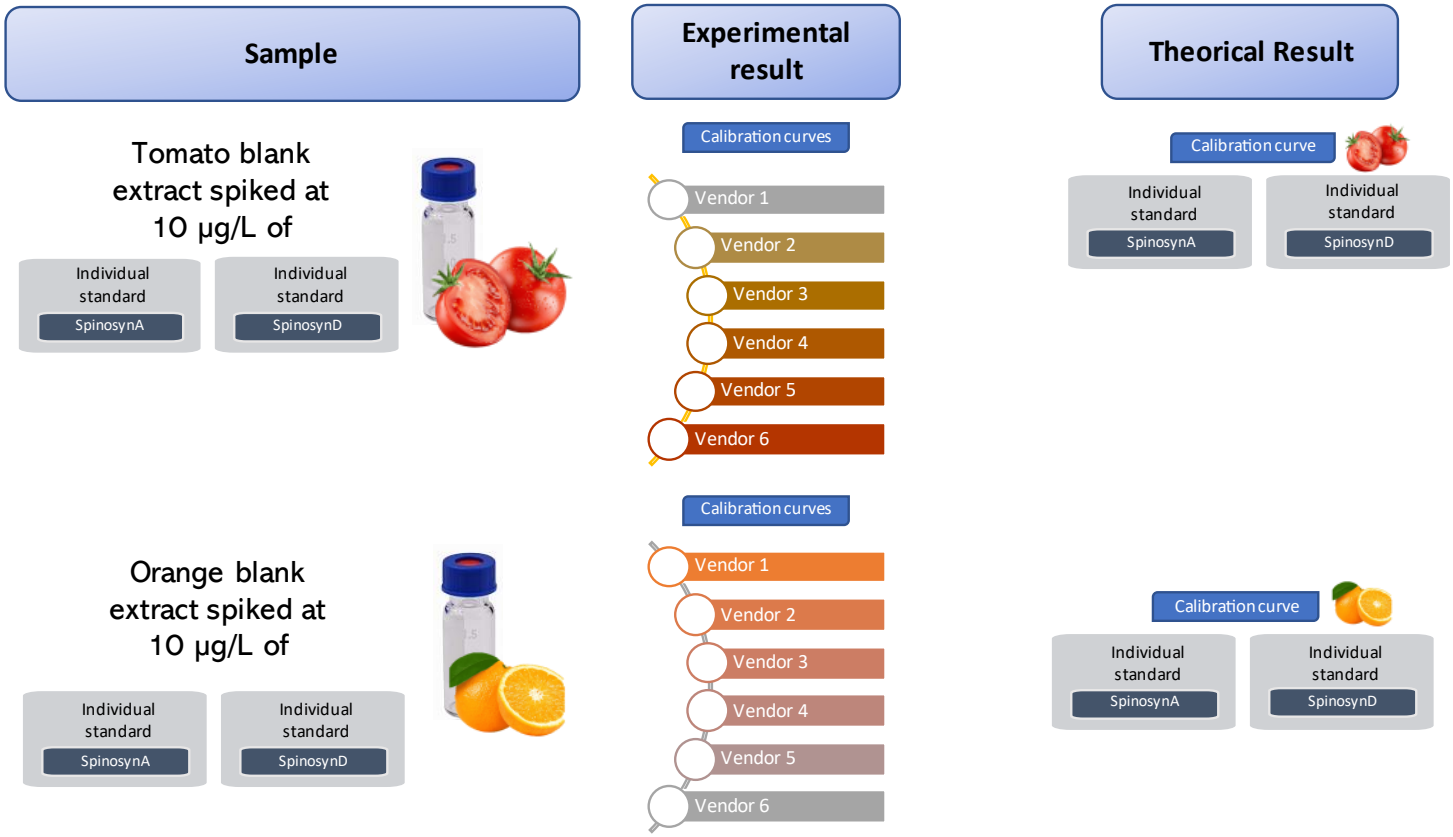


Figure 3: Different calibration curves from six commercial vendors were used to quantify the samples.

Table 2: Purity and isomer ratio found on the certificate of analysis of each commercial vendor.

Vendor	Purity (%)	Purity of Spinosyn A (%)	Purity of Spinosyn D (%)
1	96.90	65.56	31.37
2	93.61	84.00	16.00
3	97.00	90.10	8.50
4	96.60	69.00	31.00
5	96.60	77.68	18.93
6	94.20	72.00	22.20

The mean values of the five replicates (all of them with RSD (%) values below 5%) for both matrices have been represented in Table 3 and Percentage Error was calculated for each vendor based on Equation 1 being the concentration calculated with individual standards as theorical concentration.

Eq.1 $Percentage\ Error: \frac{Calculated\ Concentration - Theorical\ Concentration}{Theorical\ concentration} \cdot 100$

Table 3: Concentration of spinosyn A, spinosyn D and Spinosad (as sum) and the percentage of error

Tomato	Concentration (µg/kg)			Error (%)		
Vendor	**SA**	**SD**	**Spinosad (sum)**	**SA**	**SD**	**Spinosad (sum)**
1	11	10	21	10	0	5
2	11	10	21	10	0	5
3	12	5	17	20	-50	-15
4	8	14	22	-19	40	11
5	10	10	20	0	-4	-2
6	9	12	21	-10	20	5

Orange	Concentration (µg/kg)			Percentage Error		
Vendor	**SA**	**SD**	**Spinosad (sum)**	**SA**	**SD**	**Spinosad (sum)**
1	10	11	21	-3	10	4
2	9	9	17	-13	-14	-13
3	10	5	15	-3	-50	-27
4	7	15	22	-28	50	11
5	10	12	22	-1	20	9
6	9	12	21	-15	20	3

These results have been compared in the following **Figure 3** where the accepted value is set to 20 % of error and it is observed that for both matrices commercial vendors 3 and 4 not provide an adequate value for Spinosyn D purity.

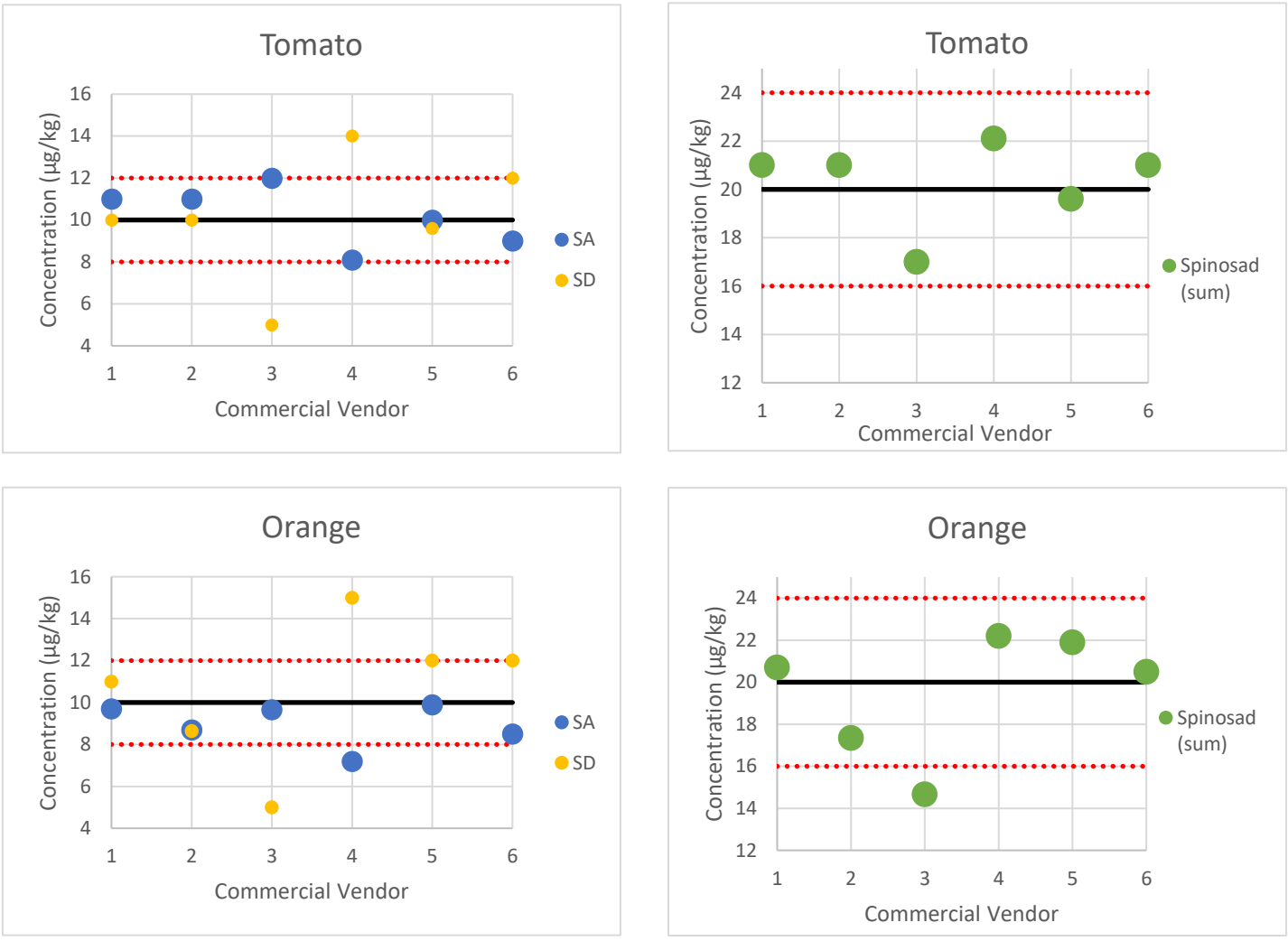


Figure 3: Control graphics for concentration of spinosyn A, spinosyn D and Spinosad (sum) for tomato and orange.

Moreover, the analysis of EUPT-FV24 tomato sample was carried out in triplicate following the extraction method previously mentioned in 3.1. Samples were quantified with calibration curves of different commercial vendor quantifying spinosyn A with the component spinosyn A of the spinosad analytical standard considering its proportion in the mix, doing the same for spinosyn D, and summing both concentrations for Spinosad. Internal injection standard (dimethoate-d6) and internal extraction standard (dichlorvos-d6 and malathion-d10) were evaluated to verify the goodness of injection and extraction.

RSD (%) values for each commercial vendor were below 5%, the percentage error was calculated with respect to the concentrations obtained with the individual standards, and as before, vendors 3 and 4 have an error of more than 20% (**Table 3**).

Table 3: Results of FV24 tomato sample

	Concentration (µg/kg)			Error (%)		
Vendor	**SA**	**SD**	**Spinosad (sum)**	**SA**	**SD**	**Spinosad (sum)**
1	138	28	166	4	0	3
2	134	26	161	0	-5	0
3	145	14	159	9	-51	-2
4	101	37	138	-24	35	-14
5	125	26	151	-6	-6	-6
6	113	32	145	-15	-15	-10

5. Conclusions

The accuracy of quantification of the individual components of spinosad, specially of spinosyn D, is greatly affected by an incorrect ratio of isomers present in the mixture provided by the vendors. However quantitation of the total sum of spinosad is not affected, with errors < 15%.