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Clean-up of cereal extracts for gas chromatography–tandem quadrupole mass spectrometry pesticide residues analysis using primary secondary amine and C18

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1 Abstract

The level of co-extracted matrix in wheat and oat extracts obtained by the QuEChERS method (EN 15662) is high and the occurrence of free fatty acids generates a major matrix peak in TIC chromatograms (rt. 13-22 min). Matrix can compromise the analytical performance in pesticide analysis using GC-MS/MS. In order to reduce the amount and the effects of matrix we tested the effect of using six different amounts of primary secondary amine (PSA)(0, 25, 50, 100, 150 and 200 mg/ml extract) with and without the addition of six different amounts of C18 (0, 25, 50, 100, 150 and 200 mg/ml extract) in the dispersive solid phase extraction (dSPE) procedure. dSPE clean-up using 25 mg/ml extract significantly reduced the major matrix peak observed for wheat extracts. Higher amounts of PSA reduced the analytical response for iprodione and malathion. For oat extract 50-150 mg PSA/ml extract was needed to obtain equally low intensity of the matrix peak. For oat the analytical responses of the target pesticides generally increased with increasing amount of PSA. C18 had no significant effect on the intensity of the major matrix peaks and even resulted in lower analytical responses for several of the target pesticides. Based on the present study it is concluded that the optimal dSPE clean-up procedure employs 25 mg PSA/ml extract for wheat and 150 mg PSA/ml extract for oat.

2 Introduction

About 1000 pesticides are available on the world market and residues of all of these may potentially occur in our foods including cereals. Multi-residue methods are a necessity in order to monitor and control residues of as many of these pesticides in our food as possible. A generic extraction method allowing efficient extraction of a wide range of analytes is needed for such multi-methods. The Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method published at the CEN method EN 15662 [1] is a generic extraction method, which has become widely used for the extraction of pesticide residues from food matrices and has also been introduced in our laboratory. During the development of the QuEChERS method several factors, as choice of extraction solvent, amount and types of partitioning conditions, extraction time and pH etc., were tested and optimised with the aim to obtain satisfactory recovery for a wide range of pesticides, that vary greatly in regard to their physical/chemical characteristic [2]. A drawback of such a generic extraction method is however, that also natural constituents of the samples are extracted, i.e. co-extracted matrix.

Occurrence of co-extracted matrix is generally unwanted because it can result in inaccurate quantification and reduced ruggedness of a method. The matrix can e.g. interact with the analyte or active sites in the inlet or gas chromatographic (GC) column to change the instrumental response or it selves produce an instrumental response. By using matrix matched external calibration for quantification, some of the matrix

effects can be compensated for. However, by this approach the amount of matrix introduced into the analytical system is also increased, and this may result in the need for more frequent instrument maintenance and thereby also more instrument down-time. Matrix induced ion suppression can also compromise the method performance in LC-MS/MS analysis. Though, because of the improved sensitivity of triple quadrupole instruments, dilution of the extract has become common practice for the reduction or elimination of matrix effects in LC-MS/MS analysis. In GC analysis, the presence of matrix can act as analyte protectants and thereby increase the instrumental response for some pesticides and consequently also the sensitivity of the method. Examples of pesticides that give higher analytical responses in the presence of matrix are azoxystrobin, phosmet, malathion, iprodion [3], acephate, dimethoate and omethoate [4]. Thus in GC the approach of diluting the samples until the matrix effects become insignificant, may not be a recommended approach, because some analytes simply never get to the mass spectrometer in the absence of matrix. Though, in cereal extracts obtained by the QuEChERS method, employing dispersive Solid Phase extraction (dSPE), clean-up with 25 mg primary secondary amine (PSA) per ml extract, the amount of matrix is so high that it increases the requirement for instrument maintenance and/or reduces the ruggedness of the method. Thus for cereal extracts a major portion of the co-extracted matrix is not removed by dSPE with 25 mg PSA per ml extract, and a reduction of the amount of co-extracted matrix will be beneficial.

By analysing QuEChERS extracts of cereals by GC-MS in scan mode we have found, that a major peak elute at rt. 13-22 min. Examples of these major matrix peaks, in wheat and oat extract, are presented in the total ion chromatograms (TIC) in Figure 1. These major matrix peaks, have by investigations at our laboratory (unpublished data), but also by Mastovska, Dorweiler, Lehotay, Wegscheid, & Szpylka, 2010 [5], been found to consist of various fatty acids and primarily linoleic acid. According to data in The National Food Institute's Food Composition Databank (www.foodcomp.dk) wheat and oat have a fat content of 20 g/kg and 65 g/kg, respectively. The fatty acids accounting for the largest fraction of this fat content is linoleic acid (44-52%), oleic acid (25-35%) and palmitic acids (15-24%) [6]. In order not to compromise the generic nature of the QuEChERS method a clean-up procedure, that specifically removes the free fatty acids, would be optimal. However to our knowledge such a procedure is not available. PSA is known to bind compounds containing carboxylic acid groups and therefore a likely candidate for the removal or reduction of the fatty acids in cereal extract. PSA will in many cases also bind acidic pesticides. PSA clean-up may therefore not always be amenable to analysis of pesticides by liquid chromatography. Mastovska et al. 2010 [5] found, that the major matrix peak of maize was significantly reduced by increasing the amount of PSA used in the dSPE, from 10 mg PSA per gram sample to 60 mg PSA per gram sample and also introducing 20 mg octadecyl (C18) sorbent per gram sample. Rice, rye and maize contain more fat than wheat but less than oat. Oat and wheat therefore represents the lower and upper extreme regarding fat content for the most common types of cereal. For fatty foods as milk, egg and avocado C18 has also been shown to reduce the amount of co-extracted matrix [7]. Thus both PSA and C18 may reduce the amount of co-extracted matrix in cereal extracts.

Thus, the amount of interfering matrix in cereal extracts obtained by our present method may be further reduced, if the amount of PSA used in the dSPE step is optimised and by also introducing C18. More extensive clean-up may, however, also remove the target analytes resulting in low recoveries. So it is often a compromise which needs to be found, i.e. an optimised clean-up procedure, by which enough of the interfering matrix is removed, in order to have a robust method, but still allows for acceptable recovery of the target analytes. Because PSA efficiently bind acids it is not feasible for analysis of acidic pesticides [8].

The aim of the present work was therefore to study whether the dSPE employed for dSPE clean-up of wheat and oat extracts obtained by the QuEChERS method could be optimised by adjusting the amount of PSA employed and by introducing C18 in the procedure. Proficiency test material was used, i.e. wheat EU proficiency test C2 (EUPT-C2) [9] and oat (EUPT-C3) [10] containing incurred and spiked pesticides. The effect of the defined test dSPE procedure, were evaluated by determining the intensity of matrix peaks in TIC

chromatograms and the analytical response obtained for the test analytes, in comparison to those obtained using no clean-up or the clean-up procedure according to the QuEChERS method, EN 15662 procedure.

3 Experimental

3.1 Chemicals

The pesticide standards (all purity >96%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). The acetonitrile (HPLC Grade S) was purchased from Rathburn Chemicals (Walkerburn, UK) and acetic acid and ammonium acetate were from Merck (Darmstadt, Germany). The magnesium sulphate was purchased from J.T.Baker, Aventor Performance Materials B.V (Center Valley, PA 18034, USA), sodium chloride from Merck & Co. (Whitehouse Station, NJ, USA), sodium citrate dehydrate and sodium citrate sesquihydrate from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany) and the clean/up sorbents PSA from Agilent Technologies (Santa Clara, CA 95051, USA), and C18 from International Sorbent Technology Ltd. (Gengoed Mid Glam, UK). Pesticide standard stock solutions of 1 mg/ml were prepared in toluene and stored at -18 °C in ampoules with argon atmosphere. A standard mixture of 10 mg/ml in acetonitrile was prepared from these stock solutions. Working solutions were prepared by dilution of this standard mixture and finally matching them 1:1 with extract of wheat and oat not containing pesticide residues obtaining a concentration range of 0.0015 to 0.05 mg/ml. The extracts used for matrix matching were obtained by the extraction and clean-up procedure described in section 3.2 as our standard procedure.

3.2 Extraction and clean-up procedure

The point of departure was the extraction and clean-up procedure employed as standard procedure for cereals at our laboratory, which is in accordance with the CEN method EN 15662. In brief, 5.0 g of milled cereal was added 10 ml of cold water and immediately extracted with 15.0 ml of acetonitrile by shaking the tube 1 min by hand. To aid the extraction a ceramic homogenizer was included. After this 6.0 g magnesium sulphate, 1.5 g sodium chloride, 1.5 g sodium citrate dihydrate and 0.75 g sodium citrate sesquihydrate was added. After 1 min of shaking by hand and centrifugation for 10 min, at 4300 g 8 ml of the supernatant was transferred to a clean tube and stored at -80°C for minimum 1 hour. The extracts were then thawed and when still very cold centrifuged at 4300 g for 5 min (freezing out step). One ml of the supernatant was cleaned by dSPE by transferring it to a centrifuge tube containing 25 mg PSA and 150 mg magnesium sulphate (i.e. 25 mg PSA/ml extract). After shaking by hand for 30 s and centrifugation for 5 min at 4300 *g* the extract was analysed by GC-MS/MS, thus, only GC amendable pesticides were included in the study.

The test material used was wheat and oat reference material from EUPT-C2 and EUPT-C3, respectively. Both of these reference materials contain incurred as well as spiked pesticides. The test materials were produced by milling of kernels in a centrifugal mill with 1 mm sieve.

The study consisted of three experiments, in which the effect of different amounts of PSA and C18 used during dSPE clean-up of the extract obtained after the freezing out step was evaluated (Table 1). The evaluation parameters were the intensity of the matrix peaks in TIC chromatograms (Figure 1) as well as the analytical result obtained by analysis in Multiple Reaction Monitoring (MRM) mode for the pesticides in the test material (Figure 3). In experiment 1 six levels of PSA were tested (0-200 mg/ml). In experiment 2 were the effect of combining the six levels of PSA (0-200 mg/ml) with equal amounts of C18 (0-200 mg/ml) studied. In experiment 3 were the effect of using a fixed amount of PSA of 150 mg/ml in combination with six levels of C18 (0-200 mg/ml) tested. All experiments were performed as double determinations.

3.3 Chromatographic separation and detection

The analysis was performed on a Quattro Micro Tandem GC-MS/MS (Waters, USA). The system consisted of a PAL-GC Auto sampler, an Agilent GC 6890N and a Quattro Micro Tandem mass spectrometer. The GC was equipped with a Gerstel PTV injector for large volume and 4 µl was injected. The injector program started with an initial temperature of 30°C for 0.8 min followed by a ramp of 480 °C/min to 290 °C, held for 2 min. Finally, the temperature was raised with 720 °C/min to 330 to clean the injector. The GC oven programme started with an initial temperature of 60 °C held for 3 min, followed by a ramp of 30 °C/min to 180 °C, held for 0.8 min, then 5 °C/min to 280 °C, held for 3 min. To clean the column, the temperature was raised with 40 °C/min to 300 °C for 10 min and 120 °C/min to 310 °C for 1 min. Chromatographic separation was performed on a RESTEK, Rxi®-5ms, 30 m., 0.25mmID, 0.25µm df column with a constant flow of 1.3 ml/min of helium as carrier gas. The temperature of the transfer line and ion source was set at 250°C and 180°C, respectively. The mass spectrometer was operated in the electronic ionization mode (EI, 70eV). Analysis in scan mode was employed to obtain TIC chromatograms for the determination of the intensities of the matrix peaks. MRM was used to perform mass spectrometric quantification of the pesticides. The employed MRM transitions, retention times and collision energies are listed in Table 2. Quantification was based on bracketing calibration curves of five matrix matched standard solutions, covering the relevant concentration range.

4 Results and discussion

The aim of the present work was to study whether the dSPE employed for clean-up of cereals extracts could be optimised by adjusting the amount of PSA employed and by introducing C18 in the procedure. The aim of such an optimization was to: 1) reduce the amount of matrix loaded into the system without significantly compromising the analytical response and thereby the sensitivity, but also 2) to increase the ruggedness of the method for pesticides co-eluting with the major matrix peak, both in term of variability of retention times and response.

4.1 Reduction of major matrix peaks

A major matrix peak was observed in TIC chromatograms of cereals extracts at rt. 13-22 min. The components accounting for this matrix peak, most likely contributes to the increased need of instrument maintenance, when analyzing cereal extracts, as well as to the reduced method ruggedness for some pesticides eluting in the same region. TIC chromatograms, obtained for wheat and oat extracts, cleaned by procedure I, II, III, IV and VI in the 3 experiments, are presented in Figure 1. Results for clean-up procedure V is not shown in order to simplify the figure and because these did not deviate from the observed pattern for the other experiments. The intensity of the matrix peak (rt. 13-22 min) for oat (Figure 1b) is significantly higher than for wheat (Figure 1a). This is in good agreement with the facts that the lipid content of oat is approximately three times higher than of wheat.

Increasing amounts of PSA (0-200 mg/ml extract) reduced the major matrix peak (rt. 13-22 min) in the wheat extracts (Figure 1a). Just 25 PSA mg/ml extract was sufficient for significant reduction. For the oat extracts on the other hand, 150 PSA mg/ml extract were needed in order to obtain correspondingly low matrix peak intensity (Figure 1b). Using more than 150 mg PSA/ml extract for the clean-up did not further reduce the matrix peak. Addition of C18 (0-200 mg/ml extract) in equal amounts to PSA (0-200 mg/ml extract) did not reduce the intensity of the matrix peak further, neither for the wheat nor the oat extracts (Figure 1c and 1d).

However, C18 (25 mg/ml) seemed to reduce the amount of co-extracted compounds in the wheat extract eluting after 30 min or later (data not shown). This effect was not observed for the oat extract. Compounds eluting this late are expected to include phytosterols. Generally, oat has a low content of phytosterols and this low level may be the reason for not observing the same effect as for wheat. Hou et al. 2013 also found that C18 (50 mg/ml extract) only have limited effect on the amount of co-extracted matrix from rice [11]. PSA would be expected to be a more efficient sorbent for the relatively polar free fatty acids (FFA), than C18 would be. C18 is a more efficiently sorbent for non-polar lipids.

Based on these results 150 mg PSA/ml extract was chosen as a fixed factor and combined with increasing amounts of C18 for clean-up in Experiment 3. As can be seen from figure 1e and 1f this experiment confirmed that including C18 in the dSPE clean-up has virtually no effect on the amount of co-extracted matrix in the region where the target pesticides elute for neither wheat nor oat extract (rt. 13-22 min).

4.2 Effect on analytical result

Clean-up may, as mentioned, not only result in removal of the co-extracted matrix but may also result in loss of analytes. Thus in order to evaluate the applicability of the different clean-up procedures (Table 1), the effect on the analytical response of the target analytes also needs to be evaluated accordingly.

The levels of the incurred and spiked pesticide residues in the test material were in the range of 0.01- 1.23 mg/kg. The assigned values are listed in Table 2. The spike procedure is described in Poulsen et al. (2009) [9].

Increasing amounts of PSA, for clean-up of wheat extracts, resulted in lower levels of iprodione and malathion (Figure 2*a*). The levels of the other pesticides in the wheat samples were reduced by less than 20% even when using 200 mg PSA. For oat, increasing levels of PSA in the clean-up procedure resulted in increasing responses for fenbuconazole and metconazole (Figure 2*b*). The levels of malathion and fludioxonil seemed to be decreased by PSA. However, the response for malathion seems to approach that of the uncleaned extract when using 150 or 200 mg PSA/ml. However, the content of malation was very low (assigned value of 0.011 mg/kg, Table 2) and the relative variability will consequently be higher. Increasing amounts of both PSA and C18, did on the other hand, generally result in lower responses (Figure 2*c* and 2*d*), except for alpha-cypermethrin in the wheat extract and flusilazole in the oat extract. This same trend was observed when increasing levels of C18 were combined with a fixed level of 150 mg PSA (Figure 2*e* and 2*f*).

By taking both the effect on the intensity of the major matrix peak and the observed effects on the analytical responses into account, it is indicated that an optimal clean-up procedure for wheat employs 25 mg PSA/ml extract and for oat 150 mg PSA/ml extract. C18 does not add to the efficiency of the clean-up and in worst case it will compromise the recovery of the pesticides. Thus it varies how much matrix is co-extracted from the different types of grain. Before we consider to change our standard procedure for extraction of pesticides from oat we need however to gain more experience on how the higher amount of PSA affect the recoveries of pesticides not represented in this study. However, because several groups of pesticides were represented in the study we expect that recoveries from oat samples for the majority GC amenable pesticides will not be compromised by introducing dSPE with 150 mg PSA/ml extract.

Though, as illustrated in Figure 3 the intensity of the major matrix peak (rt. about 13-22 min) may also vary for the same flour sample depending on the storage conditions. We have found that the major matrix peak is more intense in extract wheat, oat, rice and maize flour stored at room temperature than in flour stored at -20°C. For this test the wheat flour had been stored at room temperature for 52 weeks, whereas the oat, rice and maize had been stored at room temperature for only four weeks. This increase in intensity may be attributed to a higher concentration of FFA in the cereal grains, which is known to increase with increasing storage time and moisture content [12]. A significant reduction of the major matrix peak in extracts of flour stored at room temperature may therefore require more extensive cleanup than freshly milled samples. Thus it may be more difficult to obtain reliable results when e.g. analyzing samples of flour, which have been stored for several months at room temperature, than when analyzing e.g. proficiency test material, which have been stored at lower temperatures and for shorter time after milling. In order to limit this non beneficial change of the matrix in samples of cereal these should be put in the freezer as soon as possible after sampling.

Other co-extracted components, which is not ionizable and consequently does not reach the detector, may also affect the analytical response of the analytes, e.g. by forming active sites in the liner or column which bind the analyte and thereby compromise the analytical response [13]. Whether the occurrences of such components are also reduced by the suggested clean-up procedure needs to be studied.

5 Conclusions

Based on the present results, 25 mg PSA provide efficient clean-up of wheat extracts, whereas 150 mg PSA is needed for efficient clean-up of oat extracts. C18 did not have any significant effect on the intensity of the major matrix peak and generally no positive effect on the analytical responses. This is based on the present results which employed test materials which was stored in freezer (-20°C) after milling. Further studies are however needed in order to evaluate whether 25 mg and 150 mg PSA is also adequate for clean-up of other samples of wheat and oat which e.g. have been stored under less optimal conditions.

6 Acknowledgements

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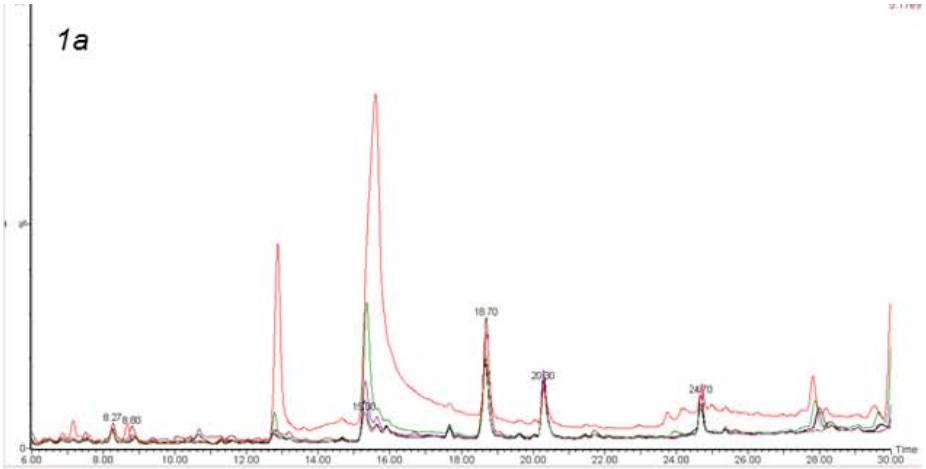
Figure captions

Figure 1. TIC chromatograms of wheat and oat extracts obtained after a range of different dSPE clean-up procedure employing PSA and C18. *1a* and *1b*: experiment 1 wheat and oat; *1c* and *1d*: experiment 2 wheat and oat; *1e* and *1f*: experiment 3 wheat and oat. The different colors represent the different clean-up procedures (Table 1); Red: I; Green: II; Purple III; Black IV; Brown VI; V is not illustrated.

Figure 2. Quantified levels after clean-up of wheat and oat extracts compared with the quantified levels in corresponding not cleaned-up extracts. The deviation is shown in percentage. The tested d-SPE procedures employed PSA and C18 at varying levels as followed: Experiment 1 with 0-200 mg PSA for wheat (2*a*) and oat (2*b*); Experiment 2 with 0-200 mg PSA and 0-200 mg C18 for wheat (2*c*) and oat (2*d*); Experiment 3 with 150 mg PSA and 0-200 mg C18 for wheat (2*e*) and oat (2*f*). Lines separate the pesticides into the groups of 1) conazoles, 2) organo phosphorous, 3) strobil ureas, 4) pyrethroids and 5) others.

Figure 3. Chromatograms different cereals stored at -20 °C (red) and the same flour stored at room temperature for 4 weeks (oat, rice and maize) and 52 weeks (wheat) (green).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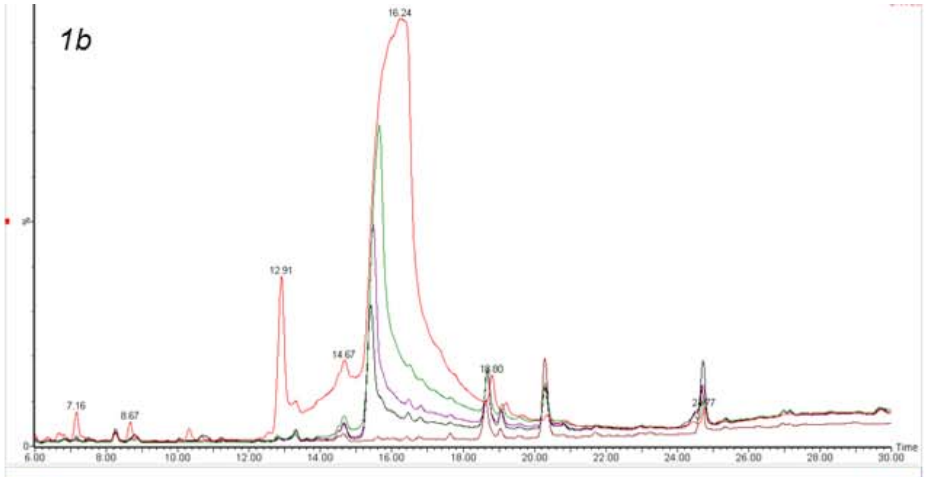
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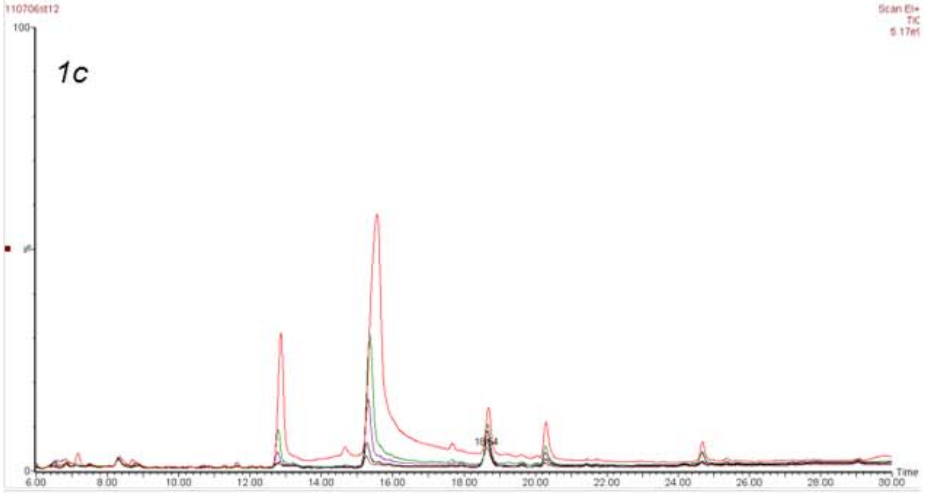
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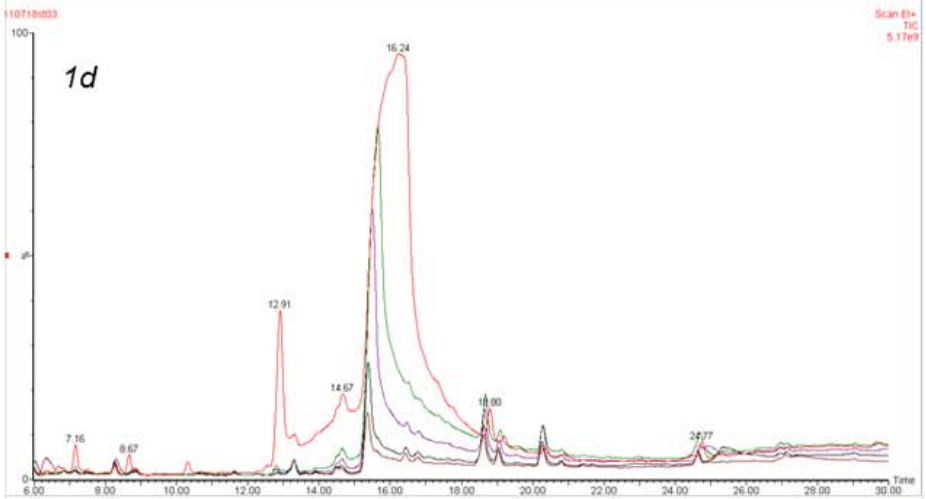
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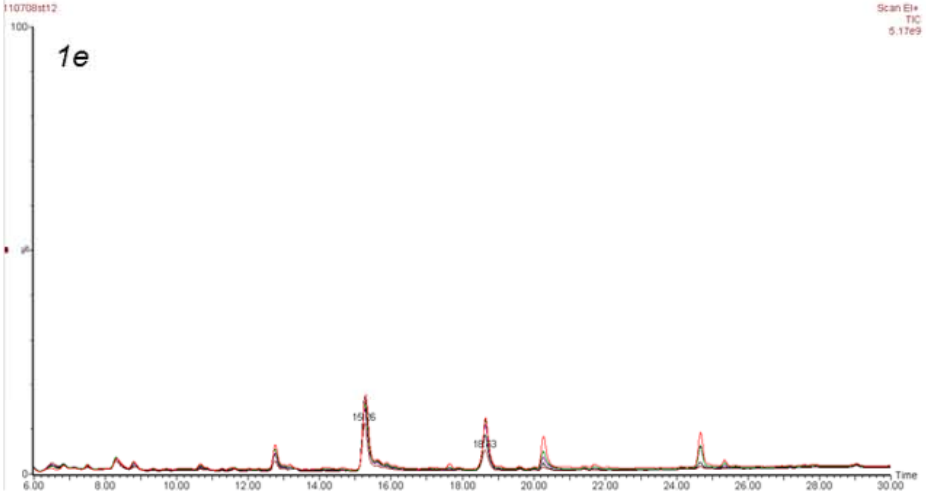
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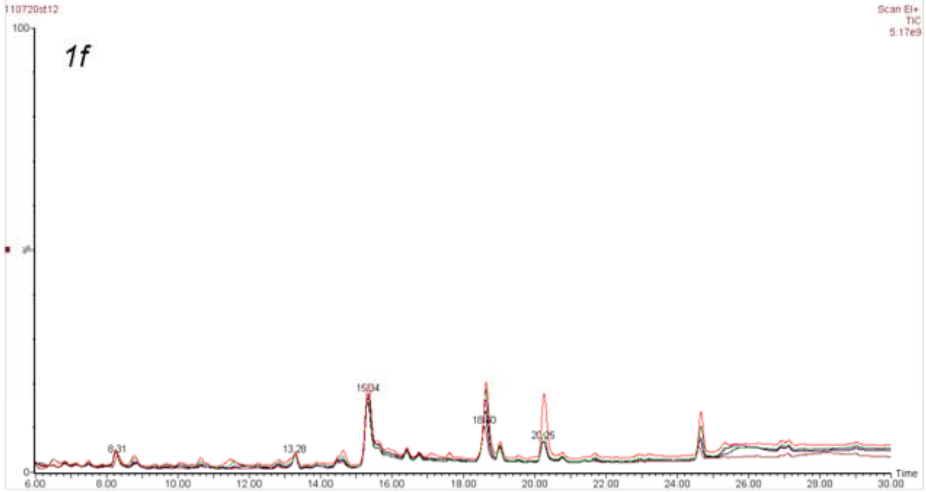
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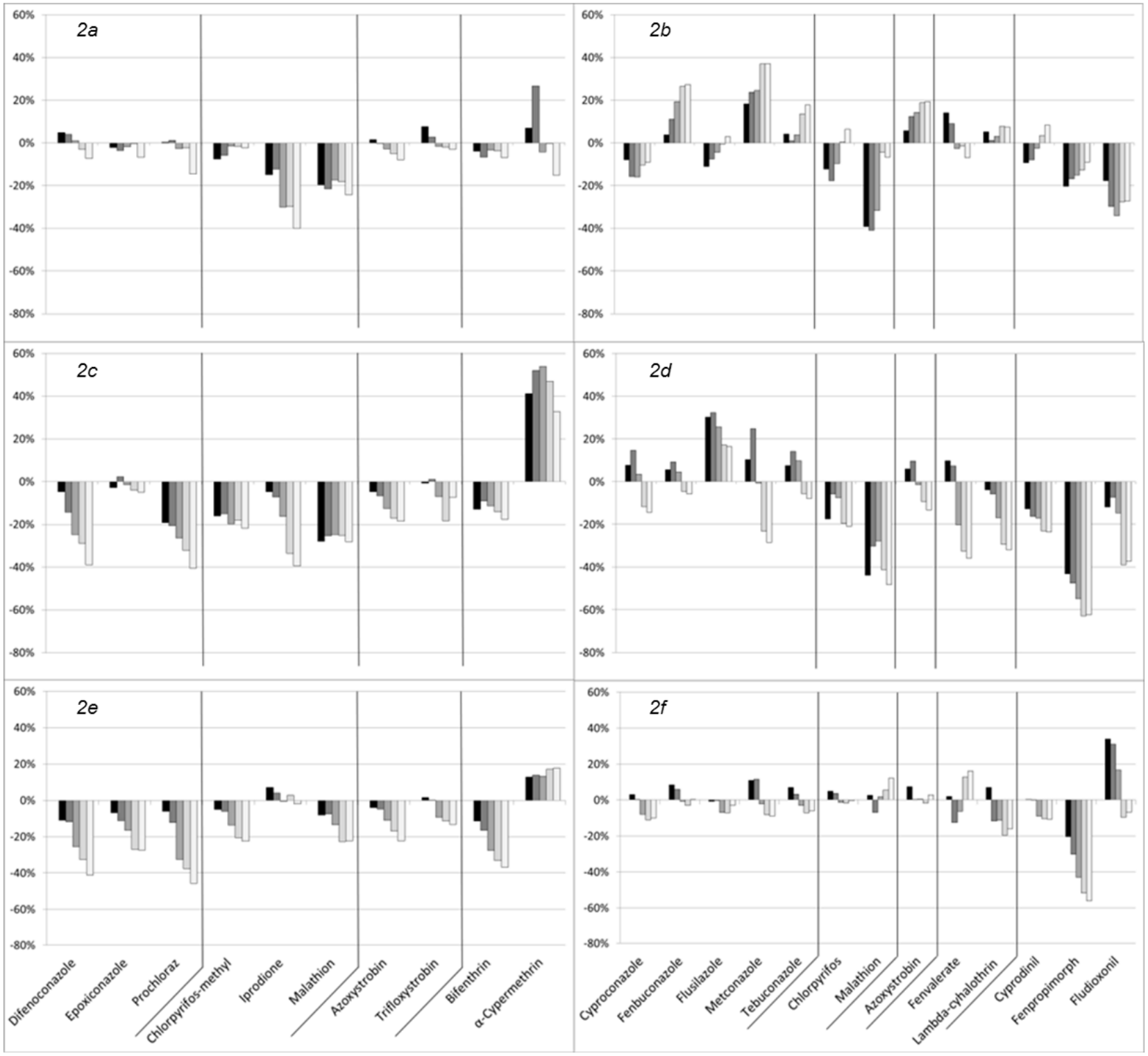
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Iprodione
Malathion
Azoxystrobin
Trifloxystrobin
Bifenthrin
α-Cypermethrin
Cyproconazole
Fenbuconazole
Flusilazole
Metconazole
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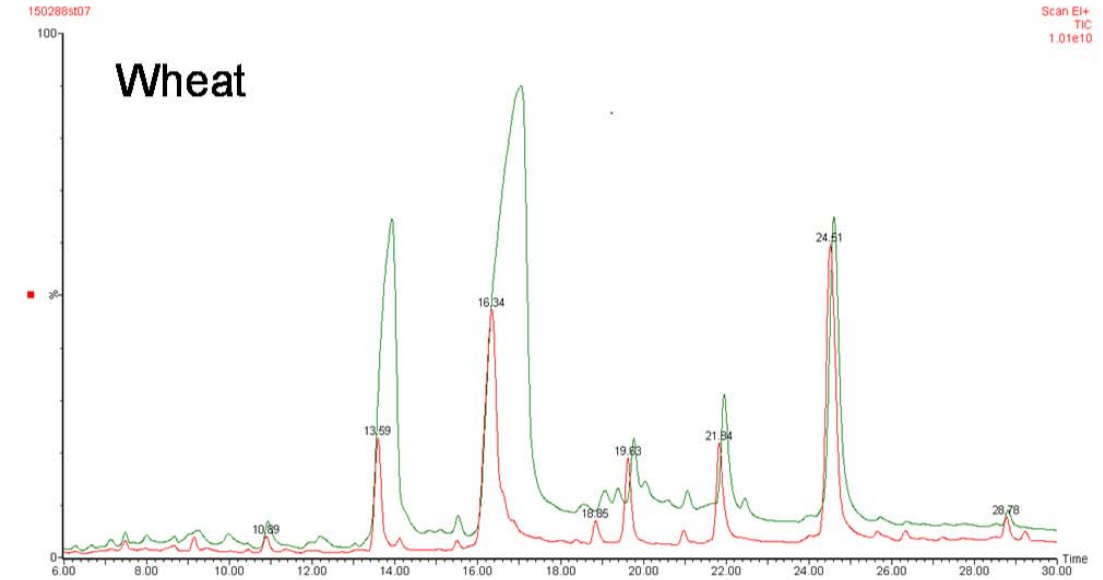
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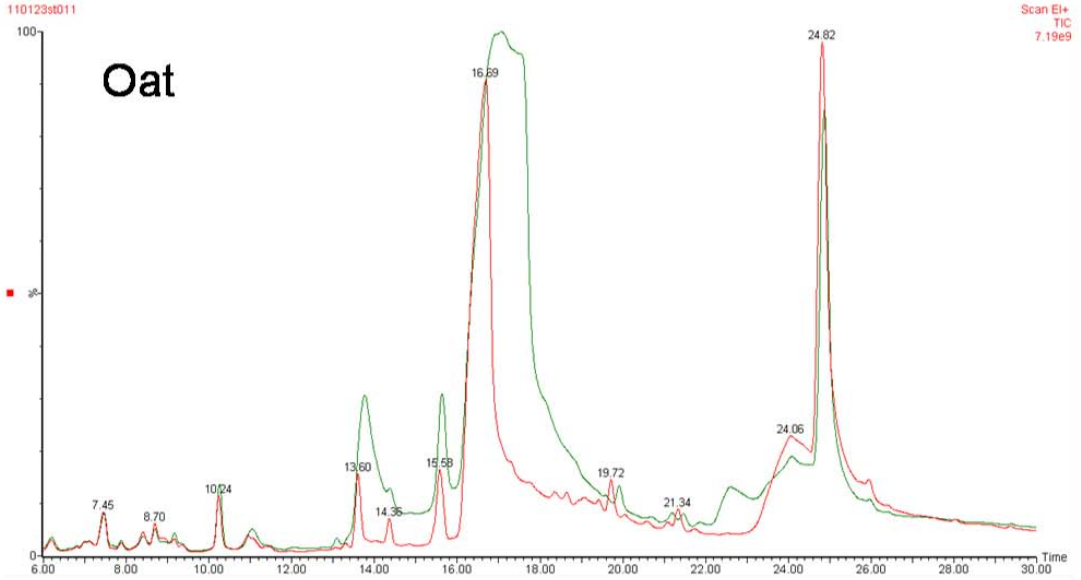
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Oat
100
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7.45
8.70
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13.60
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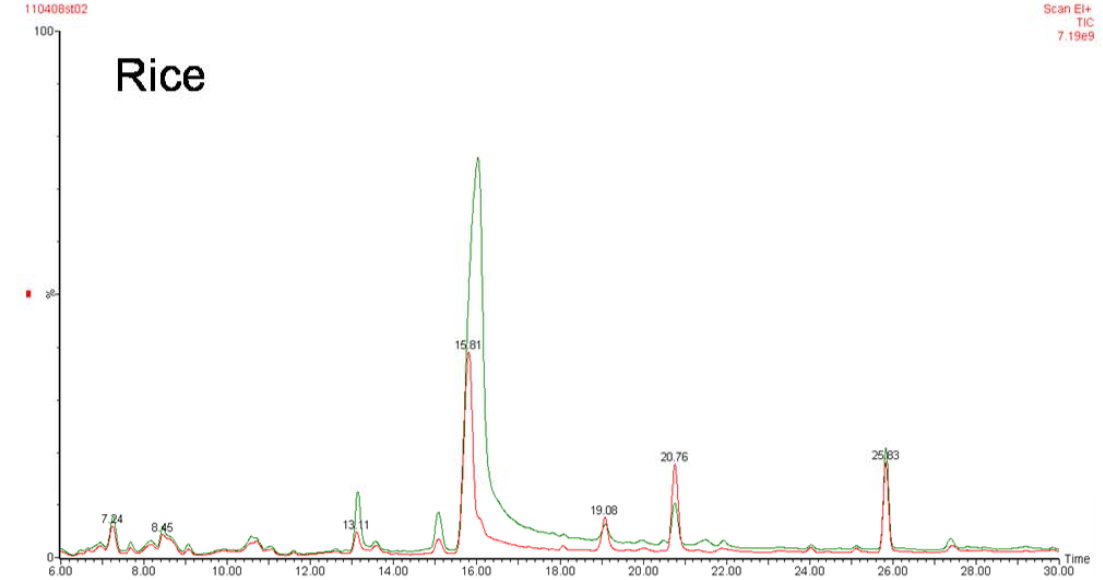
Time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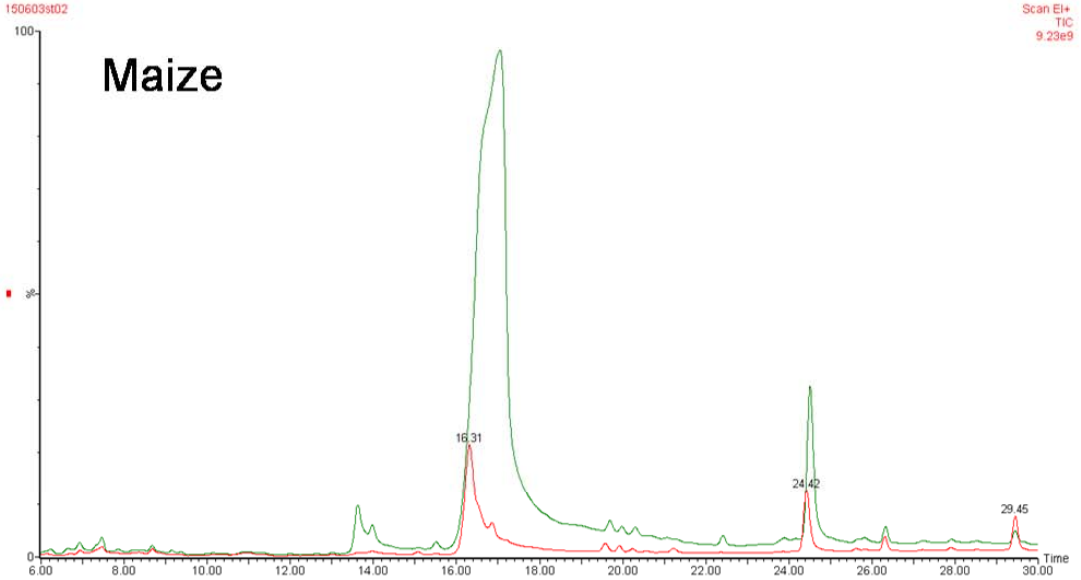
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Scan EI+
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Time

Table 1. Overview of the three experimental setups outlining the amount of PSA and C18 added to the extracts during dSPE clean- up. All experiment were performed as double determination on EUPT-C2 wheat and EUPT-C3 oat

Test materials	Clean-up procedure	Experiment 1 Increasing amount of PSA, mg/ml extract		Experiment 2 Increasing amount of PSA and C18, mg/ml extract		Experiment 3 Fixed amount of PSA and increasing amount of C18 mg/ml extract	
		PSA	C18	PSA	C18	PSA	C18
EUPT-C2	I	0	0	0	0	150	0
and	II	25	0	25	25	150	25
EUPT-C3	III	50	0	50	50	150	50
	IV	100	0	100	100	150	100
	V	150	0	150	150	150	150
	VI	200	0	200	200	150	200

Table 2. GC-MS/MS conditions including Retention times (rt.), transitions for quantifier and qualifier and collision energy (CE) as well as assigned values for the pesticides in test material EUPT-C2 and EUPT-C3.

	GC-MS/MS conditions					Assigned value in test material (mg/kg)	
Pesticide	**rt.**	**Transition 1 (quantifier)**	**CE**	**Transition 2 (qualifier)**	**CE**	**EUPT-C2 (wheat)**	**EUPT-C3 (oat)**
Alpha-cypermethrin	25.69	163>127	10	181>152	20	0.079	
Azoxystrobin	29.06	344>329	15	388>360	15	0.239[1)]	0.175
Bifenthrin	20.46	181>166	10	165>115	20	0.087	
Chlorpyrifos	13.32	197>169	10	314>258	12		1.04
Chlorpyrifos-methyl	12.06	286>93	20	125>79	5	0.130	
Cyproconazole	16.87	222>125	15	139>111	15		0.453
Cyprodinil	14.05	226>225	15	223>208	15		0.076
Difenoconazole	27.79+27.92	323>265	15	325>267	15	0.169[1)]	
Epoxiconazole	19.63	192>138	10	206>165	5	0.176	
Fenbuconazole	24.68	198>129	10	129>102	15		0.508
Fenpropimorph	13.29	303>128	5	117>115	10		0.121
Fenvalerate	27.06+27.45	167>125	10	125>99	10		0.097
Fludioxonil	16.09	248>127	20	154>127	5		0.078
Flusilazole	16.48	314>233	20	206>137	20		0.728
Iprodione	20.03	314>245	10	216>187	5	0.289	
Lambda-cyhalothrin	21.96+22.33	197>141	10	208>181	10		0.050
Malathion	13.02	173>99	10	173>127	5	0.168[1)]	0.011[2)]
Metconazole	20.84	125>89	10	127>89	10		0.478
Prochloraz	24.11	180>138	10	310>268	5	0.239[1)]	
Tebuconazole	19.08	250>125	15	125>89	10		1.23
Trifloxystrobin	18.73	222>190	5	186>145	10	0.439	

1) Pesticides spiked in laboratory

2) Residue was too low to establish an assigned value, so the value is the median of the reported results