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Determination of glyphosate, glufosinate, and metabolites in honey based on different detection approaches supporting food safety and official controls

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ABSTRACT

Honey, a component of the European diet, faces contamination challenges that impact both consumer and bee health. Glyphosate and glufosinate-based herbicides are widely used in both agriculture and domestic settings, posing potential threats to humans and bees. This study addresses the need for robust analytical methods to detect glyphosate, glufosinate, and their metabolites in honey, considering the complexities of the matrices. Advanced techniques, such as Liquid Chromatography-Mass Spectrometry (LC-MS/MS) and Ion Chromatography-High-Resolution Mass Spectrometry (IC-HRMS) were employed for method validation and monitoring across 97 honey samples. The extraction procedure was optimized, and the validation procedure followed EU Regulation 808/2021 and SANTE 11312/2021 guidelines. LC-MS/MS and IC-HRMS demonstrated comparability and high sensitivity, with RSD_r and RSD_R values falling within the range of 3%–18% and 6%–22%, respectively, for all analytes considered except for AMPA. AMPA showed CV% values > 25% at the concentration of 5 and 10 ng/g in LC–MS/MS. For IC-HRMS RSD_r and RSD_R values for all analytes fall within the 3%–14% and 4%–14% range, respectively. Glyphosate was quantified in 12% of the samples, with one sample exceeding Maximum Residue Level (MRL). Glufosinate and its metabolites were not detected in any of the samples.

1. Introduction

Honey holds a significant place in the European diet, with an average consumption of 0.7 kg per person annually, owing to its recognized health benefits and culinary versatility (Testa, Asciuto, Schifani, Schimmenti, & Migliore, 2019). European Union, as the world's second-largest honey producer, imports a substantial portion of its honey, as it is only 60% self-sufficient. These imports mainly come from countries such as China and Ukraine (European Commission, 2023). This dependence on imports exacerbates concerns for beekeepers already dealing with higher expenses and increased bee mortality resulting from declining floral resources, urbanization, and agricultural chemicals (Kleisiari, Kleftodimos, & Vlontzos, 2022).

Glyphosate (Gly) and glufosinate (Glu) are widely employed herbicides in agriculture, with their usage having significantly increased since the 1990s. The total Gly volume applied by farmers rose 14.6-fold, from 51 million kg in 1995 to 747 million kg in 2014. Global non-agricultural uses have increased five-fold since the introduction of genetically engineered Gly-tolerant crops, from 16 million kg in 1995 to 79 million kg in 2014 (Benbrook, 2016). Estimated Glu use increased more than five-fold from 1996 to 2016 in the United States, and it will likely continue to increase in the near future (Takano & Dayan, 2020). Gly and Glu-based herbicides are widely used in both agricultural and non-agricultural settings, including lawns and gardens (Annett, Habibi, & Hontela, 2014). Inadequate application practices, including excessive use of herbicides or non-compliance with guidelines, increases the risk of honey contamination (Annett et al., 2014; Farina, Balbuena, Herbert, Mengoni Goñalons, & Vázquez, 2019) Moreover, the application by untrained individuals may also contribute to the contamination of surface and groundwater (Annett et al., 2014).

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Bee exposure to Gly-based herbicides can affect their cognitive abilities, potentially contributing to Colony Collapse Disorder (Battisti et al., 2021; Johnson, 2015). Although the International Agency for Research on Cancer (IARC) classified Gly as a "probable carcinogen" in 2015, subsequent evaluations by the European Food Safety Authority (EFSA) and the US Environmental Protection Agency (EPA) have concluded that Gly is unlikely to pose a cancer risk to humans (Benbrook, 2019; European Food Safety Authority, 2017). In December 2022, the EU Commission extended Gly's approval by one year to facilitate a comprehensive EFSA peer review. EFSA's assessment in July 2023 concluded that Gly is unlikely to pose health risks but identified "unresolved issues" due to insufficient data, particularly concerning residue magnitude (Rampazzo, Zironi, Depau, Pagliuca, & Gazzotti, 2024; European Food Safety Authority, 2017). Despite the data gaps highlighted by EFSA, in November 2023 the European Commission extended Gly's authorization for the next 10 years (European Commission 2023). Nevertheless, debates persist regarding the safety of this herbicide (Casassus, 2023).

Glu also poses health risks when ingested or in contact with the skin (IUPAC Pesticides Properties DataBase). Glu was approved for use in Europe until 2018; however, the European Commission opted not to renew its registration, due to concerns about its toxicity (Regulation (EU) 2020/1068; Takano & Dayan, 2020). Nonetheless, Glu remains extensively employed in the United States, South America, and various other regions globally (Takano & Dayan, 2020).

Honey contamination from herbicides can occur through multiple pathways. Herbicide drift, a result of spray applications in crop fields, disperses minute droplets beyond the intended area, ultimately settling on flowers where bees gather pollen and nectar for honey production (Bonerba et al., 2021; Zawislak, Lorenz, Adamczyk, Wiedenmann, & Joshi, 2021). Since herbicides can persist in the environment, soil and water, used by bees, represent additional sources of contamination (Annett et al., 2014). Monitoring contaminant residues in food items, particularly honey, is essential for safeguarding human health. Given the widespread consumption of honey across various demographic groups, including vulnerable populations like children and the elderly, ensuring the absence of health risks is paramount (Panseri et al., 2020).

Maximum residue levels (MRLs) for these pesticides in honey and apiculture products have been established. Specifically, for Gly, the MRL is set at 50 ng/g (European Commission 2013). In Italy, concerning organic farming products, the maximum limit is set at 10 ng/g (Italian Minister of Agriculture, 2011). It is crucial to underline that in Europe, there are no established maximum residue levels for Gly active metabolites, such as aminomethylphosphonic acid (AMPA) and N-acetyl glyphosate. In the case of Glu, including Glu isomers, its salts, and metabolites (3-methylphosphinicopropionic acid [MPPA] and N-acetyl-glufosinate), the MRL is set at 50 ng/g under Commission Regulation (EU) 2016/1002 (European Commission, 2016).

Determining Gly, Glu, and their metabolites in honey and hive products poses challenges due to their small size and unique physicochemical properties (Verdini & Pecorelli, 2022). These properties, including high polarity, high water solubility, low ionization, and low volatility, make selective extraction difficult, leading to a significant matrix effect (Verdini & Pecorelli, 2022). Tandem mass spectrometry (MS/MS) systems coupled with liquid chromatography (LC), are commonly employed for analyzing polar herbicide analysis in hive products (Rampazzo, Gazzotti, Zironi, & Pagliuca, 2023). These systems effectively meet the contemporary requirements for sensitivity and selectivity (Neufang, Scheibner, & Jensen, 2022). Furthermore, an alternative method to enhance performance in terms of specificity and selectivity is the Ion Chromatography (IC) coupled with High-Resolution Mass-Spectrometry (HRMS). This approach could contribute to further progress in these analyses, enabling exceptional sensitivity to be achieved (Rampazzo et al., 2023).

Efficient, cost-effective, and reliable methods for detecting these substances in various types of honey are essential for monitoring, consumer protection, and ensuring the safety of the bee chain. In this context, the ICQRF-MASAF Italy (Department of Central Inspectorate for Fraud Repression and Quality Protection of the Agri-Food Products and Foodstuffs, Ministry of Agriculture, Food Sovereignty and Forests), plays a strategic role in safeguarding consumers and protecting producers from unfair competition. Recent advancements in analytical methodologies have led to an increased focus on pesticides, yet there remains a scarcity of methods capable of simultaneously analyzing multiple highly polar pesticides, especially their metabolites. Only the most recent studies in the literature examining polar pesticides in honey and hive products have also incorporated their comprehensive metabolic profiles into the research (Butovskaya et al., 2023; Jesús, Rosa García, Stecconi, Cutillas, & Rodríguez Fernández-Alba, 2023). Nevertheless, these studies both use solid-phase extraction cartridges to achieve adequate sample cleanliness for high sensitivities. This approach makes the analysis more time-consuming and more expensive, characteristics that could adversely affect efficiency, especially analyses conducted on a large number of samples, such as official controls.

This study aims to optimize cost-effective, rapid, and sensitive analytical methods for monitoring Glyphosate (Gly), Glufosinate (Glu), and their metabolites using Liquid Chromatography-Mass Spectrometry and Ion Chromatography-High-Resolution Mass Spectrometry. Through the analysis of 97 honey samples with diverse characteristics, origins, and production methods, the research aims to contribute to the development of efficient analytical protocols for simultaneously detecting these polar herbicides and their metabolites in honey.

2. Materials and methods

2.1. Chemicals and reagents

Glyphosate (purity 98%), glyphosate-2-^{13}C,^{15}N (purity 97%) (IS), aminomethylphosphinicopropionic acid (purity 99%), aminomethylphosphinicopropionic acid^{13}C,^{15}N (purity ^{13}C, 99%;^{15}N, 98%) (IS), N-acetyl-glyphosate (purity ≥95%), N-acetyl-AMPA (purity ≥95.0%), glufosinate ammonium (purity >98.0%), glufosinate-d3-hydrochloride (purity >98.0%) (IS), N-acetyl-glufosinate sodium (purity ≥95.0%), N-acetyl-glufosinate-d3 (purity ≥95.0%) (IS), 3-methylphosphinicopropionic acid; 3-methylphosphinicopropionic acid-d3 sodium salt (MPPA-d3) (IS) were purchased from TRC (Toronto Research Chemicals Inc., Canada). Acetonitrile, methanol, and formic acid, all LC-MS grade, were acquired by Merck (Darmstadt, Germany). Ultrapure water was produced from a Milli-Q® water purification system (Merck, Darmstadt, Germany). Polytetrafluoroethylene (PTFE) syringe filters (13 mm, 0.2 μm) were purchased from Waters Corp. (Milford, MA, USA). The single stock solution of glyphosate, AMPA, N-acetyl-glyphosate, glufosinate ammonium, N-acetyl-glufosinate sodium, and MPPA at a concentration of 100 μg/mL and relative internal standards at a concentration of 40 μg/mL in water were prepared in plastic flaks. Appropriate volumes of each stock solution were diluted to create a working solution containing all the analytes at a concentration of 1 μg/mL for both analytes and internal standards. All the stock and working solutions were stored and refrigerated at 4 °C.

2.2. Sampling

A total of 97 honey samples, originating from different botanical origins and productive methods (conventional and organic) were collected with the support of ICQRF through an official collaboration as presented in Table 1. Before analysis, the samples were stored under ambient conditions (+20 °C) and in darkness.

2.3. Sample size

The 97 honey samples were provided, and to verify the suitability of the sample size, the formula reported below was applied (Nobile, et al.,

Table 1
Honey samples analysed involved in the present research.

Honey botanical origin	Number	Production method
Multiflower	55	Conventional
Multiflower	4	Organic
Citrus	7	Conventional
Acacia	12	Conventional
Clover	1	Conventional
Sulla	1	Conventional
Heather	1	Conventional
Dandelion	1	Conventional
Eucalyptus	2	Conventional
Lucerne	1	Conventional
Chestnut	6	Conventional
Linden	3	Conventional
Adamesque	1	Conventional
Coriander	1	Conventional
Alpine flower	1	Conventional
Total	97	

2023):

$$N = Z^2 \times [P \times (1 - P)]/D^2$$

where Z has a value of 1.96 for a 95% confidence limit, P is the expected prevalence set at 0.5 (50 percent), and D is the precision of the estimate. This conservative approach allowed a precision value of 10% to be considered satisfactory, based on the average European honey production of 230,000 tons per year (Kleisiari et al., 2022).

2.4. Sample preparation

Two grams of honey were weighed into a 15 mL polypropylene falcon and 100 μL of internal standards working solution were added. Following this, 3 mL of methanol and 7 mL of water containing 1% formic acid were added according to the protocol developed by Chiesa, Nobile, Panseri, and Arioli (2019). The sample was subjected to vortex until complete dissolution, then sonicated for 15 min, and subsequently centrifuged at 2500 g at 4 °C for 10 min. Finally, 1 mL of the prepared sample was filtered into plastic vials, making it ready for LC-MS/MS analysis. The extract underwent a 1:1 dilution with ultrapure water before analysis in IC-HRMS.

2.5. UHPLC-MS/MS analysis

The detection of polar pesticides in honey was achieved using ultrahigh-performance liquid chromatography coupled with triple-quadrupole mass spectrometry (UHPLC-MS/MS). This analytical system included a Waters Acquity UHPLC binary pump in conjunction with a Waters Xevo TQ-S micro triple-quadrupole mass spectrometer (Waters Corporation, Milford, Massachusetts, USA). The instrument was equipped with an electrospray ionization source (ESI).

The analyses were performed using the negative electrospray ionization (ESI-) mode and followed a multiple reaction monitoring (MRM) approach, as detailed in Table 2, for the target compounds. The ESI capillary voltage was set at +3.00 kV, the cone voltage was maintained at 20.00 V, the desolvation temperature was set to 600 °C, and the source temperature was regulated at 150 °C. The desolvation and cone gas flow rates were established at 1000 and 150 L/h, respectively, with argon serving as the collision gas.

The chromatographic separation was conducted using an "Anionic Polar Pesticide" column (5 μm, 2.1 mm × 150 mm) (Waters Corporation), which was thermostat at 50 °C. The chromatographic conditions were configured as follows: mobile phases included 1.2% formic acid in water (A) and acetonitrile acidified with 0.5% formic acid (B). The gradient began at 10% phase A at 0 min, increased linearly to 80% within 1.5 min, further elevated to 95% in 1.5 min, and then returned to 10% within 17 min, with 1 min to rebalance the column. The entire run duration was 21 min, the flow rate was maintained at 0.500 mL/min, and 10 μL was the volume injected. The autosampler was held at 20 °C throughout the analysis. The acquisition and processing of data were carried out using Waters MassLynx 4.1 software, provided by Waters Corporation, based in Milford, Massachusetts, USA.

Table 2
MS/MS detection parameters.

Compound	Adduct	Precursor Ion (m/z)	Product Ion 1 (m/z)	CE 1 (eV)	Product Ion 2 (m/z)	CE 2 (eV)
Glyphosate	-H	167.89	62.88	30	149.96	13
Glyphosate-2-$^{13}C,^{15}N$	-H	170.90	62.85	20	80.88	15
AMPA	-H	109.79	62.90	15	80.90	9
N-acetyl-AMPA	-H	151.90	62.88	20	109.91	10
AMPA-$^{13}C^{15}N$	-H	111.85	62.90	14	80.90	9
N-acetyl-glyphosate	-H	209.90	62.90	24	150.01	10
Glufosinate	-H	179.95	62.90	30	94.99	15
Glufosinate-d3	-H	182.97	62.89	30	97.94	15
MPPA	-H	150.90	132.93	12	62.88	25
MPPA-d3	-H	153.90	135.95	12	62.90	27
N-acetyl-glufosinate	-H	222.02	58.93	16	136.01	20
N-acetyl-glufosinate-d3	-H	225.07	62.03	11	137.05	20

2.6. IC-HRMS analysis

An Ionic Chromatography (IC) Dionex ICS-5000+ system (Sunnyvale, CA, USA) made up of a Dual Pump (DP), a Conductivity Detector (EG), a Detector/Chromatography Module (DC), and an Autosampler (AS-AP) was used for chromatographic separation. The column was a Thermo Scientific Dionex IonPac AS19- 4 μm (2 × 250 mm, 4 μm particle size) with a guard column Dionex IonPac AG19-4 μm (2 × 50 mm, 4 μm particle size) maintained at 30 °C. The eluent flow rate was 0.30 mL/min with a gradient from 15 mmol/L KOH (aq), held for 8 min, increased to 55 mmol/L KOH (aq) at 20 min, held in these conditions for 4 min, and back to 15 mmol/L KOH (aq) at 24.1 min up to 30 min. The KOH eluent was neutralized using a Dionex ADRS 600, 2 mm electrolytically regenerated suppressor (Thermo Scientific). The injection volume was 10 μL. Fig. 1 represents a comparison of chromatograms of glyphosate, glufosinate and their metabolites by LC and IC approaches.

The detector was an Orbitrap Exploris 120™ (Thermo Scientific, San Jose, CA, USA), equipped with a heated electrospray ionization (HESI) source. The ion transfer tube temperature and vaporizer temperature were set at 320 °C and 280 °C, while the electrospray voltage was set at 3.00 kV operating in negative mode. Sheath and auxiliary gas were set at 45 and 15 arbitrary units, with an S lens RF level of 70. Instrument calibration was done every 2 days with a direct infusion of a PierceTM FlexMixTM calibration solution (Pierce Biotechnology Inc., Rockford, IL, USA). The Full Scan acquisition (FS) was combined with a product ion scan mode for the confirmatory response, based on an inclusion list. The resolving power of FS was set at 60,000 Full Width at Half Maximum (FWHM), a scan range of m/z 50–250 was selected, the automatic gain control (AGC) was set as standard, and the maximum injection time was set in the auto mode. The MS2 operated at 15,000 FWHM. The AGC target was set in the standard mode, with an auto maximum injection time. The Q1 resolution was set at 1 m/z. Fragmentation of precursors was optimized as two-stepped normalized collision energy (NCE) (35 and 60 eV). The formula of the compound, with the exact theoretical mass of the parents and the diagnostic transition used to confirm Gly, Glu, and their metabolites are reported in Table 3. XcaliburTM 4.5 was the software used (Thermo Fisher Scientific, Waltham, United States).

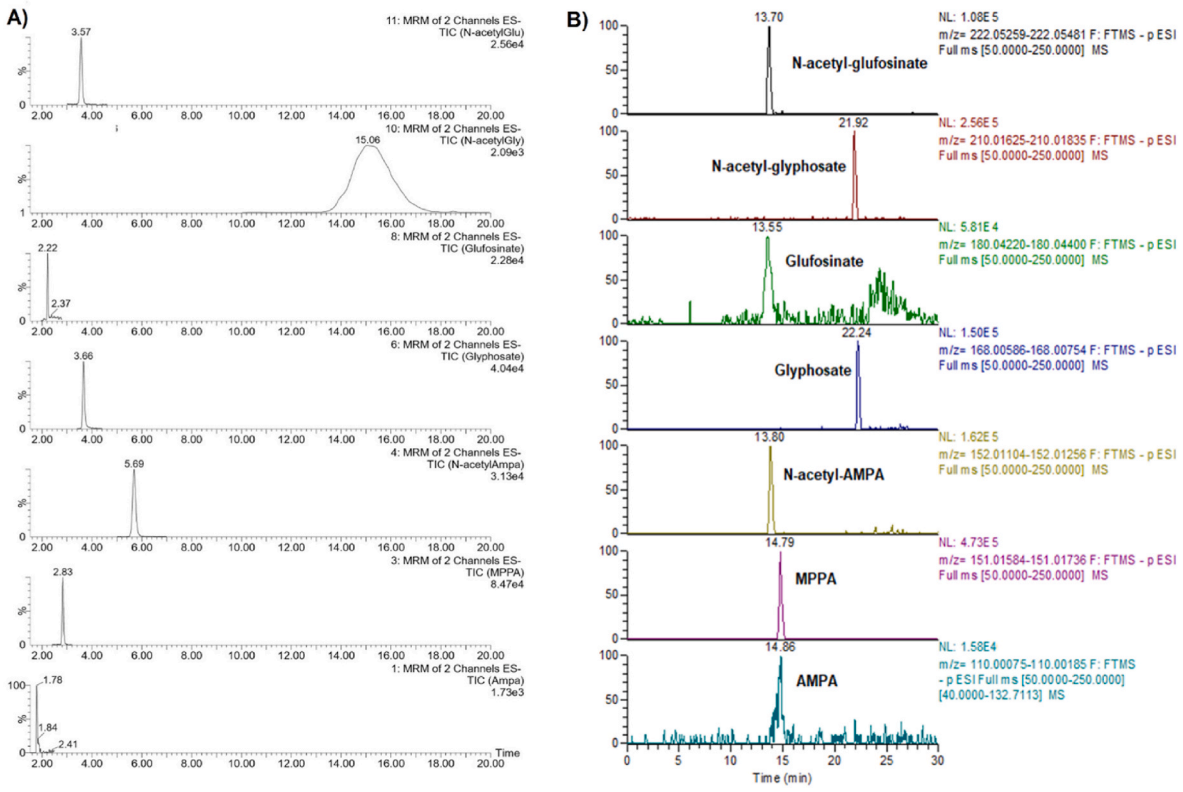

Fig. 1. Comparison of chromatograms of glyphosate, glufosinate and their metabolites at the concentration of 50 ng/g by LC (A) and IC (B) approaches.

Table 3
HRMS detection parameters.

Compound	Adduct	Formula	Precursor Ion (m/z)	Product Ions (m/z)
Glyphosate	-H	$C_3H_8NO_5P$	168.0067	62.96417, 124.01687, 149.99612
Glyphosate-2-^{13}C,^{15}N	-H	$^{13}C2C^{15}NH_8O_5P$	171.0105	62.96423, 80.97488, 153.00058
AMPA	-H	CH_6NO_3P	110.0013	62.96417, 78.95904, 80.97468
N-acetyl-AMPA	-H	$C_3H_8NO_4P$	152.0118	62.96422, 110.0138, 134.00165
AMPA-$^{13}C^{15}N$	-H	$^{13}CH_6^{15}NO_3P$	112.0016	62.96429, 80.97503
N-acetyl-glyphosate	-H	$C_5H_{10}NO_6P$	210.0173	62.96421, 124.01717, 149.99636
Glufosinate	-H	$C_5H_{12}NO_4P$	180.0431	85.02955, 94.99042, 136.05329
Glufosinate-d3	-H	$C_5H_9D_3NO_4P$	183.0619	62.96429, 98.00951, 139.07257
MPPA	-H	$C_4H_9O_4P$	151.0166	62.96404, 107.02647, 133.00574
MPPA-d3	-H	$C_4H_6D_3O_4P$	154.0354	62.96433, 110.04537, 136.02491
N-acetyl-glufosinate	-H	$C_7H_{14}NO_5P$	222.0537	59.01393, 136.05352, 180.04348
N-acetyl-glufosinate-d3	-H	$C_7D_3H_{11}NO_5P$	225.0725	62.03248, 137.05921,181.8164

2.7. Method validation

Acknowledging guidelines on analytical quality control and validation procedures in food pesticide residue analysis, alongside European regulations for animal-derived substances, an experimental validation method was designed, ensuring compliance with Regulation (EU) 2021/808 - on "The performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results" and Guidance SANTE 11312/2021 – "Analytical quality control and method validation procedures for pesticide residues analysis in food and feed". The method's performances were assessed by using blank honey that had been previously analysed to confirm the absence of residues. The absence of signals exceeding a signal-to-noise ratio of 3 at the expected retention times of the target compounds served as the criterion for confirming the absence of any interferences. To generate six-point matrix-matched calibration curves, 2 g of the matrix were spiked with an appropriate volume of the standard working solution, covering a concentration range from 5 to 100 ng/g. Repeatability (RSD_r), calculated as a coefficient of variation (CV%), was established by analysing six replicates at four different fortification levels (5, 10, 25, and 50 ng/g). The inter-day reproducibility (RSD_R) was evaluated by analysing six replicates of the four distinct levels over three separate days. Recoveries were determined by comparing the concentrations of the compounds spiked before extraction with those spiked at the end of the extraction process, at two fortification levels (10 and 50 ng/g) for all compounds. The method's limit of quantification (LOQ) was established as the lowest validated spiked level meeting the criteria of recovery falling within the range of 70–120% and a Relative Standard Deviation (RSD) of $\leq$20% and $\leq$25% for concentration under 10 ng/g. Additionally, the matrix effect was evaluated by comparing the peak areas of standards spiked into the blank extracts with those obtained from neat solution standards at concentrations of 10 and 50 ng/g, expressed as a percentage.

2.8. Statistical analysis

To assess the comparability of the instrumental approaches and their respective results, a statistical analysis of the data was conducted using

SPSS® version 29.0.2.0. This analysis aimed to demonstrate that the two instrumental platforms, LC-MS/MS and IC-HRMS, are comparable in analysing polar pesticides in the honey matrix. This assessment considered the contamination levels recorded for Gly in the analysed honey samples through both approaches. To conduct a more thorough statistical analysis, the middle-bound approach was employed, assigning a value equal to ½ LOQ at all samples where Gly was detected in trace amounts (EFSA, 2022). Thus, to evaluate the normal distribution of the parameters the Mann-Whitney test was conducted. Subsequently, the Spearman correlation index was calculated to evaluate the linear correlation between the two sets of results. p-value $\leq$0.05 was considered statistically significant for a confidence interval of 95%.

3. Results

3.1. UHPLC-MS/MS validation parameters

The six-point matrix-matched calibration curves (0, 5, 25, 50, 75, 100 ng/g) demonstrated a good linearity ($R^2 > 0.99$) for all analytes considered. The method exhibited satisfactory repeatability and reproducibility in interlaboratory settings, with RSD_r and RSD_R values falling within the range of 3%–18% and 6%–22%, respectively, for all analytes considered except for AMPA. AMPA showed CV% values > 25% at the concentration of 5 and 10 ng/g. However, all analytes displayed robust average recovery rates, ranging from 83% to 106%, at concentration levels of 10 and 50 ng/g.

As for the matrix effect, all the compounds showed a matrix effect within the range of 80%–104%, at the concentration levels of 10 and 50 ng/g, except for AMPA and Glu. AMPA and Glu exhibited a more pronounced matrix effect, measuring at 3% and 54% at a concentration level of 10 ng/g and 3% and 48% at a concentration level of 50 ng/g. This decrease in signal for AMPA and Glu is likely due to matrix interferents causing ion suppression. Nevertheless, the method maintains satisfactory accuracy for these analytes, making it suitable for its intended purpose, thanks to the use of the labelled internal standards.

Based on the same rationale, the limit of quantification (LOQ) for all analytes was set at 5 ng/g, except for AMPA. For AMPA, the LOQ was increased to 25 ng/g due to its failure to meet the precision criteria (RSD_r and RSD_R) within the $\leq$25% range at the 5 ng/g concentration level. Validation parameters results are shown in Table 4.

3.2. IC-HRMS validation parameters

During the validation process using IC-HRMS, the calibration curves, including data points at 0, 5, 25, 50, 75, and 100 ng/g, demonstrated strong linearity ($R^2 > 0.99$) for all the substances in question. The method consistently delivered reproducible results across the laboratories, with RSD_r and RSD_R values falling within the 3%–14% and 4%–14% range, respectively. Additionally, favorable recovery rates were achieved, ranging from 79% to 105%, at concentration levels of 10 and 50 ng/g for all the analytes. Concerning the matrix effect, all the compounds fell within the 70–120% range at the concentration of 10 and 50 ng/g, the only exception was AMPA at a concentration of 10 ng/g, which showed a pronounced matrix effect equivalent to 61%. This reduction in signal for AMPA, as anticipated in Section 3.1, can be attributed to the presence of co-eluting interferents within to the matrix. In this case, the method enables the establishment of LOQs for all the analytes at 5 ng/g.

3.3. Application of the methods to real honey samples

The results from the analyses conducted simultaneously on the two instrumental platforms demonstrated a high degree of comparability of the analysis outcomes. Specifically, in both IC-HRMS and UHPLC-MS/MS monitoring, 12% of the samples exhibited quantifiable levels of Gly (>LOQ). The contamination range spanned from 7.06 to 118 ng/g. Notably, one sample exceeded the MRL for Gly by more than double. In an additional 37 samples, traces of Gly were detected using both approaches simultaneously. Therefore, Gly was detected at quantifiable levels or in traces in 50% of the samples. Complete data are provided in Tables S1 and S2. Instead, IC-HRMS and UHPLC-MS/MS monitoring revealed no traces of AMPA, N-acetyl-AMPA, N-acetyl-glyphosate, Glu, MPPA, and N-acetyl-glufosinate.

3.4. Statistical analysis

The mean, median and standard deviation values are shown in Table S3 and demonstrate the equivalence of the results.

The Mann-Whitney test was conducted to evaluate the normal distribution of the parameters. The P-value of the test was >0.05 and indicated a non-normal distribution. Non-parametric Spearman correlation coefficient (rs) = 0.999. The two-tailed P value is < 0.001, considered extremely significant. Concluding, the coupling is shown to be effective.

4. Discussion

4.1. Extraction procedure optimization

In the initial stages of the project, the QuPPe (Quick Method for the Analysis of Highly Polar Pesticides in Food, version 12) extraction method was initially employed, as recommended by the EU Reference Laboratories for Pesticide Residues (Anastassiades et al., 2021). However, the QuPPe procedure didn't provide specific and detailed guidance for handling different types of honey matrices, as its primary focus was on the extraction process for plant matrices. During the implementation of the QuPPe extraction procedure, it became evident that certain steps

Table 4
UHPLC-MS/MS validation parameters.

Compound	LOQ (ng/g)	Linearity (R^2)	Matrix effect %		Recovery %		RSD_r%[b] (At 4 spike levels[a])			RSD_R %[c] (At 4 spike levels[a])
			10 ng/g	50 ng/g	10 ng/g	50 ng/g	Day 1	Day 2	Day 3	
Glyphosate	5	0.997	100	93	92	105	8-6-7-5	8-8-5-7	6-9-6-13	12-13-6-9
AMPA	25	0.998	3	3	83	87	64-23-14-9	66-61-10-8	57-21-11-18	63-42-11-14
N-acetyl-AMPA	5	0.999	86	105	105	105	10-12-13-8	11-3-10-6	7-3-3-13	10-12-8-10
N-acetyl-glyphosate	5	0.999	91	91	92	106	7-7-11-7	11-4-4-8	17-6-4-11	13-10-7-8
Glufosinate	5	0.999	54	48	87	103	17-19-16-10	12-10-8-9	7-10-8-12	15-22-10-12
MPPA	5	0.998	91	80	112	102	17-13-7-6	10-3-3-14	7-3-3-15	12-14-6-12
N-acetyl-glufosinate	5	0.996	90	104	96	100	12-13-13-4	12-13-13-4	9-9-6-13	15-11-9-8

[a] 5,10,25,50 ng/g.
[b] n = 6 replicates for each spike level.
[c] n = 18 replicates each for spike levels.

and additives, which are necessary for plant matrices, might not be required when applied to honey matrices.

As a result, the protocol proposed by Chiesa et al. (2019) was adopted, which streamlined the process with fewer steps and reduced time requirements. This sample preparation procedure was originally developed for the detection of Gly and its metabolites in animal-derived food. To tailor the method to the study's objectives and the diverse honey types while ensuring the required sensitivity, particularly for UHPLC-MS/MS analysis, it was necessary to double the initial matrix quantity from 1 to 2 g. However, in the case of IC-HRMS analysis, the extracts were diluted 1:1 with water before the injection. Using water as extraction solvent leads to the co-extraction of a considerable amount of polar matrix components that may co-elute with the analytes. This raises the possibility of interferences and affects the generation of free analyte ions within the ESI source, potentially leading to reduced signal intensity target analytes. Furthermore, the presence of these abundant co-extractives poses a risk of system contamination (Jesús et al., 2023). However, in the present study, the majority of the analysed compounds showed minimal matrix effect, with the exception of AMPA and, to a lesser extent, Glu.

As demonstrated by Jesús et al. (2023), analysing the total ion count (TIC) of a blank honey extract injected (ESI -) in full scan mode (m/z 50–650) enables the evaluation of a matrix profile. Fig. 2 shows the highest concentration of co-extractives eluted between a retention time of 1 and 2.5 min, which may cause ion suppression for the analytes eluting within this timeframe during LC-MS/MS analysis. This assessment can explain the minor sensitivity observed in the detection of AMPA and Glu, characterized by their respective retention time of 1.78 min and 2.23 min, as shown in Fig. 2. In IC-HRMS the impact of ion suppression from the matrix appears to have a relatively minor effect on the detection of AMPA and Glu. This is evidenced by the different matrix effects percentages reported in both Tables 4 and 5.

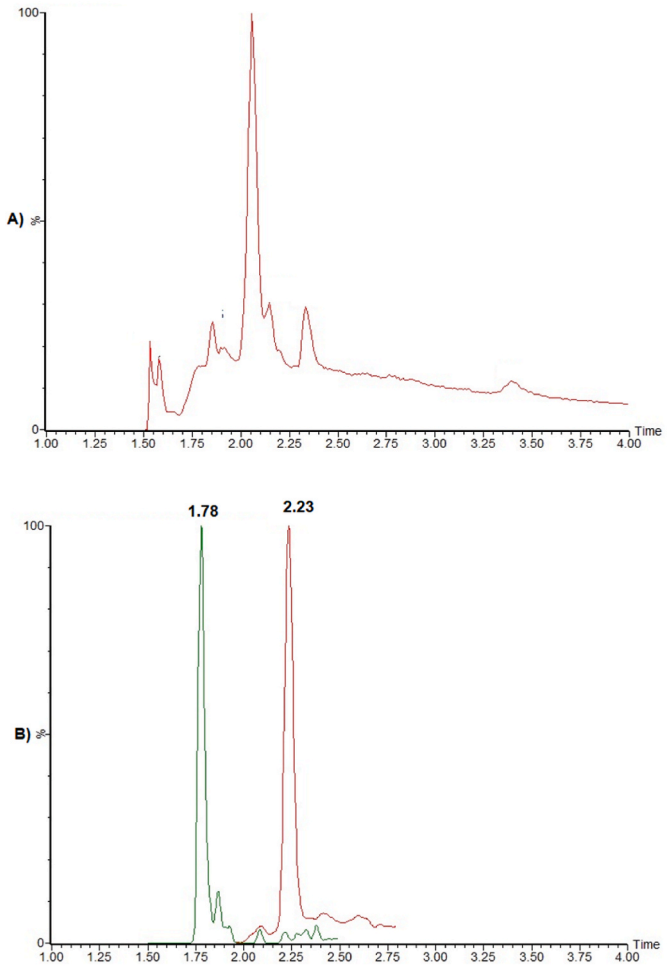

Fig. 2. (A) Total ion count (TIC) of a blank honey extract injected in RADAR mode (mass range m/z 50–650) in UPLC-MS/MS (B) Extracted ion chromatograms of standards of AMPA (RT 1.78) and Glu (RT 2.23) in LC-MS/MS.

Honey is primarily composed of sugars and other minor compounds proteins, nitrogenous substances, organic acids, minerals, polyphenols, and hydrosoluble vitamins. However, its specific composition can vary depending on the raw materials used in its production. Research suggests that the matrix effect can be influenced by the floral origin of honey (Souza Tette, Rocha Guidi, De Abreu Glória,& Fernandes, 2016). This highlights the potential for significant variability among individual honey samples.

4.2. Comparison of method validation performances of IC-HRMS and UPLC-MS/MS for polar pesticide and metabolite detection

The comparison of data derived from the method validation experiments employing the two instrumental platforms demonstrates their comparability in analysing polar pesticides regulated at the European level. Therefore, both platforms have proven suitable for use in official control activities.

The study findings suggest that the IC-HRMS approach may demonstrate slightly higher sensitivity than LC-MS/MS in detecting certain polar metabolites of pesticides, particularly AMPA, even at extremely low concentrations. This capability can be attributed to the superior resolution and, consequently, higher accuracy in determining molecular masses. Thus, through IC-HRMS analysis, it was possible to set a LOQ of 5 ng/g for AMPA in comparison with 25 ng/g for LC-MS/MS. These advantages are particularly crucial when addressing the complexities of the honey matrix and the need to identify and measure minute traces of metabolites. This will become particularly important if the decision is made to include AMPA in the assessment of the maximum residual level of Gly, as has already been established in other countries. In Australia and New Zealand, the MRL for the sum of Gly, N-acetyl-glyphosate, and AMPA in honey is set at 200 ng/g, expressed as Gly (Rampazzo et al., 2023). Furthermore, assessing contamination levels by metabolites becomes especially pertinent in toxicity studies, given the simultaneous presence of various pesticides and their metabolites within a matrix. Indeed, in recent years, the EFSA has developed a harmonized framework for evaluating the potential 'combined effects' of mixtures of chemicals in food and feed (EFSA Scientific Committee et al., 2019).

Finally, the use of IC-HRMS permits the injection of diluted matrix quantities and enables the assessment of an extraction process involving smaller initial matrix amounts. The decision to increase the matrix quantity from 1 g to 2 g compared to the original extraction protocol, was driven by the need to achieve adequate sensitivity levels with the triple quadrupole. By introducing smaller matrix quantities into the instrument, the matrix effect induced by co-eluting interfering compounds is minimized. Furthermore, this approach reduces the risk of contamination. However, in the case of triple quadrupole analysis, this risk can be mitigated through diligent routine maintenance practices, such as washing and cleaning the cone after each analysis batch, and performing regular wash injections to maintain the system in optimal condition.

4.3. Application of the method to real honey samples

Based on the results obtained, the observed status of honey contamination remains reassuring. The data obtained are consistent with those reported in the literature regarding Gly contamination levels in honey, as recently reviewed by Rampazzo et al. (2023). A notable issue identified in the literature concerns the lack of methods that encompass the simultaneous analysis of various highly polar pesticides and their metabolites. Typically, the majority of published studies focus

Table 5
IC-HRMS validation parameters.

Compound	LOQ (ng/g)	Linearity (R^2)	Matrix effect %		Recovery %		RSD_r%[b] (At 4 spike levels[a])			RSD_R %[c] (At 4 spike levels[a])
			10 ng/g	50 ng/g	10 ng/g	50 ng/g	Day 1	Day 2	Day 3	
Glyphosate	5	0.991	109	103	105	104	10-6-6-6	11-8-5-4	12-6-4-3	11-6-5-4
AMPA	5	0.991	61	83	79	104	9-13-11-6	11-8-6-6	11-7-7-6	10-9-8-6
N-acetyl-AMPA	5	0.991	94	98	99	102	9-8-5-8	7-9-7-6	5-8-7-4	6-8-7-6
N-acetyl-glyphosate	5	0.991	89	102	90	97	5-5-6-5	10-3-6-5	5-9-5-6	6-8-7-5
Glufosinate	5	0.993	77	81	81	98	12-7-9-8	13-5-8-4	14-11-5-7	14-8-8-7
MPPA	5	0.997	91	99	92	100	10-11-5-5	4-5-3-5	9-8-7-3	9-12-5-5
N-acetyl-glufosinate	5	0.991	81	94	91	103	4-10-6-4	7-10-8-5	5-10-5-6	6-10-6-6

[a] 5,10,25,50 ng/g.
[b] n = 6 replicates for each spike level.
[c] n = 18 replicates for each spike levels.

on the analysis of Gly, frequently incorporating its metabolite AMPA (Jesús et al., 2023). However, only recently have studies been published that analyze and monitor polar pesticides in honey and hive products, incorporating their complete metabolic profiles (Butovskaya et al., 2023; Jesús et al., 2023).

In summary, the spectrum of Gly contamination in honey ranges from 2.0 ng/g to 5500 ng/g, with notable concentrations observed in Pakistan (3500 ng/g) and Europe (5500 ng/g) (Bergero et al., 2021; El Agrebi et al., 2020; Karise et al., 2017; Thompson, van den Heever, & Limanowka, 2019; Zoller, Rhyn, Rupp, Zarn, & Geiser, 2018). Few studies concurrently explore the presence of AMPA concentrations, ranging from 5 ng/g to 100 ng/g (Jesús et al., 2023; Rampazzo et al., 2023). In recent studies, Jesus et al., (2023) identified Gly in 16 honey samples (84%) with concentrations ranging from 5 to 42 ng/g, all falling below the EU-MRL of 50 ng/g. The highest concentration, 42 ng/g, was observed in a commercial eucalyptus mixed honey from Spain and Uruguay, where the AMPA metabolite was also detected below the LOQ (ng/g). While, during a three-year monitoring of polar pesticides in Italian honey, Butovskaya et al. (2023) reported the presence of Gly in 37 samples (23.8%) from the Lombardy region, and in 25 samples (37.9%) from the Emilia Romagna region. During the monitoring period, Gly was detected twice at concentrations exceeding the MRL, specifically at 310 and 250 ng/g, approximately five times the MRL. The contamination of honey by Gly can be described as widespread or frequently occurring contaminations.

While Gly contamination in honey receives limited monitoring and research attention, exploration of Glu is notably scarce. Glu made a rare appearance, with Thompson et al. (2019) revealing its presence in 39% of 125 Canadian honey samples in 2019, with contamination levels ranging from 1 ng/g to 33 ng/g. The scarcity of data on Glu contamination can be attributed to factors such as its reduced usage compared to Gly, rapid degradation in soil, and the non-renewal of registration in Europe since 2018.

4.4. Statistical analysis

As anticipated in Section 2.8, the comparability of Gly contamination results in honey samples analysed using two instrumental platforms was assessed through rigorous statistical analysiss. Mean, median and standard deviation analyses demonstrate result equivalence. The Mann-Whitney test was conducted to evaluate the normal distribution of the parameters and indicated a non-normal distribution. Thus, a nonparametric index, Spearman's correlation, was then chosen to measure the strength and direction of the relationship between the two quantitative variables. The outcomes from this assessment robustly indicated a strong correlation between the analyses conducted via both platforms.

5. Conclusions

In this study, the challenges associated with the detection of Gly, Glu, and their metabolites in honey were rigorously investigated. The research encompassed comprehensive examination and validation procedures, along with monitoring across 97 honey samples using both LC-MS/MS and IC-HRMS.

This comparative analysis aimed to assess the advantages and disadvantages of analysing polar pesticides and their metabolites via mass spectrometry, employing different instrumental approaches. The results offer valuable insights to assist in the selection of the most appropriate technique, considering experimental purposes, scientific criteria, and compliance with regulatory requirements. The optimization of extraction procedures highlighted the importance of comprehending the matrix profile and its potential for ion suppression effect on the analytes under investigation. Our findings revealed a high level of comparability between the two instrumental platforms for the analysis of polar pesticides regulated at the European level. This underscores their appropriateness for official controls purposes. Moreover, the optimization process enabled the validation of a method capable of extremely low residual levels of analytes, well below the established maximum residue levels, using both approaches. The IC-HRMS approach exhibit slightly higher sensitivity than LC-MS/MS in the detection of AMPA at extremely low concentrations. This becomes particularly pertinent if AMPA is included in assessing of the maximum residual level of Gly, as in Australia and New Zealand. However, achievable sensitivity levels may vary depending on the type of honey.

This holds significant importance for accurately assessing consumer risks, ensuring alignment with EFSA's harmonized framework for assessing potential 'combination effects' from chemical mixtures in food and feed.

In the monitoring of real samples, quantifiable levels of Gly were observed in 12% of samples, with one sample exceeding the MRL by more than double. Gly was detected in 50% of the samples, while Gly metabolites, Glu, and its metabolites were not detected. These findings are consistent with the current literature, confirming the presence of Gly in honey.

In conclusion, it's clear that the discussion on the safety of polar pesticides, specifically Gly, persists, emphasizing the importance of ongoing research and surveillance. Our study provides valuable insights into advanced analytical approaches, highlighting the significance of considering metabolites and employing techniques that achieve a balance among sensitivity, selectivity, and efficiency in the assessment of pesticide residues in honey. These results provide crucial insights into the analytical efficiency and monitoring of polar pesticides in honey, aiming to achieve an accurate risk assessment of consumer exposure, and enhancing the safety of the beekeeping industry.

CRediT authorship contribution statement

Giulia Rampazzo: Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation. **Teresa Gazzotti:** Writing – review & editing, Visualization, Supervision, Data curation. **Giampiero Pagliuca:** Writing – review & editing, Supervision, Resources, Data curation, Conceptualization. **Maria Nobile:** Writing –

review & editing, Writing – original draft, Validation, Data curation. **Luca Chiesa:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Stefania Carpino:** Supervision, Resources, Conceptualization. **Sara Panseri:** Writing – review & editing, Supervision, Resources, Data curation, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116159.

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