EXPOSURE AND RISK ASSESSMENT OF GLYPHOSATE-BASED HERBICIDE AMONG FARMERS IN NORTHERN CYPRUS: A PILOT STUDY

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Abstract. Glyphosate (GLY) is an organophosphate herbicide that shows its effect by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) in the shikimate biosynthetic pathway. After its marketing, it became the most widely used herbicide worldwide. Since its classification is "probably carcinogenic to humans", debates are ongoing about its health outcomes. Thus, this study aims to assess occupational exposure and risk related to GLY and aminomethyl phosphonic acid (AMPA). Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) was used to analyse 30 pre and 30 post-application urine samples. Besides, a commercial enzyme-linked immunosorbent assay (ELISA) kit was assayed to determine 8-hydroxy-2' -deoxyguanosine (8-OHdG) levels in urine samples. Results are then used to calculate the percentage of Acceptable Occupational Exposure Level (AOEL) to perform a risk assessment. 7 samples contained GLY. There was no difference between pre-, and post-application samples ($p > 0.05$). AMPA was also observed but the levels were below LOQ values. No statistical differences were observed between 8-OHdG values or demographic and analytical data. Risk assessment values were below AOEL. During the study, no risk was calculated regarding the exposure to GLY. A larger sampling size and multiple post-application samples will aid in the evaluation of the risk assessment of GLY in a more definitive capacity.
Keywords: *GBHs, human biomonitoring, occupational exposure, risk calculation, ELISA*

Introduction

N-(phosphonomethyl) glycine is commonly known as glyphosate (GLY). The applications of GLY are not limited to agriculture but also include roadside, public

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areas, parks and gardens, and even paved surfaces. GLY disturbs the shikimate biosynthetic pathway (*Fig. 1*) in plants by inhibiting the enzyme enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) (Jensen et al., 2016).

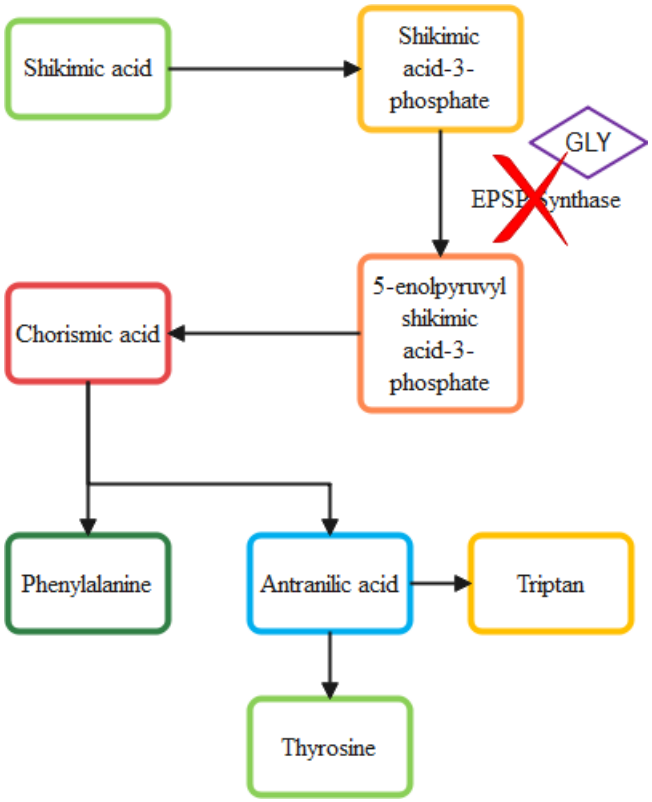

Figure 1. *Shikimate biosynthetic pathway.*

Since it was first marketed in 1974, GLY has remained the most widely used herbicide across the globe. In response to its ubiquitous use, many studies were conducted on possible health outcomes of GLY and glyphosate-based herbicide (GBH) exposure. The International Agency for Research on Cancer (2015) categorised GLY in "Group 2A – probably carcinogenic to humans". Following this classification, GBHs became a highly debated topic for both the public and the authorities. Nevertheless, carcinogenicity is not the only health effect of GLY exposure. Studies claimed that exposure to GLY is linked to various disorders such as kidney damage, mental and neurological disorders, reduction of sperm motility in men and an increased rate of miscarriages in women (Anifandis et al., 2018; van Bruggen et al., 2018).

Possible human exposure to GBHs can happen in two ways. The first is through occupational exposure during handling, preparation, and applying pesticides of concern. The second manner is through the ingestion of contaminated food. The European Food Safety Authority (EFSA) set safe values for both routes of exposure. Acceptable Daily Intake (ADI), which is the maximum amount of residue that can be ingested every day without any unwanted health effects over a person's lifetime (EFSA, n.d.), of GBHs, is 0.5 mg/kg body weight per day (mg/kg BW/day) and Acceptable Occupational Exposure Level (AOEL) of GBHs is currently set to 0.1 mg/kg BW/day (EFSA, n.d.).

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Since GBHs are associated with carcinogenesis, the mechanism of toxicity is a great concern. It is known that carcinogenesis induced by GLY is partially caused by oxidative stress (Guyton et al., 2015). Based on various human and animal studies, earlier evidence suggested a strong correlation between oxidative stress and GLY, GBHs and AMPA (IARC Working Group on the Evaluation of Carcinogenic Risks, 2017; Wang et al., 2022). Various studies show that pesticide exposure is linked to oxidative stress via the production of free radicals which might cause alterations in antioxidant defence mechanisms (Koureas et al., 2014). Furthermore, reactive oxygen species (ROS) might interact with the genetic material of cells. Oxidative damage of DNA yields more than 20 forms of oxidised DNA adducts. 8-hydroxy-2′-deoxyguanosine (8-OHdG), one of these DNA adducts, is believed to be the main form of DNA damage and the most frequently used biomarker of oxidative stress (Zhang et al., 2013).

Biomonitoring is a vital technique that is necessary for risk assessment. It is an exposure assessment method and is very beneficial in cases where the pesticide of concern has known pharmacokinetic parameters. This method of study involves the analysis of a biomarker, or the xenobiotic itself, in a biological matrix (Connolly et al., 2017). Application of a suitable form of risk assessment to the results obtained in a biomonitoring study facilitates the understanding of the risk and enlightens the decision-making pathway for regulatory authorities.

In light of previously discussed knowledge, this study aims to conduct exposure and risk assessment and measure possible oxidative damage of GBH-exposed farmers in Northern Cyprus in urine as the sampling matrix.

Materials and methods

Ethical approval

Ethical approval for the study was obtained from the Near East University Joint Committee the Research and Ethics Committee (YDU/2020/78-1046). The “Agreement for Transfer of Biological Material to be Used in Clinical Trials” was approved by the Ministry of Health of the Turkish Republic of Northern Cyprus (SAB.0.00-605/02-22/E.313).

Urine sample collection and storage

Human subjects were selected from the Morphou region of Northern Cyprus due to its extensive farming activities. The exclusion criteria were as follows: (1) being under 18 years of age, (2) not being present on the field during the pesticide application, (3) being unvaccinated/not fully vaccinated against COVID-19, and (4) not being tested for COVID-19 regularly (less than twice a week). During the sample collection, all COVID-19 pandemic preventive measures were followed. After the Informed Consent Forms were signed by the participants, they were asked to fill out study questionnaires. The questionnaire form was composed of 2 sections, a demographic information section (10 questions) and a study-oriented section (19 questions). The samples were collected purposefully in December 2021 following the first seasonal rainfalls when weeds complete their germination and were in the state of growth. The samples were collected in three days from two spots. Day 1 and Day 3 samples were collected from Tepebaşı (35.2965° N, 33.0226° E) and Day 2 samples were collected from Gemikonağı

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(35.1428° N, 32.8113° E). The weather conditions were recorded on each sampling day. Day 1 was 18°C, partly sunny with 11 km/h wind and 61% humidity. Day 2 was 16°C, partly sunny with 9 km/h wind and 64% humidity and Day 3 was 14°C, partly sunny with 8 km/h wind and 60% humidity.

A total of 60 spot urine samples (pre-application samples n = 30, post-application samples n = 30) were collected from 30 participants during the study. Pre-application samples were collected before the pesticide application and post-application samples were collected 1 h after the completion of the pesticide application (Connolly et al., 2018a, 2017). The application time was approximately 4 h. Participants used manual knapsack sprayers filled with pesticide solution (300 mL pesticide, 20 L water). Samples were then divided into three as follows; 1) 2 mL for creatinine analysis, 2) 2 mL for ELISA assay and 3) 7 mL for LC-MS/MS analysis. One of the 2 mL aliquots was directly transferred to the medical laboratory for creatinine analysis; others were transferred to Near East University Toxicology Laboratory and stored at -20°C.

Chemicals and reagents

During the study, only chromatography-grade chemicals were used. Artificial urine was obtained from Sigma- Aldrich (St Louis, MO, USA). Glyphosate and Glyphosate 13C, 15N (internal standard) were obtained from LGC standards (TRC, Canada). Aminomethyl phosphonic acid (AMPA) was purchased from Dr Ehrenstorfer GmbH (Germany). Water (18.2 MΩ) treated with Millipore (Simplicity, 185) Milli-Q water purification system (Elga Lab water Veolia, Anthony, France) was used in the preparation of all aqueous solutions.

Creatinine analysis

Creatinine analysis in spot urine was conducted by a fully automated device Roche Cobas Integra 400 (Roche Diagnostics, Rotkreuz, Switzerland). The reagents used were Cobas Integra 400 Creatinine Plus Version 2 and Jaffe Gen.2 (Roche Creatinine plus/Roche Diagnostics GmbH/Mannheim, Germany).

Glyphosate and AMPA determination using LC-MS/MS

Sample preparation and pre-treatment

To prepare the samples, 800 µL UltraPure Water that contains 0.1% formic acid was added to the 2 mL Eppendorf tubes. Pre-homogenised 150 µL urine samples were added to the tube with 50 µL GLY internal standard and vortexed for 1 min. The tubes were then centrifuged at 10,000 rpm. 750 µL of supernatant was collected and filtered using a 0.45-micron injector. 10 µL of the filtered sample was added to the LC-MS/MS vial system. These steps were repeated for blind samples for calibration and samples for validation.

Chromatographic and mass spectrometric conditions

An Agilent 1260 Series 6460 triple quadrupole LC-MS/MS with Jet Stream (ESI-MS/MS; Waghausel-Wiesental, Germany) equipped with a binary pump, a vacuum degasser, low carryover autosampler, a thermostated column compartment and a Mass Hunter data system was used to identify and quantify the compounds under consideration. 10 µL of the extract was injected into a Thermo Scientific Hypercarb

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column (100 mm × 2.1 mm, 5 μm particle size). The column temperature was kept at 40°C. The gradient mobile phases A and B consisted of 1% acetic acid in water and methanol, respectively. MS 2 Segment was used for analyses. To avoid contamination of the MS source, the diverter valve was switched to the waste position. The tandem mass spectrometry (MS-MS) was run with positive electrospray ionisation in the multiple reaction monitoring (MRM) modes. Nitrogen was used as both curtain and collision gas. Optimal conditions for ESI operation were as follows: sheath gas and auxiliary gas (N_2, 99.995%) flow rates 11 L/min; sheath gas at 400°C; the auxiliary gas temperature at 270°C; 3000 V positive capillary voltage; 750 V positive nozzle voltage. The peak areas of analytes and their ISs were studied using the Mass Hunter software version 4.0. *Table 1* summarises the HPLC-MS/MS flow gradient.

Table 1. *HPLC-MS/MS flow gradient*

HPLC	
Flow program	0 min- 5% B flow 0.2 mL/min 2 min- 5% B flow 0.2 mL/min 3.5 min- 10% B flow 0.2 mL/min 3.6 min- 10% B flow 0.4 mL/min 5 min- 95% B flow 0.4 mL/min 8 min- 95% B flow 0.4 mL/min 8.1 min- 5% B flow 0.4 mL/min 12 min- 5% B flow 0.4 mL/min 12.1 min- 5% B flow 0.2 mL/min 14 min- 5% B flow 0.2 mL/min

Validation

The HPLC-ESI-MS/MS method was validated in compliance with regulations of the FDA Guidance for Industry: Bioanalytical Method Validation (FDA and CDER, 2018), including selectivity, linearity, carryover, the limit of detection (LOD) and limit of quantification (LOQ), precision, accuracy, extraction, accuracy, and matrix effect. Artificial urine was used in the validation step. Calibration curves were developed by spiking the artificial urine with known concentrations of the analytes (1, 5, 10, 50, and 100 ng/mL). Urine samples and solvent-fortified with known masses of analytes were used to prepare the calibration curves. Each sample was individually spiked with IS. To prepare the calibration curves, the ratios of the peak area of each target analyte to the area of IS versus the nominal concentration of the analyte were plotted. The recoveries were found as a result of comparing the peak areas measured after the analysis of spiked urine samples with those found after the direct injection of standard solutions at identical concentrations. The LOD was defined as the lowest amount of GLY and AMPA in one sample the bioanalytical procedure can consistently distinguish from the background noise. LOQ, on the other hand, is similar to LOD with exceptions, such as the range of the upper and lower bounds that can be quantified with a predetermined acceptance level of precision, accuracy, and specificity. Precision was calculated as the coefficient of variation (CV%) between the measurements. The accuracy was defined as the deviation from the nominal value expressed in percentage.

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ELISA assay

A commercially available ELISA kit was performed to quantify the urinary concentrations of 8-OHdG (Cayman Chemical, USA). The immunoassay can detect all three oxidised guanine species (8-hydroxy-2′-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA and 8-hydroxyguanine from either DNA or RNA). The urine samples were diluted by a ratio of 1:250 to perform the procedure. The assay was run according to the instructions of the manual (Cayman Chemical, n.d.). The contents of 8-oxo-dG in urine samples were normalised with creatinine concentration.

Risk assessment

An internal dose of glyphosate is recognized as a biomarker of exposure, and it indicates the systemically available amount of GLY. The following equation (*Eq. 1*) can be used to calculate the internal glyphosate dose (Niemann et al., 2015; Kougias et al., 2021):

$$Internal\ Dose = \frac{(C_{urine} * V_{urine})}{BW} \quad \text{(Eq.1)}$$

where C_{urine} is the urinary concentration of GLY in mg/L, and V_{urine} stands for the daily urinary output. During the study, the V_{urine} value was assumed to be 1.5 L per person (CDC, n.d.; Niemann et al., 2015). Bodyweight (BW) in kg was obtained from the questionnaire.

As of now, there is no set value for human biomonitoring of GLY or AMPA for risk assessment. Thus, (Buekers et al., 2022a), proposed an equation that compares the predicted daily intake of glyphosate versus the ADI. This equation was rearranged on behalf of the scope of this study as follows (*Eq. 2*):

$$\%AOEL = \frac{Internal\ Dose}{F_{UE} * AOEL} \quad \text{(Eq.2)}$$

where the internal dose is calculated from *Equation 1*, F_{UE} is the urinary excretion fraction, set at 0.57% as it is the lowest observed fraction in a study (Zoller et al., 2020), and the acceptable operator exposure level (AOEL) is 0.1 mg/kg BW (EFSA, 2015).

Statistical analysis

Continuous data were presented as mean ± standard deviation, median, minimum, and maximum data. Categorical variables were presented as frequency and percentage values. Due to the suitability of the Central Limit Theorem, parametric tests were performed without normality tests (Norman, 2010). Student's t-test was used for the comparison of independent data groups. Paired data were compared using paired t-tests. Statistical significance was accepted at a p-value < 0.05. Statistical analyses were performed using www.e-picos.com (New York) software and MedCalc statistical package.

Results

LC-MS/MS method validation

Optimisation of the ion source-mass spectrometry parameters

During the optimisation, the positive mode was the most effective. *Table 2* summarises the optimisation parameters, MRM transitions and voltage.

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Table 2. *MRM transition and voltage values*

Compound	Precursor ion (m/z)	Product ion (m/z)	Reading time (milliseconds)	Fragmentor (V)	CE* (V)	Cell Acc** (V)
Glyphosate_C13N15	173	91.0	75	58	4	4
Glyphosate	170	88.1 60.2 42.2	75 75 75	55 55 55	10 30 30	4 1 1
AMPA	110	30.3	75	55	4	7

*Collision energy
**Cell accuracy

Optimisation and development of the chromatographical method

The separation column and the qualitative and quantitative reproducibility of detection were studied to perform the chromatographic analysis. The mobile phase A and B consisted of 1% acetic acid in UltraPure water and methanol, respectively. Using the program in *Table 1,* the analysis of GLY and AMPA in urine samples was studied.

Method validation

Under the abovementioned chromatographic conditions, the retention times of GLY and AMPA were ~2.2 and 1.7 min, respectively. The LODs of GLY and AMPA were found as 3 ng/mL and 5 ng/mL, respectively and the LOQ of GLY and AMPA were established as 10 ng/mL and 20 ng/mL, respectively. The coefficient of variation for both substances was below 15%. Recoveries were between 70-120%. The calibration range for GLY and AMPA were 1.5, 10, 50, 100 ng/mL and 2, 4, 8, 20, and 200 ng/mL respectively. Accuracy was 99.1% at 10 ng/mL for GLY and 107.3% at 8 ng/mL for AMPA.

Demographic and study-specific data

To conduct the study, a total of 60 urine samples were collected from 30 farmers. 30 of the samples were collected before the pesticide application and the other 30 were collected 1-h post-application. The participants were all present in the field during the application. The mean age of the participants is 46.4 ± 15.6. 56.7% of the participants were male and 43.7% were female.

Participants were asked to complete the questionnaire with study-specific questions (*Table 3*). According to the results obtained, the mean years of working as an agricultural worker was 13.4 ± 11.6. All participants were following a Mediterranean diet. Participants were asked if they had another occupation apart from being an agricultural worker. GBH application refers to doing the task of spraying personally. Duties about pesticides were divided into three categories as pesticide preparation, purchasing, and cleaning of applicators. There is an overlap between these categories. GBH knowledge refers to the basic knowledge of GBH and/or GLY such as what it is and what it is used for. Knowledge of exposure refers to the participant's awareness of the risk of exposure.

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Table 3. *Demographic and study-specific data of participants*

	Mean ± SD	**Min-Max**	
Age	46.4 ± 15.6	23-70	
Height	168.3 ± 7.39	153-180	
Weight	75.63 ± 17.31	53-130	
BMI	26.5 ± 4.5	20.52-41.03	
		n	**%**
Gender	Women	13	43.7
	Men	17	56.7
Education	No education	3	10
	Primary school	8	26.7
	High school	9	30
	University	10	33.3
Smoking	Yes	7	76.7
	No	23	23.3
Alcohol	Yes	2	6.7
	No	28	93.3
Disease	Yes	2	6.7
	No	28	93.3
Medication	Yes	5	16.7
	No	25	83.3
		n	%
Non-agricultural secondary occupation	Yes	20	66.7
	No	10	33.3
GBH application	Yes	12	40
	No	18	60
Pesticide preparation	Yes	16	53.3
	No	14	46.7
Pesticide purchasing	Yes	17	56.7
	No	13	43.3
Cleaning of applicators	Yes	5	16.7
	No	25	83.3
GBH knowledge	Yes	11	36.7
	No	19	63.3
Personal protective equipment	None	21	70
	Gloves	7	23.3
	Mask	2	6.7
Awareness of exposure	Yes	22	80
	No	6	20

GLY and AMPA analysis in human urine samples

A total of 60 urine samples from 30 participants (30 pre- and 30-post application) were collected and GLY and its major metabolite, AMPA were analysed. GLY was quantified in 9 samples 2 of which are pre-application samples. 2 of the samples were

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excluded from the calculations as they have too high levels of GLY. On the other hand, AMPA was detected in 15 samples but since the levels were below the LOQ, they cannot be quantified. There was no statistically significant difference ($p = 0.14$) between the mean levels of pre- (0.0005 ± 0.0023 mg/L) and post-GLY (0.3207 ± 1.1425 mg/L) values. *Figure 2* shows the chromatogram of a positive sample.

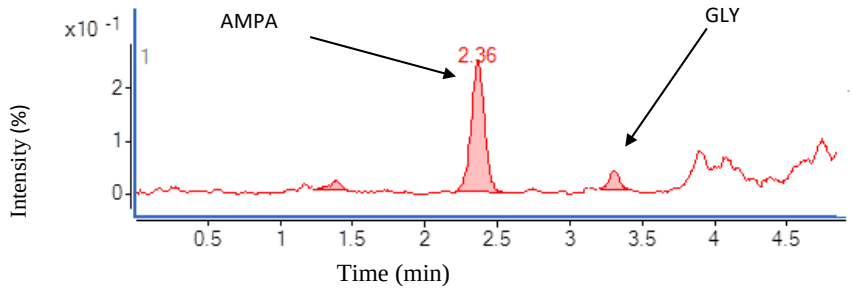

Figure 2. *A positive GLY chromatogram was obtained from a positive sample*

ELISA assay

ELISA assay was used to detect the levels of an oxidative stress biomarker, 8-OHdG. Online software was used to calculate the readings from the spectrophotometer. The values were then standardised with creatinine. the mean 8-OHdG levels in pre- and post-application urine samples were 0.0920 ± 0.1481 mg/g creatinine and 0.1376 ± 0.1719 mg/g creatinine, respectively. There was no statistical difference between the values ($p > 0.05$).

Statistical analyses

Statistical analyses were run to establish a correlation between data. In response to the statistical test, pre-application GLY values and pre-application 8-OHdG values were found to be statistically significant ($p < 0.005$). There was no significant difference between the post-application parameters. According to the student's t-test, there was no correlation between gender and levels of GLY or 8-OHdG. The values were compared concerning PPE usage and no statistical significance was detected ($p > 0.05$). 8-OHdG and GLY values were compared to medication, but no difference was observed. Therefore purchasing, preparing, spraying or cleaning pesticides likely did not affect the GLY and 8-OHdG levels.

Risk assessment

Findings suggested that relatively low GLY exposure was profound during this study. The HBM4EU studies were taken as guidelines and the risk assessment procedure of these studies was modified to fulfil the objective of this study. During the calculations, only individual data was taken into consideration. *Table 4* shows the percentages of AOEL after one application of spraying.

AMPA was also detected in multiple samples, however, the levels were all below the LOQ and therefore cannot be quantified. Thus, risk assessment cannot be performed on AMPA levels.

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Table 4. *Risk assessment calculation of GLY data*

	Sample number	%AOEL
Pre-application	Pre-2	0.863%
	Pre-6	0.726%
Post-application	Post-6	1.717%
	Post-22	13.789%
	Post-23	4.952%
	Post-24	1.190%
	Post-30	52.392%

Discussion and conclusion

GLY is an organophosphate herbicide that inhibits the key enzyme in the shikimate biosynthetic pathway in plants (Jensen et al., 2016). Following its marketing, it became the most widely used herbicide all around the globe (Kocadal et al., 2022). International Agency for Research on Cancer classified GLY in "Group 2A-probably carcinogenic to humans" (International Agency for Research on Cancer, 2015) relying mostly on the correlation between GLY exposure and non-Hodgkin's Lymphoma (Guyton et al., 2015; Davoren and Schiestl, 2018). Nevertheless, there are ongoing debates on the carcinogenic potential of GLY. US EPA considers GLY as unlikely to be a carcinogen (US EPA, 2022). Recently, the European Chemical Agency's Committee Risk Assessment decided that the existing scientific data were not sufficient enough to classify GLY as carcinogenic, mutagenic or reprotoxic (ECHA, 2022). Like any other substance, exposure to GLY was controlled by limit values. EFSA set the ADI value for GLY as 0.5 mg/kg bw/day and AOEL as 0.1 mg/kg bw/day. These values were set to avert any possible health problems since GBH exposure is associated with many adverse health outcomes such as reduced sperm motility in men, kidney damage, mental and neurological disorders, and increased risk of miscarriages in pregnant women (Anifandis et al., 2018; van Bruggen et al., 2018).

The values that are set to preserve public health are important however, without actual monitoring, exposure to GLY cannot be controlled. In GLY's case, occupational and non-occupational exposures are equally important. However, this study only focused on occupational GLY exposure. Using biomonitoring as an exposure assessment tool, it is possible to estimate the actual exposure of GLY. Thus, one of the main aims of this study is to biomonitor GLY and its major metabolite AMPA in the urine of occupationally exposed agricultural workers.

Urine was chosen as a biological matrix. During the selection, multiple factors were taken into consideration. Primarily, the collection of urine is non-invasive. Participants can easily provide urine samples without the need for any qualified medical personnel, unlike blood sample collection (Kocadal et al., 2022). Furthermore, GLY is an ionic, water-soluble substance (Nagatomi et al., 2013) allowing for excretion in unchanged form in urine (Brewster et al., 1991). It is known that GLY excretion peaks at 5 h following exposure. Thus, sampling time was calculated as 5 h (4 h application and 1 h post-application). Apart from that, the urinary excretion coefficient (F_{UE}) is well-studied (Zoller et al., 2020) allowing for the calculation of the estimated internal dose.

Even though there are many developed and validated methods for GLY and AMPA detection in different biological matrices, this study required a new method making use

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of the available equipment at hand. During the method development, an Agilent 1260 HPLC coupled with Agilent 6460 Triple Quadrupole (QQQ) Mass Spectrometer was used. The LODs and LOQs for GLY and AMPA were as follows; 3 ng/mL, 5 ng/mL, 10 ng/mL, and 20 ng/mL, respectively which gave sufficient sensitivity to carry out the experiments.

The demographic data were collected to allow comparisons of variables and produce detailed information about the subjects. It was observed that only 36.7% (n = 11) of the participants have basic information about the main ingredient of GBHs which is low in number. This may be due to the lack of information on different platforms or different ranges of education levels which also affect access to information. EPA suggests wearing gloves, goggles, and boots, and choosing calm weather during the applications (EPA, nd.). However, 70% of the participants claimed that they did not use any PPEs during the pesticide application.

GLY and AMPA were both analysed and GLY was found in 7 samples. 2 of the samples were excluded due to very high values of GLY. This was thought to be hand-to-container contamination or there might be a chance of drift-off to the container if the sample collection container lid was open during the pesticide application. To keep the samples stable, they were transferred to aliquots and stored at 4°C until transferred to the laboratory. GLY was found in 2 pre-application samples and 5 post-application samples. p-value between the pre and post was 0.14 indicating no significant difference. Even though no significant rise was observed, there was still an increase in the post-application samples. AMPA on the other hand has been found in fifteen samples but the levels were below LOQ. AMPA is the major metabolite of GLY, however, the photodegradation of amino phosphonates in water also produces AMPA (Grandcoin et al., 2017). Amino phosphonates consist of multiple phosphonic acid groups and at least one amine group. They are very abundant as they are found in detergents, fire retardants, anti-corrosives and many more (Grandcoin et al., 2017). Since only 0.3% of GLY is metabolised into AMPA (Lemke et al., 2021) and AMPA is abundant in the environment (Grandcoin et al., 2017), it is possible to be exposed to AMPA but not GLY. During the applications, all participants used a manual knapsack to apply pesticides. This was no intention of using a manual knapsack during the study, instead, participants were asked to operate the procedure like a normal working day. According to a study conducted by Connolly et al., there was no correlation between pre- and post-application levels of GLY from the data obtained from manual knapsack applicators (Connolly et al., 2017). Furthermore, during the sampling days, weather conditions were optimal. The wind speed was below 15 km/h, the humidity was above 40% and the temperatures were below 25°C. These conditions reduce the likelihood of drift due to temperature inversions or evaporation. They also increase target deposition and coverage (Deveau, 2009). Thus, these optimum spraying conditions and using a manual knapsack as an applicator might reduce the level of exposure.

Oxidative damage is one of the most important effects of xenobiotic exposure. Oxidative damage can occur to lipids of cellular membranes, proteins, and DNA. 8-OHdG is a widely studied biomarker for oxidative stress since it is one of the most prevalent forms of free-radical-induced oxidative lesions (Valavanidis et al., 2009). To assess GBH-exposure-induced oxidative damage, a competitive ELISA assay was used. Although the mean values of 8-OHdG from pre- and post-application samples showed no significant difference ($p = 0.31$), the mean 8-OHdG obtained from the post-samples was higher than that of pre-samples.

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GLY and oxidative stress data were compared to both demographic and study-specific parameters. It was found that pre-application levels of GLY and 8-OHdG are significantly correlated ($p < 0.005$) but not in post-application data. Since the time window was limited during this study, there is a possibility that post-application effects of GLY on oxidative damage could not be observed in this limited time. According to a study, 8-OHdG levels increased over time when measured weekly in cases of Bisphenol A (BPA) (Budiawan et al., 2022). No other correlation was found significant according to the statistics. Nevertheless, the number of participants is 30, which is relatively low for a biomonitoring study. The present study was originally designed as a pilot study. Thus, the number of participants was decided in accordance with previously published studies (Pierce et al., 2020; Bootsikeaw et al., 2020; Perry et al., 2019; Connolly et al., 2018b) and the questionnaires were filled out by participants. Unfortunately, the accuracy of responses is highly subjective and strongly dependent on the participants having an understanding of the topic and responding accurately.

Risk assessment is a complex, yet necessary process which involves hazard identification, dose-response assessment, exposure assessment, and risk characterisation (USEPA, n.d.). One of the objectives of this study was to apply risk assessment to the results obtained. Since there was no recommended risk assessment procedure for GLY and/or AMPA, an HBM4EU study was adopted (Buekers et al., 2022b). Risk assessment was only applied to the individual GLY levels as AMPA levels were all below LOQ. As a result of the risk assessment, the internal dose was used to calculate %AOEL. According to the findings, the highest value obtained was 52.392%. This percentage was calculated after the completion of one round of application. Thus, following a full day of application, the numbers are subject to change. Furthermore, GLY may exert its health effects below regulatory limits (Mesnage and Antoniou, 2017).

GBHs are not only used in farming but also in public places, for instance, common parks, gardens and even roadsides. Thus, environmental monitoring of glyphosate, as well as AMPA is very crucial to preserve both human and ecological health.

In conclusion, this study concentrated on occupational GBH exposure and risk assessment. During the study, no risk was calculated regarding acute exposure to GLY. The results of our pilot study will be utilised to assist in the design of a more comprehensive prospective study, with much larger sampling sizes, environmental matrices, and longer-duration post-exposure sample collection to establish more strongly a positive correlation between GLY exposure and the oxidative stress biomarker 8-OHdG.

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