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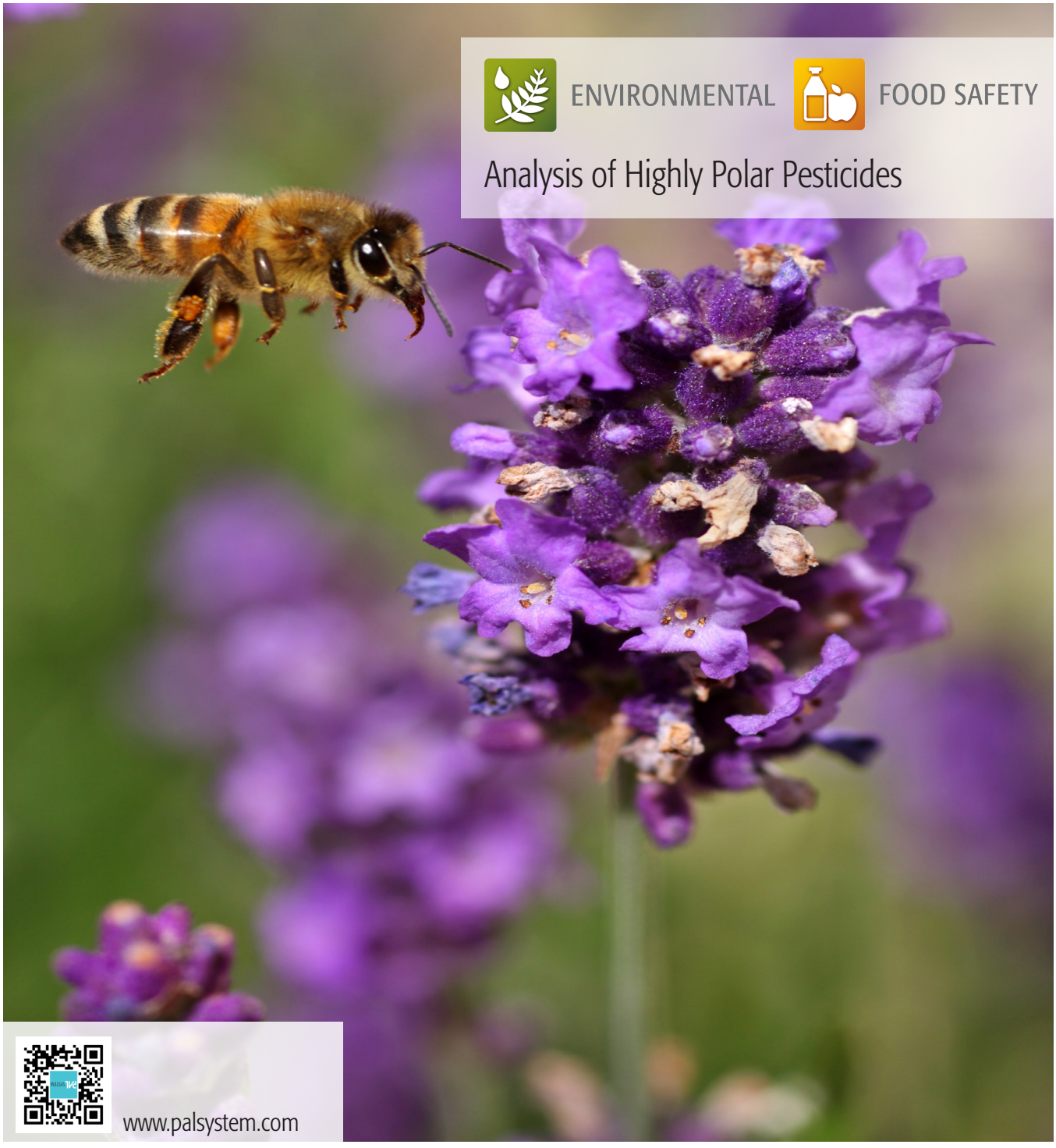

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ENVIRONMENTAL FOOD SAFETY

Analysis of Highly Polar Pesticides

Automated Micro-SPE Clean-up of Complex Food Matrices for LC-MS/MS

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Introduction

Micro-SPE (µSPE) emerged as an automated green micromethod for sample preparation and clean-up in food safety, proteomics, forensic, environmental and analysis since more than ten years. Applications are wide-ranging and cover drugs, environmental contaminants, and, in particular, the extract clean-up in multiresidue pesticide analysis. The automation of the µSPE sample preparation steps led to the desired standardization of the applied sorbent materials for extract clean-up for the large variety of food commodities, an increase in sample throughput, and the welcome potential for offline and online hyphenation with GC-MS, LC-MS or even IC analysis.

Highly Polar Pesticides Analysis

The application of "highly polar pesticides" (HPPs) in agricultural production is increasing due to their low cost and high efficiency, with glyphosate as the well-known active ingredient in the popular herbicide Roundup™. The EFSA reports compounds like fosetyl-Al, etephon, chlormequat and glyphosate within the top ranking of frequent maximum residue limit exceedances (MRL) in Europe[1,2].

Such highly polar pesticides are not amenable for extraction and clean-up by the widely used QuEChERS protocol. This group of pesticides is mainly characterized by its high water solubility. Glyphosate as the best-known pesticide in this group serves well as a lead substance for method development. Due to the high polarity and dissociation in aqueous media LC-MS/MS is the method of choice for high-throughput multi-residue analysis and simultaneous quantification.

In this application report the analysis of eleven HPPs including glyphosate, glufosinate, ethephon, fosetyl-aluminium, and their related metabolites is described for analysis from complex food matrices such as honey and pollen. For the first time the micro-SPE clean-up is introduced also for HPPs to overcome the known analytical limitations by removal of polar co-extracts with automated clean-up processing of the sample extracts[3].

Offline Sample Pretreatment: Extraction of raw extract from different matrices

The extraction of the HPPs from a representative test portion of fruit and vegetables is described with acidified methanol in the Quick Polar Pesticides Method QuPPe[4]. After centrifugation a filtered aliquot is transferred to autosampler vials for direct LC-MS/MS analysis. For the described analysis of honey and pollen samples the applied extraction method is modified as follows:

Honey: To 5 g of homogenized sample 9 mL of water is added and spiked with 100 µL of a mixed solution of isotopically labelled glyphosate-$^{13}C_2$,^{15}N and AMPA-^{13}C,^{15}N at 1 mg/L. After vortexing 10 mL of MeOH is added, and the tubes are shaken at 1000 rpm for 10 min and centrifuged at 3220 g for 5 min. The sample concentration in the extract is 0.25 g/mL.

Pollen: To 2 g of homogenized sample 10 mL of water is added and spiked with 100 µL of a mixed solution of isotopically labelled glyphosate-$^{13}C_2$,^{15}N and AMPA-^{13}C,^{15}N at 1 mg/L, and 100 µL of a solution of fosetyl-Al-D15 at 1 mg/L. After vortexing the tubes are centrifuged at 3220 g for 30 min. A 5 mL aliquot of the supernatant is then treated with 250 mg of C18 material and 5 mL of acetonitrile, vortexed, and again centrifuged at 3220 g for 5 min. The sample concentration in the extract is 0.1 g/mL.

For both types of sample the supernatant is then applied for a manual SAX SPE to compare with the automated µSPE clean-up on the PAL System.

Fully automated raw extract clean-up from complex matrix samples using PAL System µSPE

A general clean-up of extracts other than dilution is not part of the above mentioned QuPPe method, except a dispersive SPE with C18 sorbent material in case of a necessary removal of protein and lipids from cereals, nuts, seeds or oily fruits. Though, with complex matrix samples, such as honey, pollen or even with coffee or tea, the co-extraction of polar matrix components was observed. These matrix compounds co-elute with the analytes of interest and interfere the peak detection and integration even in LC-MS/MS analysis, hence limiting the identification and detection sensitivity.

Solid-phase extraction (SPE) and micro solid-phase extraction (µSPE) employing a strong anion exchange (SAX) sorbent material were implemented for a clean-up of the primary extracts. The automation and miniaturization of the SAX clean-up for HPP compounds is demonstrated in this application for the first time.

Manual SAX SPE clean-up

Honey: An aliquot of 2.4 mL extract is diluted to a final volume of 10 mL with MeOH. The sample is then loaded on an SPE cartridge (HyperSep™ SAX 500 mg/6 cc, previously conditioned with 10 mL of MeOH), then washed with 6 mL of MeOH, and the water content removed by a vacuum pump for 3 min. The analytes are then eluted with 3 mL of MeOH:HCl 1M (9:1). Sample concentration in the extract is 0.2 g/mL.

Pollen: An aliquot of 6 mL extract is diluted to a final volume of 10 mL with MeOH. The sample is then loaded on an SPE cartridge (HyperSep™ SAX 500 mg/6 cc, conditioned with 10 mL of MeOH), then washed with 6 mL of MeOH, and dried from water by a vacuum pump for 3 min. The analytes are then eluted with 3 mL of MeOH:HCl 1M (9:1). Sample concentration in the extract is 0.2 g/mL.

Automated SAX µSPE clean-up

For the micro-solid-phase extraction (µSPE) clean-up, a PAL RTC System was used as a benchtop device for automated sample preparation. The benchtop installation allows the distribution of the cleaned extracts to different analysis systems like LC-MS or IC.

µSPE uses SPE cartridges in the dimensions of 35 mm hight and 8.5 mm OD. These novel PAL µSPE cartridges contain for this application a layer of SAX sorbent material, shown in Figure 1.

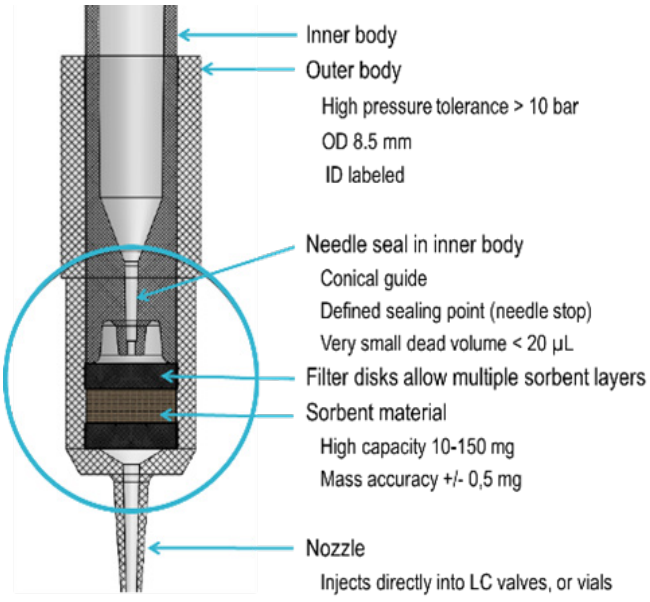

Figure 1: The novel PAL µSPE cartridge (cross section).

A syringe is used to load the raw extract, wash the sorbent bed and elute the analytes applying a precise extract and solvent volume with a predefined and constant flow of 5 µL/s, as illustrated in Figure 3. The syringe is also used for the cartridge transport between the conditioning rack, elution rack and to the waste container. The needle transport works with the needle solidly sticking in the cartridge needle seal. The executed clean-up steps are highly reproducible using the automated workflow.

The µSPE configuration uses a trayholder with three dedicated racks, as shown in Figure 2.

The vials with raw extracts are placed into position 1, the one closest to the rail. The elution rack is positioned in the center of the trayholder in position 2 with empty vials under the shown aluminium vial cover. The cartridge tray in position 3 is used also for the cartridge conditioning. A solvent station with three glass bottles of 100 mL each provides different solvents employed for cartridge conditioning, elution, and sample dilution. MeOH:H_2O (1:1 v/v), and MEOH p.a. for a fast and thorough syringe washing are provided by a Fast Wash Station with an integrated solvent pump connected to external solvent reservoirs.

A dedicated µSPE tool with a 1000 µL smart syringe with flat-tipped 22 gauge needle is used for the sample preparation and clean-up workflow. The principle of the automated µSPE operation on the PAL System is illustrated with Figure 3.

For the automated µSPE clean-up the novel septumless µSPE cartridges are containing 50 mg of strong ion exchange (SAX) sorbent material.

For the automated SAX µSPE clean-up 360 µL of honey or 900 µL of the pollen extracts was diluted to a final volume of 1500 µL with MeOH. The diluted extracts are placed in 2 mL PP vials on the dedicated PAL µSPE clean-up tray in rack position 1 (Figure 2).

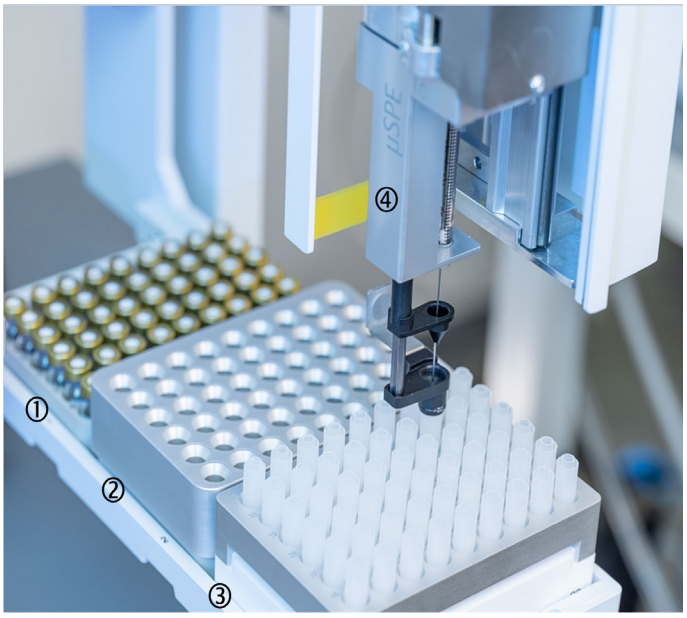

Figure 2: µSPE rack on a PAL RTC system: ① raw extract vials (Pos.1), ② the eluate rack, gets the cleaned extract (Pos.2), and ③ the conditioning rack connected to waste (Pos.3), ④ µSPE tool with 1000 µL prep syringe

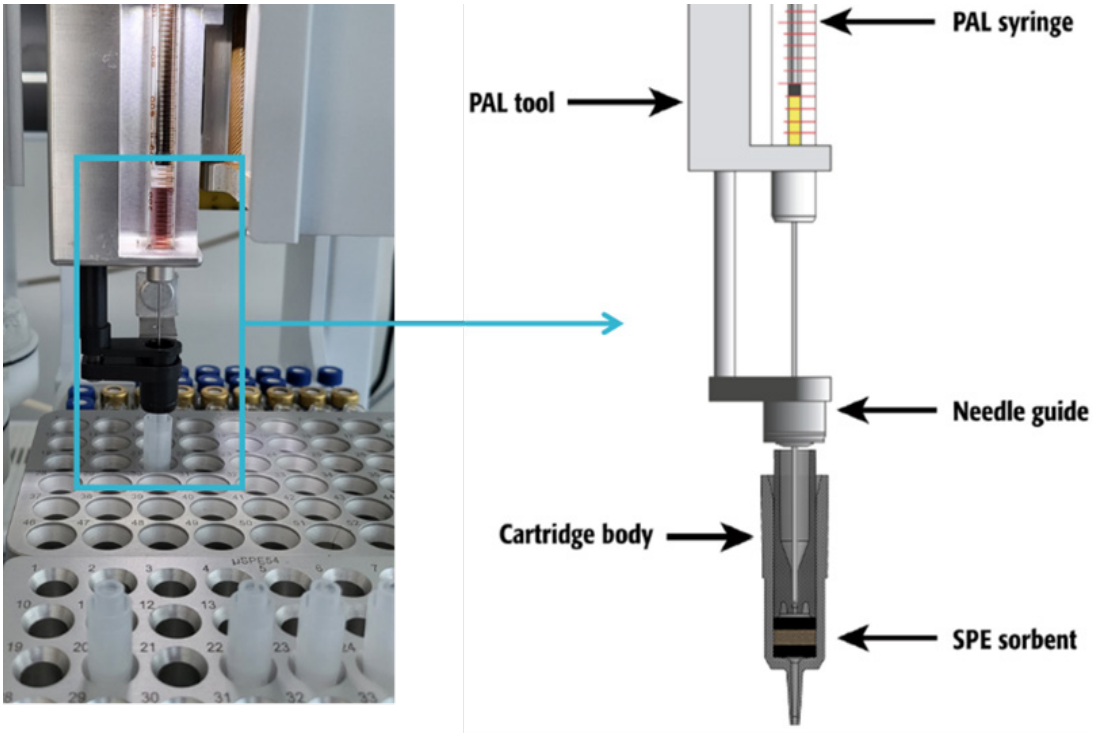

Figure 3: Principle of the PAL µSPE operation

The Automated µSPE Workflow

The automated µSPE clean-up workflow uses the classical enrichment mode, shown in the sequence of steps in Figure 4. The prepared raw extracts, empty collection vials for the cleaned extract, and the appropriate number of cartridges are loaded to the dedicated racks on the PAL µSPE trayholder. In a first step the prep syringe is selected and thoroughly cleaned with methanol from the solvent reservoirs. For every raw extract vial a cartridge and an empty collection vial are held ready in their respective racks in Pos. 2 and 3 (Figure 2). The automated workflow processes the prepared number of samples fully unattended. Using a benchtop installation the cleaned extracts are then ready for analysis by LC-MS or IC. In online installations the PAL RTC System can change the syringe to an injection syringe for applying the cleaned extract via a 6-port injection valve to LC-MS analysis.
For the detailed parameters used in this workflow see Table 1.

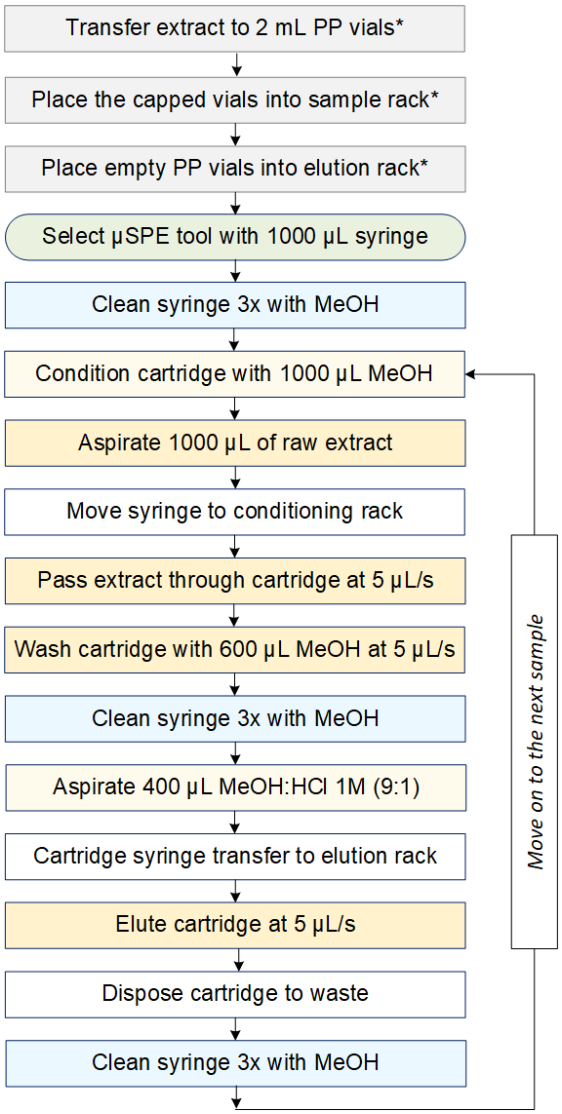

Figure 4: Sequence of the automated PAL workflow for bench-top extract clean-up using SAX µSPE cartridges (*manual steps)

Table 1: SAX µSPE clean-up workflow parameter

Activity	Description	Parameter	Flow	Source	Target
Select syringe	Liquid tool	1000 µL		Tool park station	
Wash syringe	Volume	1000 µL		Solvent station	Waste
	Cycles	3		Methanol	Waste
Conditions cartridge	Liquid tool	1000 µL	5 µL/s	Methanol	Conditioning rack
Load sample	Liquid tool	1000 µL	5 µL/s	Raw extract	Conditioning rack
Wash syringe	Volume	1000 µL		Solvent station	Waste
	Cycles	3		Methanol	Waste
Wash cartridge	Liquid tool	600 µL	5 µL/s	Methanol	Conditioning rack
Wash syringe	Volume	660 µL		Solvent station	Waste
	Cycles	3		Methanol	Waste
Take elution solent	Liquid tool	400 µL		Solvent station	
Move cartridge	Liquid tool			Conditioning rack	Elution rack
Elute analytes	Liquid tool	400 µL	5 µL/s		Elution rack
Move cartridge	Liquid tool			Elution rack	Waste container
Wash cartridge	Volume	440 µL		Solvent statio	Waste
	Cycles	3		Methanol	Waste

LC/MS Analysis

For the LC analysis, a Thermo Scientific™ Transcend™ DUO LX-2 (Thermo Scientific™, Germering, Germany) was used. Chromatographic separations were performed on an anionic polar pesticide column (APP column) from Waters™ (Milford, MA, USA) at a constant temperature of 50 °C. Mobile phase A was water containing 1.2% formic acid, mobile phase B was acetonitrile containing 0.5% formic acid. The mobile phase flow was set at 0.5 mL/min throughout the analysis. A gradient elution started at 10% A and held for 0.5 min, then ramped up to 80% A at 1.5 min, and to 90% A at 3 min. The final mobile phase composition was maintained until 16.5 min. After completed analysis the column was re-equilibrated again to 10% A to for the next injection. The injection volume was 10 µL.

A TSQ Altis LC-MS/MS system (Thermo Scientific™, San Jose, USA) equipped with an OptaMax™ NG (H-ESI II) ion source operated in negative ionisation mode was used. The source parameters were set as follows: ion spray voltage: 2500 V, sheath gas: 60 (arbitrary units), aux gas: 15 (arbitrary units), sweep gas: 0 (arbitrary units), ion transfer tube temperature: 325 °C; vaporiser temperature: 350 °C. The triple quadrupole was operated in the SRM mode for the target compounds listed in Table 2. The cycle time of the MS was set to 0.8 s. Resolution of Q1 was 0.7 u, Q3 was set to 1.2 u. As CID gas argon was used at a constant pressure of 1.5 mTorr.

Table 2: Compounds analysed with optimised SRM transitions and collision energy.

Compound	SRM 1	CE	SRM 2	CE
	(m/z > m/z)	(V)	(m/z > m/z)	(V)
AMPA	110 > 63	20	110 > 79	28
AMPA-$^{13}C,^{15}N$	112 > 63	20	112 > 107	28
Ethephon	143 > 107	7	145 > 79	7
Fosetyl-Al[a]	109 > 81	12	109 > 63	29
Fosetyl-Al-D15[b]	114 > 82	13	114 > 63	30
Glufosinate	180 > 63	40	180 > 95	17
Glyphosate	168 > 150	10	168 > 63	22
Glyphosate-$^{13}C_2,^{15}N$	171 > 153	10	171 > 63	22
HEPA	125 > 79	21	125 > 95	14
MPPA	151 > 133	13	151 > 63	32
N-Acetyl-AMPA	152 > 110	13	152 > 63	23
N-Acetyl-glufosinate	222 > 79	22	222 > 180	17
N-Acetyl-glyphosate	110 > 136	13	210 > 148	16
Phosphonic acid	81 > 79	15	81 > 63	27

a Detected as fosetyl

b Detected as fosetyl-D

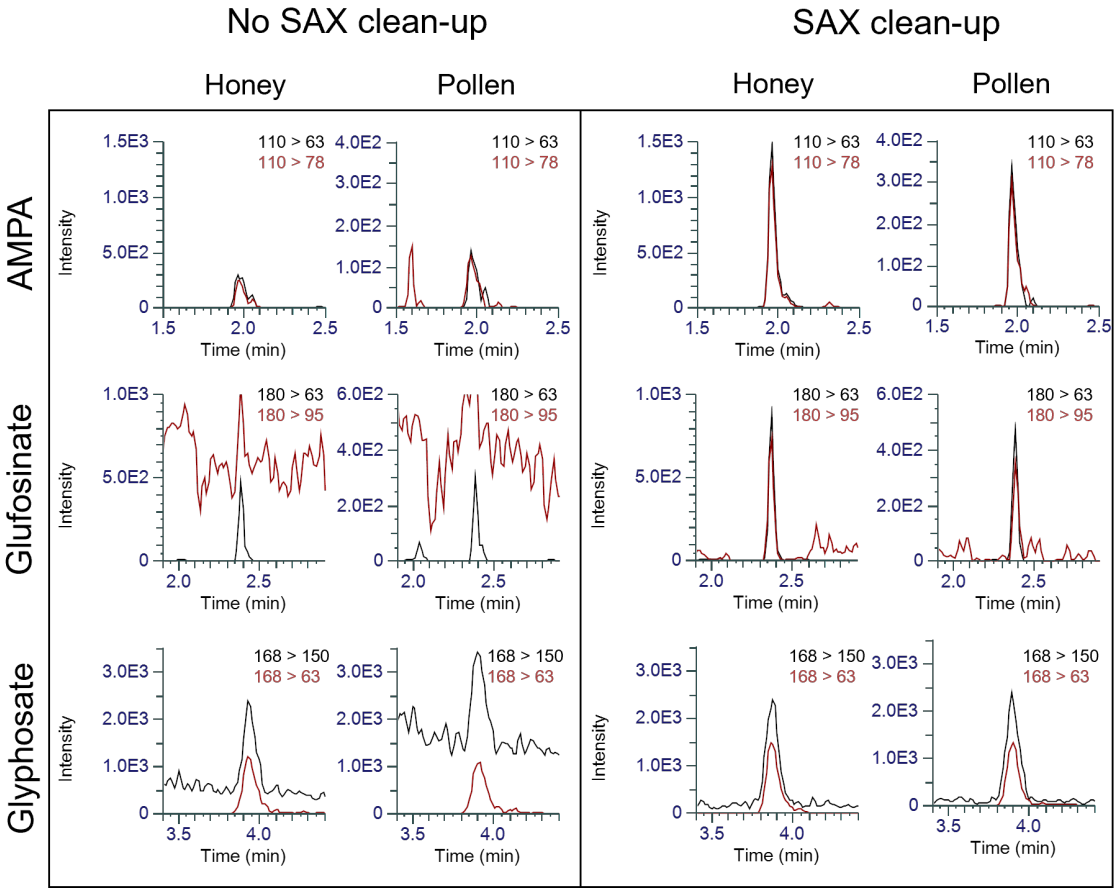

Figure 5: Extracted LC-MS/MS ion chromatograms of standards of AMPA, glufosinate, and glyphosate at 0.010 mg/kg spiked to honey and pollen matrix without (left) and after SAX clean-up (right).

Results and discussion

The effect of the SAX clean-up of honey and pollen extracts on the removal of matrix components is observed by the extracted ion chromatograms of analytes such as AMPA, glufosinate, and glyphosate as it is shown in Figure 5. After the automated SAX µSPE clean-up the compounds are detected with improved selectivity and sensitivity for a more reliable identification and peak integration, a higher signal-to-noise ratio for the analytes was achieved. The reduction of the signal suppression by the matrix, particularly for AMPA and glufosinate, is seen with the significantly reduced baseline and a signal enhancement on their peak heights.

Compared to the previously used and above described manual extract clean-up procedure significant advantages are achieved with the automated PAL System µSPE clean-up:

- Instead of 500 mg SAX material only 50 mg sorbent material is applied leading to reduced cost and faster processing.
- Also, the solvent and extract volumes and the time required for conditioning, sample loading, and washing are reduced by a factor of 10.
- The time-consuming drying step of the manual SPE method before and after the elution using a vacuum pump can be omitted.
- The recovery results obtained with the automated SAX µSPE clean-up are comparable or slightly higher to those obtained for the manual clean-up (see Table 3).
- In particular, the recoveries from honey for AMPA were between 98 and 113% (manual 70%). MPPA showed improved recoveries of 74–86%, both with acceptable RSDs.
- Overall recoveries for the HPP compounds investigated are between 70 and 120%, with RSDs below 20%, and LODs from 0.005 to 0.020 mg/kg. A linearity is achieved from 0.002 to 0.200 mg/kg.
- The considerably reduced manual labour allows the increased sample throughput for routine work.
- The automated workflow prevents human bias and leads to improved reproducibility of the results.

For the quantitative calibration the matrix-matched calibration points are also submitted to the automated clean-up. This calibration approach, called "semi-procedural standard calibration" (because the spiking was done just prior to clean-up, and not prior to the whole extraction method), was previously employed and recommended by Hakme and Poulsen[5] and by Manzano et al.[6] for the accurate multiresidue analysis of more than 200 pesticides in tomato, orange, rice, avocado, and black tea using automated µSPE clean-up for LC-MS/MS to compensate for potential analyte losses from the clean-up step.

Table 3: Recoveries, relative standard deviations (RSDs), limits of quantitation (LOQs), linear ranges, and matrix effects (MEs) obtained for honey with manual and automated SAX SPE cleanup.

Manual SAX SPE clean-up method

	0.005 mg kg^{-1}		0.010 mg kg^{-1}		0.020 mg kg^{-1}		0.050 mg kg^{-1}				
Compound	**Rec. (%)**	**RSD (%)**	**Rec. (%)**	**RSD (%)**	**Rec. (%)**	**RSD (%)**	**Rec. (%)**	**RSD (%)**	**LOQ (mg kg^{-1})**	**Linear range (mg kg^{-1})**	**ME (%)**
AMPA	75	12	73	5	68	5	73	0	0.005	0.002 – 0.200	-41
Ethephon	89	1	85	2	79	1	84	2	0.005	0.002 – 0.200	-8
Fosetyl-Al	86	2	90	2	82	0	84	3	0.005	0.002 – 0.200	-2
Glufosinate	nd	-	86	7	94	5	93	7	0.010	0.010 – 0.200	-41
Glyphosate	89	3	87	8	95	1	91	1	0.005	0.002 – 0.200	-12
HEPA	nd	-	79	4	88	2	82	0	0.010	0.010 – 0.200	-12
MPPA	78	3	63	6	58	7	61	4	0.005	0.002 – 0.200	-30
N-acetyl-AMPA	74	1	88	0	87	2	82	3	0.005	0.002 – 0.200	-14
N-acetyl-glufosinate	83	6	75	3	83	7	85	4	0.005	0.002 – 0.200	-21
N-acetyl-glyphosate	73	6	85	1	81	2	81	1	0.005	0.002 – 0.200	-15
Phosphonic acid	100	1	99	1	96	1	92	0	0.005	0.002 – 0.200	-5

Automated SAX µSPE clean-up method

	0.005 mg kg^{-1}		0.010 mg kg^{-1}		0.020 mg kg^{-1}		0.050 mg kg^{-1}				
Compound	**Rec. (%)**	**RSD (%)**	**Rec. (%)**	**RSD (%)**	**Rec. (%)**	**RSD (%)**	**Rec. (%)**	**RSD (%)**	**LOQ (mg kg^{-1})**	**Linear range (mg kg^{-1})**	**ME (%)**
AMPA	113	10	105	12	98	6	109	6	0.005	0.002 – 0.200	-71
Ethephon	80	4	94	1	94	2	99	3	0.005	0.002 – 0.200	6
Fosetyl-Al	89	5	86	2	105	7	93	3	0.005	0.002 – 0.200	9
Glufosinate	nd	-	78	14	101	9	91	3	0.010	0.010 – 0.200	-30
Glyphosate	107	9	101	7	98	9	91	4	0.005	0.002 – 0.200	21
HEPA	nd	-	115	2	109	3	99	5	0.010	0.010 – 0.200	-3
MPPA	82	2	84	8	74	4	86	5	0.005	0.002 – 0.200	-10
N-acetyl-AMPA	82	6	90	3	96	4	91	3	0.005	0.002 – 0.200	2
N-acetyl-glufosinate	97	11	101	5	96	3	98	4	0.005	0.002 – 0.200	-3
N-acetyl-glyphosate	88	2	79	4	84	4	82	2	0.005	0.002 – 0.200	24
Phosphonic acid	107	3	112	2	103	4	104	5	0.005	0.002 – 0.200	4

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