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Glyphosate – Determination of glyphosate and AMPA in urine by GC-MS/MS

Biomonitoring Method – Translation of the German version from 2023

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Keywords

glyphosate; AMPA; biomonitoring; urine; GC-MS/MS

Citation Note:
Hoppe H-W, Fest U, Eckert E, Müller J, Göen T, Hartwig A, MAK Commission. Glyphosate – Determination of glyphosate and AMPA in urine by GC-MS/MS. Biomonitoring Method – Translation of the German version from 2023. MAK Collect Occup Health Saf. 2023 Jun;8(2):Doc053. https://doi.org/10.34865/bi107183e8_2or

Manuscript completed:
29 Oct 2020

Publication date:
30 Jun 2023

Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented biomonitoring method. Glyphosate (*N*-phosphonomethylglycine) is a synthetic phosphonic acid derivative which has been used as a broad-spectrum herbicide since 1974. Its only known metabolite is aminomethylphosphonic acid (AMPA). Exposure in occupational settings is predominantly due to inhalation and dermal contact with glyphosate. The general population is exposed to glyphosate and AMPA via both dietary (plant and animal products) and environmental (soils, surface water, and groundwater) exposure.

The aim of this work was to develop a selective method for the determination of glyphosate and AMPA in urine. The method has been comprehensively validated, and the reliability data have been confirmed by replication and verification of the procedure in a second, independent laboratory. The analytes are directly derivatised in the dried urine sample with trifluoroacetic anhydride and trifluoroethanol without an initial extraction step. Calibration is performed using aqueous calibration standards processed analogously to the samples. As internal standards, glyphosate-d_2 and $^{13}C,^{15}N$-AMPA are added to the urine samples and calibration standards. The derivatives are measured after capillary gas-chromatographic separation with tandem mass-spectrometric detection (GC-MS/MS) using negative chemical ionisation (NCI).

The good precision and accuracy data show that the method provides reliable and accurate analytical results. The method is both selective and sensitive, and the quantitation

limit of 0.1 µg/l urine for glyphosate and AMPA is sufficient to determine occupational exposure as well as higher background levels in the general population.

1 Characteristics of the method

Matrix	Urine
Analytical principle	Gas chromatography with tandem mass spectrometry (GC-MS/MS)

Parameters and corresponding hazardous substances

Hazardous substance	CAS No.	Parameter	CAS No.
Glyphosate (*N*-Phosphonomethylglycin)	1071-83-6	Glyphosate	1071-83-6
Potassium glyphosate	70901-12-1; 39600-42-5	Aminomethylphosphonic acid	1066-51-9
Sodium glyphosate	34494-03-6		
Glyphosate sodium salt (2:3)	70393-85-0		
Ammonium glyphosate	40465-66-5		
Diammonium glyphosate	69254-40-6		
Triammonium glyphosate	114370-14-8		
Dimethylammonium glyphosate	34494-04-7		
Ethanolammonium glyphosate	40465-76-7		
Isopropylammonium glyphosate	38641-94-0		
Trimethylsulfonium glyphosate	81591-81-3		

Reliability data

Glyphosate

Within-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.1 µg, 0.5 µg, 1.0 µg, or 5.0 µg glyphosate per litre of urine and n = 10 determinations	s_w = 9.13%, 2.68%, 2.70%, or 4.74% u = 20.7%, 6.06%, 6.11%, or 10.7%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.5 µg or 2.5 µg glyphosate per litre of urine and n = 15 determinations	s_w = 5.15% or 3.35% u = 11.1% or 7.19%
Accuracy:	Recovery rate (rel.) at a spiked concentration of 0.5 µg, 2.5 µg, or 5.0 µg glyphosate per litre of urine and n = 10 determinations	r = 95.0%, 96.9%, or 101%
Detection limit:	0.03 µg glyphosate per litre of urine	
Quantitation limit:	0.1 µg glyphosate per litre of urine	

AMPA

Within-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.1 µg, 0.5 µg, 1.0 µg, or 5.0 µg AMPA per litre of urine and n = 10 determinations	s_w = 8.91%, 4.37%, 2.70%, or 2.41% u = 20.2%, 9.88%, 6.11%, or 5.45%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.5 µg or 2.5 µg AMPA per litre of urine and n = 15 determinations	s_w = 4.32% or 3.03% u = 9.27% or 6.50%
Accuracy:	Recovery rate (rel.) at a spiked concentration of 0.5 µg, 2.5 µg, or 5.0 µg AMPA per litre of urine and n = 10 determinations	r = 95.0%, 98.3%, or 106%
Detection limit:	0.03 µg AMPA per litre of urine	
Quantitation limit:	0.1 µg AMPA per litre of urine	

2 General information on glyphosate and AMPA

Glyphosate (see Figure 1 for structural formula) is a chemical compound derived from phosphonic acid which was first synthesised by the Swiss pharmaceutical company Cilag AG in 1950. The MONSANTO company recognised the biological activity of the compound, obtained its patent as a broad-spectrum herbicide in 1974, and launched glyphosate as an active substance of Roundup® the same year (Dill et al. 2010). Later on, glyphosate developed into the active substance with the highest worldwide production volume for weed control in the agricultural, gardening, and wine-cultivation sectors as well as for desiccation (Dill et al. 2010; Duke and Powles 2008; Jaworski 1972). In addition, the substance is used in forestry, along railway lines, on roadsides, on public greens, and in private gardens.

In the shikimate pathway, glyphosate specifically inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which only occurs in plants and is essential for the biosynthesis of the amino acids phenylalanine, tyrosine, and tryptophan. In this way, protein biosynthesis is inhibited, and the plant dies shortly after glyphosate uptake (Dill et al. 2010; Jaworski 1972).

Glyphosate is primarily marketed in the form of its salts. The salts most frequently used as active substances in herbicidal products are summarised in Table 1.

Tab. 1 Glyphosate salts used as active substances in glyphosate formulations (according to ATSDR 2020)

Substance	CAS No.	Cation
Potassium glyphosate	70901-12-1; 39600-42-5	K^+
Sodium glyphosate	34494-03-6	Na^+
Glyphosate sodium salt (2:3)	70393-85-0	Na^+
Ammonium glyphosate	40465-66-5	NH_4^+
Diammonium glyphosate	69254-40-6	NH_4^+
Triammonium glyphosate	114370-14-8	NH_4^+
Dimethylammonium glyphosate	34494-04-7	$NH_2(CH_3)_2^+$
Ethanolammonium glyphosate	40465-76-7	$NH_3(CH_2CH_2OH)^+$
Isopropylammonium glyphosate	38641-94-0	$NH_3CH(CH_3)_2^+$
Trimethylsulfonium glyphosate	81591-81-3	$S(CH_3)_3^+$

Each year, about 800 000 t of glyphosate are used worldwide, of which about 7% is applied in Europe. Agriculture accounts for 90% of worldwide glyphosate application (Antier et al. 2020), the majority of which is used outside of Europe for the cultivation of genetically modified, glyphosate-resistant plants (Transparency Market Research 2014). In Germany, glyphosate sales fell from 7600 t in 2008 to 4400 t in 2017 (Antier et al. 2020).

Experimental animals excreted 20% (19–34%) of orally administered doses of glyphosate with the urine (EFSA 2015 a; IARC 2017). More recent human studies (Zoller et al. 2020), however, indicate a much lower excretion rate of about 1%. In mammals, glyphosate is only metabolised to a very low extent. Its only known metabolite, AMPA (see Figure 1 for structural formula), accounts for less than 1% of the administered dose of glyphosate. Within seven days, absorbed glyphosate is almost completely (99%) excreted with the urine and displays no potential for accumulation. Excretion kinetics are biphasic with a more rapid phase (half-life of 2.1–7.5 h) and a slower phase (half-life of 69–337 h) (EFSA 2015 a).

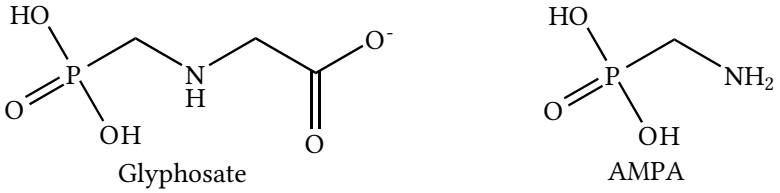

Fig. 1 Structural formulas of glyphosate and AMPA

AMPA is also the main degradation product of glyphosate in plants and in the environment. As such, glyphosate and AMPA are found not only as residues in grains, soy, and other crops after Roundup® treatment, but can also be detected in soil, surface water, and groundwater (Borggaard and Gimsing 2008). Background exposure of the general population to glyphosate and AMPA can therefore result from both dietary and environmental exposure. The correlation between glyphosate and AMPA concentrations in human urine is generally low. This finding is interpreted to mean that AMPA detected in the urine of non-specifically exposed individuals is derived secondarily from food products (EFSA 2015 a, b) and not from metabolised glyphosate. Furthermore, studies show that AMPA in the environment forms not only via the microbial decomposition of glyphosate but also through the microbial and photochemical degradation of various aminophosphonic acids (as a component of industrial chemicals, flame retardants, and surfactants) (Grandcoin et al. 2017; Struger et al. 2015). These processes, combined with the rather slow degradation of AMPA in the environment, may lead to its accumulation in plant and animal products (Van Bruggen et al. 2018).

A number of human-biomonitoring (HBM) studies have investigated background exposure to glyphosate and AMPA and indicate exposure in the general population, with very low levels in the range of the quantitation limits in the majority of samples. To present some examples, Table 2 shows the results of three German studies which use GC-MS/MS and LC-MS/MS analysis and demonstrate the magnitude of glyphosate and AMPA exposure in the general population.

Tab. 2 Glyphosate and AMPA concentrations in urine samples from the German general population

Collective (Number of persons)	Analytical method	Analyte	LOQ [µg/l]	Concentration [µg/l]		Detection frequency [%]	Reference
				Median	Maximum		
KarMeN study, adults (301), 24-h urine	LC-MS/MS	Glyphosate	0.2	< LOQ	1.36	8.3	Soukup et al. 2020
		AMPA	0.2	< LOQ	1.53	8.3	
German Environmental Specimen Bank, 20–29 a (399 samples), 24-h urine	GC-MS/MS	Glyphosate	0.1	< LOQ–0.18[a)]	0.11-2.80[a)]	10–57.5	Conrad et al. 2017
		AMPA	0.1	< LOQ–0.18[a)]	0.18-1.88[a)]	15–60	

Tab. 2 *(continued)*

Collective (Number of persons)	Analytical method	Analyte	LOQ [µg/l]	Concentration [µg/l]		Detection frequency [%]	Reference
				Median	Maximum		
Children, 2–6 a (250 samples), spot urine	GC-MS/MS	Glyphosate	0.1	0.14[b)]	3.7	63	LANUV 2016
		AMPA	0.1	0.13[b)]	2.2	58	

LOQ: Limit of quantitation
[a)] Values over the years 2001–2015
[b)] Values < LOQ were included in the calculation with half the value of the determination limit

Recent reviews have summarised and discussed the comprehensive biomonitoring data for glyphosate as well as some data for AMPA both within Europe and abroad. According to Gillezeau et al. (2019), the average urinary glyphosate concentration of the general population lies between 0.16 and 4 µg glyphosate/l, whereby there are considerable inter-regional differences. In particular, in regions where glyphosate-containing herbicides are applied by aerial spraying, the average glyphosate concentration increases up to 7.6 µg/l (Gillezeau et al. 2019; Varona et al. 2009). Niemann et al. (2015) compared urinary glyphosate concentrations from seven studies completed in Europe and the USA. In farm families, the maximum values lied between 9.5 and 233 µg/l; those of city dwellers were between 0.65 and 18.8 µg/l. For the purposes of comparability, it must be noted that these data are based on analytical methods of different sensitivity.

Workplace exposure occurs mainly via inhalation and dermal contact with glyphosate. Table 3 shows representative data from five occupational studies (forestry workers, farmers, and landscape gardeners), all of which were evaluated in the review by Gillezeau et al. (2019). The average glyphosate concentrations (above the detection limit) varied from 0.26 to 73.5 µg/l urine. The maximum value was 233 µg/l for a farmer and his family (Acquavella et al. 2004). Zhang et al. (2020) reported on a biomonitoring study in a factory in East China which produces glyphosate. In this study, 134 urine samples from employees of various production areas (crystallisation, centrifugation, desiccation, and packaging) were investigated. Glyphosate and AMPA concentrations were found to be < 20–17 200 µg/l and < 10–2730 µg/l, respectively. Workplace air concentrations correlated well with the concentrations determined in the urine samples.

Tab. 3 Glyphosate and AMPA concentrations in urine samples following occupational exposure

Collective (Number of persons)	Analytical method	Analyte	LOD [µg/l]	Concentration [µg/l]		Detection frequency [%]	Reference
				Geometric mean (Range)	GSD		
Farmers, specimen collection on date of application (48)	HPLC with postcolumn reaction and fluorescence detection	Glyphosate	1	3.2 (1–233)	6.4	60	Acquavella et al. 2004
Farmers (76)	ELISA	Glyphosate	0.05	0.334 (0.064–0.875)	–	68.4	Rendon-von Osten and Dzul-Caamal 2017
Landscape gardeners, after application (17)	LC-MS/MS	Glyphosate	0.5	0.66	1.11	–	Connolly et al. 2017
Landscape gardeners, after application (20)	LC-MS/MS	Glyphosate	0.5[a)]	1.17	–	–	Connolly et al. 2018
Employees of a glyphosate-producing company (134)	GC-MS	Glyphosate	20	262 (20–17 202)	–	86.6	Zhang et al. 2020
		AMPA	10	72 (10–2730)	–	81.3	

GSD: geometric standard deviation; LOD: limit of detection
[a)] Limit of quantitation

The acute toxicity of glyphosate is low. Based on developmental toxicity, the European Food Safety Authority (EFSA) has derived an acceptable daily intake (ADI) value of 0.5 mg glyphosate/kg body weight per day. Since AMPA and glyphosate exhibit similar toxicological profiles, the evaluation of AMPA is based on the assessment values for glyphosate (EFSA 2015 a). The International Agency for Research on Cancer (IARC) has designated glyphosate as "probably carcinogenic to humans" (Group 2A) (IARC 2017). In contrast, other expert panels have concluded that no carcinogenic

risk can be expected for humans when the substance is applied as intended (ECHA 2017, 2022; EFSA 2015 b). The discussion on potential carcinogenicity and other health risks (teratogenicity and hormonal disorders) is ongoing (US EPA 2017; Van Bruggen et al. 2018). Glyphosate has not yet been evaluated by the Commission.

3 General principles

Without an extraction step, glyphosate and its metabolite AMPA are directly derivatised in the dried urine sample with trifluoroacetic acid anhydride (TFAA) and trifluoroethanol (TFE). Calibration is carried out with aqueous standards which are treated analogously to the samples. Glyphosate-d_2 and ^{13}C,^{15}N-AMPA are added to the urine samples and calibration standards as internal standards (ISTDs). The derivatives are measured by GC-NCI-MS/MS.

4 Equipment, chemicals, and solutions

4.1 Equipment

- Gas chromatograph with an autosampler, a split/splitless injection system and a tandem mass-spectrometric detector and with a data-evaluation system (e.g. Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- Capillary gas-chromatographic column (length: 30 m; inner diameter: 0.25 mm; film thickness: 0.25 µm; stationary phase: polyethylene glycol) (e.g. HP INNOWax, No. 19091N-133, Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- Liner (e.g. Topaz 4 mm single-taper liner with wool, No. 23303.5, Restek GmbH, Bad Homburg vor der Höhe, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Heating block with drilled holes for sample vials (e.g. Barkey GmbH & Co. KG, Leopoldshöhe, Germany)
- Laboratory centrifuge (e.g. Heraeus Deutschland GmbH & Co. KG, Hanau, Germany)
- Vortex mixer (e.g. Multi-Tube Vortexer, VWR International GmbH, Darmstadt, Germany)
- Vacuum centrifuge (e.g. Jouan GmbH, Unterhaching, Germany)
- Handheld dispenser for volumes of 10 µl, 20 µl, 25 µl, 50 µl, and 100 µl (e.g. Multipette®, Eppendorf AG, Hamburg, Germany)
- 10-ml volumetric flasks (e.g. VWR International GmbH, Darmstadt, Germany)
- 2-ml autosampler vials (e.g. Klaus Ziemer GmbH, Langerwehe, Germany)
- 250-µl micro inserts (e.g. Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- 10-ml polypropylene tubes (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- 2-ml microcentrifuge tubes (e.g. Safe-Lock Tubes, Eppendorf AG, Hamburg, Germany)
- 10-ml screw-top glass vials (borosilicate) with Teflon sealing (e.g. SCHOTT AG, Mainz, Germany)
- Various piston-stroke pipettes (0.5–10 µl, 10–100 µl, and 100–1000 µl) (e.g. Eppendorf AG, Hamburg, Germany)
- Urine cups (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Urine Monovettes® (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be a minimum of *pro analysi* grade.

- Acetonitrile (e.g. No. 11317080, Fisher Scientific GmbH, Schwerte, Germany)
- Methanol (e.g. No. 136806, Biosolve BV, Valkenswaard, Netherlands)
- Trifluoroacetic acid anhydride, 99% (e.g. No. 91719, Merck KGaA, Darmstadt, Germany)
- 2,2,2-Trifluoroethanol, 99% (e.g. No. T63002, Merck KGaA, Darmstadt, Germany)
- Aminomethylphosphonic acid (AMPA), 10 µg/ml in water (e.g. No. L10205000WA, Dr. Ehrenstorfer™, LGC Standards GmbH, Wesel, Germany)
- ^{13}C,^{15}N-Aminomethylphosphonic acid (^{13}C,^{15}N-AMPA), 100 µg/ml in water (e.g. No. XA10205100WA, Dr. Ehrenstorfer™, LGC Standards GmbH, Wesel, Germany)
- Glyphosate, 10 µg/ml in water (e.g. No. L14050000WA, Dr. Ehrenstorfer™, LGC Standards GmbH, Wesel, Germany)
- Phosphonomethyl-d_2-glycine (glyphosate-d_2); pure substance, 99% isotopic purity (e.g. No. D-8030, C/D/N Isotopes, Pointe-Claire, Canada)
- Ultra-pure water (e.g. Milli-Q® Direct Water Purification System, Merck KGaA, Darmstadt, Germany)

4.3 Internal standards (ISTDs)

- Glyphosate-d_2 stock solution (1000 mg/l)
  10 mg of glyphosate-d_2 are weighed exactly into a 10-ml volumetric flask, which is made up to the mark with ultra-pure water and mixed by swivelling; the solution is then transferred into a 10-ml polypropylene tube.
- ISTD working solution (10 mg/l)
  A little ultra-pure water is placed in a 10-ml volumetric flask; 100 µl of the glyphosate-d_2 stock solution and 1 ml of the ^{13}C,^{15}N-AMPA solution are added by pipetting. Subsequently, the volumetric flask is made up to the mark with ultra-pure water, mixed by swivelling, and the solution is transferred into a 10-ml polypropylene tube. The final concentrations for glyphosate-d_2 and ^{13}C,^{15}N-AMPA are each 10 mg/l.

At –18 °C, the solutions can be stored for 12 months without analyte loss.

- ISTD spiking solution (0.01 mg/l)
  About 5 ml of ultra-pure water are placed in a 10-ml polypropylene tube, 10 µl of the ISTD working solution are added, and the tube is made up to 10 ml with ultra-pure water. The final concentrations of glyphosate-d_2 and ^{13}C,^{15}N-AMPA are each 0.01 mg/l.

In the ISTD spiking solution, glyphosate-d_2 and ^{13}C,^{15}N-AMPA are stable at 2–8 °C for at least four weeks. At –18 °C, the solutions can be stored for 12 months without analyte loss.

4.4 Calibration standards

- Spiking solution I (0.05 mg/l)
  A little ultra-pure water is placed in a 10-ml volumetric flask; 50 µl each of the standard solutions for glyphosate (10 mg/l) and AMPA (10 mg/l) are added by pipetting. Subsequently, the flask is made up to the mark with ultra-pure water and the solution is transferred into a 10-ml polypropylene tube. The final concentrations of glyphosate and AMPA are each 0.05 mg/l.

In spiking solution I, glyphosate and AMPA are stable at 2–8 °C for at least four weeks. At –18 °C, the solutions can be stored for 12 months without analyte loss.

- Spiking solution II (0.005 mg/l)
  In a 2-ml microcentrifuge tube (Safe-Lock Tube), 50 µl of spiking solution I are pipetted into 450 µl of ultra-pure water. The final concentrations of glyphosate and AMPA are each 0.005 mg/l.

Spiking solution II must be freshly prepared each workday.

The calibration standards are prepared in glass vials in which 1 ml of acetonitrile has been placed. The acetonitrile serves to prevent adsorption of the analytes to the glass surface. Calibration solutions with analyte concentrations of 0.1 to 10.0 µg/l (normalised to the water content) are prepared by adding water and spiking solution by pipetting, according to the pipetting scheme given in Table 4. The calibration standards are processed analogously to the urine samples according to Section 5.2, starting with the addition of the ISTD.

Tab. 4 Pipetting scheme for the preparation of calibration standards for the determination of glyphosate and AMPA in urine

Calibration standard	Acetonitrile [µl]	Water [µl]	Spiking solution I [µl]	Spiking solution II [µl]	Water content [µl]	Concentration in water content [µg/l]
0	1000	50	–	–	50	0.0
1	1000	49	–	1	50	0.1
2	1000	48	–	2	50	0.2
3	1000	45	–	5	50	0.5
4	1000	40	–	10	50	1.0
5	1000	32.5	–	17.5	50	1.75
6	1000	25	–	25	50	2.5
7	1000	12.5	–	37.5	50	3.75
8	1000	–	–	50	50	5.0
9	1000	40	10	–	50	10.0

5 Specimen collection and sample preparation

5.1 Specimen collection

Urine is collected in sealable urine cups and, if necessary, drawn into Urine Monovettes®. The urine samples are stable for at least one year when stored at –18 °C.

5.2 Sample preparation

The urine samples are thawed and intensely shaken. An aliquot of 50 µl is taken and transferred into a 10-ml screw-top vial in which 1 ml of acetonitrile has been placed. After adding 25 µl of the ISTD spiking solution and a short homogenisation step, the solution is evaporated to dryness in a vacuum centrifuge. First 0.5 ml of TFE, then 1 ml of ice-cold (–18 °C) TFAA are added to the mostly oily residue; the preparation is then mixed briefly on the vortex mixer. Depending on the amount of residual water, significant heat generation may occur. For derivatisation, the sealed vial is incubated at 80–85 °C for one hour in a heating block. Subsequently, the tube is opened and the solution is evaporated to 50–100 µl at 80–85 °C without a nitrogen stream. To avoid AMPA losses, the solution should not be evaporated completely to dryness at this point. The cooled residue is diluted in 50 µl of methanol and transferred into an autosampler vial with a micro insert.

6 Operational parameters

Analytical determination was carried out using a gas chromatograph with a tandem mass spectrometer.

Gas chromatography

Capillary column:	Stationary phase:	Polyethylene glycol
	Length:	30 m
	Inner diameter:	0.25 mm
	Film thickness:	0.25 µm
Temperatures:	Column:	Initial temperature of 75 °C, 0.5 min isothermal, increase to 170 °C at a rate of 20 °C/min, 5 min isothermal, increase to 265 °C at a rate of 40 °C/min, 3.5 min at final temperature
	Injector:	255 °C
	Transfer line:	280 °C
Carrier gas:	Helium 4.6	Flow rate: 1.2 ml/min, 2.0 ml/min starting at 10 min
Injection:	Injection volume:	1 µl (pulsed splitless, 120 kPa for 30 s), split on after 0.5 min
Liner:		4 mm inner diameter with quartz wool

Tandem mass spectrometry

Ionisation:	Negative chemical ionisation (NCI)
CI gas:	Methane 4.5
Ionisation energy:	240 eV
Source temperature:	150 °C
Quadrupole temperature:	150 °C
Collision gas:	Argon 5.0
Collision energy:	see Table 5
Electron multiplier:	1700 V

All other parameters must be optimised according to manufacturer specifications.

7 Analytical determination

For analytical determination, 1 µl of each sample processed according to Section 5.2 is injected into the gas chromatograph. At least one quality-control sample (see Section 10) and a reagent blank are included in each analytical run. For the latter, ultra-pure water is processed as described above and analysed in place of a urine sample.

Ionisation and fragmentation of the analytes in the ion source of the mass spectrometer is carried out using negative chemical ionisation with methane as the reaction gas. Using argon as collision gas, collision-induced fragmentation of selected precursor ions leads to the formation of specific product ions, which are then detected. Table 5 shows the time courses of the ion transitions recorded in MRM (Multiple Reaction Monitoring) mode of the triple-quadrupole mass spectrometer.

Tab. 5 Retention times, registered ion transitions, and collision energies used for the determination of glyphosate and AMPA in urine

Parameter	Retention time [min]	Precursor ion [*m/z*]	Product ion [*m/z*]	Collision energy [eV]	Detected fragment
Glyphosate[a)], Transition 1	8.4	370	245	10	$(CF_3CH_2\text{-}O)_2\text{-}P{=}O]^-$
Glyphosate, Transition 2	–	351	268	5	$351 - CF_3CH_2]^-$
Glyphosate-d_2	8.4	372	245	10	$(CF_3CH_2\text{-}O)_2\text{-}P{=}O]^-$
AMPA[a)], Transition 1	10.4	351	268	5	$351 - CF_3CH_2]^-$
AMPA, Transition 2	–	271	188	5	$271 - CF_3CH_2]^-$
$^{13}C,^{15}N$-AMPA	10.4	353	270	5	$353 - CF_3CH_2]^-$

[a)] Transition used for quantitative evaluation

The retention times given in Table 5 are only intended as a point of reference. The user must ensure the separation performance of the capillary column used and the resulting retention behaviour of the analytes. The MRM parameters are instrument-specific and must be adjusted by the user. Figures 2 and 3 show representative chromatograms on the Q_2 mass traces of glyphosate and AMPA. The chromatograms shown are not smoothed.

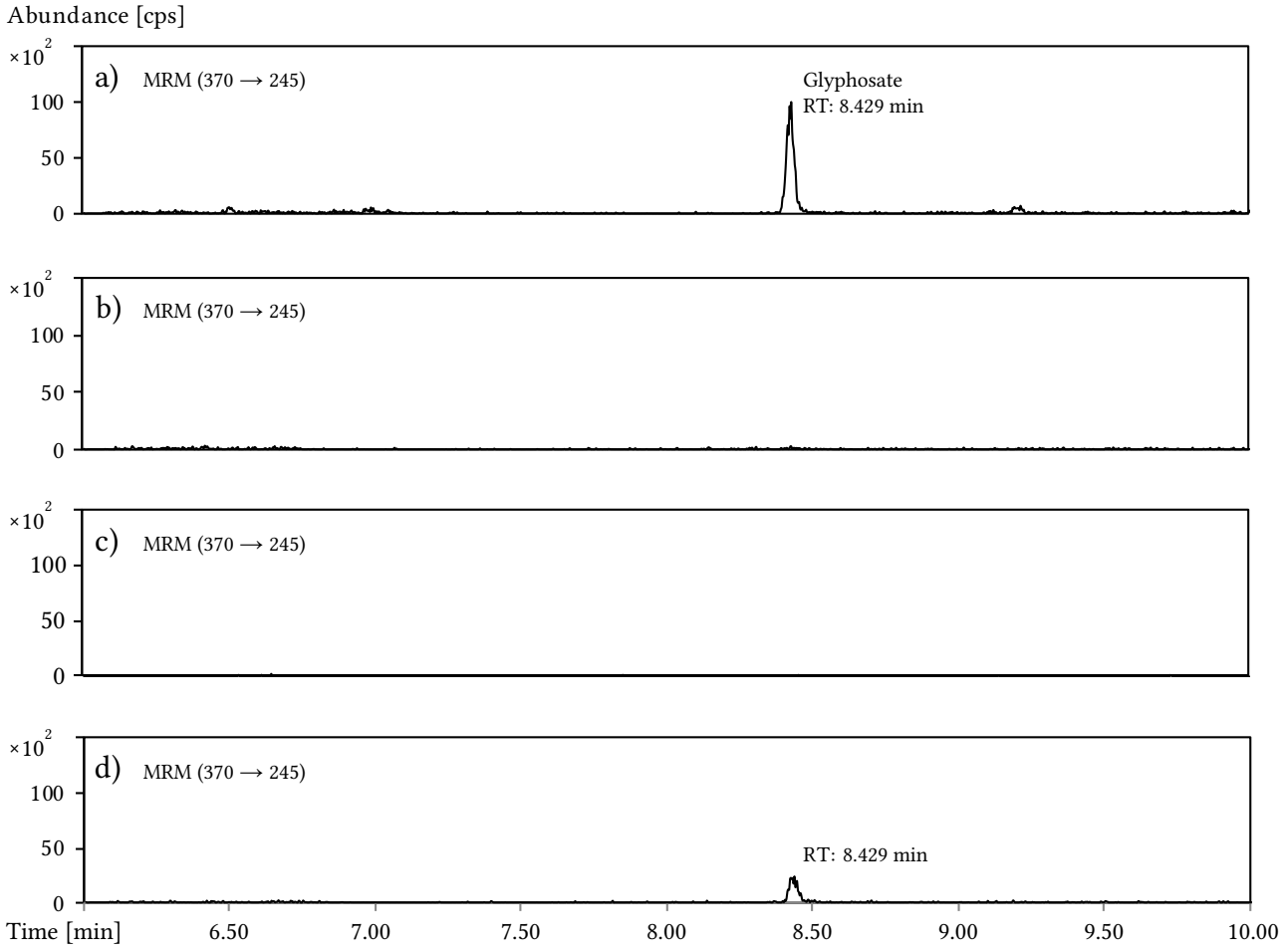

Fig. 2 Representative chromatograms of processed samples on the Q_2 mass trace of glyphosate (MRM 370 → 245): a) exposed urine sample (0.53 µg/l), b) non-exposed urine sample, c) reagent blank, d) calibration standard (0.1 µg/l; LOQ)

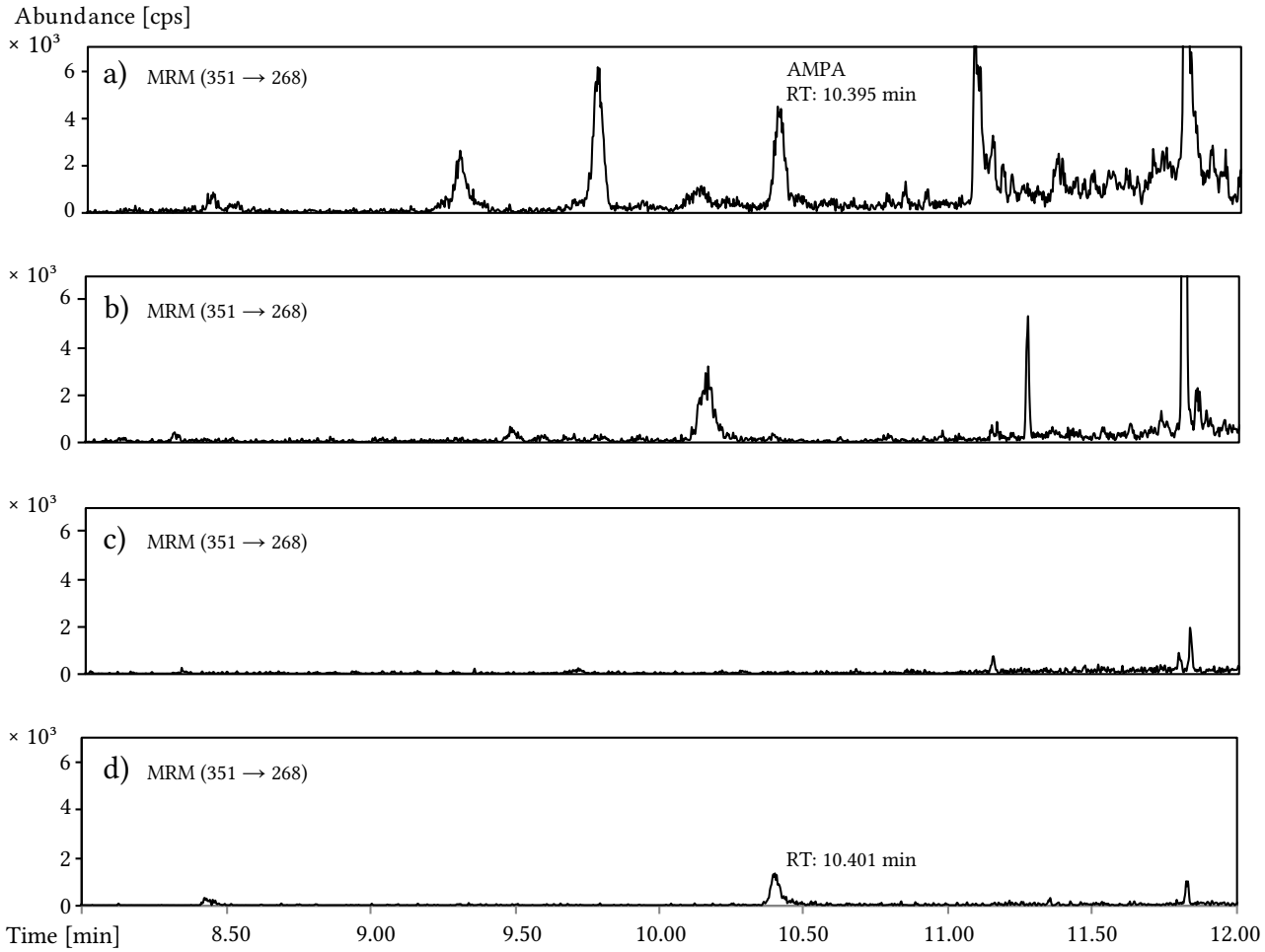

Fig. 3 Representative chromatograms of processed samples on the Q_2 mass trace of AMPA (MRM 351 → 268): a) exposed urine sample (0.16 µg/l), b) non-exposed urine sample, c) reagent blank, d) calibration standard (0.1 µg/l; LOQ)

8 Calibration

A calibration series should be included in each analytical run and the calibration curves should be newly generated.

To generate the calibration curves, the calibration standards are prepared according to Section 4.4 and analysed by GC-MS/MS. The calibration curves are generated by plotting the peak-area ratios of the analytes and the ISTDs against the corresponding analyte concentrations. The slope and axis intercept of the calibration curves are calculated by linear regression.

The calibration curves are linear from the quantitation limit (0.1 µg/l) up to 10 µg/l. Figure 4 shows representative calibration curves of glyphosate and AMPA, which were prepared in water and in pooled urine. The calibration curves in both matrices are nearly parallel. Under routine conditions, calibration on the basis of water is preferred, as pooled urine may exhibit background levels of glyphosate and AMPA.

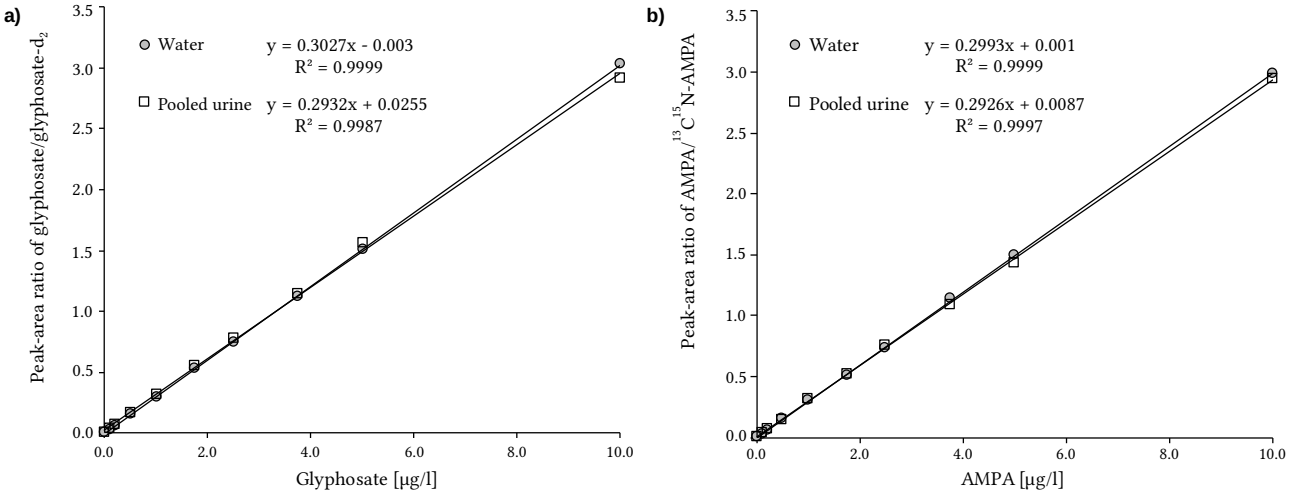

Fig. 4 Calibration curves for a) glyphosate and b) AMPA prepared in water and in pooled urine, respectively

9 Calculation of the analytical results

The glyphosate and AMPA concentrations in the analysed samples are calculated using the calibration function of the analytical run in question (Section 8). The peak-area ratios of the individual analytes and ISTDs determined in the samples are entered into the calibration functions by the evaluation software of the GC-MS/MS system, whereby the analyte concentrations are calculated in µg/l. Either no or only a low blank value (< 0.03 µg/l) is to be expected for both analytes.

If the measured value lies above the calibration range (> 10 µg/l), the urine sample in question is diluted with ultra-pure water, reprocessed, and newly analysed.

10 Standardisation and quality control

Quality assurance of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) and in a general chapter published by the Commission (Bader et al. 2010; Bundesärztekammer 2014).

To assure precision, at least one quality-control sample with a constant concentration of glyphosate and AMPA is included in each analytical run. Since commercial material is not currently available, the control material must be prepared by the in-house laboratory. To this end, individual urine samples are pooled to yield analyte concentrations within the expected concentration range. If necessary, the pooled urine may be further spiked with appropriate amounts of the analytes. A year's supply of this control material is prepared, aliquoted into 2-ml microcentrifuge tubes, and stored frozen at –18 °C. The nominal value and tolerance ranges of the quality-control material are determined as part of a pre-analytical period (singular daily analysis of the control material on 20 days).

11 Evaluation of the method

The reliability of the method was confirmed by comprehensive validation as well as by replication and verification in a second, independent laboratory as well as by successful participation in the German External Quality Assessment Scheme (G-EQUAS; https://app.g-equas.de/web/).

11.1 Precision

Within-day precision

To determine within-day precision, urine samples from persons not occupationally exposed to glyphosate were pooled and spiked with glyphosate and AMPA at concentrations of 0.1 µg/l, 0.5 µg/l, 1.0 µg/l, and 5.0 µg/l each. Ten of each of these samples were processed and analysed in one day. The precision data thus obtained are given in Table 6. Background levels of glyphosate and AMPA were not detectable in the pooled urine used (<0.03 µg/l).

Tab. 6 Within-day precision for the determination of glyphosate and AMPA in urine (n = 10)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Glyphosate	0.1	9.13	20.7
	0.5	2.68	6.06
	1.0	2.70	6.11
	5.0	4.74	10.7
AMPA	0.1	8.91	20.2
	0.5	4.37	9.88
	1.0	2.70	6.11
	5.0	2.41	5.45

Day-to-day precision

Day-to-day precision was determined as well. Pooled urines were spiked at concentrations of 0.5 µg/l and 2.5 µg/l, processed, and analysed on 15 different days. The precision data thus obtained are given in Table 7. Background levels of glyphosate and AMPA were not detectable in the pooled urines used (<0.03 µg/l).

Tab. 7 Day-to-day precision for the determination of glyphosate and AMPA in urine (n = 15)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Glyphosate	0.5	5.15	11.1
	2.5	3.35	7.19
AMPA	0.5	4.32	9.27
	2.5	3.03	6.50

11.2 Accuracy

Recovery

Recovery experiments were performed to verify the accuracy of the method. For this purpose, urine samples from non-exposed persons were pooled, spiked with glyphosate and AMPA at concentrations of 0.1 µg/l, 0.5 µg/l, 1.0 µg/l, and 5.0 µg/l each, processed ten times in parallel, and analysed. The mean relative recovery rates are summarised in Table 8.

Tab. 8 Mean relative recovery for the determination of glyphosate and AMPA in urine (n = 10)

Analyte	Spiked concentration [µg/l]	Rel. recovery *r* [%]	
		Mean	Range
Glyphosate	0.1	98.1	85.5–115
	0.5	110	104–116
	1.0	86.6	79.5–102
	5.0	102	98.6–106
AMPA	0.1	100	89.2–116
	0.5	111	102–121
	1.0	99.4	94.4–106
	5.0	99.0	95.2–105

In addition, recovery was verified using spiked individual urines. The results are presented in Section 11.3.

Interlaboratory method comparison

With the GC-MS/MS method hereby described (MVZ Medical Laboratory Bremen GmbH) as well as an LC-MS/MS method (Health and Safety Executive, Harpur Hill, Buxton, UK), 33 native urine samples were analysed as part of an interlaboratory method comparison. Twenty samples had glyphosate concentrations above the quantitation limits of both methods and the measurement results of these samples showed a strong correlation ($R^2 = 0.9891$, see Figure 5). AMPA was not included in the interlaboratory method comparison, as this analyte was not determined by LC-MS/MS.

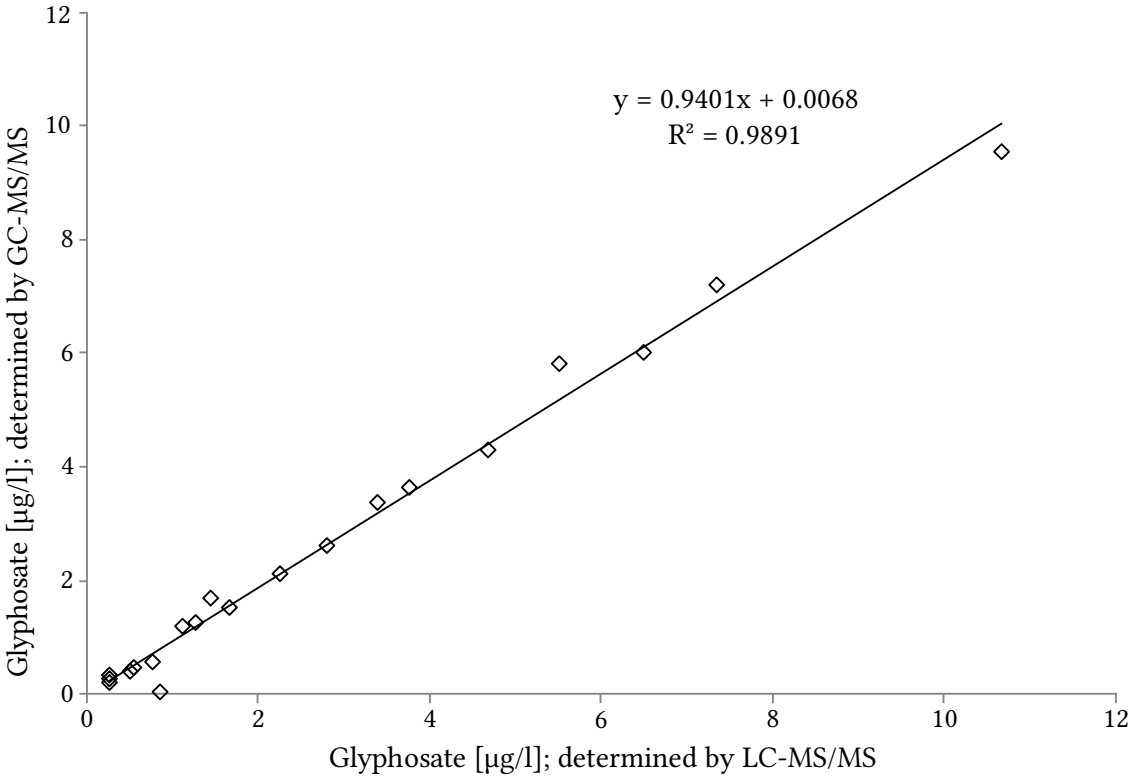

Fig. 5 Results of the interlaboratory method comparison using GC-MS/MS (MVZ Bremen, DE) or LC-MS/MS (HSE, Buxton, UK) for glyphosate determination

G-EQUAS interlaboratory comparisons

Using the method presented herein, the developer of the method consistently participated successfully in trials 64–70 of the G-EQUAS interlaboratory-comparison program (Hoppe 2021 a), whereby the control materials contained glyphosate but no AMPA. Table 9 lists detailed results from trials 64–67. The control materials (urines) were measured in parallel with the current ISTD, glyphosate-d_2, and its predecessor, 1,2-$^{13}C_2$,^{15}N-glyphosate. Deviations from the nominal values are small and prove the accuracy of the method as well as the equivalency of both ISTDs.

Tab. 9 Results of the G-EQUAS interlaboratory comparisons for glyphosate determination in urine

Sample	Nominal value [µg/l]	Tolerance range [µg/l]	Glyphosate-d_2 as ISTD		1,2-$^{13}C_2$,^{15}N-Glyphosate as ISTD	
			Result [µg/l]	Deviation [%]	Result [µg/l]	Deviation [%]
64 A	0.88	0.52–1.24	0.87	–1.1	0.86	–2.3
64 B	3.73	2.47–4.99	3.73	0.0	3.74	0.3
65 A	0.42	0.27–0.57	0.45	7.1	0.45	7.1
65 B	1.78	1.33–2.23	1.88	5.6	1.81	1.7
66 A	0.64	0.49–0.79	0.67	4.7	0.62	–3.1
66 B	1.20	0.93–1.47	1.25	4.2	1.17	–2.5
67 A	0.30	0.21–0.39	0.27	–9.3	0.28	–6.7
67 B	2.39	1.85–2.93	2.39	0.0	2.49	4.2

11.3 Matrix effects

To check for matrix effects, calibration standards for glyphosate and AMPA were prepared in both pooled urine and in water in a concentration range of 0.1–10 µg/l (see Section 4.4), processed, and analysed. It is evident from Figure 4 (see Section 8) that the calibration curves of both matrices are nearly parallel; isotope-labelled ISTDs are therefore sufficient to compensate for potential matrix effects. For this reason, calibration under routine conditions can be performed in ultra-pure water rather than in urine.

To further check for matrix effects, ten individual urines were processed and analysed both before and after spiking with 0.5 µg, 2.5 µg, or 5.0 µg glyphosate and AMPA per litre, respectively. The glyphosate and AMPA concentrations of the unspiked samples lied in the range of < LOD–0.08 µg/l and < LOD–0.09 µg/l, respectively. The mean relative recoveries are presented in Table 10 and support the conclusion that no relevant matrix effects are present.

Tab. 10 Mean relative recovery for the determination of glyphosate and AMPA in individual urines (n = 10)

Analyte	Spiked concentration [µg/l]	Rel. recovery *r* [%]	
		Mean	Range
Glyphosate	0.5	95.0	86.9–105
	2.5	96.9	92.7–105
	5.0	101	95.9–109
AMPA	0.5	95.0	89.2–103
	2.5	98.3	93.1–101
	5.0	106	99.5–110

11.4 Limits of detection and quantitation

Per the given operational parameters and based on a signal-to-noise ratio of 10 to 1, the quantitation limits for glyphosate and AMPA were each calculated to be 0.1 µg/l (Table 11). For both analytes, the coefficient of variation (the precision) at the quantitation limit was found to be 9% and the recoveries were in the range of 85–116%. The detection limits were each calculated to be 0.03 µg/l (signal-to-noise ratio of 3 to 1). The limits of detection and quantitation were confirmed using the calibration-curve method as described by DIN 32645 (DIN 2008).

Tab. 11 Limits of detection and quantitation for the determination of glyphosate and AMPA in urine

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
Glyphosate	0.03	0.1
AMPA	0.03	0.1

11.5 Sources of error

The determination of glyphosate and AMPA in human urine is challenging. The following aspects of the method described herein require special attention.

In order to avoid the adsorption of underivatised analytes to glass surfaces in samples and calibration standards, the urine samples and standard solutions should always be pipetted into glass vials already containing acetonitrile. The evaporation of these solutions is not critical, as the highly polar analytes are not volatile. In order to avoid AMPA losses, however, the samples should not be evaporated to dryness after derivatisation. In contrast, there is no problem with the derivatised glyphosate. After the evaporation step, methanol is added to the residue in order to eliminate any excess TFAA by conversion to trifluoroacetic acid methyl ester.

High demands are also placed on the instrumentation itself. After only a few injections of the matrix-containing measurement solutions, adsorption effects may arise on borosilicate liners. The resulting peak broadening and tailing then lead to an impairment of detection limits and recoveries. Moreover, AMPA is especially sensitive to impurities at the column head and in the injection system. These problems can be reliably solved by using highly deactivated liners (e.g. Topaz liner from Restek GmbH, Bad Homburg vor der Höhe, Germany) which contain a little quartz wool to support evaporation.

The selection of the separatory column is similarly important. With its high separation performance and long column life (about 600–800 injections), the polar HP INNOWax (Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany) has proven suitable under routine conditions as long as the column head is regularly shortened. Initially, the non-polar Zebron™ ZB-5 column (Phenomenex Ltd. Deutschland, Aschaffenburg, Germany) also displays high separation performance, but due to the high matrix load of the measurement solutions, this column is only stable in the short term and proved unsuitable for routine analysis.

12 Discussion of the method

Due to the good water solubility, polarity, and low molecular masses of the analytes, methods for the quantitation of glyphosate and AMPA in human biological materials are relatively demanding. The majority of older, published HPLC methods are based on fluorescence detection after protein precipitation and subsequent derivatisation with *o*-phthaldialdehyde (OPA) or fluorenylmethoxycarbonyl (Fmoc) (Acquavella et al. 2004; Hori and Fujisawa 2005). Enrichment and matrix separation are performed primarily by solid-phase extraction using cation exchangers or a Chelex resin (Freuze et al. 2007). The detection limit for urine and serum is usually given as 1 µg/l for these methods. Only the modern and more sensitive LC-MS/MS methods enable direct chromatography without derivatisation. Retention of both analytes is achieved on anion-exchange columns, on HILIC columns, or via ion-pair chromatography

using urine (Jensen et al. 2016), serum (Wang et al. 2008), or breast milk (Jensen et al. 2016; Steinborn et al. 2016). Sample workup includes protein precipitation, ultrafiltration, or solid-phase extraction (Wang et al. 2008). Quantitation limits in the range of 0.1–10 µg/l are given.

The GC-MS methods published so far are from the field of clinical chemistry and use serum as the test material. Since glyphosate and AMPA are too polar for gas chromatography, both analytes must be derivatised. After protein precipitation and solid-phase extraction using styrene-divinylbenzene (Motojyuku et al. 2008) or anion-exchange cartridges (Hori et al. 2003), the analytes are silylated and chromatographically analysed on non-polar GC columns (Type DB5). The detection limits of the GC-MS methods are relatively high at about 100 µg/l (Hori and Fujisawa 2005).

The GC-MS/MS presently described is based on an approach by Alferness and Iwata (1994) for the direct determination of glyphosate and AMPA in aqueous samples. Originally developed for food analysis (Alferness and Wiebe 2001; Kittlaus et al. 2009), the method can also be used for the highly sensitive determination of glyphosate and AMPA in human urine (Hoppe 2013; Krüger et al. 2014) and breast milk (Steinborn et al. 2016). Laborious extraction and purification steps are not necessary. In order to increase the volatility of the polar analytes, the functional groups (phosphonic acid, amino, and carboxyl group) are derivatised with TFE/TFAA directly in the concentrated urine sample. The reagents are added in excess in order to bind residual water. When using a polar capillary column (e.g. HP INNOWax, Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany), derivatised glyphosate and AMPA yield narrow, symmetrical peaks.

The added trifluoroalkyl groups further enable the high sensitivity and selectivity of the hereby presented GC-MS/MS method in NCI mode. Figure 6 shows the structures of the derivatised analytes and their isotope-labelled ISTDs.

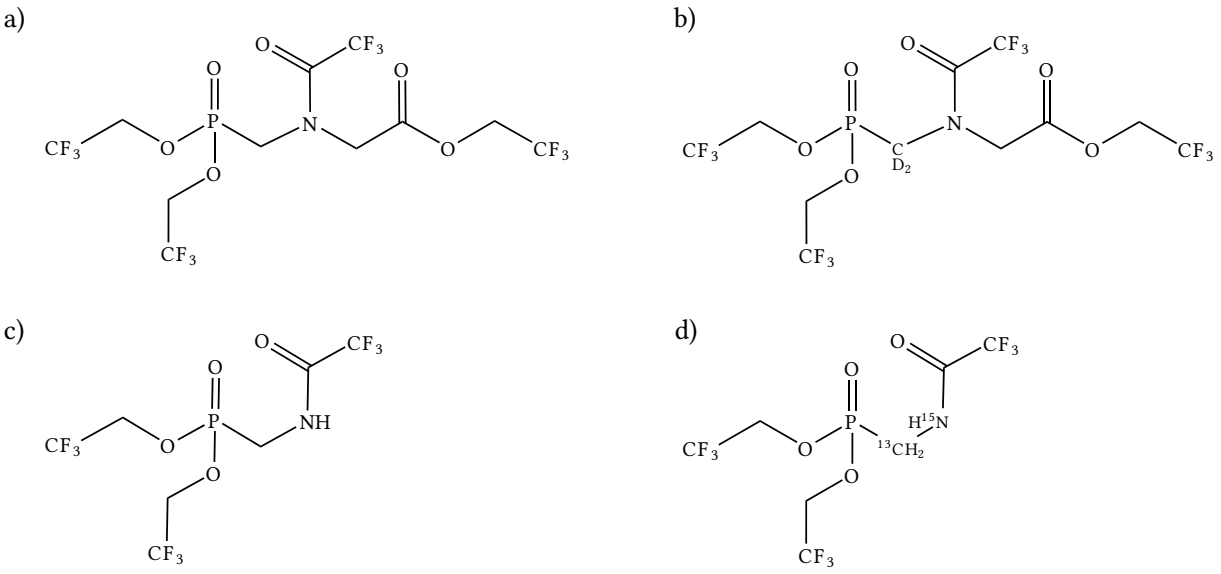

Fig. 6 Structures after derivatisation with TFE/TFAA of a) glyphosate (511 g/mol), b) glyphosate-d_2 (513 g/mol), c) AMPA (371 g/mol), and d) ^{13}C,^{15}N-AMPA (373 g/mol)

Under NCI conditions, derivatised glyphosate and AMPA are subject to very low fragmentation and form intense precursor ions at *m/z* 370 or *m/z* 351. Resulting from the natural share of ^{13}C, their M+1 satellite peaks at *m/z* 371 or *m/z* 352 are separated from the precursor ions of the isotope-labelled standards, glyphosate-d_2 and ^{13}C,^{15}N-AMPA, at *m/z* 372 or *m/z* 353. The mass spectra of the precursor ions of glyphosate and glyphosate-d_2 are presented in Figure 7, the corresponding spectra of AMPA and ^{13}C,^{15}N-AMPA in Figure 8, and the spectra of the respective product ions in Figures 9 and 10.

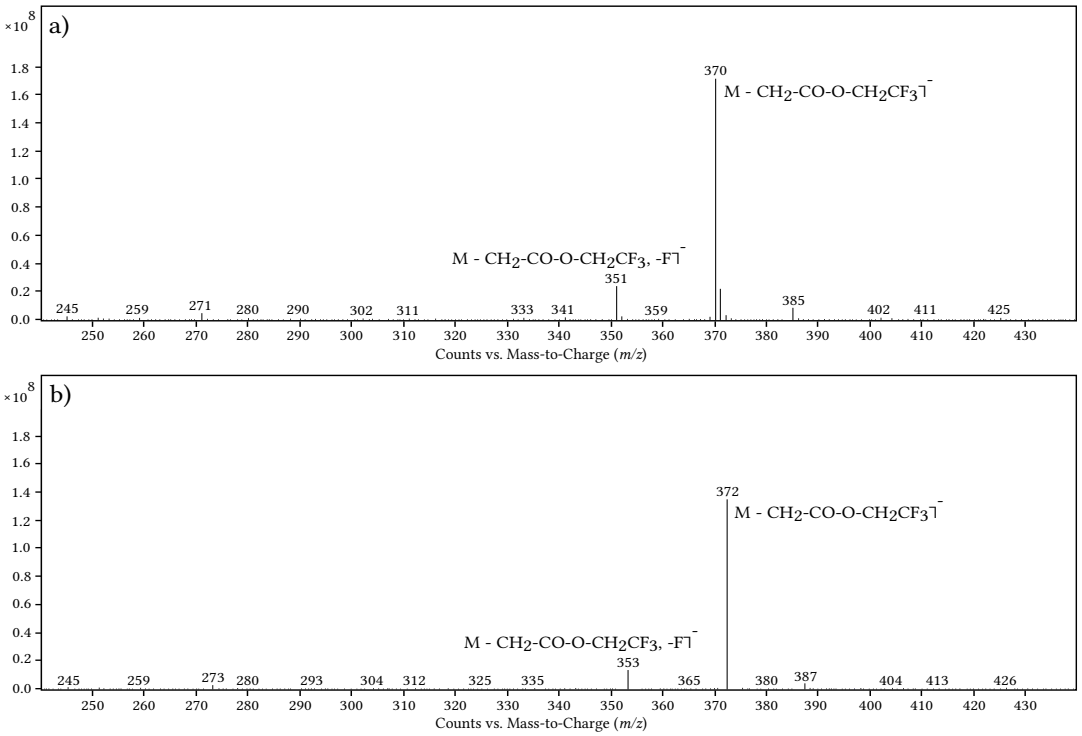

Fig. 7 Precursor-ion mass spectra (NCI mode) of derivatised a) glyphosate and b) glyphosate-d_2

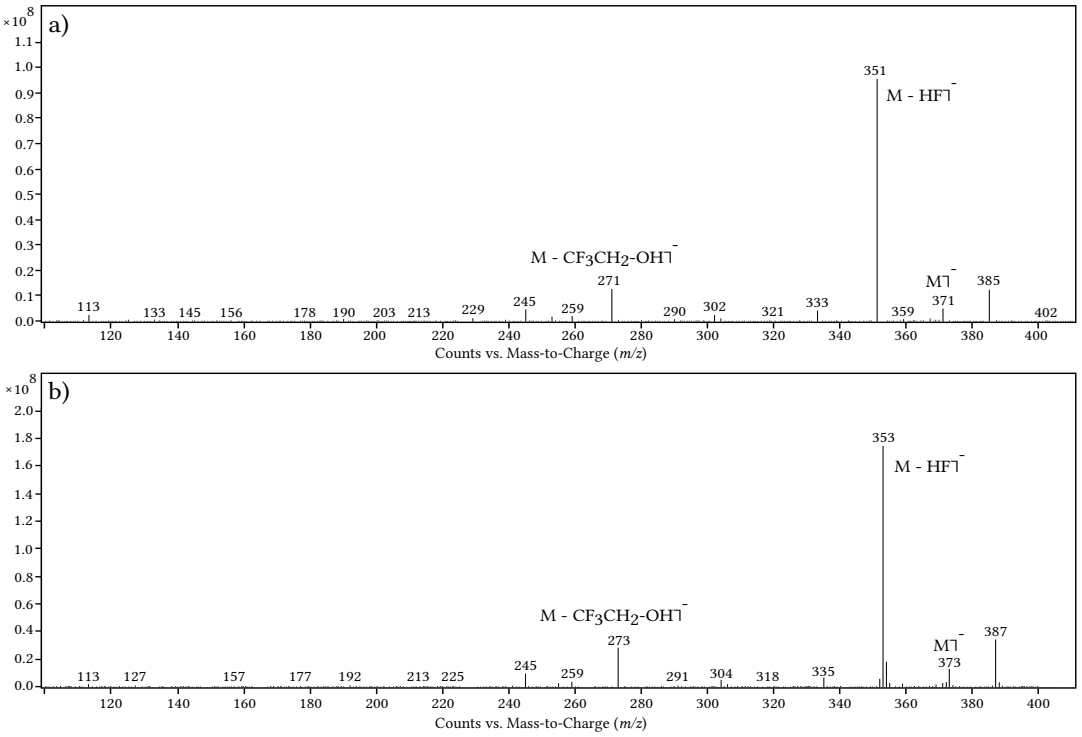

Fig. 8 Precursor-ion mass spectra (NCI mode) of derivatised a) AMPA and b) $^{13}C,^{15}N$-AMPA

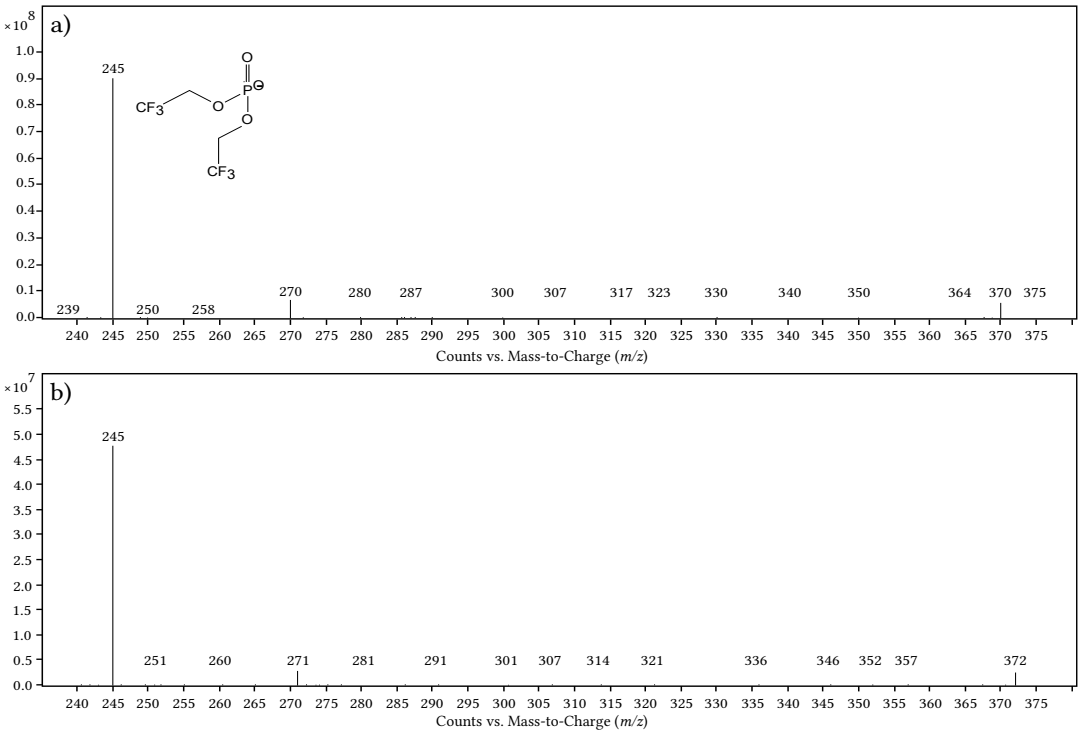

Fig. 9 Product-ion mass spectra of derivatised a) glyphosate and b) glyphosate-d_2

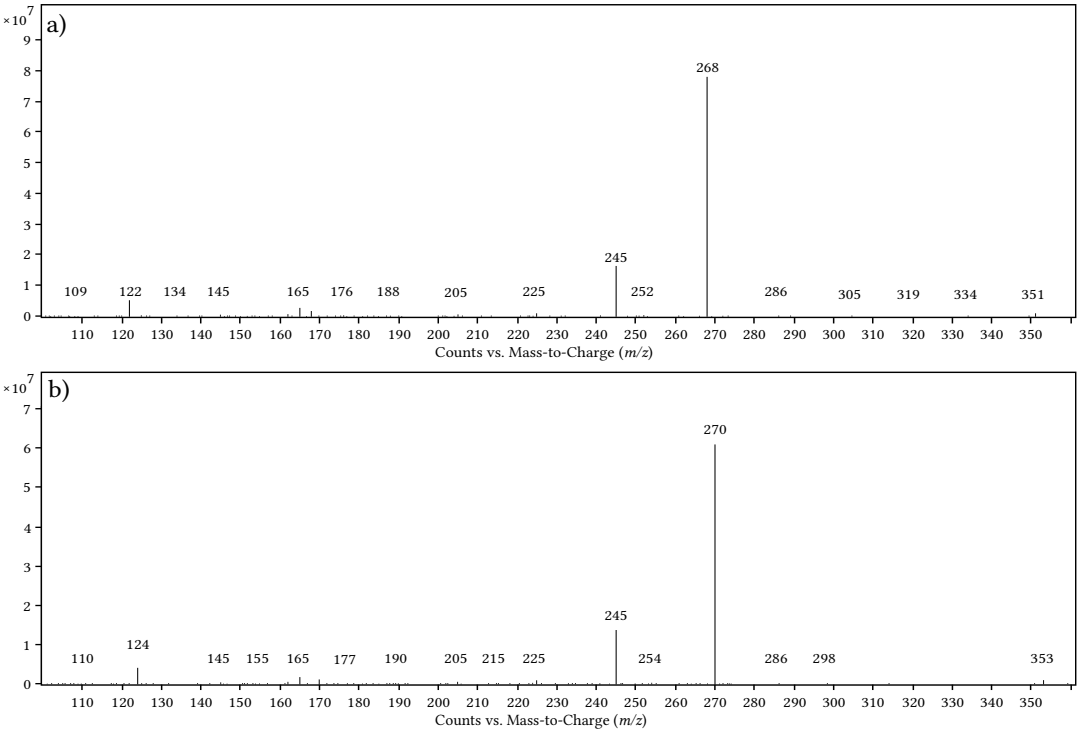

Fig. 10 Product-ion mass spectra of derivatised a) AMPA and b) $^{13}C,^{15}N$-AMPA

The basis peak *m/z* 370 in the glyphosate mass spectrum represents the cleavage of a [CH_2-CO-O-CH_2CF_3] fragment from the non-detectable molecular ion. The molecular ion *m/z* 371 is barely visible in the AMPA mass spectrum. The basis peak *m/z* 351 forms by the cleavage of HF; the low-intensity fragment *m/z* 271 forms by the cleavage of trifluoroethanol from the molecular ion.

The product ions, which form by collision-induced fragmentation, are listed in Table 5 (Section 7). In practice, the qualifier transitions 351 ⟶ 268 and 271 ⟶ 188 have proven to be of minimal use in the targeted low concentration range, since they only yielded reliable information at higher concentrations (> 10 µg/l). For this reason, these qualifier transitions were not included in the process of method validation.

In the process of method optimisation, glyphosate-d_2 was used to replace 1,2-$^{13}C_2$,^{15}N-glyphosate as ISTD. 1,2-$^{13}C_2$,^{15}N-Glyphosate is fragmented under loss of two isotope labels into the precursor ion at *m/z* 371, which interferes with the equal-mass satellite peak of native glyphosate. The influence of this spectral overlap on the ISTD at MRM trace *m/z* 371 ⟶ 245 was compensated mathematically. Parallel measurements of G-EQUAS samples with both ISTDs yielded equivalently low deviations from the nominal values and prove their equivalency (see Table 9, Section 11.2) as well as the accuracy of the GC-MS/MS method. The advantage of glyphosate-d_2 is that the undesired interference with native glyphosate is no longer an issue. Furthermore, due to the larger distance of the precursor mass from glyphosate (*m/z* 370) to glyphosate-d_2 (*m/z* 372), a reagent blank is virtually no longer detectable.

It is known that glyphosate and AMPA can easily adsorb to glass surfaces (Alferness and Wiebe 2001). The sample workup for our method nevertheless uses glass tubes as the derivatisation step with TFE/TFAA at 80 °C requires inert containers. Possible adsorption effects are prevented by placing acetonitrile in the glass containers in advance. Preliminary trials with three common polypropylene reaction vessels exhibited a series of potential problems. In vials from BRAND (No. BR780755, BRAND GMBH + CO KG, Wertheim, Germany) and Roche (No. 07857551001, Roche Diagnostics Deutschland GmbH, Mannheim, Germany), considerable amounts of AMPA formed under reaction conditions. In reaction vessels from MACHEREY-NAGEL (No. 702500, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), as used by Connolly et al. (2020), significantly lower peak areas of glyphosate and AMPA (about 63% and 32%, respectively) were observed in comparison to glass tubes. The observed formation of AMPA in the empty tubes as well as the considerable decrease in the signal intensities of both analytes may point to disturbances by plastic additives extracted into the reaction mixtures.

The reliability criteria of this method for the determination of glyphosate and AMPA can be described as excellent. Accuracy was proven by high recovery rates in both pooled urine and individual urines after spiking. Moreover, the selectivity of the GC-MS/MS method is very high. Significant interfering peaks were not observed on the mass transitions of either analyte. For glyphosate, a complete series of successfully passed interlaboratory comparisons (G-EQUAS, see Table 9, Section 11.2) is available and, in an interlaboratory method comparison, a good correlation was determined between the analytical results of the presented GC-MS/MS method and an independent LC-MS/MS method (see Figure 5, Section 11.2).

In order to estimate background exposure to glyphosate and AMPA, in 2012 a study was conducted on non-occupationally exposed employees of MVZ Medical Laboratory Bremen GmbH (Hoppe 2021 b). Table 12 lists the 95th percentiles determined in this study, which were established as in-house reference values as means of orientation.

Tab. 12 Glyphosate and AMPA concentrations in urine samples from an in-house laboratory study

Collective (Number of persons)	Analytical method	Analyte	LOQ [µg/l]	Concentration [µg/l]		Detection frequency [%]
				95th Percentile	Maximum	
In-house laboratory study, adults (90)	GC-MS/MS	Glyphosate	0.15	0.8	1.0	66
		AMPA	0.15	0.5	2.8	22

Over 2200 urine samples have been measured in the following years, primarily in non-specifically exposed individuals, using the GC-MS/MS method hereby described (Conrad et al. 2017; Hoppe 2013; Lemke et al. 2021). The sensitivity

is sufficient to determine higher levels of environmental exposure. The 95th percentiles are generally between 0.1 µg/l and 1 µg/l. In principle, this method is characterised by a low sample requirement of 50 µl and relatively simple sample workup, since an analyte extraction step is not necessary. About 40 urine samples can be processed and analysed per day.

The instrumental prerequisites are much lower than those of comparable LC-MS/MS methods (Jensen et al. 2016). The somewhat higher time expenditure due to derivatisation only comes into effect in cases of a larger number of samples.

Instruments used Gas chromatograph with a split/splitless injection system and a tandem mass-spectrometric detector, an autosampler, and a data-evaluation system (Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany); capillary gas-chromatographic column (length: 30 m; inner diameter: 0.25 mm; film thickness: 0.25 µm; stationary phase: polyethylene glycol) (HP INNOWax, No. 19091N-133, Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany), liner (e.g. Topaz Liner from Restek GmbH, Bad Homburg vor der Höhe, Germany)

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (www.dfg.de/mak/conflicts_interest) ensure that the content and conclusions of the publication are strictly science-based.

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