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Analysis of glyphosate and aminomethylphosphonic acid in leaves from *Coffea arabica* using high performance liquid chromatography with quadrupole mass spectrometry detection

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ABSTRACT

Glyphosate is a commonly applied herbicide in coffee plantations. Because of its non-selective mode of action it can damage the crop exposed through spray drift. Therefore, it is of interest to study glyphosate fate in coffee plants. The aim of this study was to develop an analytical method for accurate and precise quantification of glyphosate and its main metabolite aminomethylphosphonic acid (AMPA) at trace levels in coffee leaves using liquid chromatography with single-quadrupole mass spectrometry detection. The method is based on a two-step solid phase extraction (SPE) with an intermediate derivatization reaction using 9-fluorenylmethylchloroformate (FMOC). An isotope dilution method was used to account for matrix effects and to enhance the confidence in analyte identification. The limit of quantification (LOQ) for glyphosate and AMPA in coffee leaves was 41 and 111 $\mu g\,kg^{-1}$ dry weight, respectively. For the method optimization a design of experiments (DOE) approach was used. The sample clean-up procedure can be simplified for the analysis of less challenging matrices, for laboratories having a tandem mass spectrometry detector and for cases in which quantification limits above 0.1 $mg\,kg^{-1}$ are acceptable, which is often the case for glyphosate. The method is robust, possesses high identification confidence, while being suitable for most commercial and academic laboratories. All leaf samples from five coffee fields analyzed ($n=21$) contained glyphosate, while AMPA was absent. The simplified clean-up procedure was successfully validated for coffee leaves, rice, black beans and river water.

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1. Introduction

Glyphosate [*N*-(phosphonomethyl)glycine] (GLY) the active ingredient in Roundup® is commonly used in coffee plantations [1,2]. The herbicide is characterized as systemic with a broad weed spectrum, inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) as its mode of action. Plant metabolism of glyphosate is usually low [3]. Unintended spray drift is a common problem [4] and damage to the coffee plants is likely to happen as shown in studies simulating glyphosate spray drift [5,6]. Besides spray drift, root uptake is an alternative exposure pathway as demonstrated for other crops [7,8]. The low metabolic degradation could lead to an accumulation in the plant after several applications. This is a globally important economical factor as coffee is the second most traded commodity worldwide [9]. Typical symptoms for glyphosate toxication in plants are chlorosis, deformation of leaves leading to a narrow, pointy shape, increased tillering, and plant growth reduction [10–12].

Glyphosate is challenging to analyze due to its small size, high polarity, complex formation with metals [13,14], sorption to glassware [15], low solubility in organic solvents [16], absence of a chromo- or fluorophore and susceptibility to matrix effects [17]. Method interferences from matrix components like amino acids, and corresponding modified derivatives, sugars and other polar compounds are suspected [15].

Many methods have been published to analyze glyphosate and its main degradation product, aminomethylphosphonic acid (AMPA) [15,18,19]. The existing wide variety of methods also indicates the difficulties with glyphosate analysis. The ideal method needs to be easy to carry out for most laboratories, and suitable for a wide range of matrices, while being fast, robust, selective,

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accurate and precise. In general tandem mass spectrometry (MS/MS) analyzers are the systems of choice for trace pesticide analysis because of their superior selectivity and sensitivity.

Because of the high polarity of glyphosate, derivatization is needed for the most common analytical techniques like reversed phase liquid chromatography and gas chromatography to obtain sufficient column retention or volatility. The most common approach for analysis of glyphosate and AMPA consists of derivatization with 9-fluorenylmethylchloroformate (FMOC) and subsequent detection with liquid chromatography tandem mass spectrometry (LC–MS/MS) [15,20,21]. Recently, attempts have been made to analyze the herbicide without prior derivatization achieving separation from matrix component by capillary electrophoresis [22], and hydrophobic interaction chromatography (HILIC) [23–25] or ion exchange columns utilized with MS compatible conditions [26,27]. To reduce method complexity, sample alteration and time consumption; these strategies appear as attractive alternatives for the analysis of glyphosate. However, currently the direct measurement has yet not replaced the derivatization methods for several reasons. These include the need of more specialized instrumentation such as capillary electrophoresis mass spectrometry (CE-MS) [28] or an ion suppression system and additional pump [29]. Other deterring issues might have been the instability of HILIC columns and poor chromatographic reproducibility [24], column care and mobile phase composition issues [29,30], or problems with the use of isotope labeled internal standards (ISTD). Thus Chen et al. 2013 [31] observed unacceptable low accuracy and precision despite the use of $^{13}C/^{15}N$ labeled glyphosate and AMPA ISTDs; probably caused by different LC elution times of the analyte and ISTD.

In general, the isotope labeled ISTD is used to account for matrix effects and losses during sample clean-up. The isotope labeled ISTD approach is especially preferred over matrix matched standard curves for laboratories targeting a wide range of sample matrices with few samples of each matrix type.

As mentioned, MS/MS detection is generally preferable for more selective detection with low LOQs; however access to such instruments is limited, especially in coffee producing countries. Single quadrupole MS systems are a suitable compromise between MS/MS and fluorescence detectors in terms of selectivity, the possibility of using stable isotope ISTDs, price and accessibility. Additionally, methods using single MS detection can be readily transferred to MS/MS systems. However, only very few methods are available using single quadrupole MS systems. Those published for plant material and known to us, suffer from a relative high limit of detection (LOD) of 0.11 mg kg^{-1} and time consuming rotary evaporation steps combined with a derivatization reaction overnight [32] or that the FMOC derivatization step is carried out in the autosampler using the LC control program to mix the derivatization reagents [21]. The latter approach can cause needle or column clogging if insoluble side-products are formed during the derivatization reaction.

The aim of this study was to develop an easy to reproduce, robust method to analyze glyphosate and AMPA in coffee leaves, with LC- single quadrupole MS detection. Other method requirements were a limit of quantification (LOQ) below the commonly established maximum residue limit (MRL) for glyphosate of 0.1 mg kg^{-1} [33] with sufficient accuracy (recovery: 80–120%), precision ($<20\%$ relative standard deviation, RSD) and high identification confidence.

We were aiming to achieve the method demands, by the use of a stable isotope labeled ISTD to increase identification confidence via retention time and peak shape comparison, analyte/ISTD adduct ratios and the M+1 isotope peak for glyphosate. The method optimization was carried out using a design of experiments (DOE) approach that additionally provides information on the robustness of the method. A method can be considered robust, when minor changes in the procedure do not influence its performance. Besides analyzing samples from different coffee fields to verify sufficiently low LOQ, we tested omitting specific clean-up and preconcentration steps. This reduction applied for coffee leaves, rice, and black beans might lead to LOQs above 0.1 mg kg^{-1}; which, however, are suitable when such low LOQs are not necessary. For example, maximum residue limits (MRL) for glyphosate in many food commodities are above 0.1 mg kg^{-1} [33,34]. Omitting clean-up steps for the analysis of less complex samples like river water was additionally tested.

2. Material and methods

2.1. Chemicals and reagents

The analytical standards glyphosate (purity: 99.5% ± 0.5) and AMPA (99% ± 0.5) were purchased from Chemservice (West Chester, PA, USA). The ISTDs 1,2-$^{13}C_2{}^{15}N$ glyphosate (98%) and ^{13}C ^{15}N AMPA (99% for ^{13}C, 34% for ^{15}N) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). The organic solvents dichloromethane, methanol and acetonitrile were all HPLC grade, LiChrosolv® from Merck (Darmstadt, Germany). Hydrochloric acid (37%, pro analysis), formic acid (98–100%, pro analysis), boric acid and potassium hydroxide were purchased from Merck (Darmstadt, Germany). Sodium hydroxide from J.T. Baker (PA, USA), ammonium formate from Fisher Scientific (New Jersey, USA), ammonium hydroxide from Riedel-de Haën (Sweden), and the 9-fluorenylmethylchloroformate (FMOC chloride; 97%) from Sigma Aldrich (St. Louis, MO, USA).

Stock solutions were prepared by dissolving 25.0 mg powder of the analytes in 25.0 mL water. To dissolve the material, the solutions were sonicated (Elmasonic S120 H, Elma, Germany) at 40 °C; 2 × 5 min for glyphosate and 1 × 5 min for AMPA. The ISTDs were obtained in aqueous solutions. Milli Q water was used for all dilution procedures.

All FMOC solutions were prepared in acetonitrile on the same day of analysis. Borate buffer solutions consisted of 500 mM boric acid pH adjusted with 1 M NaOH to pH 9. The ammonium formate (AmFm) solutions were adjusted to pH 9 using 0.5 M NH_4OH.

To generate a standard curve seven calibration levels were prepared by derivatizing 300 μL of aqueous solutions of glyphosate and AMPA in the range of 0.02–1.5 mg L^{-1} with 300 μL 10 mM FMOC for one hour at room temperature and 300 μL borate buffer. From this solution, 200 μL were diluted with 300 μL MilliQ water and 1000 μL mobile phase (solvent A). The solutions were filtered (0.45 μm PTFE filter, Agilent, Santa Clara, CA, USA) prior to injection into the LC–MS. For the simplified method a mixture of 1.2 mL aqueous analyte solution, 1.2 mL borate buffer and 600 μL 10 mM FMOC solution was allowed to react for one hour at room temperature and subsequently 2 mL dichloromethane were added, vortex (Vortex, Scientific Industries INC, Bohemia, NY, USA) mixed, centrifuged at 4000 rpm and filtered. The range of the standard solution was 1–70 μg L^{-1}; including 13 and 4 μg L^{-1} glyphosate and AMPA stable isotope labeled ISTD respectively.

2.2. Sample preparation

The method development was partially based on work from Hanke et al. 2008 [20] and Goscinny et al. 2012 [35]. Three similar method versions were developed (Fig. 1). A full method to analyze low concentrations of glyphosate and AMPA in coffee leaves, a simplified version for samples where high detection limits are not problematic, or alternatively a MS/MS detector is available. The simplified version was tested for coffee leaves, rice grain and black

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