QUALITY OF PRODUCTS OF ANIMAL ORIGIN

GLYPHOSATE RESIDUES IN BRAZILIAN BEESWAX: OPTIMIZATION AND VALIDATION METHOD BY HPLC/FLD

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ABSTRACT

In Brazil, one of the world's largest agricultural producers, beehives have declined due to the mismanagement of pesticides, affecting pollination and compromising food availability. For this purpose, bee products are used as bioindicators to define environmental impacts resulting from anthropogenic changes in biodiversity. Thus, an analytical method to quantify glyphosate (GLY) and its main metabolite aminomethylphosphonic acid (AMPA), in beeswax was validated using a fluorescence detector and postcolumn reaction. The quantification limit was 0.05 mg/kg, and the recoveries ranged from 75% to 93% with a coefficient of variation of less than 11% for GLY and AMPA. The validated method was applied to 18 beeswax samples from areas with extensive agriculture and forest. None of them showed GLY or AMPA contamination. The fact that no contamination was found in Brazilian samples, even with the widespread use of GLY, emphasizes the significance of continuous studies and monitoring to protect bee health and ensure apiculture product safety.

Keywords aminomethylphosphonic acid, bee products, contamination, pesticides .

RESÍDUOS DE GLIFOSATO EM CERA DE ABELHAS BRASILEIRAS: OTIMIZAÇÃO E VALIDAÇÃO DE MÉTODO POR HPLC/FLD

RESUMO

No Brasil, um dos maiores produtores agrícolas do mundo, colmeias têm sido perdidas devido ao manejo inadequado de pesticidas, afetando a polinização e comprometendo a disponibilidade de alimentos. Com o objetivo de definir os possíveis impactos ambientais resultantes de mudanças antrópicas na biodiversidade, os produtos apícolas são utilizados como bioindicadores. Assim, um método analítico para quantificar o glifosato (GLY) e seu principal metabólito, o ácido aminometilfosfônico (AMPA), na cera de abelha foi validado utilizando um detector de fluorescência e reação pós-coluna. O limite de quantificação foi de 0,05 mg/kg, e as recuperações variaram de 75% a 93%, com um coeficiente de variação inferior a 11% para GLI e AMPA. O método validado foi aplicado a 18 amostras de cera de abelha provenientes de áreas com agricultura extensiva e florestas. Nenhuma delas apresentou contaminação por GLI ou AMPA. A ausência de contaminação nas amostras brasileiras, apesar do uso extensivo de GLY no país, destaca a importância de estudos contínuos e monitoramento para garantir a segurança dos produtos apícolas e a saúde das abelhas.

Palavras-chave ácido aminometilfosfônico, produtos de abelha, contaminação, pesticidas

INTRODUCTION

Pollinating insects, mainly bees, have a very close relationship with plants, providing a fundamental environmental service in natural and agroecological ecosystems. They are responsible for balancing biodiversity and maintaining the ecological system. In addition, several wild plants depend on these insects for their reproduction (FREITAS et al., 2020). These insects are also economically important since approximately 75% of crops require animal pollination to produce fruits and seeds (SAMANTHA et al., 2025). In Brazil, the economic impact of pollination activity in agriculture is approximately US$41.46 billion out of the total US$131.35 billion, representing one-third of the agriculture earnings of Brazil in 2021 (OLIVEIRA et al., 2021).

The global decline of pollinators is highlighted in different countries (CASTILHOS et al., 2019; CASTILHOS et al., 2021; GRAY et al., 2019; BRUCKNER et al., 2018; PIRES et al., 2016; USDA, 2015). In the US, during 2017-2018, approximately 30% of colonies were lost. Similar results have also been reported in some European countries (e.g., Portugal, Spain). In Brazil, CASTILHOS et al. (2021) conducted a survey with beekeepers, and during the 2018 -2019 period, colony losses of up to 20.5% were observed. The main factors are pathogens, habitat degradation, and the use of pesticides and expansion of exotic monocultures in Brazil's agricultural areas (CASTILHOS et al., 2021; OLIVEIRA et al., 2021; PIRES et al., 2016).

Agricultural practices expose bees to a wide variety of pesticides while searching for food, such as nectar, water and pollen. The suspended particles of contaminants can become trapped in the bodies of the bees, or the bees can inhale the particles (FOREZI, 2022). The bee products are widely used as bioindicators to define the environmental impacts arising from anthropogenic changes in biodiversity, but beeswax is still scarcely studied.

Glyphosate (N-phosphonomethylglycine, GLY) is a nonselective, systemic, and postemergent herbicide that inhibits the synthesis of 5-enolpyruvyl-shikimate-3-phosphate (EPSP), an important enzyme that acts in the shikimic acid pathway in plants and bacteria, inhibiting the biosynthesis of aromatic amino acids. The main metabolite of glyphosate biotransformation is aminomethylphosphonic acid (AMPA) (AHUJA et al., 2024).

With a specific mechanism of action, glyphosate does not present relevant acute toxicity for bees (LD50 > 100 μg/bee), but in recent studies with bees exposed to sublethal doses of this herbicide, they showed loss of sensitivity and decreased associative memory, which compromises the return of bees to their hives after foraging (LOPES et al. 2024; NOCELLI et al. 2019; EFSA, 2015).

Beeswaxes are complex matrices containing more than 300 different substances, mainly saturated and unsaturated hydrocarbons and long-chain mono- and di-unsaturated esters. Produced by bees or added by the beekeeper to the hive, the accumulation of chemical substances applied in agriculture can occur in waxes, given their characteristics (FOREZI, 2022). Beeswax is commonly used in the cosmetics industry, for example, in creams, treatment oils, and lotions, as well as in the preparation of pharmaceutical products and candles. Due to its biodegradable characteristics, beeswax can also be used to attract swarms to empty hives and maximize honey production through the recycling of honeycomb (PINTO et al., 2017; FRATINI et al., 2016).

The beeswax market in Brazil is difficult to measure, with the most recent official data being from the 2017 Agricultural Census, which reported that 4,369 establishments commercialized approximately 387 tons of beeswax (IBGE, 2018).

Although GLY is nonlipophilic, with a very low Log P (log Kow = -3.2), it can still be absorbed by plants and other organisms via other mechanisms, potentially affecting the environment and agriculture. EL AGREBI et al. (2020) determined that glyphosate residues in beeswax from Belgium were present in nearly 26% of the samples (n=100) at concentrations of up to 320 ng/g, and PIERRE et al. (2024) reported residues of up to 150 ng/g in beeswax from Martinique, France. Therefore, the beeswax matrix is a main route of bees' exposure to different classes of pesticides, making this preliminary study on Brazilian waxes an important step in the evaluation, monitoring, and implementation of appropriate protective measures in Brazil.

MATERIALS AND METHODS

Chemicals, reagents, and standard solutions

HPLC-grade methanol was purchased from JT Baker. The salts used in the derivatization reaction, o-phthaldialdehyde (OPA) and calcium hypochlorite, were purchased from Sigma (USA). Monopotassium phosphate, 2-mercaptoethanol, sodium chloride, sodium hydroxide, hydrochloric acid, and phosphoric acid 85% were purchased from Merck (Germany). The Fe^{3+} complexation resin Chelex 100-200 mesh and the chloride resin AG1 X8 mesh 200-400 were provided by Bio-Rad (USA).

The GLY and AMPA reference standard was purchased from Sigma–Aldrich (USA), with purities of 99.8 and 98.7%. The stock solutions were prepared in water obtained from a Milli-Q water system (Millipore, Bradford, MA, USA).

Instrumentation and HPLC conditions

HPLC analysis was performed on a Shimadzu LC-20A system with an RF 10 AxL fluorescence detector (Tokyo, Japan). The analytical column, an A-9 ionic exchange column in potassium form (150 × 4.6 mm, 5 µm), was kept at 50 °C. The postreaction coil was used to provide the fluorescence at 38 °C. The excitation and emission wavelengths used were 330 nm and 465 nm, respectively. The mobile phase was 0.005 mol/L monopotassium phosphate (KH_2PO_4) with 4% methanol, the pH was set at 1.9, and a flow rate of 0.6 mL/min was used. The injection volume was 50 µL.

Optimization of the sample preparation step

The sample preparation steps were based on De Souza et al. (2021) and optimized by a factorial 2^3 design. The variables agitation time, agitation equipment, and sample amount were studied. All modifications were analyzed by observing the recovery rates after the extraction. The data were submitted to statistical analysis using *Statistica 7.0* (StatSoft Inc., Oklahoma, USA).

Sample preparation

The samples were homogenized according to Svečnjak et al. (2019). Then, 10.0 g was extracted with 80 mL of 0.1 mol/L HCl by vortexing for 2 min. The pH of the aqueous phase was set between 3 and 4 with 85% phosphoric acid. The clean-up step was perfomed in a Chelex resin impregnated with Fe^{3+} solution (DE SOUZA et al. 2021). The extracts with GLI and AMPA were submitted to a glass column with 15 mL of Chelex resin and washed with 0.1 mol/L HCl solution. Then, GLY and AMPA were eluted with 25 mL of HCl 6 mol/L solution. Then, the extract was applied to another glass column with AG1X8 resin. After the elution, the clean extract was evaporated in vacuum and suspended in mobile phase, filtered in Millex HV (0.45 µm, Millipore) and analyzed by HPLC-FLD.

HPLC-FLD method validation and quality control

Method validation and performance for quantitation of residues were carried out in accordance with quality control standards. The figures of merit studied were linearity, limit of detection (LOD) and limit of quantitation (LOQ), precision (intraday and interday) and accuracy.

Linearity and sensitivity were evaluated by analytical curves in triplicate in solvent. The data were analyzed by *Statistica* 7.0 software (StatSoft Inc., Oklahoma, USA).

The limits of detection (LOD) and quantification (LOQ) were established by analyzing blank samples spiked with analyte solutions in solvent. The LOD was obtained considering the signal-to-noise ratio of 3 times the baseline, and the LOQ was determined by the signal-to-noise ratio (S/N) of 10 times.

For intraday precision studies, extractions of the sample spiked with standard analytical solution at two different concentration levels, LOQ (low level) and 10x LOQ (high level), were performed with five individual replicates per level. At each level, the coefficient of variation (%CV) was calculated. Interday precision was performed on two different days. The accuracy of the method will be evaluated by recovery tests due to the lack of certified material.

Blank samples spiked with the analytes at the validation levels were used as quality controls. In addition, the blank solvent and blank sample were analyzed to ensure that no contamination occurred during sample preparation or instrumental analysis.

Samples

A total of 18 beeswax samples from São Paulo State were analyzed. Thirteen (13) samples were provided by beekeepers from areas with extensive agriculture, and six (6) samples were collected from forests (Table 1).

Table 1: Location of sampling points and landscape description.

Location	City	Coordinates	N° Samples	Landscape description
01	Ribeirão Corrente	20° 27′ 25″ S, 47° 35′ 24″ W	05	small city (5.000 population) in a metropolitan region, agricultural area (coffee)
02	Ribeirão Preto	21° 10′ 40″ S, 47° 48′ 36″ W	06	metropolitan city (650,000 population), high urban density, agricultural area (sugarcane, corn)
03	Aguaí	22° 3′ 32″ S, 46° 58′ 44″ W	01	small city (37,000 population) with an extensively agricultural area (soybean, corn, cotton, sorghum
04	Morungaba	22° 52′ 48″ S, 46° 47′ 3 1″ W	02	small city (14,000 population) in a metropolitan region, mainly a green area
05	Apiaí	24° 30′ 32″ S, 48° 50′ 3 4″ W	04	small city (24,000 population) near to forest reserve, small agricultural area (tomato)

RESULTS AND DISCUSSION

Optimization of the sample preparation step

Beeswax is a complex matrix, and GLY is a hydrophilic molecule. The sample preparation was based on De Souza et al. (2021), and some modifications were made to achieve extraction in beeswax. Thus, a Plackett–Burman screening design was used to evaluate sample preparation variables that could influence herbicide extraction.

The experimental design was conducted according to Rodrigues and Iemma (2014) and resulted in recoveries from 22% to 102%. The main effects (%) were calculated at 95% confidence. The main effects of the evaluated parameters on the recovery (%) of GLY estimated according to the 2^3 experimental designs.

The variation in the sample amount from 5 g to 15 g resulted in no significant effect ($p > 0.05$) on the recovery of GLY. The same conclusion was obtained using different equipment (vortex, shaker, and ultraturrax) to extract the herbicide from beeswax samples, whereas an increase in the amount of extraction time from 30 s to 120 s was observed. Therefore, 10 g of sample (central point condition) using the vortex (easy to handle) and an extraction of 2 min were chosen as conditions for the extraction step (Table 2).

Table 2: Main effects of evaluated parameters on the recovery (%) of GLY estimated according to the Plackett–Burman design

Factor		Effect estimates
agitation time	Effect (%)	-14.00
	Standard error	10.70
	t(2)	-1.31
	p value	0.32
agitation equipment	Effect (%)	-13.00
	Standard error	10.70
	t(2)	-1.21
	p value	0.35
sample amount	Effect (%)	2.50
	Standard error	10.70
	t(2)	0.23
	p value	0.84

HPLC-FLD method validation and application

In method validation, selectivity, linearity, and sensitivity, matrix effect, precision (intraday and interday), accuracy, detection and quantification limits (LOD and LOQ) were evaluated. A blank sample free of GLY and AMPA contamination was chosen for validation procedures.

The selectivity of the method was verified by analyzing the blank of the sample, and no matrix interference was observed close to the retention time of GLY and AMPA. Representative chromatograms of the blank and spiked samples are shown in Figure 1. The chromatographic conditions allowed a total run time of 20 minutes, and the retention times of GLY and AMPA were 6.6 and 11.8 minutes, respectively.

Linearity was evaluated with five concentration levels in triplicate in the solvent and in the matrix in the range of 0.05 to 1 μg/mL. The curves were submitted to ANOVA, and no linearity deviation was evidenced ($p<0.05$). The correlation coefficients were greater than 0.999, and the residual distribution was randomly distributed and less than ± 20%.

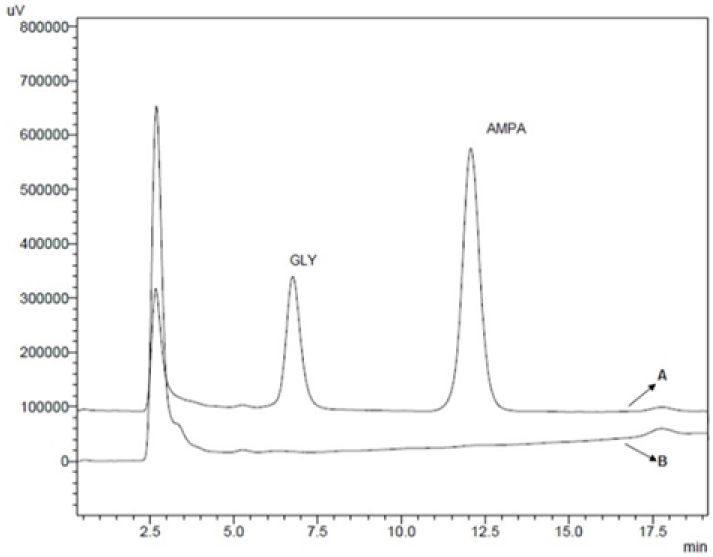

Figure 1. Chromatograms of (a) a beeswax sample fortified in 1 mg/kg, (b) blank sample.

For both analytes, the average recovery values ranged from 79% and 87% at the LOQ and were between 89% and 90% at the high concentration. For intraday precision, the highest RSD value was 10% for both analytes, whereas for interday precision, the RSD maximum value was 11% (Table 3). All these values are according to the SANTE Validation Guideline 11312/21 (SANTE, 2022).

Table 3. Validation parameters of the analytical method for the determination of glyphosate and AMPA residues in beeswax.

Ana-lyte	LOD[i] (mg/kg)	LOQ[ii] (mg/kg)	Linearity (R)	Recovery (%)		Precision			
				Low[iii]	High[iv]	Intraday RSD[v] (%)		Interday RSD[v] (%)	
						Low	High	Low	High
GLY	0.02	0.05	0.9885	87	89	10	6	11	6
AMPA	0.02	0.05	0.9997	79	90	10	3	9	3

iLOD = limit of detection; iiLOQ = limit of quantitation; iiiLow= LOQ; ivHigh = 10 x LOQ; vRSD = relative standard deviation.

Applying this validated method to 18 samples, we did not identify GLY or AMPA residues, regardless of the beeswax origin (forest or agricultural areas). These results contradict the expectation of finding residues, especially in samples from agricultural areas. The absence of detectable residues can be explained by several factors: GLY and its metabolite AMPA were not transferred to the beeswax; the bees did not collect contaminated water, pollen, or nectar; and/or the wax used in the hives was not previously contaminated (e.g. recycled clean wax).

The number of samples analysed in this study is comparable to that in other studies, such as PIERRE et al. (2024), who analysed 16 samples and detected GLY in samples from agricultural and urban areas. The absence of pesticide residues in our study can be explained by three main factors. First, seasonality may have influenced the collected samples, as production and sampling may have occurred during periods of lower pesticide use when agricultural spraying cycles did not coincide with bee activity. Second, the type of predominant crop in the bees' foraging area is crucial. If the bees visited areas with little or no agrochemical application, such as natural pastures or forests, residues are expected to be absent. Finally, the use of recycled wax may have contributed to the purity of the samples if beekeepers used good quality or purified wax, thereby preventing the introduction of contaminants into the hives. However, the presence of GLY and AMPA residues in apiculture products, such as beeswax, represents a growing environmental concern because of the widespread use of this herbicide (DE SOUZA et al. 2021; ZOLLER et al. 2018).

PIERRE et al. (2024) and EL AGREBI et al. (2020) have already detected the presence of GLY in beeswax, highlighting the need for continuous monitoring. In this context, Brazil lacks studies evaluating the presence of GLY in beeswax, despite the fact that several authors, considering the high consumption of this herbicide in the country (LOPES et al., 2024; NOCELLI et al., 2019; LOURENCETTI et al., 2023), have investigated the environmental impacts of this herbicide on bees.

Our study has two important limitations: the small sample size (n=18) and its geographical restriction to a single Brazilian state. This approach prevents the conclusions from being generalized to a national context, limiting the representativeness of the results. It is suggested that future work includes a larger number of samples, collected from different regions of Brazil, in order to obtain a more comprehensive and representative overview. Additionally, investigating a longer time period could offer insights into the temporal dynamics of the phenomena studied, complementing the approach used here.

Even with the lack of residues found in our specific samples, the ongoing monitoring of GLY concentrations in beekeeping products is crucial to protect bee health and maintaining product safety.

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DECLARATIONS

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