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1 **Sensitive and selective quantification of glyphosate and**
2 **aminomethylphosphonic acid (AMPA) in urine of the general population by**
3 **gas chromatography-tandem mass spectrometry**

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12

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16 **Keywords:**

17 Glyphosate

18 Aminomethylphosphonic acid

19 AMPA

20 Urine

21 Human biomonitoring

22 Environmental exposures

23

24 **Abstract**

25 Glyphosate is the highest volume herbicide used worldwide, and its main biodegradation
26 product is aminomethylphosphonic acid (AMPA), both are listed as priority substances in the
27 Human Biomonitoring for Europe initiative (HBM4EU) which aims at improving policy by
28 filling knowledge gaps by targeted research. The objective of the current study was to
29 advance the sensitivity of an existing gas-chromatography tandem mass spectrometry
30 analytical method to measure environmental population exposures. A 50% lower limit of
31 quantification of 0.05 µg/L was achieved for both analytes by slight modifications in sample
32 work-up, and use of another isotope labelled internal standard. In a pilot study on 41 urine
33 samples from the general German population, glyphosate and AMPA could be quantified in
34 66% and 90% of the samples respectively, which is a sufficient number to reliably describe
35 distributions of urinary concentrations in the non-occupationally exposed population.

36 1. Introduction

37 Glyphosate (N-(phosphonomethyl)glycine) is a broad-spectrum herbicide, its main
38 environmental biodegradation product is aminomethylphosphonic acid (AMPA), and both are
39 often detected as residues on crops and plants, and in foodstuff [1]. There are over 750
40 herbicidal products containing glyphosate; it is the highest volume used herbicide in the
41 world, with over 6 million metric tons applied worldwide in the past decade [2, 3].
42 Glyphosate is classified as ‘Group 2A – probably carcinogenic to humans’ by the
43 International Agency for Research on Cancer [4], a classification refuted by international
44 regulatory agencies [5-7].

45 Human biomonitoring (HBM) studies indicate a ubiquitous exposure to glyphosate [8-19].
46 However, existing analytical methodologies vary considerably in both their sensitivities and
47 specificities, depending on the underlying analytical principles (e.g. LC-MS/MS, GC-MS/MS,
48 ELISA), their limits of detection/quantification (LOD/LOQ), and their use of appropriate
49 isotope labelled internal standards, all of which make comparisons between study results
50 difficult. Depending on their purpose, some methods have been developed to cover
51 specialised exposures (e.g. occupational), or are adapted for use on human matrices, e.g. from
52 water or food surveillance methods, or do not include AMPA and glyphosate simultaneously.
53 To date, only ten HBM exposure studies on glyphosate have also analysed for AMPA. When
54 quantified, however, AMPA was found in similar concentration ranges as glyphosate. Urinary
55 AMPA moderately correlates with urinary glyphosate [11], but it is not fully understood if (or
56 how much of) urinary AMPA stems from human metabolism of glyphosate or what
57 proportion is due to AMPA uptake concurrently (e.g. via food) with glyphosate [20]. AMPA
58 has been reported to be of toxicological relevance as well [21] and measurement of both
59 compounds allows for a more comprehensive exposure assessment. Consequently, both
60 glyphosate and AMPA have been selected as priority substances in the European project
61 HBM4EU [22] (www.HBM4EU.eu) with the intention to establish an EU-wide HBM survey
62 for these substances, including thorough quality assessment of applied methods and
63 continuous quality assurance to increase data comparability [23].

64 After screening existing analytical methods and previously published HBM data the need for
65 more sensitive analytical methods to quantify both glyphosate and AMPA in urine below 0.1
66 µg/L was identified, in order to capture the median and reliably describe distributions in the
67 general population. Gas-chromatography tandem mass spectrometry in combination with
68 isotope dilution quantification is likely to be the most appropriate method, allowing sensitivity
69 and selectivity. Thus, we decided to advance a previously published analytical method [11] to
70 fulfil these aims. We demonstrate the applicability of this advanced method by the analysis of
71 urinary glyphosate and AMPA background levels in individuals from the general German
72 population.

73

74 2. Materials and methods

75 2.1. Chemicals and reagents

76 Water, methanol and acetonitrile (all LC-MS grade) were purchased from Honeywell
77 Research Chemicals (Neu Wulmstorf, Germany). Trifluoroethanol (TFE) and trifluoroacetic
78 anhydride (TFAA) (both >99%) were purchased from Merck/Sigma-Aldrich (Darmstadt,
79 Germany). Glyphosate (98% purity), (phosphonomethyl)glycine- $^{13}\text{C}_2$ - ^{15}N ('glyphosate-
80 $^{13}\text{C}_2$, ^{15}N '; 99% isotopic purity; product no. G765002), AMPA (95% purity), and *P*-
81 (aminomethyl)phosphonic acid- ^{13}C , ^{15}N ('AMPA- ^{13}C , ^{15}N '; 98% isotopic purity; product no.
82 A617342) were purchased from TRC (North York, Ontario, Canada). *N*-Phosphonomethyl- d_2 -
83 glycine (glyphosate- d_2 ; 99% isotopic purity; product no. D-8030) was obtained from C/D/N
84 Isotopes (Point-Claire, Quebec, Canada) and helium (6.0), nitrogen (5.0), argon (6.0), and
85 methane (5.5) were obtained from Air Products (Hattingen, Germany).

86 2.2. Standard solutions

87 All standard and internal standard solutions were prepared and stored in polypropylene
88 containers. Ten calibration solutions ranging from 0.01 to 5 $\mu\text{g/L}$ in water were prepared from
89 an aqueous stock solution containing 50 $\mu\text{g/L}$ each of glyphosate and AMPA. An internal
90 standard working solution containing 8 $\mu\text{g/L}$ AMPA- ^{13}C , ^{15}N , as well as 4 $\mu\text{g/L}$ glyphosate-
91 $^{13}\text{C}_2$, ^{15}N or glyphosate- d_2 (to compare these two internal standards; **Figure 1**), was prepared
92 in water. Aliquots of 1 mL of each calibration solution were transferred into 2 mL
93 polypropylene N9 screw cap vials (Macherey-Nagel, Düren, Germany) immediately after
94 preparation. All solutions were stored at -18 °C until use. For the final validated method, we
95 used glyphosate- d_2 instead of glyphosate- $^{13}\text{C}_2$, ^{15}N , contrary to Conrad et al. (2017).

96 2.3. Sample preparation

97 Sample preparation was performed as described in Conrad et al. (2017)[11] with two
98 modifications: in the initial concentration step, 2.0 mL polypropylene (PP) HPLC vials were
99 used instead of 10 mL screw-capped glass tubes, and after derivatization, the mixture was not
100 evaporated to full dryness before adding methanol. 50 μL of urine (or calibration solution)
101 and 25 μL of internal standard working solution were added to 1 mL acetonitrile in a 2.0 mL
102 PP vial. The solution was homogenised by vortex mixing and then evaporated to dryness at
103 0.1 mbar and 50 °C for 22 min using a vacuum centrifuge (Christ RVC 2-33 CDplus vacuum
104 centrifuge; Osterode, Germany). Afterwards, 0.5 mL TFE and 1 mL -18 °C cold TFAA were
105 added, the vial was capped, and the mixture was briefly homogenised by vortex mixing.
106 Derivatization was then performed for 1 h at 80-85 °C in a heating block thermostat (VWR,
107 Darmstadt, Germany). After cooling down to ambient temperature, the reaction mixture was
108 concentrated to approx. 50 μL at 85 °C. After cooling down again, the solution was mixed
109 with 50 μL methanol and transferred into a GC vial with a glass micro insert.

110 2.4. Instrumental analysis

111 A 7890B GC coupled to a 7000A triple quadrupole-MS (both Agilent, Waldbronn, Germany)
112 was used for GC-MS/MS analysis. Chromatographic separation was performed on a Zebtron

113 ZB-WAX column (30 m x 0.25 mm ID, 0.25 μm film thickness, Phenomenex, Offenbach,
114 Germany) with chromatographic conditions as reported by Conrad et al. (2017)[11]. A sample
115 volume of 1 μL was injected in pulsed splitless mode (0.5 min splitless time, 120 kPa; vent
116 flow 50 mL/min to split). A special deactivated 4 mm inner diameter liner with quartz wool
117 was used (Sky[®] liner; Restek, Bad Homburg, Germany). Injector and transfer line temperature
118 were 255 and 240 $^{\circ}\text{C}$, respectively. An initial GC oven temperature of 75 $^{\circ}\text{C}$ was kept for
119 0.5 min. Then, the temperature was increased to 170 $^{\circ}\text{C}$ at 20 $^{\circ}\text{C}/\text{min}$ and kept for 5 min.
120 Finally, the oven temperature was increased to 250 $^{\circ}\text{C}$ at 40 $^{\circ}\text{C}/\text{min}$ and kept for 3.5 min. The
121 carrier gas (helium) was kept at 1.2 mL/min during the analysis and increased to 2.0 mL/min
122 after 10 min. Ionization of the analytes was performed in negative chemical ionization mode
123 (NCI) with methane as CI gas and an ionization energy of 240 eV. The ion source was kept at
124 150 $^{\circ}\text{C}$. Argon was used as the collision gas. Analyte retention times and further MS
125 conditions are shown in Table 1. MassHunter GC/MS Acquisition software (B.05.02) was
126 used for instrument control, and MassHunter Workstation Software Quantitative Analysis
127 (B.07.00) was used for quantitative data analysis (Agilent, Waldbronn, Germany).

128 2.6. Calibration, validation and quality control

129 Calibration was performed by linear regression (ratio of analyte and internal standard peak
130 area vs concentration). Intraday and interday reproducibility were studied by repeated analysis
131 of two different pooled urine samples ('Q_{low}' and 'Q_{high}'), containing native concentrations of
132 glyphosate and AMPA. This material was also used as quality control material for routine
133 analysis. For the evaluation of the method accuracy, eight different urine samples (0.4 – 2.6
134 g/L creatinine) were analysed, spiked at two concentrations (0.2 and 1.0 $\mu\text{g}/\text{L}$), as well as
135 without spiking. Relative recoveries were calculated for glyphosate and AMPA after
136 subtraction of their respective native concentrations.

137 2.7. Study subjects

138 To demonstrate the applicability of the method, 41 convenience spot urine samples from the
139 German general population (29 females, 12 males; aged 23-61 years), collected by IPA in
140 2016 and 2017, were analysed. Of the participants, 31 were non-smokers. Creatinine levels
141 ranged from 0.12 to 2.31 g/L. No information on pesticide use was available on this sample
142 population. The study protocol was registered with the Ethics Commission of the Faculty of
143 Medicine of the Ruhr-University Bochum, Germany (Registry Numbers: 3867-10).

144 3. Results and discussion

145 3.1. Method advancement

146 A LOQ for both glyphosate and AMPA of 0.05 $\mu\text{g}/\text{L}$ was achieved, an improvement of a
147 factor of 2 compared to the original method of Conrad et al. (2017)[11]. Demonstrative GC-
148 MS/MS chromatograms for both glyphosate and AMPA are shown in *Figure S1*,
149 *supplemental material*. The method's precision and accuracy data are presented in **Table 2**
150 and **Table 3**. For the intra- and inter-day precision, relative standard deviations (RSD) of <6%
151 and <11%, respectively, were achieved.

152 The LOQ was determined based on a signal-to-noise ratio of 10 in a matrix (as previously
153 described in Bury et al. 2019 [24]), in accordance with the guidelines of the working group
154 “Analyses in Biological Materials” of the Permanent Senate Commission for the Investigation
155 of Health Hazards of Chemical Compounds in the Work Area of the German Research
156 Foundation [25].

157 Method accuracy, calculated as relative recoveries from native, spiked urine samples at two
158 concentration levels (0.2 and 1.0 µg/L) was on average 109 and 115% for glyphosate and 104
159 and 116% for AMPA, with single values in the range of 83-124% and 91-123%, respectively.
160 Recoveries were not matrix-dependent (creatinine content of urine samples).

161 During sample work-up, two modifications were essential to improve method sensitivity and
162 ruggedness while keeping reproducibility and accuracy high. Glyphosate has been shown to
163 adsorb onto glassware [26, 27]. We also made similar observations (data not shown), when
164 comparing the original method with the current one. Exchanging non-silanised glass vials for
165 PP vials improved the LOQ by a factor of 2. This improvement was also observed for AMPA.
166 Consequently, we replaced glassware with polypropylene (PP) material where
167 possible/appropriate and saw considerable improvements after replacement. Additional to
168 sample work-up, the use of glassware should also be avoided during preparation and storage
169 of the stock and working solutions.

170 Secondly, but of equal importance, was the choice of the standard. Glyphosate-d₂ was used as
171 an internal standard, replacing glyphosate-¹³C₂,¹⁵N used by Conrad et al. 2017 and others.
172 Initially, glyphosate-¹³C₂,¹⁵N looks more appropriate than glyphosate-d₂, considering the shift
173 in molecular mass of +3 u instead of +2 u. However, the formation of the quantifier precursor
174 ion via dissociative electron capture is accompanied by the elimination of a radical containing
175 the two glycine carbons of glyphosate, and thus the ¹³C₂ label in case of glyphosate-¹³C₂,¹⁵N,
176 resulting in a mass shift of only +1 u (**Figure 2**). Naturally occurring glyphosate-¹³C₁ yields a
177 precursor ion of the same nominal mass and both glyphosate-¹³C₂,¹⁵N and glyphosate-¹³C₁
178 form the same quantifier fragment ion at *m/z* 245, consequently resulting in an interference on
179 this mass transition for the internal standard caused by glyphosate itself. In the case of the
180 glyphosate-d₂ used in the final method, the mass of the precursor ion is shifted by +2 u,
181 eliminating this interference (see Figure S2).

182 The German External Quality Assessment Scheme for analyses in Biological Materials (G-
183 EQUAS) (<http://www.g-equas.de>) currently includes only glyphosate but not AMPA. With
184 the presented method we received certification of successful participation in the two most
185 recent G-EQUAS rounds for glyphosate (G-EQUAS 64 and 65). Additionally, with this
186 method we successfully participated in all three rounds of the HBM4EU
187 (<http://www.hbm4eu.eu>) interlaboratory comparison investigations of selected pesticide
188 biomarkers in human urine for both glyphosate and AMPA.

189 **3.2. Biomonitoring results**

190 The applicability of this method was demonstrated through the analysis of 41 spot urine
191 samples from the general German population, collected in 2016 and 2017. Glyphosate was
192 detected at concentrations at or above the LOQ in 66% (n=27) of the samples, with a median

193 of 0.09 µg/L and a maximum concentration of 0.33 µg/L. For AMPA, 90% (n=37) of the
194 samples were above the LOQ, with a median of 0.20 µg/L and a maximum of 2.54 µg/L. In
195 principle, these concentrations are comparable to levels found in a previous environmental
196 study of the German population (Conrad et al. 2017)[11]. However, due to the improved
197 sensitivity, the range of quantifiable data now is extended, allowing us to derive more
198 informative median concentrations, for glyphosate and AMPA (for glyphosate, using a
199 method with a LOQ of 0.1 µg/L would have resulted in only 24% (n=10) samples above the
200 LOQ, making this dataset unsuitable for calculating actual median concentrations). Having a
201 higher fraction of measured values should assist with further elucidating sources of exposures.

202 **4. Conclusions**

203 Method modifications resulted in a 50% lower limit of quantification for both glyphosate and
204 AMPA (0.05 µg/L) [11] without impacting performance or quality. This increase in
205 sensitivity is essential for the analysis of environmental exposures of populations, to have
206 more values above the LOQ and thus for deriving more informative median levels in human
207 biomonitoring studies in Europe. Glyphosate and AMPA have been selected as priority
208 substances within the EU-funded European Joint Programme HBM4EU illustrating the need
209 for sensitive and reliable analytical methods for both substances. This method successfully
210 passed all three rounds of the HBM4EU interlaboratory comparison investigations for both glyphosate
211 and AMPA.

212 **Declaration of Competing Interest**

213 None.

214

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325

326 **Tables and Figures:**327 **Tables:**

328 **Table 1:** Further MS parameters of the final method. Analyte retention times (t_R) and
 329 precursor and product ions with corresponding collision energies are given. Qualifier
 330 transitions in parentheses (no appropriate qualifier available for AMPA- ^{13}C - ^{15}N).

331

Analyte	t_R [min]	Precursor ion [m/z]	Product ion [m/z]	Collision energy [eV]
Glyphosate	6.8	370 (351)	245 (268)	10 (5)
Glyphosate- d_2	6.8	372 (353)	245 (268)	10 (5)
AMPA	7.9	351 (271)	268 (188)	5 (5)
AMPA- ^{13}C - ^{15}N	7.9	353	270	5

332

333 **Table 2:** Method precision data for glyphosate and AMPA. Quality control materials (Q_{low}
 334 and Q_{high}) were prepared from pooled urine samples containing native concentrations of
 335 glyphosate and AMPA.

	Glyphosate		AMPA	
	Q_{low}	Q_{high}	Q_{low}	Q_{high}
Intra-day (n=10)				
Measured conc. ($\mu\text{g/L}$)	0.19	0.85	0.38	0.90
RSD (%)	5.2	4.8	2.6	3.1
Inter-day (n=10)				
Measured conc. ($\mu\text{g/L}$)	0.21	0.83	0.42	0.90
RSD (%)	10.4	4.9	7.5	6.9

336

337 **Table 3:** Accuracy of the method for glyphosate and AMPA. Calculated as relative recoveries
 338 from eight different urine samples varying in creatinine concentration (0.4 – 2.6 g/L),
 339 analysed without spiking and spiked with 0.2 µg/L and 1.0 µg/L.

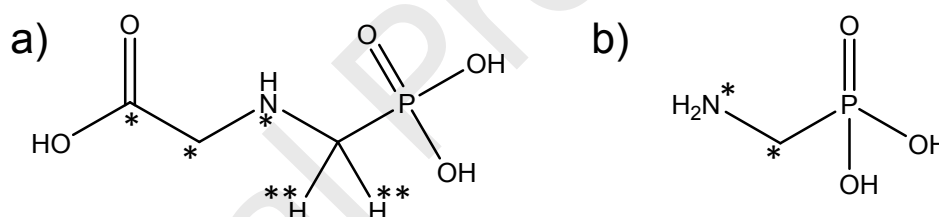
Spiked concentration (µg/L)	Glyphosate		AMPA	
	0.2	1.0	0.2	1.0
Mean native concentration measured (range) (µg/L)	0.11 (<LOQ - 0.40)		1.14 (0.15 - 3.40)	
Mean concentration measured (Σ native and spiked; range) (µg/L)	0.32 (0.22 - 0.64)	1.26 (1.13 - 1.56)	1.34 (0.35 - 3.61)	2.3 (1.34 - 4.63)
Spiked conc. calculated (µg/L)				
Mean	0.22	1.15	0.21	1.16
Range	0.17 – 0.24	0.99 – 1.24	0.18 – 0.24	0.94 – 1.23
RSD (%)	12.2	7.2	10.2	8.3
Accuracy (range) (%)	109 (83 – 122)	115 (99 – 124)	104 (91 – 120)	116 (94 – 123)

340

341 **Figures:**

342

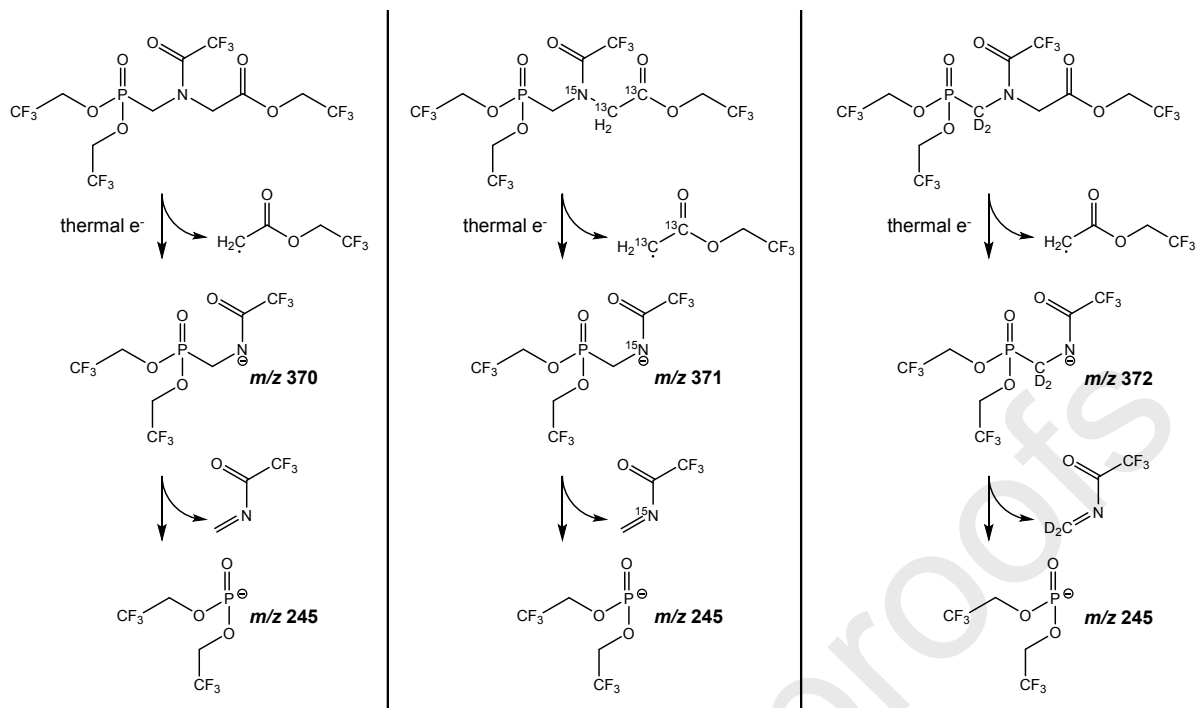
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344

345 **Figure 1:** Chemical structures of (a) glyphosate, glyphosate- $^{13}\text{C}_2,^{15}\text{N}$ (isotopic labels marked
 346 with one asterisk), and glyphosate- d_2 (isotopic labels marked with two asterisks), as well as
 347 (b) AMPA and AMPA- $^{13}\text{C},^{15}\text{N}$ (isotopic labels marked with one asterisk).

348



349

350 **Figure 2:** *Quantifier mass transitions of glyphosate (left), glyphosate-¹³C₂,¹⁵N (middle) and*
 351 *glyphosate-d₂ (right). Ionization of the internal standards by dissociative electron capture*
 352 *results in the formation of precursor ions differing from non-labelled glyphosate by +1 u*
 353 *(glyphosate-¹³C₂-¹⁵N) and +2 u (glyphosate-d₂), respectively.*

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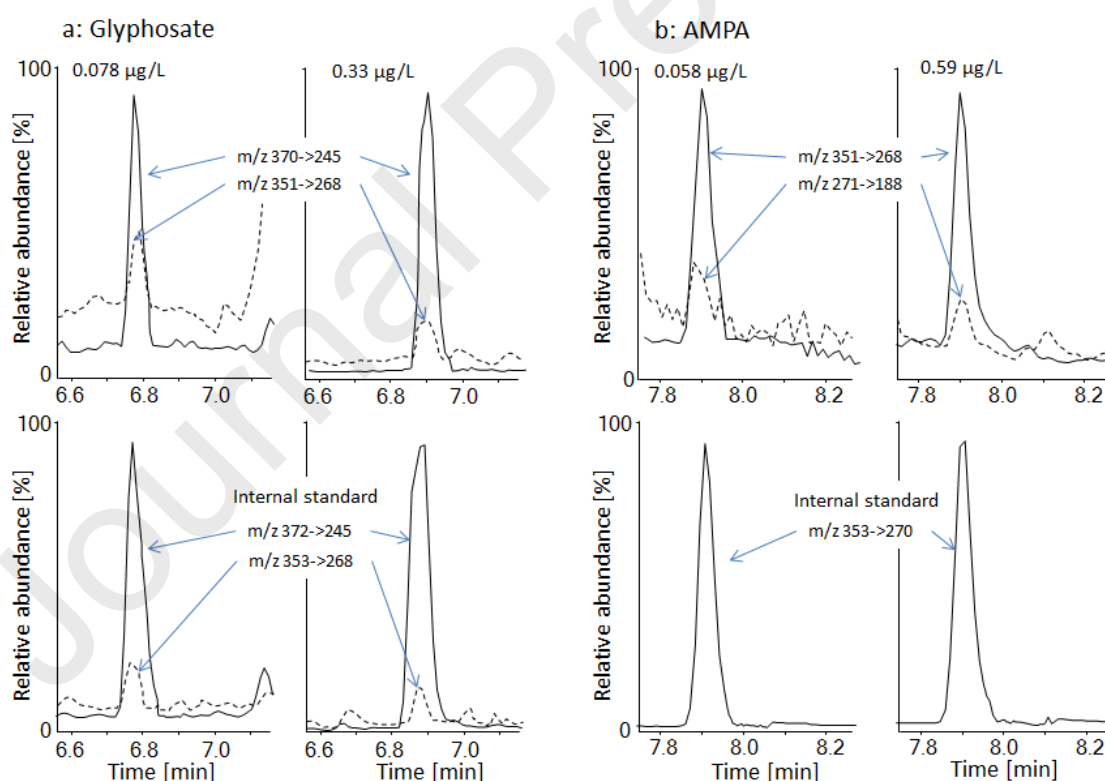
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358 **Supplementary Data**359 **Sensitive and selective quantification of glyphosate and**
360 **aminomethylphosphonic acid (AMPA) in urine of the general population by**
361 **gas chromatography-tandem mass spectrometry**362 Alison Connolly^{a,b*}, Stephan Koslitz^a, Daniel Bury^a, Thomas Brüning^a, André
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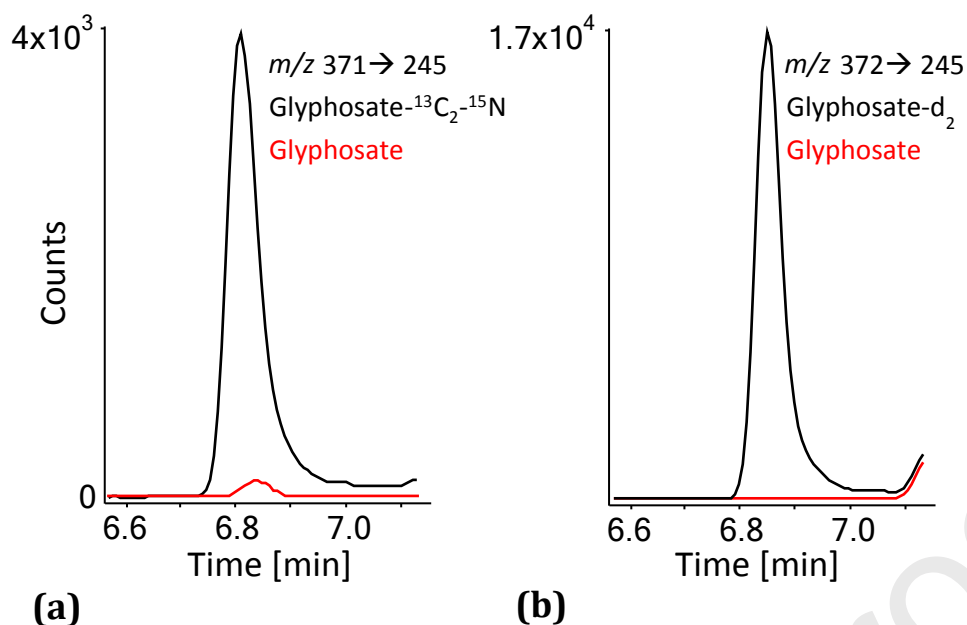
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374



375

376 **Figure S1:** Chromatograms of native urine samples showing a) glyphosate and b) AMPA.
377 The upper row shows the target analytes with respective concentrations, the lower row shows
378 the respective internal standards; quantifier transitions as continuous lines and qualifier
379 transitions in dotted lines.



380

(a)

(b)

381

382 **Figure S2:** Interference for glyphosate- $^{13}\text{C}_2$, ^{15}N caused by glyphosate. Extracted ion
383 chromatograms for the quantifier mass transitions of (a) glyphosate- $^{13}\text{C}_2$ - ^{15}N and (b)
384 glyphosate- d_2 in urine. Chromatograms of 2.5 $\mu\text{g/L}$ glyphosate (red; without internal
385 standard) showing the interference on the (a) glyphosate- $^{13}\text{C}_2$ - ^{15}N quantifier mass trace in
386 comparison to 4 $\mu\text{g/L}$ (i.e., the concentration used in the method) of glyphosate- $^{13}\text{C}_2$, ^{15}N
387 (black). No significant interference was observed for (b) glyphosate- d_2 .

388