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RESEARCH ARTICLE

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Quantification of glyphosate and aminomethylphosphonic acid from microbiome reactor fluids

Katarina Fritz-Wallace[1] | Beatrice Engelmann[1] | Jannike L. Krause[2] | Stephanie S. Schäpe[1] | Judith Pöppe[3] | Gunda Herberth[2] | Uwe Rösler[3] | Nico Jehmlich[1] | Martin von Bergen[1,4] | Ulrike Rolle-Kampczyk[1]

[1]Department of Molecular Systems Biology, Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany

[2]Department of Environmental Immunology, Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany

[3]Institute for Animal Hygiene and Environmental Health, Freie Universität Berlin, Berlin, Germany

[4]Institute of Biochemistry, Faculty of Life Sciences, University of Leipzig, Leipzig, Germany

Correspondence
U. Rolle-Kampczyk, Department of Molecular Systems Biology, Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany.
Email: ulrike.rolle-kampczyk@ufz.de

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Rationale: Glyphosate is one of the most widely used herbicides and it is suspected to affect the intestinal microbiota through inhibition of aromatic amino acid synthesis via the shikimate pathway. *In vitro* microbiome bioreactors are increasingly used as model systems to investigate effects on intestinal microbiota and consequently methods for the quantitation of glyphosate and its degradation product aminomethylphosphonic acid (AMPA) in microbiome model systems are required.

Methods: An optimized protocol enables the analysis of both glyphosate and AMPA by simple extraction with methanol:acetonitrile:water (2:3:1) without further enrichment steps. Glyphosate and AMPA are separated by liquid chromatography on an amide column and identified and quantified with a targeted tandem mass spectrometry method using a QTRAP 5500 system (AB Sciex).

Results: Our method has a limit of detection (LOD) in extracted water samples of <2 ng/mL for both glyphosate and AMPA. In complex intestinal medium, the LOD is 2 and 5 ng/mL for glyphosate and AMPA, respectively. These LODs allow for measurement at exposure-relevant concentrations. Glyphosate levels in a bioreactor model of porcine colon were determined and consequently it was verified whether AMPA was produced by porcine gut microbiota.

Conclusions: The method presented here allows quantitation of glyphosate and AMPA in complex bioreactor fluids and thus enables studies of the impact of glyphosate and its metabolism on intestinal microbiota. In addition, the extraction protocol is compatible with an untargeted metabolomics analysis, thus allowing one to look for other perturbations caused by glyphosate in the same sample.

1 | INTRODUCTION

Glyphosate is one of the most commonly used herbicides worldwide, and since the introduction of glyphosate-resistant crops in 1996 its use has dramatically increased.[1] Although possible health effects of glyphosate are vigorously discussed, there have been no credible reports of proven adverse effects of glyphosate on human and animal health.[1]

Glyphosate was originally designed and patented as an antibiotic. It inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase

Katarina Fritz-Wallace and Beatrice Engelmann contributed equally

(EPSPS), which is present in most bacteria as part of the shikimate pathway, and disrupts the production of aromatic amino acids.[2] As the shikimate pathway is present in higher plants, glyphosate was found to be an efficient general herbicide.[3] Since the shikimate pathway is absent in humans and animals, there is no mechanistic explanation for the observed effects of glyphosate on the redox status in mammalian cells[4-6] or its possible carcinogenicity.[7]

However, several microorganisms express a glyphosate-sensitive EPSPS, and thus the molecule may influence the gut microbiome of animals[8] and thereby mediate an adverse effect on a host. A recent study showed that glyphosate affects honey bees by altering their microbial community composition, and this may be a threat to bee health due to a greater susceptibility to pathogens.[9] For the mammalian microbiome, Lozano et al found a gender-specific effect on the composition of intestinal microbiota in rats[10] and Mao et al revealed effects in rats on the bacterial composition in the F1 generation.[11]

Apart from the inhibitory effect of glyphosate on EPSPS, some microorganisms are able to metabolize glyphosate. The first biodegradation route relies on the cleavage of the C&bond;P bond by carbon–phosphorus lyase, resulting in sarcosine and inorganic phosphorus. The second glyphosate degradation route is widespread and better understood. Glyphosate oxidoreductase catalyzes the cleavage of the C—N bond yielding aminomethylphosphonic acid (AMPA) and glyoxylate. While glyoxylate can be used as an energy substrate, AMPA is often exported to the extracellular space, as it can only be degraded by a few microorganisms.[12] Wang et al showed the degradation of glyphosate into amino acids in a water–sediment system,[13] suggesting a role of sediment during microbial degradation. In systems where AMPA is not further degraded, it may serve as an indicator for glyphosate degradation.[14] In order to study the effects of glyphosate on the microbiome, but also to estimate the actual exposure of the herbicide, methods for accurate quantitation in complex matrices are required.

One option for the determination of glyphosate concentrations is by enzyme-linked immunosorbent assay.[15] However, due to the higher flexibility of chromatographic methods in conjunction with mass spectrometry (MS), this approach is more widely used. The identification and quantitation of pesticides and herbicides from different matrices using gas chromatography or liquid chromatography (LC) coupled to MS have been proven to be precise[16-18] and thus useful. Due to the polar nature of glyphosate and the need to quantify small concentrations, there are reports of a variety of approaches, which all have specific advantages and drawbacks.

Chromatographic methods usually require purified samples, and therefore several extraction techniques for glyphosate and related metabolites have been developed. Due to the requirement for low detection limits, various enrichments based on solid-phase extraction (SPE) have been used.[19,20] SPE enables the enrichment of compounds from large sample volumes allowing for the detection of lower original concentrations of glyphosate. Mostly, SPE has been reported for the purification of glyphosate in a combination of different steps. However, every step in sample preparation potentially reduces reproducibility. Furthermore, as far as we know, there have been no reports of glyphosate extraction with SPE from complex bioreactor media.

One widely used method is based on the derivatization of the glyphosate molecule with fluorenylmethyloxycarbonyl chloride. The derivatization enables detection based on the fluorescence of the derivatized molecule.[21] Although LC/MS allows a more specific detection than UV, standard reversed-phase LC/MS on a C18 column of non-derivatized glyphosate cannot be applied due to the polar nature of the molecule. Hence, alternatives to the standard reversed-phase LC are needed. However, so far, literature reporting this has been sparse.[22] One alternative approach has used hydrophobic interaction LC coupled to MS for the analysis of glyphosate in various food items.[23]

Currently, glyphosate quantification methods have been established in water, soil, food products and body fluids such as urine and breast milk.[20,23-26] To the best of our knowledge, there are no studies reporting the quantitation of glyphosate from bacterial culture media used in bioreactors. Bioreactors can be used to simulate conditions found in the intestinal tract of animals and humans *in vitro*, but the media are accordingly complex. The options of *in vitro* gut fermentation models range from simple batch cultures to single- and multistage continuous flow models.[27] The latter permit a close evaluation while operating under well-defined culture conditions.[28] Environmental parameters such as temperature, anaerobiosis, pH and flow rate of the medium are closely monitored and controlled. However, changes in the microbiota community structure following inoculation are difficult to adjust.[29] When using liquid inoculation, a rapid washout of less competitive bacteria is generally experienced, restricting the experiment time to less than 4 weeks.[30]

In contrast to *in vivo* conditions, bioreactors are usually based on a homogeneous diet which realistically does not occur in microbiome hosts, especially not in humans. Another potential limitation is the missing interaction of the microbiome with the immune or neuroendocrine system of the host.[30] Nevertheless, the innovative technology of gut fermentation models facilitates a higher throughput of different conditions such as community structure and diet. In addition, exposure to other substances such as xenobiotics can be analyzed.[31] In view of social and ethical aspects, human studies are primarily limited to the analysis of fecal samples which do not necessarily reflect the community structure and function in the colon.[32]

The effect of herbicides like glyphosate on the microbiome of pigs is relevant as stock animals are exposed to higher amounts of glyphosate due to the higher maximum residue levels in animal feed. It has been reported that glyphosate concentrations in tested companion animal feed were higher than in human diets.[33] The impacts of a high exposure of pig colonic microbiota to glyphosate are currently unknown.

The aims of the study reported here were therefore (i) to develop a time- and cost-effective and reliable method for the extraction of glyphosate and its degradation product AMPA from a complex

bioreactor medium, (ii) to quantify glyphosate and AMPA at exposure-relevant concentrations from a complex intestinal medium and (iii) to be able to combine the measurement with an untargeted approach in order to allow for a high degree of multiplicity.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Acetonitrile, methanol, ammonium acetate and ammonium hydroxide were all purchased from Sigma Aldrich (St Louis, MO, USA). All solvents for MS were of analytical grade purity. Experimental water (resistivity of 18.2 MΩ cm) was purified using a Milli-Q system (Millipore, Milford, MA, USA).

Roundup® unkrautfrei LB plus (Monsanto Agrar Deutschland GmbH, PZN 024142–00), simply called "Roundup" in the following text, was used as the glyphosate-based product to be analyzed. A standard stock solution of Roundup (10 μg/mL) was prepared in Milli-Q water.

Glyphosate (*N*-(phosphonomethyl)glycine) was obtained from Glentham Life Sciences Ltd (Corsham, UK) and AMPA was purchased from Sigma Aldrich (Darmstadt, Germany). Standard stock solutions for both (10 μg/mL) were prepared in Milli-Q water and stored at −20°C. Working dilutions were prepared in Milli-Q water immediately before use.

Phosphate-buffered saline (PBS) constituents NaCl, Na_2HPO_4 and KH_2PO_4 were all purchased from Merck (Darmstadt, Germany). Complex intestinal medium (CIM) pig, CIM human and brain–heart infusion (BHI) medium were used as matrices. Chemical compositions and corresponding suppliers of these bioreactor media are included in Tables S1–S3 (supporting information).

2.2 | Bioreactor model of swine colon

Three parallel 250 mL vessels (A, B and C) of a Multifors2 bioreactor system (Infors, Bottmingen, Switzerland) were inoculated with 0.5 g of colonic bacteria from two 8- to 9-week-old German Landrace pigs on day 0 (pig 1, bioreactors A and B; pig 2, bioreactor C). The bacteria were cultivated under anaerobic conditions, with constant stirring at 150 rpm and at 37°C. The pH was kept at 6.5 by automatic addition of 1 M NaOH and an average retention time of 48 h was chosen.[30] To prevent washing out of slow-dividing bacteria, continuous cultivation was started on day 1 with a dilution rate of 0.02. The bioreactors were then run for 25 days. After ten bioreactor turnovers, the communities were considered as stable. Thus, days 20 to 22 were considered as the control phase, as the community should not change unless there are external perturbations. The treatment phase lasted from days 23 to 25 when the bioreactors were treated with 10.7 mM Roundup. Roundup was directly spiked into the bioreactor vessels, and simultaneously the medium supply was changed to a medium containing 10.7 mM Roundup.

2.3 | Sampling and extraction

For biomass determination, 1 mL of bioreactor medium was pelleted (3200 *g*, 4°C, 10 min). The supernatant was discarded and the bacteria pellets were washed twice with PBS (140 mM NaCl, 10 mM Na_2HPO_4, 7 mM KH_2PO_4). The pellets were dried completely in a vacuum concentrator (MicroCenvac NB-503CIR, Acondor) at 45°C. Bacterial dry weight was determined using a precision scale (AC 210S, Sartorius).

Samples for glyphosate measurement were taken daily at 24 h intervals on days 20 to 25. The bacterial suspension was centrifuged (5000 *g*, 5 min, 4°C) and the supernatant was stored at −80°C until sample preparation. Extraction of glyphosate and AMPA was performed by adding 1000 μL of methanol:acetonitrile:water (2:3:1) to 100 μL of specimen. Samples were vortexed for 5 min, sonicated for 5 min and finally centrifuged at 14 000 × *g* for 10 min at room temperature. The supernatant was dried in a vacuum centrifuge (Concentrator Plus, Eppendorf AG Hamburg, Germany). The dried extract was dissolved in 100 μL of Milli-Q water and immediately used for LC/MS/MS analysis. The procedure was the same for all matrices, in particular water and the bioreactor media CIM pig, BHI and CIM human.

Samples with a concentration above the upper limit of quantitation were diluted with the appropriate media.

2.4 | Replicates

For the validation of the LC/MS method, we used five repeat injections as technical replicates. In order to show the robustness of the extraction method we used three biological replicates (i.e. three different bioreactors). From each bioreactor three aliquots were extracted separately and finally each extract was measured twice.

2.5 | LC/MS/MS analysis for selected reaction monitoring (SRM) method

For the analysis of glyphosate and AMPA using LC/MS/MS, 10 μL of resuspended extract was injected onto a BEH amide column (2.1 × 100 mm, 1.7 μm; Waters, Milford, MA, USA). The autosampler was kept at 10°C and the column oven was run at 30°C. The following solvents were used for the LC program. Solvent A: 66% H_2O, 33% acetonitrile, 10 mM ammonium acetate, 0.04% ammonium hydroxide, pH 9; solvent B: 10% H_2O, 90% acetonitrile, 10 mM ammonium acetate, 0.04% ammonium hydroxide, pH 9. The LC program was performed at a constant flow rate of 0.4 mL/min. The injection volume was 10 μL. Both glyphosate and AMPA were eluted with 0% B which was initially used to equilibrate the column. Afterwards, the column was cleaned with a gradient from 0% B to 100% B within 2.5 min. Then 100% B was held for 2 min and finally there was an equilibration step at 0% B for 3.4 min. Identification and quantitation of AMPA and glyphosate were based on specific SRM

traces for both analytes measured using a QTRAP® 5500 (Sciex, Framingham, MA, USA) in negative mode electrospray ionization. The ionization source settings were as follows: ion spray voltage, −4.5 kV; temperature, 450°C; curtain gas flow rate, 35 arbitrary units; collision gas, medium; ion source gases, 40 and 60 arbitrary units. The transitions and the specific corresponding declustering potentials and collision energies are provided in Table 1. Parameters were evaluated *a priori* using flow injection analysis. Data acquisition and analysis were performed in Analyst® software (version 1.6.2, Sciex).

2.6 | Method validation

The method was applied to water as a reference matrix and to various complex bioreactor media. Standard curves for all matrices were prepared with spiked amounts of glyphosate and AMPA and with addition of known amounts of Roundup for the measurement of bioreactor samples. The standards were then extracted in the same way as described above (section 2.3).

Method validation included the measurement of specificity, linearity, limit of detection (LOD), lower limit of quantification (LOQ), accuracy and precision (intra- and inter-assay variation). The specificity of the method was evaluated by comparing a blank matrix sample and a glyphosate and AMPA spiked sample (50 ng/mL). Standard curves were generated by a linear regression ($y = ax + b$) for all four matrices. The linearity was assessed at six concentrations, 2, 5, 20, 40, 200 and 500 ng/mL ($n = 5$), in all four matrices. The signal-to-noise ratio required for LOD and LOQ determination was established using the signal-to-noise script implemented in Analyst 1.6.2 software. A time window of 30 s before the peak of interest was defined as noise and the peak itself was selected in a time window of 0.1 min and defined as the signal. LOD and LOQ were estimated by the lowest concentration of spiked sample with a signal-to-noise ratio of at least 3 and 10, respectively. The accuracy (defined as the percentage recovery) and precision (defined as intra-assay variation and inter-assay variation measured as relative standard deviation (RSD)) were calculated at different concentration levels in each matrix. We considered a recovery of 70–120% as acceptable.

TABLE 1 SRM transitions and settings for glyphosate and AMPA measurement

Q1 *m/z*	Q3 *m/z*	Time (ms)	ID	DP (V)	CE (V)
110.0	79	50	AMPA_1	−60	−60
110.0	63	50	AMPA_2	−60	−60
168.0	79	50	Glypho_1	−110	−110
168.0	63	50	Glypho_2	−30	−30
168.0	150	50	Glypho_3	−30	−30
168.0	124	50	Glypho_4	−30	−30
168.0	81	50	Glypho_5	−30	−30

2.7 | Untargeted metabolomics

Extraction of bioreactor samples for untargeted metabolomics was performed as described in section 2.3.

For LC/MS/MS measurement, 10 μL of resuspended extract was injected into a high-performance LC quadrupole time-of flight instrument (6540 UHD Accurate-Mass Q-TOF LC/MS, Agilent Technologies, Santa Clara, CA, USA). Metabolites were separated on a C18 column at a flow rate of 0.3 mL/min with the following gradient of running solvent A (0.1% formic acid in water) and running solvent B (2% isopropanol, 0.1% formic acid in acetonitrile): 0–5 min, 1% B; 5.1–20 min, 1–100% B; 20.1–25 min, 1% B. The mass spectrometer was set up in centroid mode and in screening mode allowing the detection of ions with a mass-to-charge ratio of between 60 and 1000. After each full scan the most intense ion (threshold 200 counts) was subjected to fragmentation.

For data analysis, raw files (.d) were converted to mzML files using ProteoWizard.[34] Following the principles described by Alonso et al, the spectral processing was carried out using XCMS adapted for use via Galaxy.[35-37] The workflow included a peak picking step (using the xcmsSet script) followed by a grouping step and retention time alignment (using xcmsGroup and xcmsRetcor scripts). Settings for xcmsSet script were as follows: extraction method for peak identification, centWave; maximum tolerated ppm *m/z* deviation, 25; minimum and maximum peak width, 10 and 35 s; signal-to-noise threshold, 10; minimum difference in *m/z* for peaks with overlapping retention time, 0.05. The workflow finished with a fillPeak script and CAMERA annotate. This resulted in a feature matrix that was used for statistical analysis. The peaks were filtered using a blank subtraction. Medium blanks were subtracted from corresponding samples (pure medium without Roundup was subtracted from bioreactor samples which did not contain Roundup; medium supplemented with Roundup was subtracted from bioreactor samples treated with Roundup). Normalization and calculation of statistics were done in R.

3 | RESULTS AND DISCUSSION

3.1 | Rapid extraction and measurement of glyphosate and AMPA

In order to estimate effects of glyphosate, e.g. on a microbiome, a reliable method for the extraction and quantitation of glyphosate and its degradation product AMPA is required. In Figure 1 an optimized workflow for the analysis of glyphosate is presented. We used a simple one-step extraction protocol, which facilitates fast and reproducible results. A mix of methanol, acetonitrile and Milli-Q water was added to the sample. After vortexing, sonication and centrifugation, the supernatant was dried in a vacuum centrifuge. The glyphosate- and AMPA-containing residue was reconstituted in water for measurement. Separation of compounds was carried out on an amide column using a gradient of diluted acetonitrile at basic pH. Although the compounds of interest elute during the isocratic

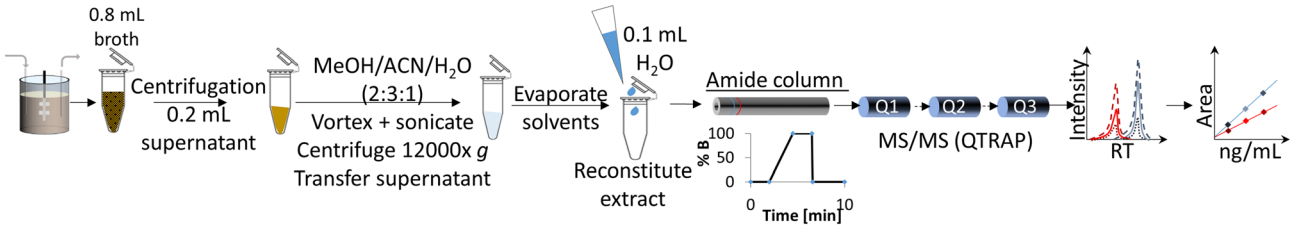

FIGURE 1 Exemplary workflow for the quantification of AMPA and glyphosate from bioreactor samples. After centrifugation of the culture broth, 100 µL of supernatant is used for the extraction with methanol, acetonitrile and water. After vortexing, sonication and centrifugation, the supernatant (containing glyphosate and AMPA) is dried in a vacuum centrifuge and finally the extract is reconstituted with 100 µL of water. AMPA and glyphosate are chromatographically separated on a BEH amide column and masses are determined with SRM using a QTRAP 5500 system (for details, see section 2)

part of the LC run, the gradient was needed to clean the column. Separation of glyphosate and AMPA was achieved (Figure 2) and unambiguous assignment of analytes due to different ion masses enabled reliable quantification.

The optimized parameters for MS/MS are described in section 2. Full-scan MS and the corresponding product ion scan for glyphosate and AMPA were performed in negative ionization mode. In full-scan MS, glyphosate produced a $[M-H]^-$ ion at m/z 168.0; the corresponding transition with the highest intensity was m/z 168.0 → 63.0 which was used for quantification. The $[M-H]^-$ ion of AMPA was at m/z 110, and the transition used for quantification was m/z 110.0 → 63.0.

3.2 | Method validation

In order to assess the applicability of this LC/MS method, standards of glyphosate and AMPA were diluted in water and analyzed. The absence of interfering peaks at the retention time of both analytes in all blank matrix samples verified the specificity of the method. A representative chromatogram of different blanks and spiked sample (in water and CIM pig) is shown in Figure S1 (supporting information). The linear response for glyphosate and AMPA ranged from 2 to 500 ng/mL in water with an average SEM of 3.15% for glyphosate and 3.69% for AMPA with four technical replicates (Figure S2, supporting information). In the studied range there was a linear correlation between intensity and concentration with R^2 values of 0.9994 and 0.9998 for glyphosate and AMPA, respectively.

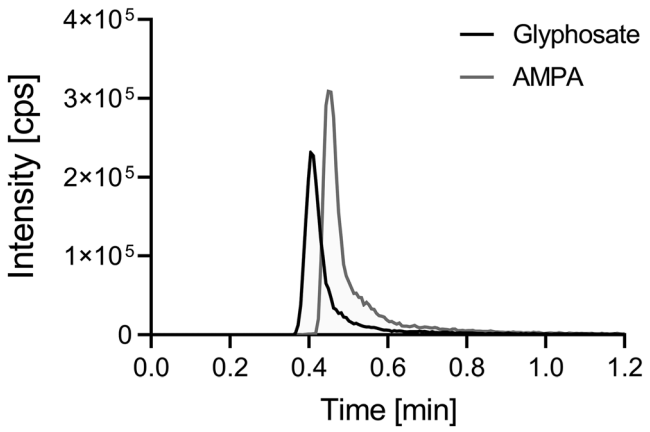

FIGURE 2 Exemplary chromatogram of the detection of glyphosate and AMPA standards with hydrophobic interaction LC/MS/MS

A common problem with the analysis of glyphosate and AMPA is the extraction from complex matrices. Standard curves for AMPA and glyphosate were prepared in water as reference and in different complex bioreactor media (CIM pig, CIM human and BHI medium). Both analytes were spiked into the media and extracted with a mix of acetonitrile, methanol and Milli-Q water. This mixture precipitates most of the proteins and at the same time enables solubilization of small polar molecules. In addition, this mixture is used for the extraction of metabolites in our untargeted metabolomics workflow. Thus, the same composition was used in order to combine untargeted metabolomics with the targeted analysis of glyphosate within the same sample. Furthermore, the drying step concentrates compounds, while omitting the need for SPE methods. The absence of interfering peaks at the retention time of both analytes in all blank matrix samples verified the specificity of the method. A representative chromatogram of both blank and spiked sample is shown in Figure S1 (supporting information).

The linearity of the glyphosate standard curves was sufficient in the range 2 to 500 ng/mL (R^2 = 1) for water, 2 to 500 ng/mL (R^2 = 0.9995) for CIM pig, 5 to 500 ng/mL (R^2 = 0.9998) for CIM human and 10 to 500 ng/mL (R^2 = 0.9991) for BHI medium. The standard curve of extracted AMPA from water showed linearity in the range 2 to 500 ng/mL (R^2 = 0.9998). Linearity was achieved from 5 to 500 ng/mL (R^2 = 0.9999) in CIM pig and from 5 to 500 ng/mL (R^2 = 0.9999) in CIM human. AMPA measurement in BHI was rather difficult showing a linearity only between 10 and 500 ng/mL (R^2 = 0.9923). Probably, the nutritional composition of BHI hinders AMPA quantification at low concentration levels. Standard curves of both analytes in CIM pig are displayed in Figure 3 and standard curves measured in CIM human and BHI medium are shown in Figure S3 (supporting information).

All calibration curves were established for five technical replicates per concentration. The focus here was on validation of the LC/MS method; thus, each sample was injected five times. LOD and LOQ, defined as the lowest concentration that can be discriminated from

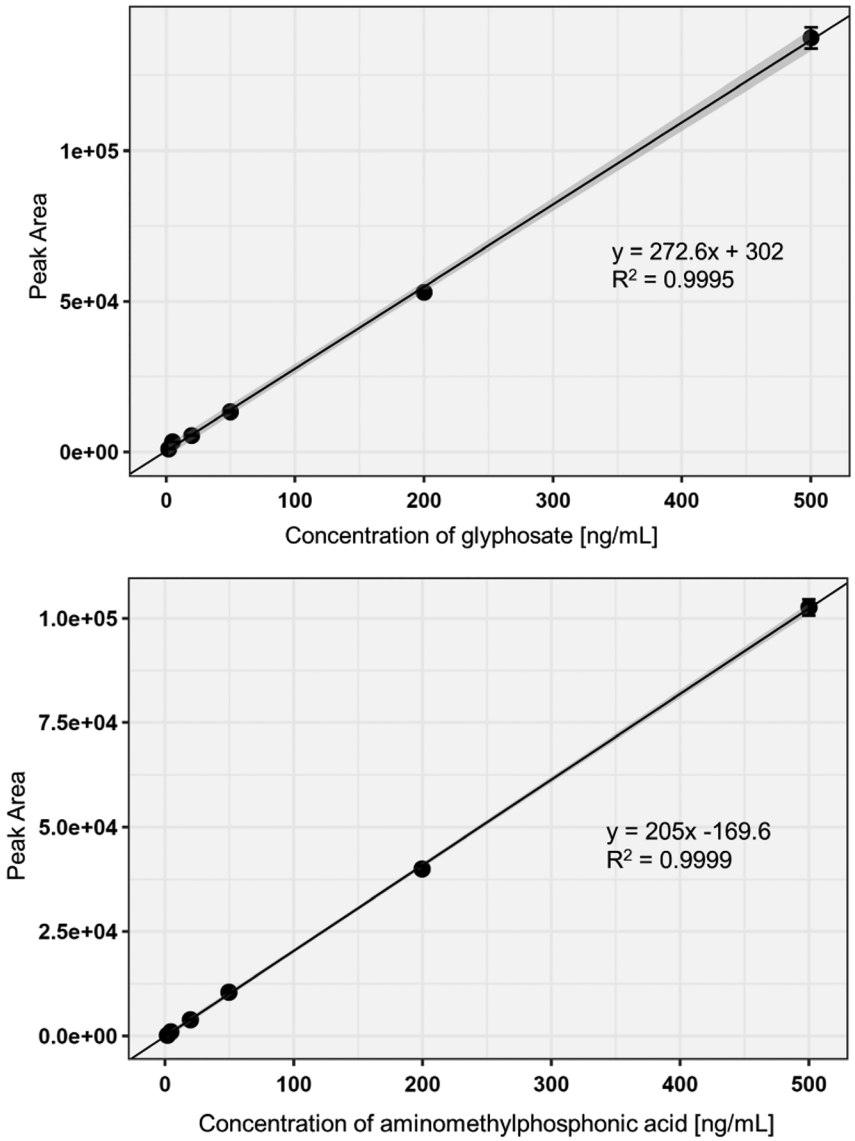

FIGURE 3 Calibration curves (n = 5) of glyphosate and AMPA in the range 2–500 ng/mL extracted from CIM pig

the background with a signal-to-noise ratio greater than 3 and 10, respectively, are presented in Table 2 for all tested matrices. As expected, LOD and LOQ were lowest for both AMPA and glyphosate when extracted from water. For glyphosate, a LOD of 0.5 ng/mL and a LOQ of 2 ng/mL were obtained. LOD and LOQ for AMPA were 0.5 and 1 ng/mL, respectively. Detection and quantification limits for glyphosate and AMPA were higher in the investigated bioreactor media (Table 2). The recovery rates are presented as mean percentages for 5 and 50 ng/mL in Table 3. For glyphosate and AMPA, the recovery rates ranged from 95.3% to 101.0% in water, from 95.4% to 227.0% in CIM pig and from 79.1% to 106.5% in CIM human. Spiked concentration of 5 ng/mL showed a recovery of 227.0% in CIM pig, failing the desired recovery. Considering the precision of the calibration curve, this measurement can be seen as an outlier.

TABLE 2 Determined LOD and LOQ of glyphosate and AMPA in all tested matrices

	Glyphosate (ng/mL)		AMPA (ng/mL)	
Matrix	LOD	LOQ	LOD	LOQ
Water	0.5	2	0.5	1
CIM	2	20	5	20
CIM human	5	20	5	20
BHI medium	10	50	10	50

Both analytes fulfilled the accuracy criterion range of 70–120% in water and two of the used bioreactor media, indicating that the presented method can be considered as reliable and reproducible. In BHI medium the signal-to-noise ratio was lower than 3 at 5 ng/mL and the recovery of glyphosate and AMPA was therefore only calculated at 50 ng/mL.

Values of RSD of less than 20% and 10% (n = 4) were defined as acceptable precisions for inter-assay and intra-assay variation, respectively. For glyphosate and AMPA, the RSD was calculated for 5 and 50 ng/mL (Table 3). In general, inter-assay variation was better

TABLE 3 Percentage recovery, intra-assay variation and inter-assay variation of glyphosate and AMPA in all analyzed matrices

Extraction matrix	Water				CIM				CIM human				BHI	
	Glyphosate		AMPA		Glyphosate		AMPA		Glyphosate		AMPA		Glyphosate	AMPA
Spiked concentration (ng/mL)	5	50	5	50	5	50	5	50	5	50	5	50	50	50
Recovery (%)	101.0	100.7	99.7	95.3	227.0	95.4	117.7	103.5	79.1	106.5	78.9	101.2	102.1	121.5
Intra-assay variation, RSD (%)	6.3	10.2	7.6	8.1	3.3	4.4	1.2	2.5	8.4	6.9	1.1	4.5	3.8	2.8
Inter-assay variation, RSD (%)	12.2	9.14	19.3	17.8	5.6	8.5	12.3	8.5	1.7	6.2	5.9	4.7	5.2	15.8

for glyphosate measurement, ranging from 1.7% to 12.3% compared with 4.7% to 19.3% for AMPA measurement across all analyzed matrices. The intra-assay variation was slightly better for AMPA measurement (1.1–8.1%) than for glyphosate measurement (3.3–10.2%). For all analyzed matrices, the RSD for inter-assay and intra-assay variation was lower than 20% and 10%, respectively (Table 3).

Previously reported approaches for detection of glyphosate include gas chromatography, high-performance liquid chromatography, ion chromatography as well as MS-coupled methods. Often, derivatization is required for analysis, thus adding a time-consuming step. In the last few years an increasing number of methods have been published omitting the tedious derivatization step.[20,38] However, pre-purification of the sample via an SPE cartridge or other enrichment strategies is often included.[20]

LC/MS/MS omits the need for a derivatization step, thus improving recovery and reproducibility. Our aim was to establish a simple extraction step without enrichment, derivatization or filtration. In order to achieve lower LOD and LOQ, direct injection methods have been applied. However, due to matrix interferences, direct injection methods are only applicable for relatively clean matrices, such as water or simple water-based matrices.[25,38,39] A glyphosate detection limit of 0.25 ng/mL has been reported in water samples without extraction but with a filtering step.[25] In urine, Sierra-Diaz et al could quantify concentrations of 0.363 μg/mL.[32] These methods are not likely to be applicable to bioreactor fluids due to their complexity and an extraction step will be needed. Our method provides this extraction step while still maintaining high recovery and reproducibility for the measurement of glyphosate and AMPA.

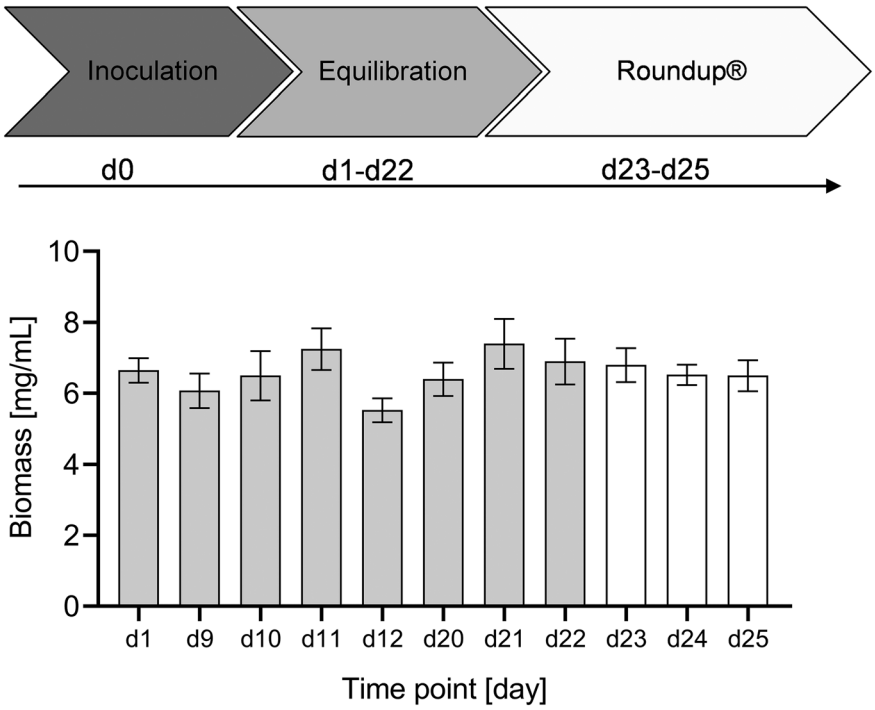

FIGURE 4 Determined mean biomass concentrations ±SEM per day are displayed. Three parallel 250 mL vessels of a bioreactor system were inoculated with 0.5 g of colonic bacteria from two 8- to 9-week-old German Landrace pigs on day 0. After 22 days of equilibration, Roundup (1.8 g/L) was spiked into the bioreactors (days 23 to 25)

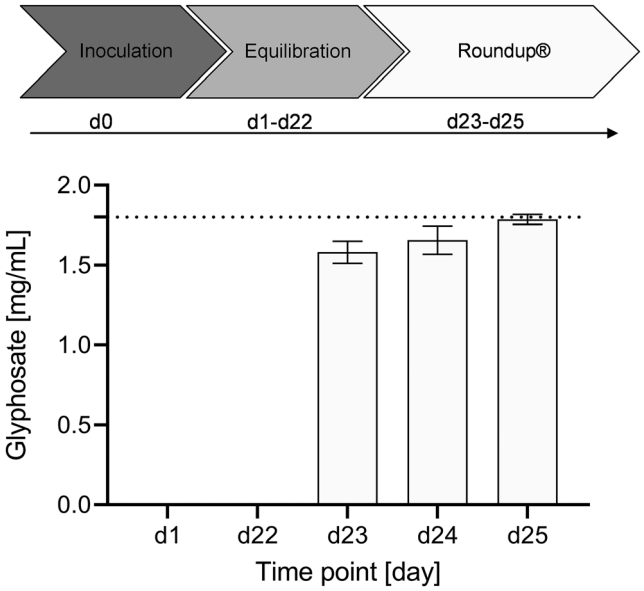

FIGURE 5 Experimental setup with measured glyphosate concentrations in bioreactor samples. After addition of glyphosate to a final concentration of 1.8 mg/mL (dotted line), a mean concentration of 1.68 mg/mL (day 23–25) was detected

3.3 | Application

In recent years, glyphosate quantification has been established in different matrices including water, soil and food.[20,23,24,26] Nevertheless, it was claimed that the current methodology for glyphosate analysis is not sufficient, stating that monitoring should be intensified.[33] Since there is an interest in the effect of glyphosate on the metabolism of intestinal microbiota, we applied the method to quantify glyphosate and AMPA in bioreactor media. The addition of Roundup to bioreactors containing porcine colonic bacteria was monitored. The bacterial communities were equilibrated for 21 days, which is equivalent to ten bioreactor turnovers. Then Roundup was spiked into the bioreactors directly after sampling and the medium supply was also exchanged to a medium including Roundup. Samples were drawn daily during the control phase (days 20 to 22) and the treatment phase (days 23 to 25). As the concentration of glyphosate added to the bioreactor was higher than accounted for by the calibration curve, samples were diluted with fresh medium before extraction to get the concentration within the linear range. Although Roundup was applied in a high concentration equal to 10.7 mM or 1.8 mg/mL glyphosate, an effect on the biomass could not be observed (Figure 4). The absence of an effect on biomass does not

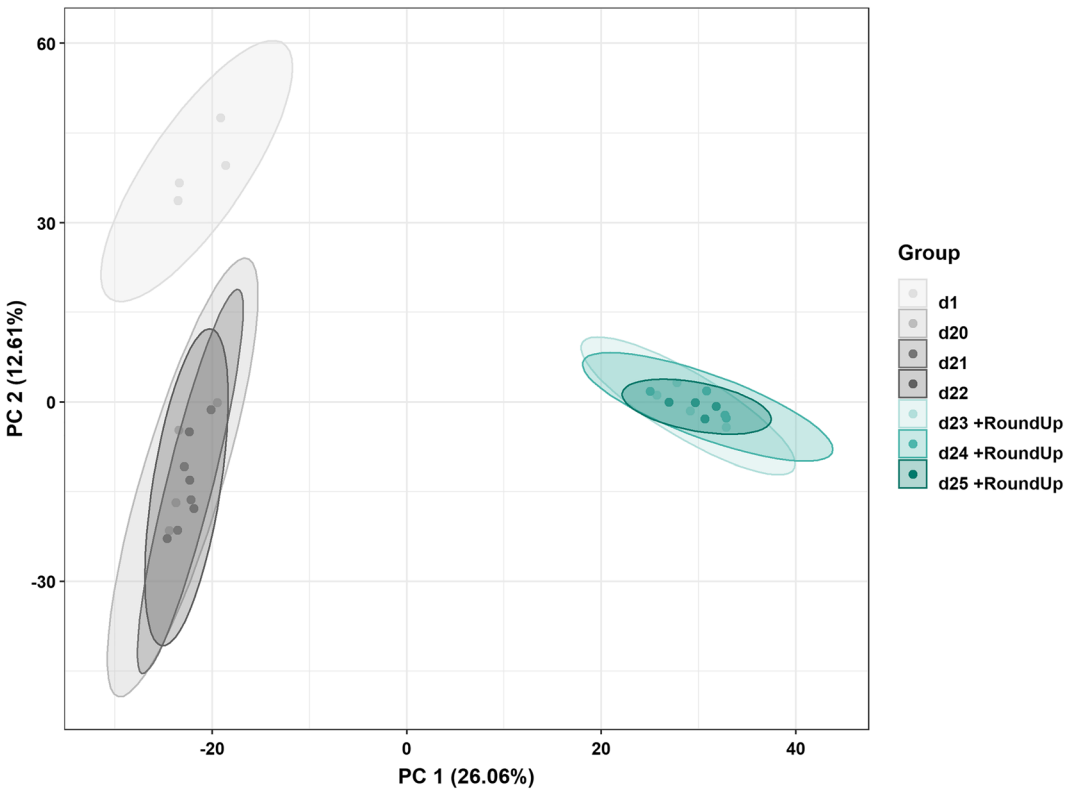

FIGURE 6 Principal component analysis of all bioreactor media samples. Each dot represents one bioreactor and color indicates the group. Each ellipse displays the 95% confidence interval per group. Performance was done using blank-subtracted and median-normalized data. Grouping is based on the day of cultivation: day 1, 24 h after inoculation; days 20–22, controls; days 23–25, Roundup addition. PC, principal component

exclude the possibility of a functional disturbance in the community. After the addition of glyphosate a mean concentration of 1.68 mg/mL (days 23–25) was detected (Figure 5), corresponding to a recovery of 93%. In the samples up to day 22 no glyphosate could be detected. AMPA was detected in the analyzed bioreactor fluid on days 23–25. However, the peak areas for AMPA were similar to those for Roundup itself, suggesting a missing metabolism of glyphosate by the intestinal microbiota during the treatment phase.

Medium complexity is a relevant topic in bioreactor cultivation because compositions of media are crucial for bacterial cultivation in batch culture and chemostat models.[40] At this point we can detect and quantify glyphosate and its main degradation product AMPA at exposure-relevant concentrations in different bacterial culture media, representative of complex culture compositions.

3.4 | Extraction method is compatible with untargeted metabolomics

Untargeted metabolomics was used to evaluate the metabolic profile of the medium before and after the addition of Roundup. Metabolic profiles of pure medium with and without Roundup were subtracted from those of the appropriate bioreactor samples to ensure that detected differences were based on the addition of Roundup itself.

Unsupervised principal component analysis of the metabolic profile was conducted to get an impression of the overall variations between the samples (Figure 6). Identification of metabolites was omitted as this was not the aim of this study, but rather to show the compatibility with the untargeted metabolomics method. Each dot represents one bioreactor medium sample and the color indicates the group. Control samples of days 20 to 22 and the samples of the days in which Roundup was supplemented to the bioreactor medium are separated based on the first two principal components. The difference between day 1 and all other later time points without Roundup was expected. In a model community, SIHUMIx takes five days to stabilize the community, and this can also be observed in the metabolic profile. Once the community is stable small perturbations like the transit time only slightly affect the community.[41,42] However, the more complex community arising from pig colon content is expected to be even more stable against external influences.

Although there was no effect on the biomass, the metabolic profile is influenced by the addition of Roundup. As Roundup is a mixture of various chemicals, the effect of glyphosate is inconclusive. However, this is not the aim of this study, but rather to show the compatibility of the two different methods. Although glyphosate could be detected in the untargeted approach, the sensitivity is not sufficient for determination of low glyphosate concentrations and thus renders necessary the targeted approach presented here.

4 | CONCLUSIONS

We present a simple method for the extraction of glyphosate and its degradation product AMPA from complex matrices such as bioreactor media. Identification and quantification were realized using a targeted LC/MS/MS method, which enables the quantification of glyphosate and AMPA at exposure-relevant concentrations. Due to the simple sample extraction and preparation procedure, the methodology allows for the possibility of robust and high-throughput detection and quantification. Negative mode electrospray ionization with SRM gives excellent sensitivity and selectivity. The establishment of the technique for other related matrices and media is still ongoing.

This extraction method is compatible with untargeted profiling of metabolites, enabling the quantification of glyphosate and AMPA and the characterization of the metabolome from the same samples, thus combining hypothesis-generating workflows with the quantification of glyphosate and AMPA. This is especially relevant, as it is still uncertain as to whether glyphosate has mechanisms of action independent of EPSPS.

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ORCID

Katarina Fritz-Wallace https://orcid.org/0000-0002-2317-9387

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