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Glyphosate and AMPA in saliva and other traditional human matrices. New findings for less invasive biomonitoring to the exposure to pesticides

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ABSTRACT

Human biomonitoring of the exposure to pesticides is usually performed in biological matrices such as urine and plasma. However, the possibility of using less invasive matrices allows the screening of large number of subjects. The objective of this study was to evaluate the body burden of the exposure to the most widely used herbicide, Glyphosate (GLY), and its main metabolite, the aminomethylphosphonic acid (AMPA), in different populations from the province of Córdoba (Argentina), and to propose the saliva as a matrix for possible implementation in biomonitoring of the exposure to pesticides.

Glyphosate and AMPA concentrations were evaluated in urine, plasma, and saliva of subjects occupationally and environmentally exposed to pesticides from one of the most important agricultural areas of Argentina. Gas chromatography coupled to tandem mass spectrometry (MS/MS) was used for the identification and quantification of the analytes.

Both GLY and AMPA were quantified in all matrices with higher detection frequency (DF) in the occupationally exposed group than in non-occupationally exposed individuals. Among evaluated matrices, the highest DF and concentration levels of GLY were found in saliva. Moreover, the only statistical difference between groups of subjects were found for GLY and AMPA concentrations in saliva, indicating the possible use of this non-invasive human matrix to evaluate different levels and scenarios of exposure. No significant correlation was found between GLY and AMPA levels in saliva and the traditional matrices (urine and blood) used to measure exposure to pesticides.

This is the first report of the presence and concentrations of GLY and AMPA in human saliva samples. Results of the present study are relevant for future biomonitoring of the exposure to GLY, but also to pesticides in general. Saliva deserved further investigation as an alternative, easy, and economical matrix involving less invasive methods for biomonitoring and screenings of large populations.

1. Introduction

Glyphosate (*N*-[phosphonomethyl]-glycine; GLY) is one of the most widely used pesticides worldwide (Duke, 2018). It is a broad spectrum, post-emergent and non-selective herbicide product used for residential, commercial, and agricultural purposes. The main biodegradation product of GLY is aminomethylphosphonic acid (AMPA). Similar toxicological effects of GLY and AMPA have been shown in toxicity studies (Van Bruggen et al., 2018).

Potential health implications in human populations, such as neurological and congenital effects, metabolic and respiratory diseases, have been assessed in epidemiological studies and described in associations with the exposure to GLY (Agostini et al., 2020; Hsiao et al., 2023). Moreover, in 2015, the evaluation of GLY toxicity by the International Agency for Research on Cancer (IARC, 2017) concluded that it is "probably carcinogenic to humans" (Group 2A). Since then, several regulatory agencies re-evaluated the toxicity of the herbicide and reaffirmed their previous conclusion that the herbicide is "unlikely to pose a

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carcinogenic risk to humans" (European Food Safety Agency (EFSA), Food and Agricultural Organization of the United Nations (FAO-JMPR), and the United States Environmental Protection Agency (USEPA)).

In any case, the rising volumes of GLY use worldwide, and the concerns about the potential adverse health effects, recommend to increasing the studies on the possible human health threats of this compound. Most of the few studies performed so far have been focused on highly exposed populations, but fewer focused on the quantification of the analytes in biological matrices due to their physical chemical properties, the complexity of the biological matrices, and the need of detecting trace levels of the compounds (Benbrook, 2016; Gillezeau et al., 2019).

Environmental contaminants can enter the body through dermal absorption, ingestion and inhalation (Esteban and Castaño, 2009). Concerning GLY, ingesting contaminated food and drinking water is the main route of exposure for the general population. Inhalation of contaminated air becomes another important way of exposure for populations living near agricultural areas whereas dermal contact seems to be the main route of exposure in occupational settings, especially for people involved in mixing, loading and application of the product (Aquavella et al., 2004; ATSDR, 2020).

According to the toxicokinetic data, a high percentage of the absorbed GLY is rapidly excreted in the urine as the parent compound (ATSDR, 2020). Then, urine seems to be a suitable matrix in biomonitoring to evaluate exposure to GLY (Bressan et al., 2021; Connolly et al., 2018). However, the intensive use of the herbicide globally, may results in continuous exposure to low doses of the population to the compound, so the presence of the parent compound and/or its metabolite in the blood cannot be neglected (Aris and Leblanc, 2011; Kongtip et al., 2017).

In the last decade, non-invasive human matrices were used to assess exposure to pesticides, such as hair, meconium and saliva (Yusa et al., 2015; Park et al., 2022; Denovan et al., 2000; Rovedatti et al., 2012; Russo et al., 2019), which show some advantages as they are easier to collect, with superior compliance and acceptance among the potential participants in biomonitoring surveys than blood and urine. Saliva has been recently considered as a suitable matrix to evaluate the exposure to pesticides (Marín-Sáez et al., 2023). An actual limitation of using saliva as a biological fluid to measure the exposure to xenobiotics is the uncertainty on the salivary gland uptake and the extent of clearance, from the blood to saliva, of the xenobiotic (Timchalk et al., 2015; Michalke et al., 2015). In this sense, the exposure to neonicotinoid insecticides in saliva and periodontal blood in people from the general population of China showed saliva/periodontal blood ratios of the insecticides higher than 1 (Zhang et al., 2021). Also, the exposure to diazinon and bromopropylate in saliva and exhaled breath condensate was investigated in an occupationally exposed population of farmers after having sprayed them. Results showed that the concentration of the pesticides was higher in saliva, and while the concentration of the analytes in the exhaled breath condensate decreased rapidly after the application, the concentration of the pesticides in saliva increased up to 1 hour after the initial exposure (Jouyban et al., 2019). Moreover, after dermal exposure to permethrine, a peak of the insecticide in saliva occurred between 6 and 24 h post-dosing, indicating dermal absorption and excretion of the circulating molecules in the matrix (Buchholz et al., 2021). Although the presence of these contaminants in saliva is indicative of exposure to these substances, correlations need to be established between the concentration of the chemicals in the traditional matrices (such as blood and urine) and the non-invasive propose matrix (such as saliva) for assessment of the usefulness of the latter (Esteban and Castaño, 2009). In the case of saliva, the physical chemical properties of the pesticide are critical as they determine their occurrence in this matrix (Michalke et al., 2015; Timchalk et al., 2015), but levels in saliva can also be influenced by gingival exudate, nasal cavity, gastrointestinal reflux and food debris (Amann et al., 2014)

This work aims to evaluate the exposure to GLY and AMPA by measuring them in urine, plasma, and saliva in populations with different scenarios of exposure (environmental and occupational) from the province of Córdoba (Argentina). The hypothesis underlying this study is that the saliva will be an alternative, easy, and economical matrix for AMPA and GLY exposure assessment, involving a less invasive methods for biomonitoring of large populations.

2. Materials and methods

2.1. Study area and population

GLY and AMPA exposure was measured in the East of the province of Córdoba, Argentina. The region (Marcos Juarez and Union County) is one of the most important agricultural areas of the country.

The population under study is part of a well-characterized cohort of subjects which is under examination since 2007 (Butinof et al., 2017a; Butinof et al.; 2017b; Lantieri et al., 2009; Lantieri et al., 2011; Filippi et al., 2021a; Filippi et al., 2021b). Two populations with different exposure levels were included: one occupationally exposed (ground pesticide applicators who perform activities of mixing, loading and/or applying pesticides), and the other, individuals with non-occupational exposure that is taken as control group. The subjects from the control group were selected from general population after consideration of the occupation, sex, age (±5 years), and household area.

Inclusion and exclusion criteria are described in Filippi et al. (2021b). Briefly, recruitment required age over 18 years old and residence in the study area for at least the last 5 years. The selected occupationally exposed subjects had to attend the mandatory applicator licence courses and to have worked for at least two consecutive years doing mixing, loading, application, and/or repairing sprayer machinery tasks. Exclusion criteria in both groups of subjects included pharmacological therapy, chemotherapy/radiotherapy treatment, recent surgeries, viral diseases, and any chronic disorder, such as, diabetes, hypertension, liver dysfunction, etc. In the case of the non-occupationally exposed subjects, having worked as applicators or being in contact with pesticides were also criterion for exclusion.

All participants were informed on the purposes of the study and signed a written informed consent. The research proposal was approved by the Ethics Review Committee of the Hospital Nacional de Clínicas, and registered by the Ethics Committee of Health Investigations of the Province of Córdoba (RePIS N°1582 y 044/10).

2.2. Sample collection

Date of sampling was established in September 2019 at the beginning of the spray season, considered a period of high exposure for the occupationally exposed subjects. All the samples were obtained during the same morning, collected in sterile tubes and containers after an overnight fasting period at a local hospital. Blood samples were obtained by peripheral venous puncture, and collected in tubes with EDTA anticoagulant. Plasma was separated by centrifugation (10 min–1500 x *g*). Saliva samples were collected by spitting in polypropylene tubes without anticoagulant after the usual oral cleaning and stored without any treatment. Study participants were asked to attend with the first-morning urine sample in a sterile container previously delivered. All samples were refrigerated at 4 °C until they reached the laboratory and then stored at −20 °C until analysis. As the general population is constantly and chronically exposed to GLY throughout their diet and the environment, a spot urine sample is suitable in this case to measure the exposure to this compound. Instead, populations occupationally exposed to pesticides, may have episodes of higher exposures due to their working conditions. Therefore, collection of serial 24 h urine samples is recommended (Solomon, 2020). However, the collection of urine for long time periods could generate discomfort among the subjects involved in the study. Then, urinary creatinine levels were used to compensate for the lack of the daily urine volume and analyte

concentrations in spot samples (Filippi et al., 2021a). Urinary creatinine levels were spectrophotometrically measured (Jaffe reaction) using a commercial kit (GT Laboratory, Santa Fe, Argentina).

2.3. Chemicals and reagents

Standards of GLY (99.5 % purity) and AMPA (99.8 % purity) were purchased from Sigma-Aldrich (St. Louis, USA) and Supelco (Bellefonte, USA), respectively. The isotope labelled 1,2-$^{13}C2^{15}N$-GLY (99 %) and $^{13}C^{15}N$-AMPA (99 %) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Derivatization agents trifluoroacetic anhydride (TFAA) (99 %) and 2,2,2-trifluoroethanol (TFE) (99.5 %), water HPLC grade, and citral were from Sigma-Aldrich (St. Louis, USA. Acetonitrile (ACN). Ethyl acetate (EtAc), acetone HPLC grade, and silica gel 60 (0.063–0.200 mm) were from Merck (Darmstadt, Germany). Synthetic urine and plasma were obtained from DYNA-TEK Industries (Lenexa, USA) and Sigma-Aldrich (St. Louis, USA), respectively.

2.4. Standards solutions

Individual stock solutions of GLY and AMPA (100 mg L^{-1}) and 1,2-$^{13}C2^{15}N$-GLY and $^{13}C^{15}N$-AMPA (1000 ng mL^{-1}) were prepared in HPLC grade water. Intermediate mixed GLY and AMPA solutions at concentrations of 1000, 100 and 10 ng mL^{-1}, and isotope labelled standards, at a concentration of 100 ng mL^{-1} were prepared by diluting the stock solution with HPLC grade water. Working calibration standard solutions of GLY and AMPA were obtained from serial dilution of the intermediate solutions to cover a concentration range of 0.05–10 ng mL^{-1}, and were prepared in synthetic urine and plasma, whereas saliva from donors with unknown exposure to pesticides was used for this matrix.

2.5. Sample treatment and extraction procedure

The analytical procedure was performed as described in Junqué et al., (2023) for urine samples, with slight modifications for plasma and saliva matrices. Briefly, 300 μL of urine sample were diluted to 1:10 with HPLC grade water, and 300 μL of the dilution were introduced into 10 mL borosilicate glass (Pyrex, Stoke on Trent, United Kingdom). Internal standard solutions were added to obtain final concentrations of 5 and 10 ng mL^{-1} for 1,2-$^{13}C_2$ ^{15}N-glyphosate and ^{13}C ^{15}N-AMPA, respectively. Then, 100 μL of acetone and 1 mL of ACN were added. Samples were vortexed and reduced to dryness under a gentle stream of nitrogen at 40 °C. Derivatization was carried out by adding 0.5 mL of TFE and 1 mL of TFAA. The mixture was vortexed, sonicated in an ultrasonic bath (10 min) and heated at 90 °C for one hour. Then, sample volume was reduced to dryness under a very gentle stream of nitrogen at 80–85 °C. The extract was dissolved in 1.5 mL of EtAc, vortex, and cleaned up by column adsorption chromatography with silica gel to remove derivatization residues. The eluate was recovered in a glass vial, evaporated under a gentle stream of nitrogen and reconstituted with 300 μL of EtAc/citral (500:1 v/v). In the case of plasma and saliva samples, 300 μL of ACN and the internal standards were added to 300 μL of the sample diluted 1:6 in water. Samples were vortexed and centrifuged (10 min at 1500 × *g*). The supernatant was transferred to a 10 mL borosilicate glass round bottom and 20 μL of HCl (0.3 M) were added. Then, the procedure followed that described in Junqué et al., (2023).

2.6. Instrumental analysis

Instrumental analysis was performed by gas chromatography (GC; Agilent 7890B GC System, Agilent Technologies, Palo Alto, CA, USA) coupled to a 7000C triple quadrupole mass spectrometry (Agilent, USA) operating in negative ion chemical ionization (NICI) mode with ammonia as reagent gas. The extracts were injected in split/splitless into a DB-5-625 capillary column (30 m × 0.25 mm, 0.25 μm i.d., Agilent Technologies), using helium as carrier gas. The temperature of the injector was 280 °C. The oven temperature programme started at 75 °C (1.5 min), and increased to 150 °C at 10 °C min^{-1}, and then to 300 °C at 50 °C min^{-1}, with a holding time of 5 min. The collision gas was nitrogen at a flow of 1.5 mL min^{-1}. Analyte determination was performed in multiple reaction monitoring mode (MRM). Spectrometric conditions are detailed in Junqué et al (2023).

2.7. Method validation and quality control

Validation of the methodology was carried out following the Guideline on Bioanalytical Method Validation of the European Medicines Agency (EMA, 2011). Synthetic urine and plasma, and saliva from donors with unknown exposure to pesticides were used to evaluate calibration curves, lower limit of quantification (LLOQ), linearity, accuracy, and precision.

Calibration curves were prepared in the corresponding matrix by spiking different amounts of the working calibrations standards to cover a concentration range of 0.05–10 ng mL^{-1}. A minimum of 3 calibration curves with at least 6 concentration levels were evaluated for each matrix during the analysis of the samples. The back-calculated concentration of the calibration standards had to be within ±20 % of the nominal concentration for the LLOQ and ±15 % for the rest of the concentration levels to be included in the calibration curve. The LLOQ was considered the lowest calibration standard in the calibration curve that could be quantified with acceptable accuracy and precision that were evaluated, at least by triplicate, in the synthetic matrices and real samples with non-detected concentrations of the analytes of interest at the lowest concentration levels (0.05 and 0.1 ng mL^{-1}). To assess accuracy, the mean concentration obtained from the calibration curves was compared with the nominal value, and reported as a percentage of the nominal value, whereas precision was evaluated by the coefficient of variation (CV). For being accepted, the results of the accuracy and precision had to be within ±20 % for concentrations at the LLOQ and ±15 % for the rest of the concentration levels.

Blank samples were analysed for each sample batches. The ratio of the two MRM transitions, in addition to the retention times, was used to identify the analytes. The ion ratio of the samples should not exceed ±20 % of the average ratio obtained from standard solutions. Isotope dilution method using labelled GLY and AMPA standards was used to perform the quantitative analysis.

2.8. Statistical analysis

The outcomes were calculated using the full analysis set (excluding subjects who violated inclusion/exclusion criteria). Baseline characteristics of the population were described as mean and standard deviation (SD), geometric means (GM), medians (M), and quantiles (Q). Detection frequencies (DF) were calculated using concentrations above the LLOQ. The concentration of the analytes in the different matrices was expressed in ng mL^{-1}. Additionally, in urine samples the concentration values were adjusted by creatinine levels in μg g^{-1} creatinine. For statistical purposes, the results of concentrations <LLOQ were used if they fulfilled the criteria of quantification. Although these values are associated with a higher uncertainty, they are more real than any other value assigned by imputation techniques or by replacing all of them by the LLOQ/2. When results did not fulfil the quantification criteria, they were reported as not detectable (ND) and replaced with the value of LLOQ/2. Student t-test and Chi^2 test were used to compare continuous variables between groups of subjects and to observe possible association between the exposure condition and categorical variables, respectively. Mann-Whitney *U* test was used to compare the median concentration of the analytes between groups of subjects. The correlations between the concentrations of the analytes in the different matrices were evaluated by means of the Spearman coefficient. *p*-values under 0.05 were considered significant. The analyses were performed using Stata© v17.

3. Results and discussion

3.1. Study area and population

Argentina is one of the main crop producers worldwide. In this country, the use of pesticides has markedly increased during the last decade, from 225 million kg in 2008 to 343 million kg in 2016 (CASAFE, 2009, 2016). Herbicides are the most important sector in the phytosanitary market and GLY has represented more than 60 % of this market over the years. The province of Córdoba is one of the three more productive provinces of the country, being the first, second, and third main producer of corn, soya, and wheat, respectively. Marcos Juarez and Union County are the provincial areas with highest yield values for all the above mentioned crops (MAGyP, 2022).

Forty individuals participated in the study. Thirty-five of them were included for statistical analysis because they fulfilled the inclusion/exclusion criteria. Fifteen subjects were occupationally exposed (terrestrial pesticide applicators), and 20 were non-occupationally exposed to pesticides. The socio-demographic characteristics of the participants are shown in Table 1. All the subjects involved were men, with no differences in age, height, and marital status. Statistical differences were found for weight between the two groups, but no differences were found for the body mass index (BMI). However, higher proportion of occupationally exposed subjects corresponds to the obesity category of the BMI. Disturbances in serum expression of lipids have recently been published for chemical factory workers exposed to GLY (Zhang et al., 2023). Around 50 % of the subjects from both groups lived within 100 to 500 m of the agricultural fields, and almost 40 % at distances >500 m (Table 1). Every subject in the study reported writing and reading skills, although educational level was higher in the control group. Domestic use of GLY was not reported by the non-occupationally exposed subjects, whereas most of the occupationally exposed individuals indicated having applied GLY during the previous week of sampling. The exact timing between application and biomonitoring was not known.

Table 1
Socio-demographic characteristics of subjects occupationally ($n = 15$) and non-occupationally ($n = 20$) exposed to pesticides from the province of Córdoba.

Socio-demographic characteristics	Non-occupationally exposed mean ± SD or %	Occupationally exposed mean ± SD or %	*p*-value[a]
Gender	Male	Male	
Age	43 ± 6	44 ± 9	0.7703
Height	1.76 ± 0.05	1.77 ± 0.08	0.501
Weight	80.08 ± 10.19	93.40 ± 17.70	0.0083
BMI[b]			0.061[c]
Normal	45	20	
Overweight	50	47	
Obesity	5	33	
Educational level[d]			0.004[c]
Elementary	5	20	
Middle	0	20	
High school	45	60	
University	50	0	
Marital status[e]			0.183[c]
Married	60	87	
Divorced or separated	10	0	
Widower	0	0	
Single	30	13	
Distance from home to the nearest crop[f]			0.896[c]
<100 m	10	7	
>100–500 m	50	47	
>500 m	40	47	
Glyphosate application[g,h]			
The day before the sampling	0	0	
Previous week	0	60	
Previously	0	27	

[a] Otherwise noted, Student *t*-test for the comparison of continue variables between non-occupationally and occupationally exposed groups.
[b] BMI (body mass index, kg/m^2).
[c] chi^2 test of the association between exposure and categorical variables.
[d] Educational level categorization: Elementary (1), Middle (2), High school (3), University (4).
[e] Marital status categorization: Married (1), Divorced or separated (2), Widower (3), Single (4).
[f] Distance from home to the nearest crop categorization: <100 m (1), >100–500 m (2), and >500 m (3).
[g] Glyphosate application: No (0), Yes (1).
[h] More than one answer was possible

3.2. Sample treatment and extraction procedure

The analysis of trace levels of GLY and AMPA in complex biological samples is challenging because of the high polarity, low mass, water solubility, amphotericity and low volatility of these compounds (Arkan and Molnár-Perl, 2015). In this study, we applied the previously reported method developed for urine (Junqué et al., 2023) with slight modifications for plasma and saliva samples. In these matrices, additional deproteinization and acidification steps were necessary because proteins could interfere with the derivatization agents, and stable complex could be formed between GLY and AMPA and multivalent cations due to their amphoteric character (Demonte et al., 2018; Zouaoui et al., 2013). Deproteinization was achieved by ACN addition to the sample. After stirring and centrifugation, the supernatant was acidified with HCl.

3.3. Method validation

Method validation performance is shown in Table 2. Calibration curves followed a linear regression model in the studied concentration range (0.05-10 ng mL^{-1}) with r^2 coefficient >0.99. The LLOQ was 0.05 ng mL^{-1} for all the matrices. Precision and accuracy were determined at the two lowest concentration levels of the calibration curve, the LLOQ, and 2 × LLOQ. Results were accepted when they were within ±20 % for the LLOQ and ±15 % for 2 × LLOQ.

3.4. Analysis of human samples

In general, biomonitoring studies are focused only in GLY exposure. However, the toxicological effects of GLY and AMPA are comparable. Therefore biomonitoring of both compounds is necessary for assessment of health deleterious effects and understanding the behaviour and relationship between them.

GLY and AMPA detection frequencies (DF) and concentrations in urine, plasma and saliva samples of the population from the province of Córdoba are shown in Tables 3 and 4.

In all matrices, detection frequencies (DF) of GLY and AMPA were always higher in the occupationally exposed group than in the non-occupationally exposed group (Table 3).

With regard to the median concentration found in each matrix (Table 4), in the non-occupationally exposed group statistical differences were found among the three matrices for GLY ($p < 0.05$) and between urinary and salivary median concentration of AMPA ($p < 0.01$). In the occupationally exposed group statistical differences were found on the median concentration of GLY between urine and saliva ($p < 0.01$) and between plasma and saliva ($p < 0.01$).

3.4.1. Urine

Detection frequency of GLY in the occupationally exposed group (20 %, Table 3) was lower than reported in the bibliography for highly exposed populations (DF over 55 %; Campbell et al., 2022; Connolly et al., 2017; Zhang et al., 2020). Detection frequency obtained for GLY in the non-occupationally exposed group (5 %, Table 3) was also lower than reported in general population from Spain (Ruiz et al., 2021), USA (Parvez et al., 2018) and France (Grau et al., 2022), but similar to the DF

Table 2
Method validation parameters. Calibration curves and r^2, precision and accuracy at level of the LLOQ and 2 × LLOQ for urine, plasma and saliva.

Matrix	Analite	Calibration curve	r^2	LLOQ ng mL^{-1}	Precision	Accuracy	2 × LLOQ ng mL^{-1}	Precision	Accuracy
Urine	GLY	$y = 0.9746\,x + 0.0294$	0.9969	0.054	4	−3	0.108	9	−5
	AMPA	$y = 1.0195\,x - 0.0014$	0.9982	0.056	18	3	0.112	15	−4
Plasma	GLY	$y = 1.1717\,x + 0.0275$	0.9988	0.054	2	−15	0.108	1	15
	AMPA	$y = 0.8202\,x + 0.0017$	0.9983	0.055	4	14	0.112	5	6
Saliva	GLY	$y = 1.2292\,x + 0.0154$	0.9992	0.054	1	−20	0.108	2	4
	AMPA	$y = 0.8318\,x + 0.0016$	0.9982	0.055	2	9	0.112	3	5

Table 3
Detection frequencies (DF) of AMPA and GLY in biological samples of subjects non-occupationally ($n = 20$) and occupationally ($n = 15$) exposed from the province of Córdoba (Argentina).

Matrix	Analite	Non-occupationally exposed population				Occupationally exposed population			
		n	DF (%)			n	DF (%)		
			ND	<LLOQ	>LLOQ		ND	<LLOQ	>LLOQ
Urine	AMPA	20	0	60	40	15	0	33	67
	GLY	20	10	85	5	15	27	53	20
Plasma	AMPA	20	50	20	30	15	20	20	60
	GLY	20	25	65	10	15	33	40	27
Saliva	AMPA	20	35	60	5	15	33	20	47
	GLY	20	10	25	65	15	0	0	100

Table 4
Geometric mean (GM), confidence interval (CI), median (M), range and percentiles (p95) in subjects non-occupationally ($n = 20$) and occupationally ($n = 15$) exposed to of GLY and AMPA from the province of Córdoba (Argentina).

Matrix	Analite	Non-occupationally exposed population					Occupationally exposed population					p-value[a] (median)
		GM	CI	Median	Range	p 95	GM	CI	Median	Range	p 95	
Urine	AMPA (ng mL^{-1})	0.489	0.393–0.607	0.442	0.211–1.377	0.856	0.589	0.426–0.814	0.634	0.205–1.38	1.15	0.2433
	GLY (ng mL^{-1})	0.158	0.105–0.237	0.191	<LLOQ–0.637	0.451	0.153	0.064–0.366	0.130	<LLOQ–3.92	1.92	0.6640
	AMPA (ng mg creatinine^{-1})	0.287	0.236–0.348	0.266	0.125–0.643	0.510	0.415	0.283–0.608	0.380	0.146–1.55	1.15	0.1096
	GLY (ng mg creatinine^{-1})	0.093	0.063–0.137	0.098	<LLOQ–0.380	0.253	0.108	<LLOQ–0.281	0.082	<LLOQ–3.92	2.16	0.7897
Plasma	AMPA (ng mL^{-1})	0.102	<LLOQ–0.218	<LLOQ	<LLOQ–1.57	1.54	0.264	0.127–0.550	0.382	<LLOQ–1.07	1.05	0.1236
	GLY (ng mL^{-1})	0.175	0.098–0.314	0.259	<LLOQ–3.10	0.464	0.135	0.069–0.263	0.245	<LLOQ–0.427	0.413	0.9597
Saliva	AMPA (ng mL^{-1})	0.069	<LLOQ–0.113	0.064	<LLOQ–0.440	0.299	0.188	0.082–0.429	0.307	<LLOQ–1.25	1.06	0.0364
	GLY (ng mL^{-1})	0.378	0.215–0.663	0.375	<LLOQ–3.52	2.86	2.87	1.41–5.84	2.87	0.438–19.3	16.3	0.0001

[a] Mann–Whitney U tests for comparisons between non-occupationally and occupationally exposed groups. Half of the LLOQ was used when the result was considered non detectable (ND).

found in Australia (Campbell et al., 2022).

Geometric mean (GM) and median (M) concentrations of GLY (Table 4) were similar in both groups and in the same order of magnitude of the mean concentrations in general population studies (Connolly et al., 2018; Gillezeau et al., 2019; Soukup et al., 2020). In contrast, most of the studies on highly exposed populations reported a mean or median urinary concentrations of GLY at least an order of magnitude higher than the observed concentrations in these regions of Cordoba (Acquavella et al., 2004; Campbell et al., 2022; Connolly et al., 2017; Perry et al., 2019; Zhang et al.; 2020; Table 4). The exact timing between application and biomonitoring was not known for the occupationally exposed subjects. Therefore, detected levels may be the consequence of environmental exposure to GLY rather than occupational one. A previous study carried out on a similarly exposed population to GLY in the north of Argentina observed DF and concentration ranges in the same order of magnitude as the present one (ng mL^{-1}; Bressan et al., 2021).

Despite similarities in toxicological effects with its parent compound, AMPA has been scarcely investigated. The results of the current study show higher DF for AMPA than GLY in both groups of subjects (Table 3) which is consistent with those reported in general population (Conrad et al., 2017; Faniband et al., 2021; Ruiz et al., 2021). Recent studies carried out on humans orally exposed to GLY and AMPA found low urinary recoveries of the precursor herbicide (1–6 %) whereas the recoveries of AMPA ranged between 10 to 33 %, indicating lower absorption rates of GLY than AMPA by the gastrointestinal tract (Faniband et al., 2021; Zoller et al., 2020). Faniband et al. (2021) also found that GLY is poorly bio-transformed to AMPA after oral exposure (<1 %). No studies evaluating absorption, metabolism and excretion related with dermal contact or inhalation of GLY and AMPA in human beings have been performed.

The geometric mean of the concentrations of AMPA ranged between 0.29 μg g creatinine^{-1} (non-occupational exposed subjects) to 0.42 μg g creatinine^{-1} (occupational exposed subjects; Table 4). To our knowledge, only two previous studies assessed the concentration of AMPA in urine of occupational exposed subjects. Perry et al. (2019) only found one sample with concentrations above quantification limit in pesticide applicators whereas Campbell et al. (2022) found a mean concentration (ng mL^{-1}) an order of magnitude higher than in this Argentinian study. Concentrations of AMPA found in this study were also significantly lower than the results found in populations from Colombian regions highly exposed to the herbicide by aerial applications (Varona et al., 2009). For the non-occupationally exposed subjects, GM (ng mL^{-1}; Table 4) concentration of AMPA was similar to that found in general population from Germany, and USA (Mills et al., 2017; Soukup et al., 2020), but at least 5 times lower than the mean concentration in general populations from different countries of Europe (Hoppe et al., 2013).

3.4.2. Plasma

As for urine samples, in plasma higher DF for AMPA than GLY was found. DF varied between 30 and 60 % for AMPA and 10 and 27 % for GLY in non-occupationally and occupationally exposed subjects, respectively (Table 3). Biomonitoring of blood samples is more invasive than other matrices such as urine. Then, most of the information about the presence of GLY and AMPA in blood samples comes from poisoning cases of accidental or intentional ingestion of GLY (Cho et al., 2019; Usui et al., 2019; Zouaoui et al., 2013).

To our knowledge, no study assessed the exposure to GLY and AMPA in plasma samples from highly exposed populations. Only two studies reported the GLY concentrations in plasma of environmentally exposed pregnant women (Aris and Leblanc, 2011; Kongtip et al., 2017), with very different results. High GLY median concentrations in Thailand, much higher than in the present study (17.5 ng mL^{-1}; Kongtip et al., 2017) and lack of samples above the quantification limit in Canada for GLY and AMPA (Aris and Leblanc, 2011). However, in this last case the methodology applied had a high limit of detection (10 ng mL^{-1}).

Glyphosate is quickly degraded in the environment, mainly to AMPA. Similar half-lives have been determined for both compounds in water samples, but AMPA shows more persistence in soils than GLY (Battaglin et al., 2014). The analysis of environmental samples showed DF and concentrations of AMPA higher than GLY in water, soil, sediments and air (Battaglin et al., 2014; Demonte et al., 2018; Ramirez Haberkon et al., 2021).

The metabolite, AMPA, is also a degradation product of several household detergents and this aspect may explain its high occurrence in the environment (Battaglin et al., 2014). Then, our findings in urine and plasma samples are in accordance with those found in environmental matrices.

3.4.3. Saliva

Detection frequency of GLY in saliva was higher than in urine and plasma and, contrary to these matrices, also higher than that of AMPA for both groups of subjects (Table 3). Statistically significant differences were found for the median concentration of GLY between groups of subjects, with levels an order of magnitude higher in the occupationally exposed group (2.86 ng mL^{-1}) than in the non-occupationally exposed individuals (0.38 ng mL^{-1}), and also an order of magnitude higher than in urine and plasma.

Statistical significant differences between studied groups were also found for AMPA. In this case, the median concentration of AMPA in saliva from the occupationally exposed group (0.31 ng mL^{-1}) was also an order of magnitude higher than for the non-occupationally exposed group (0.06 ng mL^{-1}), but similar to the levels found in urine and plasma samples.

These results show that GLY and AMPA are present in the saliva, and that this fluid can be used to assess the presence of GLY and AMPA in different exposure scenarios. Samples were taken in the morning after an overnight fasting period and after the usual oral cleaning. Hence, saliva results are unlikely to come neither from recent inhalation nor from the ingestion of contaminated air, food or drink, respectively.

This is the first report on the presence of GLY and AMPA in saliva from a population environmentally and occupationally exposed to pesticides. It is worthy to mention that some of the excluded occupationally exposed subject presented the highest GLY concentration levels in saliva (data not shown).

3.5. Correlations

Results of the Spearman correlation are shown in Table 5. We found positive and statistically significant correlations between GLY and AMPA concentrations in urine and saliva of both groups of subjects (p-value <0.05). These results are consistent with other studies reporting positive correlations between GLY and AMPA concentrations in urine from the general population (p-value <0.05; Conrad et al., 2017; McGuire et al., 2016). On the contrary, no correlations between these compounds were observed in plasma (Table 5). The lack of correlations may be due to exposure to AMPA coming from sources of contamination different from GLY application, direct exposure to environmental AMPA coming from the degradation of GLY, because of the inter-individual differences in metabolism and elimination, or simply because of the low number of positive samples (Faniband et al., 2021; Gillezeau et al., 2019). Concerning the correlations of the concentrations between matrices, no statistically significant correlation was found at 95 % confident level (Table 5). However, a positive relationship were found between concentrations of GLY in urine and saliva, and negative for AMPA in plasma and saliva, for the non-occupational exposed group (p-value <0.1). These borderline associations highlight the importance to keep on investigating the possible use of the saliva as a sensible matrix for biomonitoring the exposure to GLY. The negative correlation of AMPA between saliva and plasma must be interpreted cautiously. It could be consequence of the passage of the absorbed compound from the blood to the saliva or, on the contrary, could be the result of the passage from the saliva to the blood.

The lack of correlation between these three fluids may indicate that each of them represents a different store of GLY and AMPA in the body, which may also be related with diverse intake pathways and degree of preservation against metabolic changes.

In occupational exposure, determinations in saliva may reflect the most immediate exposure through respiration or unwanted ingestion through mouth and throat which is supported by the higher concentrations of the precursor compound (GLY) than the metabolite in this fluid.

4. Conclusions

The present study is the first in evaluating the exposure to GLY and AMPA of non-occupationally and occupationally exposed populations by analysis in urine, plasma and saliva. The validated methodology was successfully applied to detect and quantify GLY and AMPA in the target matrices. This study is the first in reporting the presence and

Table 5
Spearman correlation coefficients (Rho) between concentration of GLY and AMPA in the different matrices of the non-occupationally and occupationally exposed population from the province of Córdoba (Argentina). Urine levels expressed in ng mg $creatinine^{-1}$

ANALYTE		Urine				Plasma				Saliva			
		AMPA		GLY		AMPA		GLY		AMPA		GLY	
		p-value	Rho	p-value	Rho	p-value	Rho	p-value	Rho	p-value	Rho	p-value	Rho
Occupationally exposed population (n = 15)													
Urine	AMPA	–	–	**0.0462**	**0.5214**	0.9192	0.0287	0.8067	−0.0691	0.9078	0.0327	0.5672	0.1607
	GLY	**0.0147**	**0.5368**	–	–	0.1757	−0.3692	0.6049	0.1455	0.4830	−0.1964	0.9496	0.0179
Plasma	AMPA	0.1209	0.3583	0.6810	0.098	–	–	0.3303	0.2701	0.4731	0.2008	0.5484	0.1685
	GLY	0.3254	0.2318	0.2235	0.2849	0.7576	−0.0737	–	–	0.1520	−0.3889	0.7083	−0.1055
Saliva	AMPA	0.3624	−0.2151	0.4059	0.1967	0.0532	−0.4383	0.3552	0.2183	–	–	**<0.0001**	**0.8620**
	GLY	0.3488	0.2211	0.0768	0.4047	0.4093	−0.1953	0.7340	0.0811	**0.0015**	**0.6609**	–	–
Non-occupationally exposed population (n = 20)													

concentration levels of GLY and AMPA in saliva matrix of human populations. The study revealed different patterns of DF of GLY and AMPA in the different matrices evaluated, being the latter detected at higher extent in urine and plasma, whereas DF of GLY was highest in saliva. Statistical significant differences for GLY and AMPA concentrations between groups of subjects found in saliva, supports the usefulness of this non-invasive matrix to evaluate different scenarios of exposure.

Finally, this study contributes with validated methods that should be considered in further and larger epidemiological investigations to increase the knowledge of human toxicokinetics of GLY and AMPA. In addition, performing more comprehensive studies with the inclusion of environmental matrices, such as water, soil, air, and food samples could help in the understanding of the exposure context and could contribute to integrated information on the population exposure.

CRediT authorship contribution statement

Iohanna Filippi: Methodology, Investigation, Writing – original draft. **Pilar Fernández:** Methodology, Data curation, Resources, Writing – review & editing, Funding acquisition. **Joan O. Grimalt:** Resources, Writing – review & editing, Funding acquisition. **Mariana Butinof:** Conceptualization, Funding acquisition. **María V. Amé:** Conceptualization, Resources, Supervision, Funding acquisition, Writing – review & editing. **Sonia E. Muñoz:** Conceptualization, Resources, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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