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Preliminary data on glyphosate, glufosinate, and metabolite contamination in Italian honey samples

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Abstract

Glyphosate and glufosinate are among the most widely used pesticides in agriculture worldwide. Their extensive use leads to the presence of their residues on crops and in the surrounding environment. Beehives, bees, and apiculture products can represent potential sources for the accumulation of these substances and their metabolites, and the consequences for bee health, as well as the level of risk to human health from consuming contaminated food, are still unclear. Furthermore, information on the contamination levels of honey and other beehive products by these compounds remains poorly documented. This study is part of a broader research effort aimed at developing specific analytical methods for monitoring the level of these contaminants in bee products. The methodology employed enabled the acquisition of preliminary information concerning the levels of glyphosate and glufosinate contamination in honey samples obtained from various retailers in Italy to assess compliance with the limits established by Regulation 293/2013. The liquid chromatography tandem mass spectrometry analysis of the 30 honey samples revealed quantifiable levels of glyphosate in eight samples, with contamination ranging from 5.4 to 138.5 ng/g. Notably, one sample of the wildflower type showed residue levels nearly three times the maximum residue limit. Additionally, trace levels of glyphosate contamination were detected in another ten samples. It is noteworthy that glufosinate and its metabolites were not detected in any of the analyzed samples within the established method's detection ranges.

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Introduction

Pesticides are the most frequently encountered environmental contaminants in apiculture products. Among these, glyphosate (Gly) and glufosinate (Glu) are some of the most used in this field. Since the 1990s, when genetically modified Gly-resistant crops were introduced, the use of Gly has significantly increased. This has led to a notable rise in both environmental risks and human exposure to these herbicides (Lajmanovich *et al.*, 2022). Their extensive use results in widespread contamination, affecting not only crops but also the surrounding environment, water sources, and potential bee foraging sites. Bees collect not only nectar from flowers, pollen, and propolis from buds but also consume water from the environment and gather suspended particles that settle on plants. The presence of these substances and their metabolites in the environment poses a risk to the bees themselves and can lead to the contamination of hive products (Rampazzo *et al.*, 2023).

Recent studies have highlighted how bee exposure to Gly and Gly-based herbicides can have toxic effects and, in some cases, lethal effects on bees, including impacts on cognitive abilities and sleep patterns. Both adult bees and larvae are sensitive to these substances, with chronic exposure being the most detrimental. This sensitivity contributes to the phenomenon of colony collapse disorder, in which the majority of worker bees abandon the hive, leaving the queen, nurse bees, and larvae behind, ultimately leading to a decline in honeybee populations (Johnson, 2015; Battisti *et al.*, 2021). The impact of these polar pesticides remains a topic of intense debate. Gly exposure has raised significant concerns due to its potential association with a range of adverse health effects, including bladder and liver toxicity, severe eye damage, and disruptions of the endocrine system. In 2015, it was declared a "probable carcinogen" by the International Agency for Research on Cancer (IARC, 2017), and subsequent assessments by the European Food Safety Authority and the US Environmental

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Protection Agency have concluded that Gly is unlikely to pose a cancer risk to humans (European Food Safety Authority, 2017; US Environmental Protection Agency, 2017; Benbrook, 2019). Nevertheless, an impurity present in Gly-based herbicide formulations has proven to be potentially clastogenic in an *in vitro* study of chromosomal aberration at Gly concentrations equal to or below the established acceptable daily intake (ADI) value (Santovito *et al*., 2018). It was found to be potentially clastogenic in an *in vitro* study of chromosomal aberration at Gly concentrations equal to or below the established ADI value (Santovito *et al*., 2018).

Glu also poses potential risks to human health when ingested or when it encounters the skin. The International Union of Pure and Applied Chemistry (IUPAC) pesticide properties database highlights Glu as a potential toxicant affecting the kidneys, bladder, blood, and lungs. While Glu was previously authorized for use in Europe until 2018, its registration was not renewed by the European Commission due to concerns about its toxicity. Despite this, Glu continues to be extensively used in the United States, South America, and various regions across the world, both in agricultural and non-agricultural settings (European Commission, 2020; Takano and Dayan, 2020). However, to ensure consumer health protection, the European Union has set maximum residue limits (MRLs) for pesticides in honey and related apiculture products. It is worth noting that Regulation 2018/62 clarifies that MRLs for honey do not apply to other apiculture products due to their distinct chemical characteristics (European Commission, 2018). Specifically, the MRL for Gly has been established at 50 ng/g following Regulation 2013/293 (European Commission, 2013). In the case of Glu, which encompasses the sum of Glu isomers, its salts, and its metabolites (3-[idrossi(metil)fosfinoil] propionic acid and N-acetyl-Glu), the MRL has been set at 50 ng/g by Regulation 2016/1002 (European Commission, 2016).

In 2017, the European Commission renewed the license for the use of Gly for 5 years, albeit with some restrictions on its use. In December 2022, the European Union extended the approval of Gly by 1 year to allow the European Food Safety Authority sufficient time to complete its new peer review (European Parliament, 2022). Recently, in July 2023, the European Food Safety Authority published the peer review assessment report on Gly, which did not identify any critical issues that would hinder the herbicide from obtaining European-level renewal. Based on the examination of current evidence, it appears "unlikely" that Gly poses risks to human health related to endocrine disruption, carcinogenesis, teratogenesis, mutagenesis, reproductive toxicity, neurotoxicity, or degenerative diseases (European Food Safety Authority, 2023). However, certain "unresolved issues" emerged during the European Food Safety Authority peer-review process due to insufficient data to prove the safety of Gly under the proposed condition of use. Among the data gaps highlighted by the European Food Safety Authority conclusion, one pertains to the incompleteness of the data set on the magnitude of residues, which makes it impossible to finalize the risk assessment for consumers, even though the current situation appears reassuring based on the available data (European Food Safety Authority *et al.*, 2023). After discussions with Member States within the Permanent Committee on Plants, Animals, Food, and Feed in September 2023, the Commission has made available an updated renewal report, considering the comments received from Member States, along with a draft regulation that establishes approval conditions. On October 13, 2023, Member States voted on the regulation draft presented by the European Commission (2023). European Union governments were unable to reach a definitive consensus on a proposal to extend approval for the use of Gly. To either support or reject the proposal, a "qualified majority" of 15 countries representing at least 65% of the European Union's population was required. The European Commission, in a statement, confirmed that no such qualified majority was achieved during the committee vote among the 27 member states. Further attempts to reach a clear consensus will be made in the coming months. If the next attempts fail to yield a decisive opinion, the final decision will be up to the European Commission (2023).

The current study is part of a broader research project aimed at identifying innovative, rapid, and specific analytical approaches for monitoring these herbicides and their metabolites in apiculture products. Our study aims to provide information on the contamination levels of these products with herbicide residues, in line with the knowledge gap identified in the European Food Safety Authority's opinion. The preliminary data presented originate from honey samples of various types and geographic origins, intending to assess compliance with the MRLs established by Regulation 2013/293 and Regulation 2016/1002 (European Commision, 2013, 2016) for Gly and Glu in honey.

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Materials and Methods

Chemicals and reagents

Gly (purity 98%), Gly $^{13}C2,^{15}N$ (purity 97%) [internal standard (IS)], Glu ammonium, Glu-d3-hydrochloride (IS), n-acetyl-Glu sodium, (3-) methylphosphinicopropionic (MPPA), (3-) methylphosphinicopropionic acid-d3 sodium salt (IS) were purchased from TRC (Toronto Research Chemicals Inc., Canada). Acetonitrile, methanol, and formic acid, all liquid chromatography-mass spectrometry grade, were acquired by Merck (Darmstadt, Germany). Ultrapure water was freshly produced from a Milli-Q® water purification system (Merck, Darmstadt, Germany). Polytetrafluoroethylene syringe filters (13 mm, 0.2 µm) were purchased from Waters Corp (Milford, MA, USA). The single stock solution of Gly, Glu ammonium, n-acetyl-Glu sodium, and MPPA at a concentration of 100 µg/mL and relative IS at a concentration of 40 µg/mL were prepared in plastic flasks. 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 IS. All the stock and working solutions were stored and refrigerated at 4°C.

Instrumentation

The monitoring of these polar pesticides in honey was achieved using ultrahigh-performance liquid chromatography coupled with triple-quadrupole mass spectrometry (LC-MS/MS) technology. The equipment employed consisted of a Waters Acquity UHPLC® binary pump coupled with a Waters Xevo® TQ-S micro triple-quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA) featuring an electrospray ionization source (ESI). Analyses were performed in negative ESI and multiple reaction monitoring modes, following specific transitions for the target analytes as reported in Table 1. The ESI capillary voltage was set at +3.00 kV, cone voltage (V) was set at 20.00, desolvation temperature was set at 600°C and source temperature at 150°C. Desolvation and cone gas flow were 1000 and 150 L/Hr, respectively, and argon was used as collision gas. The chromatographic separation was achieved on an anionic polar pesticide column (5 µm, 2.1×150 mm) (Waters Corporation, Milford, MA, USA) thermostated at 50°C. The chromatographic conditions were settled as

follows: mobile phases were 1.2% formic acid in water (A) and acetonitrile acidified with 0.5% formic acid (B). The gradient started at 0 minutes with 10 % phase A; this increased linearly to 80% in 1.5 minutes and 95% in 1.5 minutes, then decreased to 10% in 2 minutes. This condition was maintained for 1 minute for the rebalancing of the column. The total run time was 6 minutes, the flow rate was 0.500 mL/min, and the volume injected was 10 µL. The autosampler was kept at 20°C. Data were acquired and processed using Waters MassLynx™ 4.1 software (Waters Corporation, Milford, MA, USA).

Sampling and sample preparation

A total of 30 honey samples of different types and geographical origins were purchased from local Italian markets. All samples were stored at room temperature and in the dark before analysis.

The sample preparation followed the extraction protocol described and validated by Chiesa *et al*. (2019), which was originally designed for the detection of Gly and its metabolites in food of animal origin based on ion-chromatography high-resolution mass spectrometry. Appropriate modifications were made to adapt the method to different types of honey, aiming to achieve adequate sensitivity, considering the different types of instrumentation used. Compared to the original protocol, it was decided to increase the initial matrix quantity, with a selection of 2 g as the initial aliquot for extraction. Briefly, 2 g of honey are weighed into a 15-mL polypropylene falcon, then 3 mL of methanol and 7 mL of water with 1% formic acid are added. The sample is vortexed until completely dissolved, then sonicated for 15 minutes and centrifuged at 5000 rpm at 4°C for 10 minutes. Finally, 1 mL of the sample is filtered into plastic vials before LC-MS/MS analysis.

Method validation

The validation procedure was carried out following Guidance SANTE 11312/2021 "Analytical quality control and method validation procedures for pesticide residues analysis in food and feed" (European Commission, 2021). The method's selectivity was assessed by injecting extracted blank honey samples. The absence of signals exceeding a signal-to-noise ratio of 3 at the anticipated retention times of the target compounds served as the criterion for demonstrating the absence of interferences. To construct the 6-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. The method's limit of quantification (LOQ) was established as the lowest validated spiked level that met the criteria of recovery falling within the range of 70-120% and a relative standard deviation (RSD) of ≤20%, following the guidelines of the European Commission (2021).

Repeatability (RSD_r), expressed as a coefficient of variation, was determined by analyzing five replicates at three different fortification levels (25, 50, and 75 ng/g). The inter-day reproducibility (RSD_R) was evaluated by analyzing five replicates of the three different levels studied over three separate days. Recoveries were calculated 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. Additionally, the matrix effect was evaluated by comparing the peak areas of standards spiked into the blank extracts with the peak areas obtained from neat solution standards at a concentration of 50 ng/g, expressed as a percentage.

Results and Discussion

Extraction procedure

In the initial phases of the work, the quick method for the analysis of highly polar pesticides in food (QuPPe) extraction method recommended by the European Union Reference Laboratories for Pesticide Residues (Anastassiades *et al*., 2020) was first adopted. Nonetheless, it was essential to consider that the QuPPe procedure lacks specific and detailed instructions regarding the treatment of different types of honey matrices, as it primarily focuses on the extraction process for plant matrices. Implementing the QuPPe extraction procedure, it became evident that several steps and additives, typically used for plant matrices, could be deemed unnecessary when applied to honey matrices. Consequently, a decision was made to adopt the Chiesa *et al*. protocol (2019), with fewer steps and reduced time requirements.

Liquid chromatography coupled with triple-quadrupole mass spectrometry validation parameters

Considering the method application to LC-MS/MS and the intrinsic characteristics of the different types of honey analyzed, the method underwent an internal validation procedure according to SANTE 11312/2021 guidelines (European Commission, 2021).

The 6-point matrix-matched calibration curves (0,5,25,50, 75,100 ng/g) showed a good linearity range (R^2>0.99). The method proved to be repeatable and reproducible under interlaboratory conditions, with RSD_r and RSD_R values ranging between 2-17% and 3-13% for all analytes considered. All analytes showed good average recovery rates, ranging from 87% to 105%, consistent

Table 1. Mass spectrometry parameters of the compounds.

Compound	Precursor Ion (m/z)	Product Ion 1 (m/z)	CE (eV)	Product Ion 2 (m/z)	CE (eV)
Glyphosate	167.89	62.88	30	149.96	13
Glyphosate $C^{13}N^{15}$	170.90	62.85	20	80.88	15
Glufosinate	179.95	62.90	30	94.99	15
Glufosinate D3	182.97	62.89	30	97.94	15
MPPA	150.90	132.93	12	62.88	25
MPPA D3	153.90	135.95	12	62.90	27
N-acetyl glufosinate	222.02	58.93	16	136.01	20

MPPA, methylphosphinicopropionic; CE, collision energy.

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with SANTE guidelines at concentration levels of 10 and 50 ng/g. When considering the matrix effect, it is essential to highlight that all the compounds exhibited a matrix effect ranging from 80% to 104%, except for Glu. Glu demonstrated a more noticeable matrix effect, corresponding to 48% at the concentration level of 50 ng/g. This decrease in signal for Glu is likely due to matrix interferents causing ion suppression. Nevertheless, the accuracy of the method remains satisfactory and suitable for its intended purpose, thanks to the use of the labeled IS. For the same underlying rationale, the LOQ for all analytes was set at 5 ng/g, except for Glu. In the instance of Glu, the LOQ was raised to 25 ng/g because it did not meet the precision criteria (RSD_r and RSD_R) exceeding ≤20% at the concentration level of 5 ng/g. The results of the validation parameters are summarized in Table 2.

Table 2. Validation parameters about glyphosate, glufosinate, and its metabolites in honey analyzed by liquid chromatography coupled with triple-quadrupole mass spectrometry.

Compound	LOQ (ng/g)	Matrix effects (%)	CV % (At 3 levels*)	Recovery % (At 2 levels**)	Linearity (R^2)
Glyphosate	5	93	6-5-2	91-105	0.997
Glufosinate	25	48	8-5-2	87-103	0.999
MPPA	5	80	8-7-3	101-103	0.998
N-acetyl glufosinate	5	104	8-6-3	91-100	0.996

LOQ, limit of quantification; MPPA, methylphosphinicopropionic; CV, coefficient of variation; *25-50-75 ng/g; **10-50 ng/g.

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Table 3. Glyphosate, glufosinate, methylphosphinicopropionic and N-acetyl-glufosinate concentration in real honey samples purchased from Italian markets.

N°	Type	Origin	Analyte concentration (ng/g)			
			Gly	MPPA	Glu	N-acetyl-Glu
1	Wildflower	Italy	11.9	ND	ND	ND
2	Wildflower	Italy	9.0	ND	ND	ND
3	Wildflower	Bulgaria	Traces	ND	ND	ND
4	Wildflower	Hungary-Ukraine	Traces	ND	ND	ND
5	Wildflower	Italy-Hungary-Moldavia-Argentina	Traces	ND	ND	ND
6	Wildflower	Mexico-Cuba-Argentina	Traces	ND	ND	ND
7	Acacia	Italy	ND	ND	ND	ND
8	Orange	Italy	Traces	ND	ND	ND
9	Chestnut	Italy	Traces	ND	ND	ND
10	Wildflower	Italy	Traces	ND	ND	ND
11	Wildflower	Italy	Traces	ND	ND	ND
12	Coriander	Italy	ND	ND	ND	ND
13	Linden	Italy	138.5	ND	ND	ND
14	Chestnut	Italy	ND	ND	ND	ND
15	Wildflower	Italy	ND	ND	ND	ND
16	Chestnut	Italy	ND	ND	ND	ND
17	Wildflower	Italy	ND	ND	ND	ND
18	Acacia	Italy	ND	ND	ND	ND
19	Orange	Italy	ND	ND	ND	ND
20	Orange	Italy	ND	ND	ND	ND
21	Chestnut	Italy	ND	ND	ND	ND
22	Orange	Italy	Traces	ND	ND	ND
23	Citrus fruit	Italy	Traces	ND	ND	ND
24	Alpine flower	Italy	ND	ND	ND	ND
25	Wildflower	Italy	14.7	ND	ND	ND
26	Wildflower	Italy	7.5	ND	ND	ND
27	Wildflower	Italy	5.4	ND	ND	ND
28	Wildflower	Italy-Hungary	5.8	ND	ND	ND
29	Wildflower	Italy	ND	ND	ND	ND
30	Wildflower	Italy	11.1	ND	ND	ND

Gly, glyphosate; MPPA, methylphosphinicopropionic; Glu, glufosinate; ND, not detected.

Application of the method to real samples

The proposed method was utilized to perform preliminary monitoring of Gly, Glu, and its metabolites in 30 real honey samples of diverse types and origins collected from the Italian markets. The results are shown in Table 3. Gly was quantifiable in 8 out of the 30 samples (27%), with contamination levels ranging from 5.4 to 138.5 ng/g. Notably, one sample exceeded the MRL set at 50 ng/g by a factor of three. Furthermore, trace amounts of Gly (limit of detection<x<LOQ) were detected in 10 samples (33%). In contrast, Glu, MPPA, and N-acetyl-Glu were not quantified in any of the samples within the limits of our methodology. Among beekeeping products, honey is the most monitored matrix, especially for the presence of Gly. However, available data on Gly residues in honey is both limited and inconsistent. Rampazzo *et al.* (2023) recently reviewed Gly and Glu residues in honey and other hive products. A comparison of the few available mean or median values shows that, in most cases, the reported concentrations are below the MRL. Nonetheless, there are cases where these concentrations far exceed the established limits, with averages reaching levels 20 or 40 times higher than the specified thresholds. Studies on Glu are even more limited. Glu was detected in just one research study, conducted by Thompson *et al.* (2019), which detected it in 125 Canadian honey samples with concentrations varying from 1 ng/g to 33 ng/g. However, this study did not investigate the presence of its metabolites. It was only recently that the first study focusing on the detection of polar pesticides in honey and pollen, including Glu metabolites, was published. However, no traces of the herbicide and its metabolites were detected (Jesús *et al.*, 2023).

Conclusions

In the present study, preliminary monitoring of Gly, Glu, and metabolites in honey was conducted. The method employed underwent an interlaboratory validation process in compliance with SANTE 11312/2021, confirming its suitability for the intended purpose. The development and validation of rapid, cost-effective, and reliable methods for detecting Gly and Glu in diverse types of honey is of paramount importance for official controls and consumer protection. These procedures play a crucial role in maintaining honey safety on the market. They enable efficient monitoring by regulatory bodies, safeguard the beekeeping industry, and economize resources.

The current situation regarding the presence of herbicide residue in honey is confirmed as reassuring. However, about one-quarter of the samples reported quantifiable levels of Gly, with one sample exceeding MRLs. Thus, considering the limited and inconsistent data available on Gly and Glu residues in honey and other hive products and the ongoing debate surrounding the potential health effects of these herbicides on humans, it is crucial to emphasize the necessity for further data and information to better define the residue profile. This would support a more comprehensive risk assessment for consumers.

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