APPLICATION NOTE 73853

Polar pesticides in honey

Optimized chromatographic workflow

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Keywords: AMPA, glufosinate, glyphosate, IC-MS, ion chromatography, mass spectrometry, pesticide residues, TSQ Altis Triple Quadrupole MS

Goal

To develop and validate an integrated sample-to-result analytical workflow with integrated sample preparation and based on ion chromatography (IC) coupled with triple quadrupole mass spectrometry (MS/MS), for the multi-residue determination of polar anionic pesticides in representative honey samples

Introduction

The Codex Alimentarius defines honey as the natural sweet substance produced by honeybees from plant nectar, from secretions of living plant parts, or from excretions of plant-sucking insects.[1] Since ancient times honey has been used for sweetening, but also in medicine to treat burns, gastrointestinal diseases, asthma, infected wounds, and skin ulcers.[2] The main components of honey are sugar (70–80%), water (15–20%), organic acids, enzymes, amino acids, pollen, minerals, and solid particles. Honey composition is influenced by the plant species, climatic and ecological conditions, and the beekeeper's contribution.[3] The global production of honey has increased in the last 20 years. According to the Food and Agriculture Organization of the United Nations (FAO), 1.85 million tons of honey were produced in 2018, with China accounting for 24% of world production, followed by Turkey, Argentina, Iran, and Ukraine.[4] In addition to the main components mentioned above, trace contaminants must also be determined to assess the quality of honey. These include, for example, pesticides whose maximum contents are regulated by the EU.[5]

There are two main contamination pathways in honey:

- Cross-contamination through the collection of contaminated pollen and nectar by bees
- Contamination through the treatment of hives with insecticides, fungicides, and acaricides to protect against parasites such as *Varroa destructor*, *Acarapis woodi*, and *Paenibacillus* larvae

Tolerance levels for glyphosate in cereal crops up to four hundred times higher than for honey[5] suggest the possibility of cross-contamination. The development of glyphosate- and glufosinate-tolerant, genetically modified crops encouraged the use of these broad-spectrum herbicides, which are still used in horticulture. As a result, these polar components occur as environmental contaminants and thus in food such as honey. The EU set the maximum residue level (MRL) for glyphosate and glufosinate in honey to 0.05 mg/kg as the lower limit of the analytical determination procedure.[5,6] In 2017 and 2018, two German consumer organizations reported glyphosate-contaminated honey, referencing LC-MS/MS as the analytical method.[7,8] The State Office for Consumer Protection and Food Safety of Lower Saxony (Germany) tested domestic honey samples in 2016. Out of the 193 samples, 94% did not contain glyphosate and 3% of the samples contained glyphosate below the permitted limit. The remaining 3% of samples were found to be above the maximum level.[9] A more recent local study reported several pesticide residues but no glyphosate.[10] The controversial debate on glyphosate in honey and the relevance of glyphosate for human health[11] suggests the need for an optimized method for the accurate determination of polar pesticides and their metabolites in honey.

The chromatographic separation of polar and ionic pesticides is one of the more challenging tasks in food evaluation. Due to their high polarity, classical reversed-phase chromatography (RPLC) of glyphosate, aminomethylphosphonic acid (AMPA), glufosinate, and other polar pesticides requires their derivatization[12] or the use of unique separation columns.[13] More recent approaches are based on hydrophilic interaction liquid chromatography (HILIC) without derivatization of the polar pesticides. Frequently reported experimental limitations in the routine use of HILIC or of special RPLC applications refer to the robustness of the columns used. Their occasional rapid aging has drastic effects, e.g., on retention time, peak efficiency, and resolution, and thus on evaluation and quantification.[14,15]

In contrast to classical RPLC and HILIC, ion chromatography (IC) is the method of choice for the separation of polar and ionic compounds. Initially designed for the analysis of inorganic ions, today IC is successfully used for the separation of, e.g., organic anions and cations, sugars, amino acids, peptides, proteins, and nucleotides.[16]

At high pH values, glyphosate, AMPA, and glufosinate are anionic, suggesting the use of anion exchange chromatography. Derivatization is not necessary, and modern analytical ion exchangers are optimized for the separation of small polar ionic compounds. Until recently, coupling IC with mass spectrometry (MS) has been considered rather unusual, due to the eluents consisting of aqueous corrosive alkalis or acids.[16] With the introduction of electrolytically regenerated membrane suppressors, however, the robust continuous desalting of the eluents, or more precisely their chemical conversion into water, is now possible before the eluent enters the mass spectrometer.[17]

Mass spectrometry has become an accepted technique for the detection of pesticides. Triple quadrupole MS/MS systems are currently in widespread use in food analysis. These systems meet the current requirements for sensitivity and selectivity in the selected reaction monitoring (SRM) mode.[18-22] Additional improvements in detection specificity and selectivity result from the use of high-resolution accurate mass spectrometry (HRAM).[23-29]

A matrix-specific challenge arises in the application of MS due to the high sugar content of honey, which can lead to contamination of the mass spectrometer inlet cone, resulting in instrument downtime.

This paper describes an IC-MS/MS method for the direct analysis of glyphosate, AMPA, and glufosinate in honey. Our evaluation is supplemented by an automated inline elimination of sugars before the mass spectrometer.

Experimental

A metal-free ion chromatograph (Thermo Scientific™ Dionex™ ICS-6000) with a Thermo Scientific™ Dionex™ AS-AP autosampler was coupled to a Thermo Scientific™ TSQ Altis™ Triple Quadrupole Mass Spectrometer (Figure 1). A Thermo Scientific™ Dionex™ IonPac AS19-4µm polymeric based separation column and guard column were used. The KOH gradient was generated in-situ with an eluent generator without the use of external chemicals (RFIC™). After separation, eluent and eluites passed through the Thermo Scientific™ Dionex™ ADRS 600 Suppressor being electrolytically regenerated in external water mode. For matrix elimination, a second valve was integrated, diverting the effluent from the MS for a selected time segment. In this state, the effluent is first collected in a loop (750 µL), the contents of which are fed separately to waste after switching back. To improve the evaporation of the effluent (desolvation), 2-propanol was added post-column before the mass spectrometer interface. The Thermo Scientific™ Chromeleon™ Chromatography Data System software was used for data acquisition and analysis. All chemicals used in these investigations were of analytical grade quality or better; the deionized water used was freshly taken from the ultrapure water system.

Equipment

- Dionex ICS-6000 HPIC™ system*, including:
  - SP Pump, Isocratic with Degas (P/N 22181-60003)
  - DC Microbore Compartment with Dual Temperature Zone, Two Injection Valves (P/N 22181-60049)
  - EG Module (P/N 22181-60019)
  - EG Degas Unit (SB/MB) (P/N 075522)
  - CD Detector (with Cell) (P/N 079829)
  - EO Eluent Organizer Tray with two 2 L bottles (P/N 072057)
  - IC PEEK Viper Fitting Kit for Dionex ICS-6000 with Conductivity Detector (Microbore 2 mm) (P/N 302965)
  - Dionex Suppressor External Regenerant Installation Kit (P/N 038018)
- Dionex AS-AP Autosampler, with Tray Temperature Control Option (P/N 074926) with three vial trays (P/N 074936)
- Thermo Scientific™ Dionex™ AXP Auxiliary Pump (P/N 063973)
- Thermo Scientific™ Dionex™ AXP-MS Auxiliary Pump (P/N 060684)
- TSQ Altis Triple Quadrupole Mass Spectrometer (P/N TSQ02-10002)
- Chromeleon Chromatography Data System software, version 7.2.9 or higher (P/N 7200.0201-ICSP) with Spectral License—3D/MS Data Acquisition (P/N 7000.0020-ICSP)
- Thermo Scientific™ Barnstead™ Pacific™ GenPure™ ultrapure water system with UV-photo-oxidation, ultrafiltration membrane, and TOC monitor (P/N 50131256) with Pacific TII 40 (UV) (P/N 50132133) and double cartridge pretreatment system (P/N 09.4000)

Reagents and supplies

- AMPA, (Aminomethyl) phosphonic acid (P/N 05164-50MG) Sigma-Aldrich
- Deionized (DI) water, (18.2 MΩ·cm, TOC < 5 ppb, 0.2 µm inline filter), Thermo Scientific (see Equipment)
- Glufosinate-ammonium, Pestanal™ (P/N 45520-100MG) Sigma-Aldrich
- Glyphosate, Pestanal™ (P/N 45521-250MG) Sigma-Aldrich
- Isopropanol, Optima™ LC/MS Grade, Fisher Chemical™ (P/N 10091304) Fisher Scientific
- Fisherbrand™ Non-sterile Nylon Syringe Filter, 25 mm, 0.2 µm (P/N 15121499) Fisher Scientific
- Vial Kit, 1.5 mL Polypropylene with Caps and Septa, 100 each (P/N 079812) Thermo Scientific

* or a Thermo Scientific™ Dionex™ Integrion™ HPIC™ system (RFIC model) with two injection valves, and CD Detector with cell.

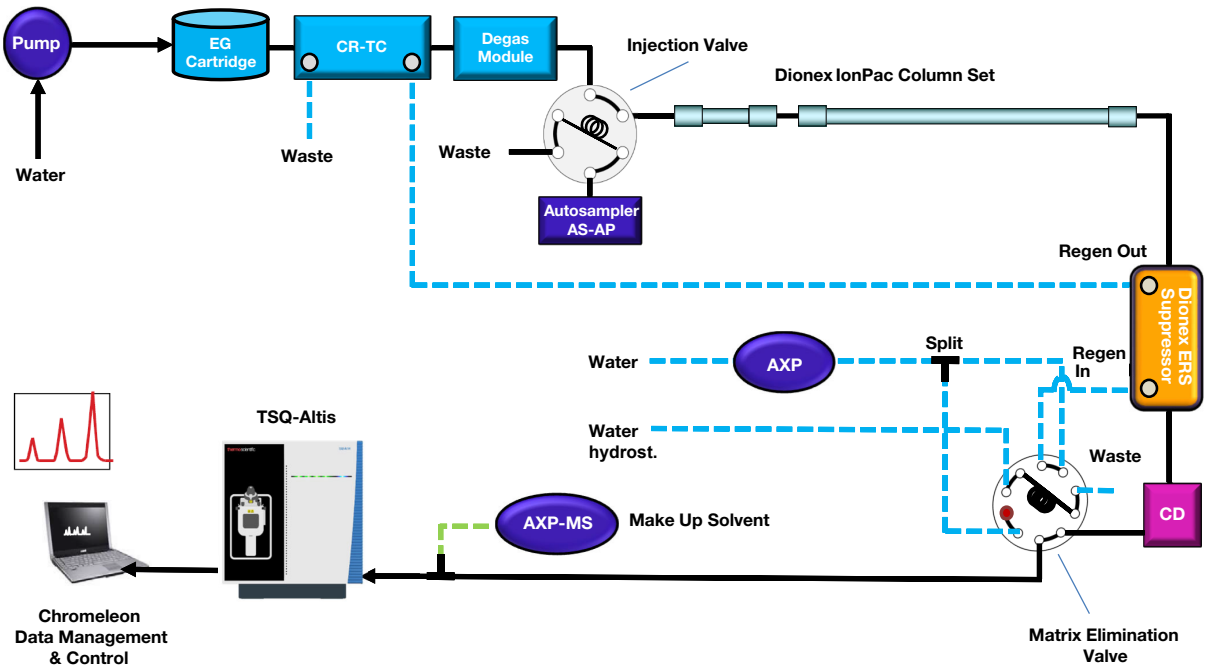

Figure 1. Schematic IC-MS/MS-configuration; Position of matrix elimination: "Off"

Table 1 (part 1). Conditions for ion chromatography

IC system:	Dionex ICS-6000 HPIC system
Columns:	Dionex IonPac AG19-4µm Guard, 2 × 50 mm (P/N 083225) Dionex IonPac AS19-4µm Analytical, 2 × 250 mm (P/N 083223)
Eluent source:	Thermo Scientific™ Dionex™ EGC 500 KOH Eluent Generator Cartridge (P/N 075778) with Thermo Scientific™ Dionex™ CR-ATC 600 (P/N 088662)

KOH gradient:

Time (min)	KOH (mM)	Matrix elimination
0.0	Start	Off*
0.0	20	
2.5		On*
4.0	20	
5.5		Off*
16.0	60	
18.0	60	
18.1	80	
19.0	80	
19.1	20	
24.0	End of Run	On*

Flow rate:	0.25 mL/min
Injection volume:	10 µL (push full mode)
Temperature:	25 °C (column compartment) 20 °C (detector compartment) 35 °C (conductivity detector cell)
System backpressure:	<3300 psi (100 psi = 0.6895 MPa)
Suppressor:	Suppressed Conductivity, Dionex ADRS 600 Suppressor (2 mm) used in the dynamic regeneration mode (3.8 V), AutoSuppression, external water mode via a Dionex AXP pump, external water flow rate (0.5 mL/min)
Background conductance:	<0.5 µS/cm
Run time:	24 min
IC-MS interface:	Tee union (PEEK, P/N 00101-18204) to combine the effluent from the conductivity detector via Thermo Scientific™ Viper™ tubing with the makeup solution. Use Viper connections between grounding union and H-ESI spray insert.
Post-suppressor makeup solution:	2-propanol at 0.15 mL/min via a Dionex AXP-MS pump

* On: Effluent to waste Off: Effluent to MS

Table 1 (part 2). Conditions for mass spectrometric detection

Ion source settings	
Ion source type:	H-ESI
Spray voltage:	Static
Negative ion:	3,500 V
Sheath gas:	30 Arbitrary units (Arb)
Aux gas:	10 Arb
Sweep gas:	0 Arb
Ion transfer tube temp.:	250 °C
Vaporizer temp.:	350 °C
Probe setting:	Vertical: L/M Horizontal: 1.1 Side-to-side: Center
MS global settings	
Start time:	0 min
End time:	24 min

Master scan	
Scan mode:	SRM
Polarity:	Negative
Use cycle time:	True
Cycle time:	0.6 s
Q1 resolution (FWHM):	0.7
Q3 resolution (FWHM):	1.2
CID gas:	2.0 mTorr
Source fragmentation:	0 V
Chromatographic peak width:	6 s
Transition conditions:	Optimized for each compound using the automated compound optimization tool (Table 2)

Table 2. IC-MS/MS parameters for selected SRM transitions for glyphosate, AMPA, and glufosinate

Compound	t_{ms} (min)*	Transition	Precursor *(m/z)*	Product *(m/z)*	Collision Energy (V)	RF Lens (V)
Glufosinate	10.3	Quantifier	180	63	40	60
		Qualifier 1	180	95	20	
		Qualifier 2	180	136	18	
AMPA	10.5	Quantifier	110	79	28	49
		Qualifier 1	110	63	25	
		Qualifier 2	110	81	14	
Glyphosate	20.6	Quantifier	168	63	24	50
		Qualifier 1	168	79	28	
		Qualifier 2	168	124	12	
		Qualifier 3	168	150	10	

*t_{ms}: Retention time

MS conditions

All precursors, quantifiers, and qualifiers were individually determined using standards. Typical conditions are summarized in Table 1 and Table 2. Because the target analytes are small molecules with low mass-to-charge (*m/z*) product-ions, the mass spectrometer was calibrated using the Thermo Scientific™ Pierce™ Triple Quadrupole Extended Mass Range Calibration Solution (P/N 88340), which contains 14 components (mass range from 69 *m/z* to 2800 *m/z*) for calibration in both positive and negative ionization modes. This solution improves mass accuracy and transmission compared to conventional polytyrosine mass calibration solution, especially in the low *m/z* range.[18]

Samples and sample preparation

The honey samples were sourced from regional commercial and private production. The samples (~2.8 g) were diluted with DI water to a volume of 25 mL, thoroughly mixed, and filtered through a nylon filter (0.2 µm pore size). The ready to inject solutions (original and spiked) were adjusted to hold 100 ± 0.5 g/L honey. Aliquots were transferred to polymeric sample vials, which prevent analyte loss, avoiding wall adsorption effects known for glass vials.[18]

Results and discussion

Direct analysis of the honey samples

For direct examination of the diluted honey samples, the setup shown in Figure 1 was chosen, and the second valve was left in the "Off"-position shown. Thus, the sugar matrix and the anions and target components reached the MS.

Based on the findings of the pilot study by Pareja et al., we decided not to use isotopically labeled internal standards (ILIS) and evaluate the honey samples using the standard addition method.[25] To determine the matrix effects (*ME*), the target components were calibrated externally with aqueous, matrix-free standards in the range of 0.1 µg/L to 5 µg/L. Analytical characteristics are listed in Table 3.

Due to the high sensitivity of the TSQ Altis MS, 10 µL of the diluted honey solution (100 g/L) was injected. The amount of sugar injected, and thus the load on the inlet cone, increases at the same time. The absolute value of *ME* (Equation 1) for glyphosate was less than 30% in our experiments and, therefore, comparable to the reported literature values.[25]

$$ME = \frac{Slope\ of\ standard\ addition - Slope\ of\ external\ calib.}{Slope\ of\ external\ calibration} \cdot 100$$

Equation 1. Calculation of the *ME*[30]

Our tests showed excellent instrument stability. Despite the reduced injection volume, deposits on the inlet cone could still form with continued analysis.

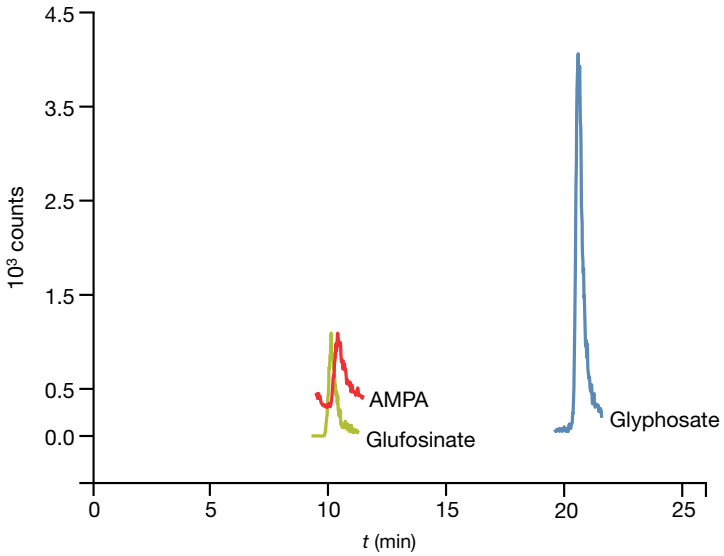

Figure 2. SRM chromatograms for glyphosate, AMPA, and glufosinate from a standard solution (ρ = 0.4 µg/L for each target component), Conditions: see Experimental section.

Table 3. Characteristics of the external calibration and retention times; calibration range for the target components: 0.1–5 µg/L

Component	Correlation coefficient (r^2)	Evaluation	Limit of detection—LOD (µg/L)	Limit of quantitation—LOQ (µg/L)	Standard deviation t_{ms} (min)*
Glufosinate[a]	>0.9999	Peak Area	0.06	0.2	<0.1
AMPA[b]	>0.9998	Peak Area	0.20	0.5	<0.1
Glyphosate[b]	>0.9997	Peak Area	0.20	0.7	<0.1

[a] Calculation of the limit of detection and limit of quantification according to ISO 8466-2.31
[b] Calculation of limit of detection and limit of quantification according to DIN 32645.32
n = 5, confidence level = 99.5%, tolerated error at the limit of quantification 33.3%
* n = 42, including real samples and matrix-free solutions

Figure 3 and Figure 4 show representative chromatograms of wild honey and blossom honey. Both figures combine the chromatogram of the conductivity detector and the SRM traces of the analytes. The method is suited for the simultaneous determination of anionic honey constituents (e.g., organic acids, inorganic anions) after appropriate peak assignment and calibration.[33,34] The glyphosate content in wild honey was below the required detection limit of 50 µg/kg. The investigated blossom honey, however, showed a glyphosate content of more than three times the permitted value (Figure 4).

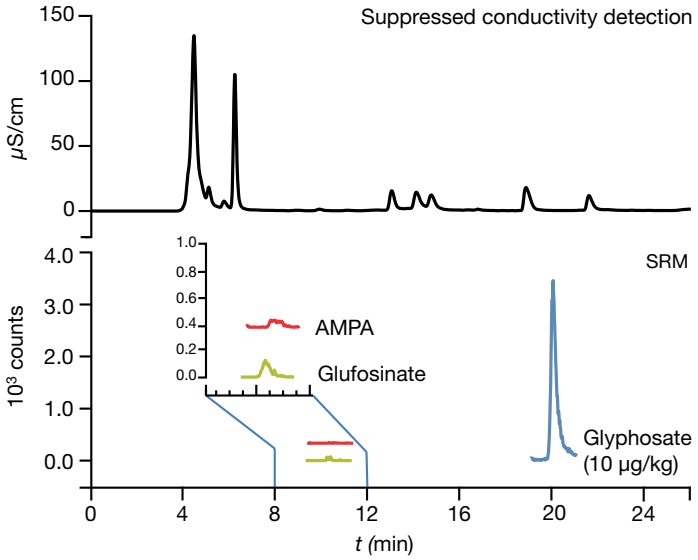

Figure 3. Representative chromatogram of diluted wild honey. Conditions: see Experimental section. Detection: conductivity after suppression (black) and SRM chromatograms for glufosinate (green), AMPA (red), and glyphosate (blue). Contents: Glufosinate (<3 µg/kg), AMPA (<2 µg/kg), glyphosate (10 µg/kg). The concentrations are those calculated for the original honey sample.

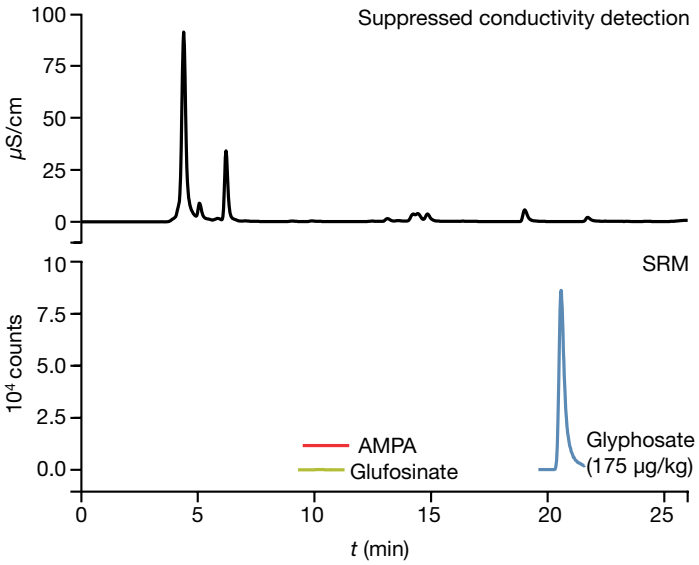

Figure 4. Representative chromatogram of diluted blossom honey showing high glyphosate content. Conditions: see Experimental section. Detection: conductivity after suppression (black) and SRM chromatograms for glufosinate (green), AMPA (red), and glyphosate (blue). Contents: Glufosinate (<1 µg/kg), AMPA (not detectable (n.n.), glyphosate (175 µg/kg). The concentrations are those calculated for the original honey sample.

Inline matrix elimination

Although only 10 µL of the diluted honey solution were injected, the high sugar load (70–80 g/L) was sufficient to lead to discoloration of the MS inlet cone. To minimize the effect, automated matrix elimination was set up. It uses a timed second switching valve, which directs the effluent to the waste instead of the mass spectrometer (Figure 1).

Determination of the switching times

Guyong et al.[35] reported mono- and disaccharides to elute from a classical Dionex IonPac anion exchange column at the beginning of the chromatogram. The appropriate switching times were determined using an amperometric detector instead of suppressed conductivity detection, allowing the carbohydrate detection at high pH.[36-39] The elution of the sugar matrix (glucose, fructose, sucrose) starts at 2.5 min, and the main part of the sugar matrix has eluted at 5.5 min (Figure 5). Through timed actuation of the matrix elimination valve (Figure 1, Table 1) the column effluent does not reach the MS, and the sugar matrix is diverted to waste.

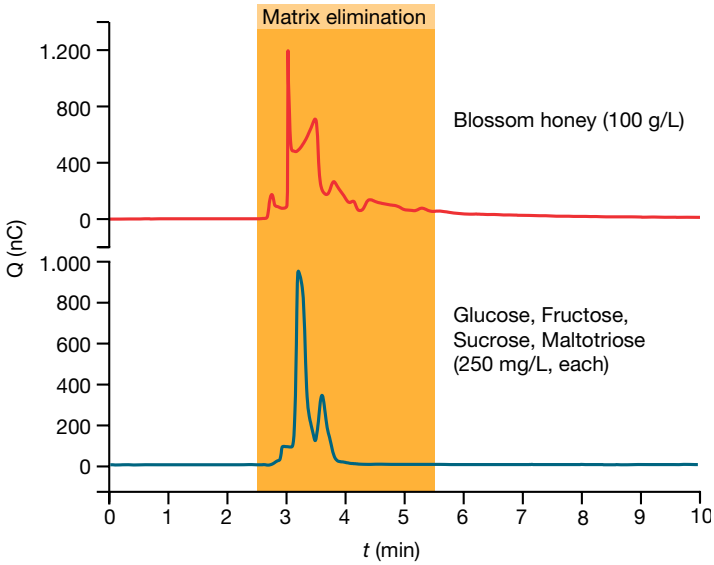

Figure 5. Chromatograms of diluted blossom honey and a sugar reference solution to determine the time segment of the matrix elimination. Conditions: see Experimental section. Detection: Pulsed amperometry on Au (four-potential pulse sequence against Ag/AgCl).[37] The first ten minutes of chromatograms are shown.

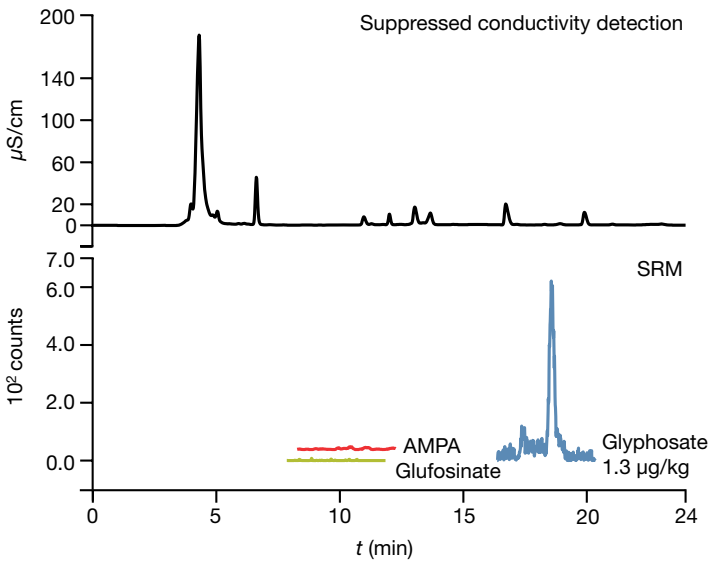

Figure 6. Representative chromatogram of diluted honey from a local beekeeper using matrix elimination. Conditions: see Experimental sections. Detection: conductivity after suppression (black) and SRM chromatograms for glufosinate (green), AMPA (red), and glyphosate (blue). Contents: Glufosinate (n.n.), AMPA (n.n.), glyphosate (1.3 µg/kg). The concentrations are those calculated for the original honey sample.

Figure 6 shows the chromatographic analysis of a local beekeeper honey, where the sugar matrix was eliminated before MS.

In addition to the LOD and LOQ calculations based on the calibration function, we determined the method detection limit (*MDL*). The sample used for the repetitive injections contained 0.8 µg/L of glufosinate, AMPA, and glyphosate.

$$MDL = t_{(n-1,\ 1-\alpha=0.99)} \cdot S$$

Equation 2. Calculation of *MDL*[40]
MDL = the method detection limit based on samples.
t (n-1, 1-α=0.99) = the Student's t-value, single-tailed 99th percentile t statistic, n-1 degrees of freedom.
S = sample standard deviation of the replicate sample analyses.

Table 4. Determination of *MDL*. Sample target concentration 0.8 µg/L, each; n = 9

	Amount (µg/L) MS quantitation peak		
Replicate	Glufosinate	AMPA	Glyphosate
1	0.80	0.63	0.83
2	0.79	0.63	0.80
3	0.85	0.77	0.76
4	0.77	0.63	0.78
5	0.80	0.80	0.75
6	0.78	0.66	0.79
7	0.79	0.71	0.83
8	0.77	0.63	0.78
9	0.75	0.57	0.83
Sample standard deviation (*S*)	0.03	0.07	0.03
MDL	0.09	0.21	0.09

$t_{(8,\ 0.99)} = 2.896$

Our results show that the original analytical characteristics of the glufosinate, AMPA, and glyphosate determination remain unchanged. The most prominent advantage of matrix elimination is, therefore, the prevention of undesirable matrix effects and matrix buildup on the MS inlet cone (Figure 7).

(A) Without matrix elimination **(B) With matrix elimination**

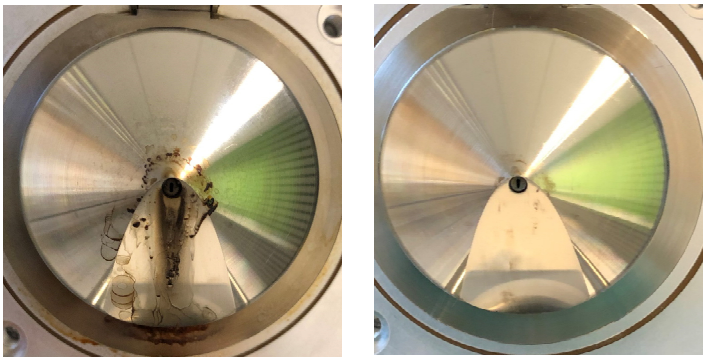

Figure 7. Comparison of inlet cone (~40 test injections): (A) Without matrix elimination, (B) With matrix elimination. Conditions: see Experimental section.

Summary

Trace levels of glyphosate, AMPA, and glufosinate can reliably be determined using IC-MS/MS in diluted honey. In the combination of IC with MS, the continuously electrolytically regenerated membrane suppressor acts as a desalter through which the alkaline eluent is converted into water. The resulting effluent is directed to the MS interface. The applied chromatographic conditions allow the automated, inline elimination of the sugar matrix. It reduces the matrix effect on the MS hardware, and the uninterrupted operating time of the analysis system increases. The LODs and LOQs are well below the values required by the EU. The method presents itself as a reliable and cost-effective analytical tool for routine analysis of glyphosate, AMPA, and glufosinate in honey.

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