Analytical and Bioanalytical Chemistry
https://doi.org/10.1007/s00216-021-03194-x

RESEARCH PAPER

Determination of glyphosate, glufosinate and their major metabolites in urine by the UPLC-MS/MS method applicable to biomonitoring and epidemiological studies

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Received: 28 September 2020 / Revised: 12 January 2021 / Accepted: 23 January 2021

Abstract

The preoccupation concerning glyphosate (GLYP) has rapidly grown over recent years, and the availability of genetically modified crops that are resistant to GLYP or glufosinate (GLUF) has increased the use of these herbicides. The debate surrounding the carcinogenicity of GLYP has raised interest and the desire to gain information on the level of exposure of the population. GLYP and aminomethylphosphonic acid (AMPA) are commonly simultaneously analysed. GLUF is sometimes also monitored, but its major metabolite, 3-[hydroxy(methyl)phosphinoyl]propionic acid (3MPPA), is rarely present in the method. Using a pentafluorobenzyl derivative to extract the analytes from human urine, we present a method that contains four important analytes to monitor human exposure to GLYP and GLUF. The use of the flash freeze technique speeds up the extraction process and requires less organic solvent than conventional liquid-liquid extraction. The limits of detection in the low μg/L range enable the use of this method for epidemiological studies. The results obtained for 35 volunteers from the Quebec City area are presented with the results from multiple interlaboratory comparisons (G-EQUAS, HBM4EU and OSEQAS). This methodology is currently being used in the Maternal-Infant Research on Environmental Chemicals (MIREC-ENDO) study and in the Canadian Health Measures Survey (CHMS).

Keywords Glyphosate · Glufosinate · PFBBr · Urine · Flash freeze

Introduction

To help feed the ever-growing population, human ingenuity created glyphosate (GLYP). GLYP is a synthetic chemical and a non-selective phosphorus-containing amino-acid-type herbicide (PAAH). In 1996 [1], the first genetically modified crop resistant to glyphosate was marketed. However, that was 1 year after the commercial availability of glufosinate (GLUF)-resistant modified canola crops. GLUF is also part of the PAAHs and can be obtained from synthetic or natural sources from the degradation of the natural compound bialaphos (BIAL) [2]. The sales report [3] for 2018 in the Province of Québec mentioned a 39% drop compared to 2017, but the amount of GLYP herbicide sold for 2018 was 943,376 kg of active ingredient. For the same year, the sales of GLUF (also known as phosphinothricin) were in the range of 1000–10,000 kg of active ingredient. A simple Internet search shows that GLYP has clearly gained worldwide attention in recent years, and there is still a debate on its carcinogenicity in humans [4, 5]. In 2017, the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) considered that the fact that GLUF was classified as a R1B for its presumed reprotoxicity could not be ruled out and decided to withdraw its marketing authorisation.

When GLYP is degraded in the environment and plants [6], the main metabolite observed is aminomethylphosphonic acid (AMPA) [7]. The International Agency for Research on Cancer (IARC) tested AMPA in five genotoxicity assays and all were positives [8]. For GLUF, the main metabolite in soil, plants [2] and human urine is 3-[hydroxy(methyl)phosphinoyl]propionic acid (3MPPA) [9] and the United States Environmental Protection Agency (US EPA) considered it less toxic than the parent compound. The average half-life of GLYP is 7.25 h in humans [10]; in one patient, the GLUF half-life was measured

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Published online: 05 February 2021

to be 9.59 h [11]. GLYP is cleared into urine from the body within 48 h via the kidneys and remains mostly unchanged.

In scientific literature, acute intoxication with GLYP- [12] and GLUF- [13] containing herbicides has been presented. More recently, the focus has shifted to chronic exposure to these compounds. In humans, methods to determine 3MPPA, AMPA, GLUF and GLYP have been presented with serum [9, 14, 15] mostly been the targeted matrix. The use of a less invasive matrix such as urine may be interesting for donors but is an augmented challenge for scientists due to its wide variability. The use of this matrix may now be preferable because urinary GLYP has been recognized as a quantitative biomarker of oral exposure [16]. To monitor the exposure to glyphosate, GLYP and AMPA are often simultaneously analysed. It may be assumed in environmental studies that the majority of AMPA comes from exposure to AMPA and not from human metabolism of GLYP [17]. AMPA may also come from the degradation of aminotrimethylene-phosphonic acid (ATMP), ethylenediaminetetra(methylenephosphonic acid) (EDTMP) and diethylenetriamine-pentamethylenephosphonic acid (DTPMP), which were used in detergents [18] before the phosphonate ban occurred in many countries.

These four compounds (3MPPA, AMPA, GLUF and GLYP) are very polar, and their analysis may become quite challenging in complex matrices. They may be directly analysed in the mass spectrometry (MS) negative mode [9, 15, 19], but the literature also showed that they might be derivatized with N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) [14] or trimethyl orthoacetate (TMOA) [20]. In the case of TMOA derivatization, the limit of detection (LOD) in urine was in the mid-high μg/L range, which makes them unsuitable for epidemiological studies. Among different chemical reagents for the analysis of the four compounds, 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr) [21] was selected due to its ability to react with different functional groups of these molecules while providing good signals in the positive mode. This chemical can form derivatives that may be easily extracted from water, but its use must be carefully monitored due to its safety issues. The availability and the stability of the PFB derivatives in a liquid chromatographic context are the reasons why PFBBr was selected over other fluorinating reagents like N-(trifluoroacetyl) imidazole (TFAI) and N-heptaflurobutyrylimidazole (HFBI) that are known to create derivatives more suitable to gas chromatography (GC).

Like the cloud-point extraction technique, freezing the aqueous layers to perform the extraction is a simple and rapid process. As noted by Kojro et al. [22], cloud-point extraction is rarely combined with liquid chromatography (LC) coupled to mass spectrometry as it requires the use of surfactants which might interfere with the ionization of the molecules and create important matrix effects. The replacement of the traditional liquid-liquid extraction by freezing the aqueous layer enables the organic layer to be easily transferred, reduces time [23] and the used amount of solvent [24] while protecting the experimenter from repeated manoeuvres that may cause problems.

In this article, we report the development of a method containing the main products GLYP and GLUF and their major metabolites AMPA and 3MPPA in human urine in a single extraction by UPLC-MS/MS with LOD in the low μg/L range, which enables its use in biomonitoring or epidemiological studies. This methodology is currently being used in the Maternal-Infant Research on Environmental Chemicals (MIREC-ENDO; https://www.mirec-canada.ca) study and in the Canadian Health Measures Survey (CHMS; https://www.statcan.gc.ca/eng/survey/household/5071).

Materials and methods

Standards and reagents

The following standards were purchased from Sigma-Aldrich Co. (Oakville, Ontario Canada): 3MPPA, AMPA (for quality controls (QCs)), GLUF ammonium and GLYP. GLUF ammonium (for QC), GLUF-d_3 hydrochloride, 3MPPA disodium salt (for QC) and 3MPPA-d_3 sodium salt were obtained from Toronto Research Chemicals (North York, Ontario, Canada). The following stable isotope-labelled internal standards (SIL-IS) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA): AMPA-^{13}C, ^{15}N, methylene-d_2 and GLYP-^{13}C, ^{15}N. AMPA was obtained from Thermo Fisher Scientific (Ottawa, Ontario, Canada). GLYP (for QC) was purchased from LGC Standards USA (Manchester, NH, USA).

Ammonium acetate and PFBBr were purchased from Sigma-Aldrich. Acetone (pesticide grade), hydrochloric acid, anhydrous potassium carbonate, methyl tert-butyl ether (MTBE) (HPLC grade) and deionized water (from a Milli-Q Advantage A10 purification system) were obtained from Thermo Fisher Scientific. The following solvents were supplied by VWR (Mississauga, Ontario, Canada): acetonitrile (OmniSolv) and methanol (LC–MS, OmniSolv).

Biological samples

Human urine samples were collected from multiple volunteer donors in a 125-mL polypropylene bottle. Donors were volunteers from the Quebec City area with ages of 4–80 years. The urine samples were frozen and stored at −20 °C.

Standard solutions

The 3MPPA, AMPA, GLUF and GLYP individual solutions were prepared at a concentration of 1 mg/mL in 10% methanol [25]. The solutions were combined and diluted in 10% methanol to a concentration of 1 mg/L. For the internal standard, individual stock solutions were ordered or prepared at 100 mg/L in water, and the working IS solution was prepared: 3MPPA-d_3 (100 µg/L), AMPA-^{13}C, ^{15}N, methylene-d_2 (200 µg/L), GLYP-^{13}C, ^{15}N (500 µg/L) and GLUF-d_3 (500 µg/L) in 10% methanol. Solutions for the QCs were prepared at identical concentrations to those for the calibration curve, but they were obtained from a different supplier. All solutions were kept at −20 °C until use.

Calibration curve and QC samples

Urine, which was previously tested for its low content of analytes, was obtained from a volunteer and spiked with working standard solutions prepared in 10% methanol, providing seven calibration levels to cover the concentration range of 0.1–50 µg/L for all analytes. The calibration curve was injected at the beginning and the end of each sequence. QC samples were prepared in human urine obtained from previously tested volunteers. The matrices were spiked at three different concentrations: low (0.45 µg/L), medium (2.5 µg/L) and high (12 µg/L). Aliquots of 0.4 mL were dispensed into 0.5-mL polypropylene microtubes and stored at −20 °C until use. The QC samples were alternated in a sequence after every ten samples. The German external quality assessment scheme (G-EQUAS) (https://www.g-equas.de) and external quality assessment scheme for organic substances in urine (OSEQAS) (https://www.inspq.qc.ca/en/ctq/eqas/oqesas/description) samples consisted of native pooled human urine spiked with defined amounts after the appropriate preparation. For the human biomonitoring project in Europe (HBM4EU) (https://www.hbm4eu.eu), we had the great opportunity to serve as an external laboratory in the project and received human urine control samples to be analysed.

Sample preparation

In polypropylene tubes (13 mL), 100 µL of urine, water or QC samples was fortified with 20 µL of SIL-IS working solution. The tubes were covered with aluminium foil and agitated 5 s on a multi-tube vortex from VWR. The following solutions were added: 0.02-M solution of potassium carbonate (1 mL) and a 1:1 mixture of acetone:acetonitrile (1 mL); the tubes were covered and agitated 5 s on a multi-tube vortex. Under a fume hood, PFBBr (10 µL) was added, and the tubes were screwed and agitated 1 min on a multi-tube vortex. The tubes were placed in a water bath (70 °C) for 4 h before being returned to room temperature for 15 min. To ensure that all the analytes were protonated, a 1% hydrochloric solution (2 mL) was added to the reaction, followed by MTBE (3 mL) for liquid-liquid extraction. The tubes were screwed, agitated for 1 min on a multi-tube vortex and centrifuged at 3000 RPM for 5 min using an Allegra 25R benchtop centrifuge (Beckman Coulter, Mississauga, Ontario, Canada). Using the flash freeze technique with dry ice and methanol, the organic layer was transferred by decantation into disposable glass tubes (16 × 100 mm). The extracted samples were evaporated to dryness with a Turbo Vap (Zymark Corp., Hopkinton, MA, USA) set at 40 °C and reconstituted with 1000 µL of 2-mM ammonium acetate in 40% acetonitrile. The tubes were covered with aluminium foil and agitated 15 s on a multi-tube vortex. For 3MPPA, 200 µL of the solution was picked up, and 200 µL of 2-mM ammonium acetate solution was added to 1.5-mL polypropylene LC-MS vials. For other analytes, the remaining solution was transferred to the same type of LC-MS vials. The reaction scheme for the analytes is presented in Fig. S1 of the Supplementary information (ESM).

Liquid chromatography

Chromatographic separation was achieved using an Acquity I-Class UPLC system (Waters, Milford, MA, USA). Mobile phase A was 2-mM ammonium acetate, and B was 2-mM ammonium acetate in 99% acetonitrile. The autosampler temperature was set at 10 °C, and the injection volume was 10 µL, while the temperature of the analytical columns was set to 50 °C, and the flow rate was 500 µL/min. For 3MPPA, the column was an HSS T3, 2.1 × 50 mm, 1.8 µm from Waters; for the other analytes, we used BEH Phenyl, 2.1 × 100 mm, 1.7 µm also from Waters. Gradient programming for 3MPPA was held for 4 min with 14% B, increased to 100% B and held for 2 min, before being returned to 14% B. For the other analytes, the gradient programming was started at 0 min with 42% B, held for 4.5 min, increased to 65% B, held for 2 min, increased to 100% B, held for 2 min and finally returned to 42% B at 8.5 min.

Mass spectrometry

The Xevo TQ-XS mass spectrometer from Waters was operated in electrospray ionization (ESI) in the positive mode and multiple reaction monitoring (MRM) mode. A post-column splitter was also used with a ratio of 2:1. The cone energy was set to 30 eV for AMPA and GLYP, 40 eV for 3MPPA and 56 eV for the analysis of GLUF. The collision gas was argon at a required flow rate to obtain a pressure of approximately 4×10^{-3} Torr in the collision cell. Table 1 summarizes the retention times, collision energies and mass transitions of each analyte and their SIL-IS.

Table 1 Retention time (RT), parent and product for the quantifier (Qn) and qualifier (Ql) ions, collision energy (CE) and ion ratio monitored for 3MPPA, AMPA, GLYP, GLUF and SIL-IS

Compound	RT (min)	Parent (*m/z*)	Product (*m/z*)	Ion	CE (eV)	Ion ratio (Qn/Ql)
3MPPA	2.71	333.03	135.00	Qn	14	
		334.03	135.00	Ql	14	13
3MPPA-d_3	2.69	336.05	138.02	Qn	14	
		337.05	138.02	Ql	14	13
AMPA	4.01	652.02	390.03	Qn	15	
		623.02	390.03	Ql	15	14
AMPA-^{13}C, ^{15}N, d_2	3.99	656.03	394.05	Qn	15	
		657.03	394.05	Ql	15	13.2
GLYP	4.20	710.02	448.04	Qn	18	
		711.02	448.04	Ql	18	14.6
GLYP-^{13}C,^{15}N	4.20	712.02	450.04	Qn	18	
		713.02	451.04	Ql	18	5.8
GLUF	6.17	722.06	236.01	Qn	40	
		722.06	416.01	Ql	36	3.4
GLUF-d_3	6.16	725.08	236.01	Qn	40	
		725.08	416.01	Ql	36	3.7

Validation

The procedures for validation in our laboratory were under the scope of the ISO/CEI 17025 accreditation and described elsewhere [26]. Briefly, for the LOD and the limit of quantification (LOQ), a urine sample containing the analyte at a concentration between 7- and 10-fold the estimated LOD was analysed 10 times on the same day, by the same analyst. The final LOD was calculated as 3 times the standard deviation (SD) obtained following the analysis of those 10 replicates, and the LOQ is equal to 10 times the obtained SD. The same process was repeated on 3 identical instruments in the lab, and highest values obtained for the LOD and the LOQ was selected as the final LOD. The linearity was defined as the concentration levels from the LOQ to the highest concentration where the slope of the curve was validated linear. *R*-squared values of the calibration curve must be greater than 0.995. The global matrix effect (ME) [27] was evaluated by extracting 6 different non spiked urine samples (set E) with different specific gravities and the same 6 samples spiked at two different concentrations (set D). The global ME (%) was calculated by the recovery of the analyte added.

Data analysis

The MassLynx version 4.1 software, SCN 909 from Waters, was used for system control, data acquisition and processing. Statistical analysis was performed with Excel 2013 (Microsoft Corp., Redmond, WA, USA).

Results and discussion

As stated by Valle et al. [28], there is a "glyphosate paradox" as it is the most widely used herbicide and one of the most hardly determined. And, as reported by Huhn [29], the analysis of GLYP in body fluids is still rare so that knowledge on exposure should be increased. One of the major reasons behind this lack of information is the physicochemical properties of GLYP. To quote Huhn [29] on glyphosate analysis, "Analytical chemists are still faced with problems in method development, reachable precision and detection limits." GLUF and AMPA also possessed the same type of physicochemical properties but 3MPPA is slightly different which is an added challenge.

So, it is understandable that there is currently no published methodology to evaluate glyphosate, glufosinate and their major metabolite (AMPA and 3MPPA) with LODs low enough to monitor the general population, and this is highlighted by the compilation available in Table 2. The different parts of this challenge will be discussed separately.

Sample preparation

To reduce and accelerate the sample preparation to extraction the derivatized analytes, the flash freeze technique [23] was used. Compared to liquid-liquid extraction (LLE), the use of methanol and dry ice to freeze the aqueous layer proved very advantageous for the recovery of the analytes without simultaneously consuming an excessive amount of MTBE and increasing the sample throughput. To obtain similar results with

Table 2 Comparison of methods presented in the literature containing all the analytes (3MPPA, AMPA, GLYP and GLUF) in biological matrices, the corresponding LODs and the percentage of detection in the population monitored

Reference	Derivatization	Technique used	Matrix	Volume (µL)	LOD (µg/L)				Population presented	Percentage of detection			
					3MPPA	AMPA	GLYP	GLUF		3MPPA	AMPA	GLYP	GLUF
[19]	None	LC-ICP-MS	Serum	1000	300	700	100	700	None				
			Urine		500	1000	200	1600	None				
[20]	TMOA	LC-MS	Serum	100	50	200	50	30	None				
			Urine		50	200	50	50	None				
[9]	None	LC-MS/MS	Serum	500	10	20	30	30	None				
[15]	None	LC-MS/MS	Blood	200	10	20	20	20	None				
[14]	MTBSTFA + TBDMCS	GC-MS	Serum	200	5	10	15	10	30 pregnant women	100%	0%	0%	0%
									39 non-pregnant women	67%	0%	5%	18%
This work	PFBBr	LC-MS/MS	Urine	100	0.095	0.086	0.077	0.084	21 women	19%	95%	81%	0%
									14 men	14%	100%	100%	0%

TBDMCS tert-butyldimethylchlorosilane

traditional LLE, we had to perform a second LLE with 2 mL of MTBE. Once the aqueous layer has frozen, the organic layer may easily be transferred by decantation into clean glass tubes ready for evaporation, which simplifies the manipulations of the LLE.

Derivatization

Since AMPA, GLUF and GLYP are zwitterions and 3MPPA is not, a strategy had to be devised to simultaneously derivatize all analytes. PFBBr was selected based on its properties to react with amines, carboxylic acids and phosphorus oxoacids on the targeted analytes (see Fig. S1 of the ESM for the reaction scheme). The conditions for the reactions were selected to maximize the synthesis of the mono-pentafluorobenzyl (PFB) derivative of 3MPPA while minimizing the di-derivatized compound that does not provide sufficient ionization potential to obtain good LODs in ESI. The other selected analytes for the analysis were derivatized with three PFB groups. The optimisation of the conditions was performed in a manner similar as presented in the paper by Vander Heyden et al. [30] which is a multivariate approach. Reaction conditions were selected to cover a range below and over the expected maximum, and the different conditions shuffled in the different test tubes. The K_2CO_3 solution concentration and volume were tested along with the ratio acetone:acetonitrile and its volume. The volume of PFBBr used, the reaction temperature and time were also tested in the same manner. The variations in the peak area percentage for the four selected analytes are available in Tables S1 to S7 of the ESM.

Chromatography

With three PFB groups attached to AMPA, GLUF and GLYP, these molecules lost their hydrophilic character and required a fair percentage of the organic mobile phase to be correctly chromatographed on a nonpolar column. Figure 1 presents a graphical display of the quantifier trace for the four analytes of interest and the associated derivatized structure. The absence of signal emanating from the complete process was observed and the chromatograms from a reagent blank (water sample) are presented in Fig. S2 of the ESM. The mono-PFB 3MPPA could have been analysed with the same type of column (phenyl) and recovery solution as the other analytes, but we decided to perform a separate chromatography for this analyte with a recovery solution closer to the initial separation conditions. Thus, it was possible to obtain a better peak shape and better separation from interferences. The initial tests performed with a phenyl column also showed interference at the same retention time as 3MPPA, while the use of an HSS T3 column eliminated this overlap. A 3MPPA chromatographic example of the same sample analysed with the phenyl (no separation) and the HSS T3 column, showing the interference that needs to be separated was made available at Fig. S3 of the ESM.

Spectrometry

We used the great sensitivity in the positive mode of the TQ-XS mass spectrometer and in adding PFB groups to the molecules to make them more prone to ionize in this mode. As previously stated, for 3MPPA, the addition of two PFBs to the

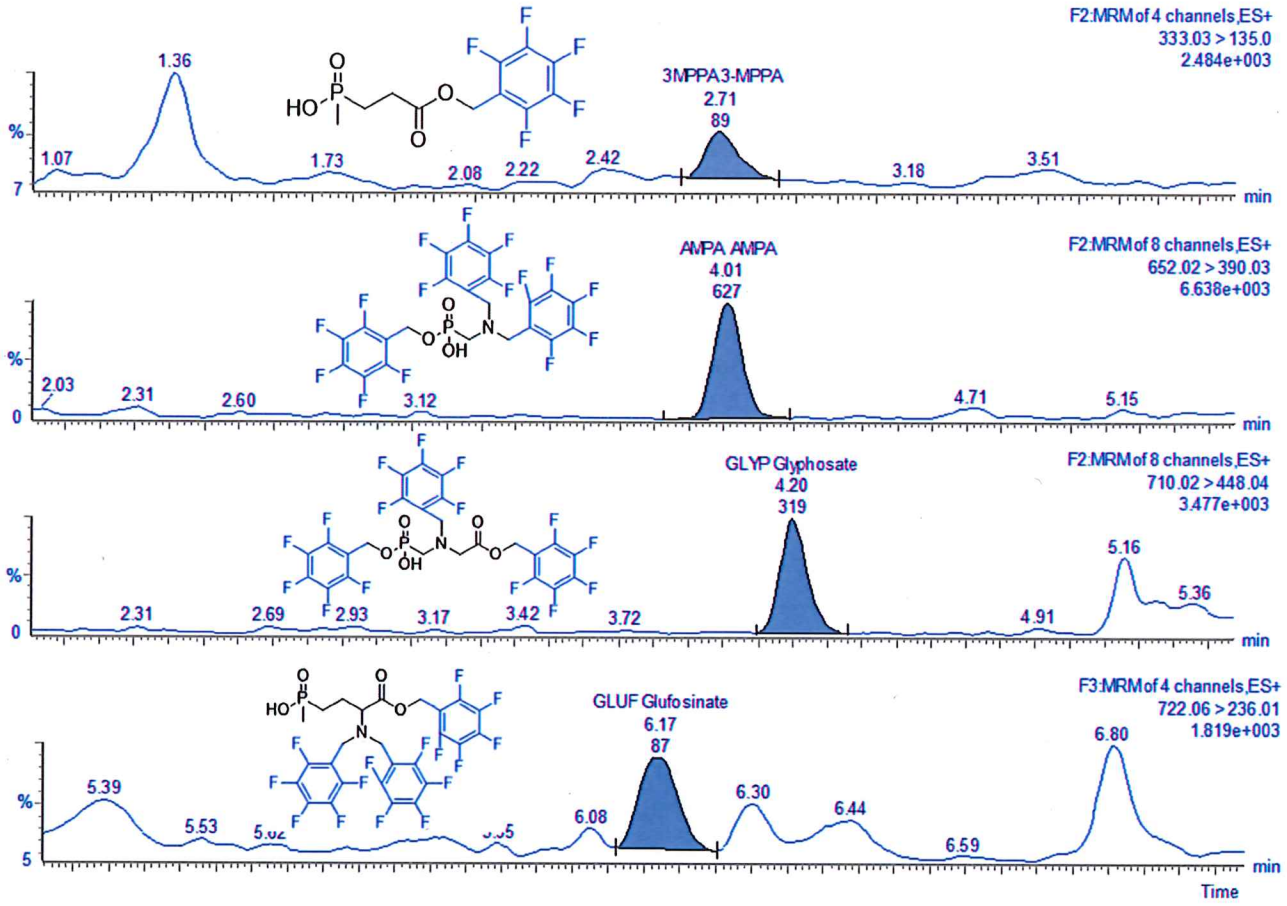

Fig. 1 LC-MS/MS quantifier traces and associated structures of the monitored compounds near the LOD level

molecule was detrimental to its ionization potential. Thus, the derivatization reaction was optimized to maximize the mono-PFB derivative.

For all analytes, one of the most intense transition was $[M+H]^+ > 181$ (corresponding to the loss of the PFB group). This transition could have been used but was not sufficiently specific compared to others. For the SIL-IS, the transitions corresponding to more specific transitions of the standards were used. Since there was no other specific transition for 3MPPA, AMPA and GLYP that satisfied our criteria, the ^{13}C transition was used as a qualifier. However, the use of this transition is not perfect because of the lack of reliable qualifiers in the low portion of the calibration curve. This is the reason why only the quantifier transition was considered for samples with concentrations below 0.25 µg/L for AMPA and GLYP, and 0.30 µg/L for 3MPPA. For GLUF, the derivatized molecule can be fragmented at two sites, which provides a more reliable ratio between the quantifier and the qualifier.

As a corroborating evidence for the structures proposed, the results of high-resolution mass spectrum experiments for all the analytes in MS and MS/MS mode are presented in Table S8 and Figs. S4–S11 of the ESM.

LOD, LOQ and linearity

To the best of our knowledge, the LODs reported for methods with the four analytes in human biological specimens were in the mid-high µg/L range (see Table 2). In addition to having GLUF, GLYP and their major metabolites (3MPPA and AMPA) in the same extraction, this method has LODs below 0.1 µg/L for all analytes (Table 3), when repeated on multiple instruments, which satisfies the requirements to use it in bio-monitoring or epidemiological studies. 3MPPA, AMPA, GLUF and GLYP had personalized SIL-IS, and they were used for the quantification. The calibration curves were matrix-matched and had a weight of 1/x, where the origin was included in the calculation. The graphical representation of the calibration curves for the linearity study (Fig. S12) and the corresponding *F*-tests (Table S9) for all the analytes are presented in the ESM.

Matrix effect

At the CTQ, the common procedure to document the effects of the matrix was reported elsewhere [27]. This methodology is

Table 3 Validation results for the LOD, LOQ, linearity and global ME on six different urine specimens of varying specific gravities

Compound	LOD* (µg/L)	LOQ* (µg/L)	Linearity (µg/L)	Global ME	
				Level (µg/L)	(%±SD)
3MPPA	0.095	0.32	LOQ to 50	5.0	94.2 ± 3.2
				50	94.2 ± 3.9
AMPA	0.086	0.29	LOQ to 50	5.0	103.9 ± 7.8
				50	97.5 ± 6.5
GLYP	0.077	0.26	LOQ to 50	5.0	98.3 ± 3.0
				50	96.7 ± 2.8
GLUF	0.084	0.28	LOQ to 50	5.0	87.3 ± 4.4
				50	87.2 ± 4.3

SD standard deviation of 6 samples

*The highest value obtained on 3 instruments was selected as the final LOD and LOQ

very effective and provides useful information, but in this case, since the derivatization reagent is added at the beginning of the sample preparation, it would have been necessary to individually obtain the derivatized compounds and subject them to the method protocol. Even then, these derivatized molecules may have behaved differently from the underivatized molecules during the sample preparation and cause false conclusions. Thus, when a derivatization reagent was added at the very beginning of the sample preparation, only sets D and E of the standardized procedure [27] were performed. We used 6 urine samples from different volunteers with specific gravities of 1.006–1.028 and varying pH (set E). These urine samples were spiked (set D) at two levels: 5.0 and 50 µg/L. The global ME provides information on the instrumental ME and recovery of the analytes. The global ME for all analytes of the method (87.2–103.9%) indicates that the SIL-IS selected behave the same way the native compounds do, and that the matrix selected for the calibration curve has the same behaviour as the real samples, making them suitable for quantification.

Accuracy and precision

To prepare the QC samples, a second source of starting material was used. The accuracy and precision obtained for the three QCs ($n = 10$) are presented in Table 4. The interday ($n = 42$) accuracy and precision were calculated using the intraday concentrations. The intraday precision of the method is 1.9–7.6%, while the interday precision is 4.0–8.3%.

Even if fair results were obtained for the accuracy of the 3MPPA QCs, the difference between the solutions for the calibration curve and the QCs makes the accuracies being approximately 90%, while in the case of AMPA QCs, the higher variability in the ME as seen by the SD of the global ME is the most likely cause of the accuracies being approximately 8% too high.

Assessment schemes

As noted by Connolly et al. [31], GLYP has been added to national biomonitoring programs in Canada, Germany and the USA. To externally verify the results of this method, we participated in the OSEQAS and HBM4EU assessment schemes (GLYP and AMPA) and G-EQUAS (GLYP). Currently, there is still no assessment scheme for GLUF and 3MPPA in urine.

G-EQUAS, based on the guidelines of the German Medical Association, which is an international external quality assessment scheme and certification provider for occupational-medical and environmental-medical toxicological analyses in biological materials, added glyphosate to their certification in 2019 (round 64). There are two rounds comprised of two samples per year. To be successful, both values have to be within the defined tolerance range.

Within the HBM4EU project was organised by the Wageningen Food Safety Research (part of Wageningen University & Research, Netherlands) an interlaboratory comparison for the determination of biomarkers in urine containing GLYP and AMPA starting in 2020. Four expert labs were selected from four different countries in Europe, and two external labs were added later. There were two rounds comprised of two samples in 2020.

OSEQAS, which started in 2015, is organised by the CTQ and follows the ISO/IEC 17043 requirements. This international external quality assessment scheme for organic substances in urine added GLYP and AMPA in 2020. Two rounds of three proficiency testing materials are available each year. To be considered comparable, *Z*-score results has to be within ±2 for HBM4EU and OSEQAS assessment schemes.

The results obtained in these assessment schemes are presented in Table 5. Even if the number of participants remains small in these proficiency tests, between 3 and 11 participants depending on the scheme and the analyte, which is probably due to the very recent availability of

Table 4 Intra- and interday accuracy/precision for the three QC levels

Compound	Spiked level (µg/L)	Intraday (n=10)		Interday (n=42)	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
3MPPA	0.45	90.6	6.1	100.7	7.9
	2.5	89.5	3.9	101.8	5.9
	12	91.6	2.3	98.2	4.9
AMPA	0.45	108.5	5.2	99.9	8.3
	2.5	107.6	7.6	103.5	7.9
	12	108.6	4.9	103.4	8.3
GLYP	0.45	93.8	5.2	98.2	6.8
	2.5	99.6	4.7	98.4	4.0
	12	100.8	3.3	96.8	4.8
GLUF	0.45	102	6.1	100.1	7.7
	2.5	104.1	3.8	98.2	6.1
	12	108.6	1.9	94.2	7.1

the tests, the results provided by some laboratories were considerably outside the accepted range. The reason behind this variability remains to be determined, but it may indicate interferences or major matrix effects in some methodologies to quantify these compounds, which can make a result comparison very difficult [32]. In our case, the obtained results are consistent with the consensus or reference values in the three external quality assessment schemes. In fact, the Z-score was in the ±0.3 range for AMPA (concentration ranged from 0.176 to 3.72 µg/L) and ranged from +0.1 to −0.6 for GLYP (concentration ranged from 0.42 to 6.09 µg/L) which is well inside the requirements of the assessment scheme providers.

Application of the method on human subjects

The results obtained for 35 volunteers in the Quebec City region are presented in Table S10 of the ESM. Only traces of 3MPPA were found in these samples, and no sample contained detectable amounts of GLUF. Most samples showed the presence of GLYP and AMPA. Only one sample did not show traces of AMPA, while 4 samples had GLYP levels below the LOD. In this population, AMPA and GLYP concentrations commonly have the same order of magnitude. This small sample set (Fig. 2) shows that most samples had concentrations below 1 µg/L for AMPA and GLYP, which makes the LODs of this method suitable for analysing the general population.

Table 5 Results, consensus or reference value, number of participants and Z-score obtained for the different assessment schemes for AMPA and GLYP

		AMPA				GLYP			
Assessment schemes		#	Result (µg/L)	Consensus value (µg/L)	Z-score**	#	Result (µg/L)	Consensus or reference value (µg/L)	Z-score**
G-EQUAS	64 A	–	–	–	–	11	0.86	0.88	−0.1
	64 B	–	–	–	–	11	4.1	3.73	−0.4
	65 A	–	–	–	–	11	0.42	0.42	0.0
	65 B	–	–	–	–	11	1.82	1.78	+0.1
HBM4EU	R1A	5	2.25	2.30	−0.1	6	2.527	2.82	−0.4
	R1B	5	0.19	0.176	+0.3	6	0.80	*	*
	R3A	5	0.896	0.901	0.0	6	0.136	*	*
	R3B	5	0.580	0.587	−0.1	6	6.25	6.09	0.1
OSEQAS	E2001	3	1.86	1.96	−0.19	4	1.53	1.77	−0.60
	E2002	3	< LOD	*	*	4	< LOD	*	*
	E2003	3	3.62	3.72	−0.13	4	3.60	3.79	−0.22
	E2004	6	3.10	3.28	−0.24	8	4.23	4.34	−0.13
	E2005	6	1.33	1.31	+0.07	7	2.54	2.49	+0.10
	E2006	6	1.95	2.07	−0.27	8	3.59	3.73	−0.18

#:number of participants; – absent from the assessment scheme; * no assigned value; ** for G-EQUAS the Z-score was calculated with the formula: (submitted result – reference value)/(tolerance range/3). For OSEQAS, the value provided is the Z'-score (details available on the provider's website)

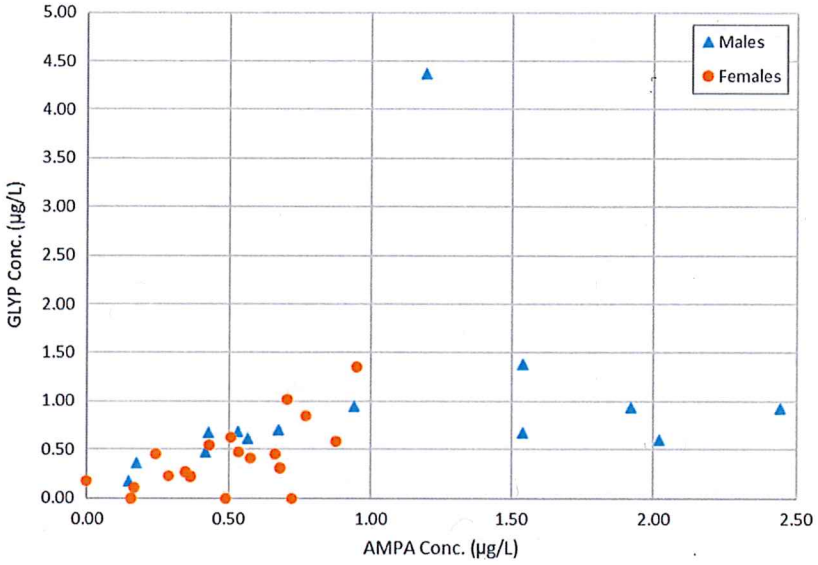

Fig. 2 AMPA and GLYP concentrations for different volunteers in the Quebec City area

Conclusion

The use of PFB derivatives and flash freeze technique helped to design an analysis method to determine covet herbicides GLYP, GLUF and their respective metabolite (AMPA and 3MPPA) in urine. The method was validated following the ISO/CEI 17025 guidelines and provided satisfactory results. The determination of the LODs was performed on three analytical instruments, thus providing a more representative and conservative estimate. So far, this method participated in three assessment schemes for GLYP and two for AMPA, and the results obtained were well inside the acceptable ranges for all the tested materials, thus providing a result of 100% for both analytes (11/11 for GLYP and 9/9 for AMPA). These results may be considered a good indication that this method provides reliable and well comparable results to other laboratories in the world. Evaluation of the concentration observed in a small population showed that the LODs selected were low enough to evaluate the exposition to glyphosate and AMPA. The addition of GLUF and its metabolite 3MPPA are added features that are not very common in the scientific literature and provide interesting insights to better understand the exposure of the general population to glyphosate and glufosinate. This method is currently in use for the MIREC-ENDO and CHMS studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-021-03194-x.

Acknowledgements The authors wish to acknowledge the contributions of Anny-France Hudon, Claudine Roussy, Nathalie Morissette and Marie-Pier Ouellet, who collaborated with us to develop and improve this analytical method.

Funding Financial support was provided by an internal budget dedicated to development and research.

Declarations

Ethics approval This study involves the use of human urine samples collected from multiple volunteer donors, including laboratory staff and members of their family. According to the rules and regulations concerning ethical review in Québec, particularly those specified in the Civil Code (http://legisquebec.gouv.qc.ca/fr/ShowDoc/cs/CCQ-1991) and in subsequent IRB practice, appropriate regulation was followed for the current project because it was considered as evaluation and therefore, no IRB was required.

Consent to participate We obtained the consent of all participants (or parental consent in case of minors) to use the urine samples for method development and validation, and they may also be used for research and publications. Participants were protected by the removal of any personal identifiers that may have linked the participant to the donation. The samples were coded to maintain the anonymity of the participants and disconnect the identity of each participant from the data generated from their urine sample. Furthermore, all the staff members of the CTQ are required to sign a confidentiality agreement upon hiring.

Conflict of interest The authors declare no conflict of interest.**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00216-021-03194-x.

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