QUANTIFICATION OF GLYPHOSATE AND ITS METABOLITE, AMINOMETHYL PHOSPHONIC ACID, IN WUYI ROCK TEA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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Abstract. Glyphosate (GLY), which is a commonly used herbicide in tea plantations worldwide, effectively controls the spread of gramineous and broadleaf weeds. However, owing to its toxicity, the maximum residue limits of GLY in food have become increasingly stringent. In this study, we developed an analytical method for accurate and precise quantification of trace levels of GLY and its main metabolite, aminomethyl phosphonic acid (AMPA), in Wuyi Rock tea using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS). The method was based on a rapid detection kit including solid-phase extraction columns and derivatisation agents. The limits of quantification of GLY and AMPA in Wuyi Rock tea samples were 0.03 and 0.05 mg/kg, respectively. Good linear calibrations curves ($R^2 \geq 0.996$) were achieved for GLY and AMPA solutions in the concentration range 2–100 ng/mL. The recovery values of the samples spiked with 16, 80, and 160 μg/kg of the analytes ranged between 89.32% and 97.62%, and the relative standard deviations ranged between 2.02% and 3.86%. Compared with the standard method currently used to analyse tea samples in China, the HPLC–MS/MS method requires lower amounts of reagents and shorter pretreatment times, involves facile operation, is highly accurate, and presents a low detection limit. Therefore, the method is suitable for batch detection and analysis of GLY and AMPA in Wuyi Rock tea samples.

Keywords: *GLY, AMPA, HPLC-MS/MS, Camellia sinensis, derivative reaction*

Introduction

Wuyi Rock tea is made using the leaves and buds of *Camellia sinensis* (L.) O. Kuntze. This is a predominant agricultural product in the Wuyi Mountain area, which has an excellent ecological environment and rich landscape resources. As the original Oolong tea, Wuyi Rock tea has been harvested and consumed for more than 1,500 years and is renowned for its rich flavour and long-lasting fragrance, termed ‘rock charm and floral fragrance’ (Xiao, 2017). Production of high-quality Wuyi Rock tea involves complex procedures, including leaf-picking, withering, partial fermentation (which involves alternating rotation and cooling steps), fixation (enzyme inactivation), twisting, drying, sorting (removal of old leaves and tea stalks), roasting, grading, blending, and packaging (Huo et al., 2021). The manufacturing process of Wuyi Rock tea has been included in the first-batch of the China’s Intangible Cultural Heritage list and was the first tea-processing method included on the list (Chen et al., 2010; Tan et al., 2013; Guo et al., 2021).

Glyphosate [N-(phosphonomethyl) glycine; GLY], which is also known as Roundup, is an absorbent, transgenic, and broad-spectrum herbicide (Zhao et al., 2007). It is the

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most commonly used herbicide worldwide, and the production and sales volume of GLY are the highest among all herbicides (Cressey, 2015). On March 2015, GLY was classified as ‘possibly carcinogenic to humans’ (Group 2B) by the International Agency for Research on Cancer (Lyon, France), the World Health Organisation’s agency for cancer research (Feitschi et al., 2015). Consequently, GLY was banned in more than 20 countries and regions worldwide (Meftaul et al., 2020); the countries that did not ban GLY implemented more stringent residue limits. The maximum residue levels (MRLs) for GLY in tea commodities in China and the European Union (EU) are 1 and 2 mg/kg, respectively (GB 2763-2019). Moreover, the EU followed the recommendations of the European Food Safety Authority and adopted low MRLs for GLY (0.1–50 mg/kg) in 153 types of agricultural products, including tea (Ministry of Agriculture and Rural Affairs of the People’s Republic of China, 2020), in addition to a low limit of quantification (LOQ) of 0.05 mg/kg (Guillarme et al., 2010). Therefore, the methods used to detect GLY residues in tea samples should be optimised to meet these standards.

Currently, the GLY residues in tea are primarily detected via water extraction, followed by purification using a cation-exchange column, concentration, derivatisation, and detection using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) or gas chromatography–mass spectrometry (Steinborn et al., 2016; Thompson et al., 2019). The target compounds lack chromophores, ultraviolet-absorbing moieties, or fluorescent groups. Therefore, derivatisation is typically performed to improve the sensitivity of the analytical methods for GLY and AMPA (Chen et al., 2013; Catrinck et al., 2014). Owing to the complex fabrication process of Wuyi Rock tea, its biochemical components are more complex than those of other teas. The large amounts of pigments, caffeine, tea polyphenols, tannins, and other substances in Wuyi Rock tea result in strong matrix effects during pesticide residue detection (Chen et al., 2018; Zeng et al., 2017). Moreover, the complex matrix of Wuyi Rock tea affects the sensitivity and accuracy of the detection method and increases testing time and cost (Guillarme et al., 2010; Xu et al., 2021).

Therefore, in this study, we developed a repeatable and robust method for the quantification of GLY and AMPA in Wuyi Rock tea samples using HPLC–MS/MS. The optimal pretreatment conditions were determined by optimising the extraction agent, elution amount, and derivatisation time using a rapid detection kit. Moreover, the LOQ of the method was lower than the commonly established MRL for GLY (0.05 mg/kg); the method presented good accuracy, excellent precision, and high identification confidence.

Materials and methods

Chemicals and reagents

GLY and AMPA reference standard solutions with concentrations of 100 µg/mL and purities of > 98% were supplied by the Agro-Environmental Protection Institute, Ministry of Agriculture and Rural Affairs, China. HPLC-grade acetonitrile and methanol were provided by Sigma-Aldrich (Merck, Germany). Analytical-grade ammonium acetate (NH_4OOCCH_3), dichloromethane (CH_2Cl_2), monopotassium phosphate (KH_2PO_4), hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from China National Medicines Corporation Ltd. (Shanghai, China). Analytical-grade formic acid (CHOOH) was purchased from Aladdin Co. Ltd. (Shanghai, China). Rapid detection kits featuring solid-phase extraction (SPE) columns and derivatisation agents were purchased from Fujian Lanhao Biological Technology Co. Ltd. (Fujian, China). Wuyi Rock tea (Dahongpao) was purchased from a local market (Wuyishan, China). Ultrapure water was obtained in our laboratory.

Instrumentation

The HPLC–MS/MS system, which comprised a HPLC (Model, 1290) column and a triple quadrupole MS (Model 6420) instrument, was purchased from Agilent Scientific Instruments Inc. (SG). A liquid chromatography column (ZORBAX Extend-C18 2.1 mm × 100 mm × 3.5 µm) was also purchased from Agilent Scientific Instruments Inc. (SG). The injection volume was 20 µL and the flow rate of the binary mobile phase was 0.4 mL/min. A gradient method was used, as follows: 8% phase B for 0 min, decrease to 35% 6 min, linear to 95% B in 7 min, this was kept at 95% for 2 min, by increasing to 8% (starting conditions) from 9 min to 9.1 min. The system was equilibrated until 14 min. The column temperature was maintained at 40 °C. The multiple-reaction mode (MRM) MS instrument was operated in the triple quadrupole mode. The MS/MS transitions and specific transition parameters are summarised in *Table 1*. A quadrupole MS instrument with an electrospray ionisation ion source in the positive mode was used as the detector. The capillary and charging voltages were set to 3.5 kV and the flowing rate of the drying gas was set to 10 L/min at a temperature of 350 °C. The typical chromatograms of solutions containing 100 ng/mL GLY and AMPA are shown in *Figure 1*.

A centrifuge (H1650, Xiangyi, Hunan, China) was used for centrifugation. A disintegrator (FW100, Tianjin Taisite Instrument Co. Ltd., Tianjin, China), a thermostatic oscillator (THZ-98AB, Shanghai Yiheng Scientific Instrument Co. Ltd., Shanghai, China), an ultrasonicator (KQ-250BB, Jiangsu Kunshan Ultrasonic Instrument Co. Ltd., Jiangsu, China), and an electronic scale (AL204, Mettler Toledo Instrument Co. Ltd., Shanghai, China) were used to prepare tea samples.

Preparation of standard substances and reagents

Stockpile standard aqueous solutions containing 4 µg/mL of GLY and AMPA were prepared. Composite working standard solutions with concentrations of 0, 2, 5, 10, 20, 40, and 100 ng/mL were freshly prepared by diluting the standard stockpile solution with water. The acidic modifier solution was prepared by dissolving 16 g of KH_2PO_4 in 160 mL of water, followed by the addition of 40 mL of methanol and 13.4 mL of HCl. Mobile phase A was prepared by dissolving 0.154 g of NH_4OOCCH_3 and 1 mL of HCOOH in water and diluting the mixture to 1 L using water. Mobile phase B was prepared by dissolving 1 mL of HCOOH in acetonitrile and diluting the mixture to 1 L with acetonitrile.

Table 1. *Mass spectrometry data; here, CE, and Rt denote collision energy and retention time, respectively*

Compound	Monitoring ion pair	Dwell time (ms)	Fragmentor (V)	CE (eV)	Rt (min)
GLY-FMOC	391.9/88* 391.9/214	20	105 105	25 7	7.870
AMPA-FMOC	333.9/179.1* 333.9/112	20	85 85	25 15	6.595

*Quantitative ion pair

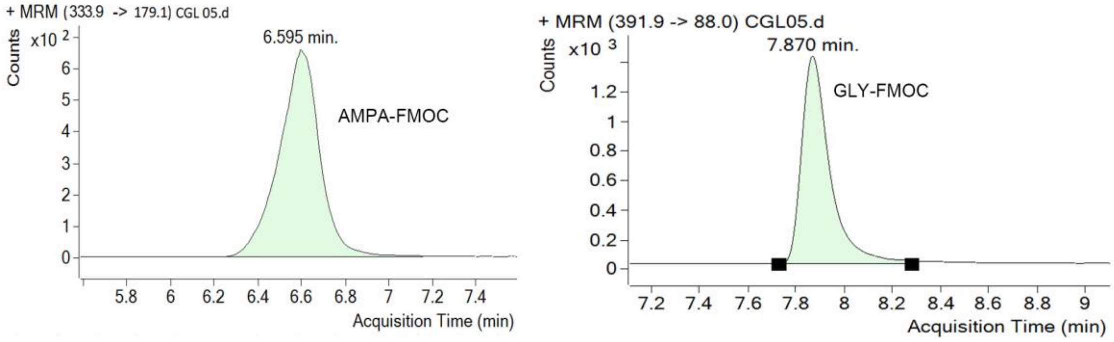

Figure 1. *Chromatograms of solutions containing 100 ng/mL GLY and AMPA*

Sample preparation and measurement

Wuyi Rock tea (200 g) was sieved through a 20-mesh sieve, mixed thoroughly, and placed in a clean container. Tea powder samples (2 g) were added to 50 mL centrifuge tubes, and 20 mL of a 0.05 mol/L NaOH solution was added to each tube. After shaking, the samples were extracted ultrasonically for 30 min and centrifuged at 8000 r/min for 5 min. Thereafter, 1.0 mL of each supernatant was collected and added to a 2 mL centrifuge tube. Acidic modifier solution (0.2 mL) was then added to each tube. After centrifugation at 16000 r/min for 5 min, the supernatant was collected and injected into the SPE column, which had been preconditioned with 2 mL of methanol and 2 mL of a 0.5% HCOOH solution. The resin in the column was saturated with eluting solution in the absence of vacuum (SN/T 1923-2007). Subsequently, the column was washed with 2 mL of a 0.5% HCOOH solution and the eluent was collected into a 5 mL test tube. After all the eluents were drained, the pH of each eluent was adjusted to 7–9 using a HCl solution. Then, water was added to each eluent to achieve a final volume of 3 mL. The as-prepared samples were maintained at room temperature and were then subjected to derivatisation.

Cleaning fluid (0.6 mL) was added to a 2 mL centrifuge tube; then, 200 µL of a 5% (v/v) borate buffer was added to the tube, and the reactants were mixed. Thereafter, 200 µL of derivatisation agent from the rapid detection kit was added to the tube. After thorough vertexing, the mixture underwent derivatisation for 10 min at room temperature. Subsequently, the derivatised mixtures were filtered through a 0.22 µm filter membrane, and the obtained solutions were used for quantitative analysis via HPLC–MS/MS.

Results and discussion

Optimisation of extraction solvent

Because GLY and AMPA are polar compounds, they are easily soluble in water and insoluble in organic solvents. Therefore, pure water (Zhang et al., 2019), water–CH_2Cl_2 mixtures (Schrübbers et al., 2016), and 0.05 mol/L NaOH solutions (Wu et al., 2015) are commonly used to extract GLY and its metabolites from samples. To achieve optimal extraction efficiency for the pesticides in Wuyi Rock tea samples, a recovery experiment was conducted to compare the results obtained using 20 mL of pure water, a mixture of 20 mL of pure water and 10 mL of CH_2Cl_2, and 20 mL of a 0.05 mol/L NaOH solution as extractants for a solution spiked with 50 µg/kg of a composite pesticide. The extraction experiments were performed in quintuplicate.

The experiment using 0.05 mol/L NaOH as the extractant yielded the highest GLY and AMPA recovery rates of 99.3% and 95.6%, respectively, and relative standard deviations (RSD) of 0.36% and 1.02%, respectively (*Fig. 2*). This was attributed to GLY and AMPA generating salts under alkaline conditions, which increased their water solubility. In contrast, tea polyphenols were more likely to be oxidised under alkaline conditions, which was equivalent to a purification step during extraction (Zhu et al., 2015).

Wuyi Rock tea is roasted during processing (Chen et al., 2010); therefore, the solutions extracted with pure water were dark coloured. The solutions purified using the SPE column still has a light colour, which lowered the recovery rates of GLY and AMPA and shortened the service life of the LC column. The recovery rates of GLY and AMPA for the extraction experiments using pure water and CH_2Cl_2 were high: 98.7% and 91.8%, respectively. However, post-extraction steps were required to recover and treat CH_2Cl_2, thereby increasing the testing time, labour cost, and cost of the experiments.

According to these results, 0.05 mol/L NaOH was selected as the optimal extractant for the Wuyi Rock tea samples.

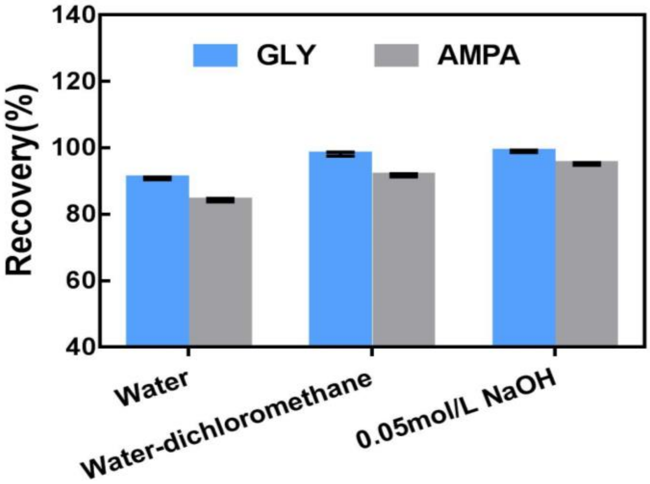

Figure 2. *Effects of water, water–dichloromethane, and 0.5 mol/L NaOH extractants on the recoveries of GLY and AMPA from Wuyi Rock tea samples at concentration of 80 µg/kg*

Optimisation of eluent volume

Currently, SPE is a commonly used pretreatment method in pesticide residue detection, and elution is a critical step of the purification process (Yan et al., 2013). The residual target compounds in SPE columns can be eluted and separated from interfering substances. The effect of the eluent volume (2, 3, 4, 5, and 6 mL) on the recovery rates of GLY and AMPA from Wuyi Rock tea samples was evaluated (*Fig. 3*). Each experiment was performed in quintuplicate.

At an eluant volume of 2 mL, the recovery rates of GLY and AMPA were 82.8% and 80.9%, respectively. At an eluent volume of 3 mL, the recovery rates of GLY and AMPA were the highest: 94.60% and 92.10%, respectively. Upon increasing the eluent volume from 3 to 6 mL, the recovery rates of GLY and AMPA decreased. This was attributed to the increase in eluent volume promoting the elution of the impurities from the SPE column and increasing the dilution ratios of GLY and AMPA in the cleaning fluid. This affected the precision of the results, especially for trace substances.

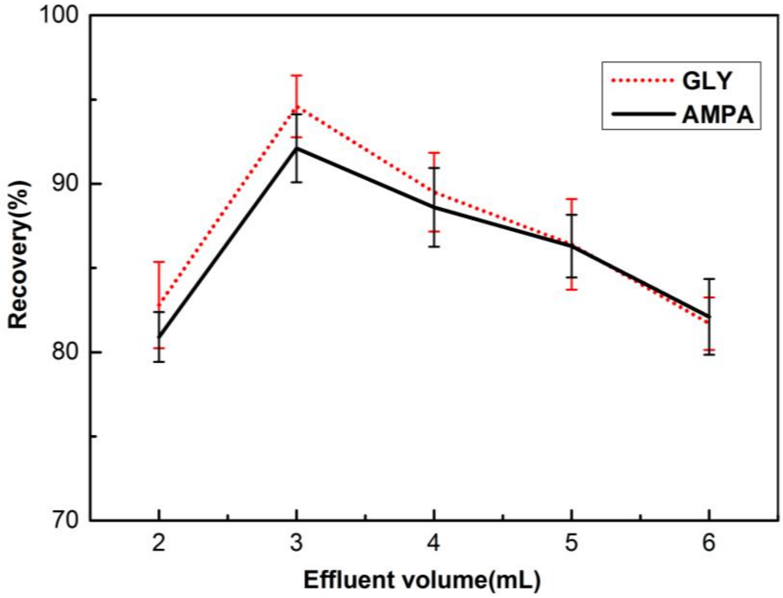

***Figure 3.** Effect of eluent volume on the recoveries of GLY and AMPA from Wuyi Rock tea samples at a concentration of 80 µg/kg*

Optimisation of derivatisation time

Because GLY and AMPA lacked necessary functional groups, HPLC–MS/MS could not be used to directly detect them. Fluorenylmethylchloroformate (FMOC) is the most common derivatisation agent for GLY and AMPA (Bernal et al., 2012; Hao et al., 2011). FMOC-derivatisation significantly lowered the detection efficiency, and previous studies have reported performing derivatisation for 1 h (SN/T 1923-2007) or 2 h (Le Bot et al., 2002) or overnight (Grey et al., 2001). The derivatisation agent from the rapid detection kit was used herein. GLY and AMPA were subjected to derivatisation for different times (5, 10, 15, 20, and 30 min), and the effect of derivatisation time on the recovery rates of GLY and AMPA was evaluated. Each experiment was performed in quintuplicate.

The results revealed that the recovery rates of GLY and AMPA increased gradually with increasing derivatisation time (*Fig. 4*). The recovery rates of GLY and AMPA were the highest at a derivatisation time of 10 min, indicating that the derivatisation reaction was completed after 10 min. Therefore, 10 min was selected as the optimal preliminary extraction time for the Wuyi Rock tea samples. The detection time for this newly developed method was significantly shorter than that of the SN/T 1923-2007 method (4 h); moreover, the detection efficiency of our method was higher than that of the SN/T 1923-2007 method.

Method validation

Validation was performed to determine whether the proposed method can be used to achieve the expected goal and whether the errors of the method were within the allowable range. Linearity in respect to a standard curve, recovery, precision, and LOQ were used as validation parameters.

The calibration curve was prepared using external standards with concentrations of 2, 5, 10, 20, 40, and 100 ng/mL. The results revealed good linearities for the recovery rates

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of GLY and AMPA with correlation coefficients (R^2) > 0.996 (*Table 2*). For the tagged recovery test, 0.1, 0.5, and 1 mL of 4 g/mL stockpile standard solutions of GLY and AMPA were added to the same Wuyi Rock tea sample to simulate recovery experiments at low, medium, and high concentrations. Each experiment was replicated six times. The recovery rates of GLY and AMPA ranged between 89.32% and 97.62% and 90.24% and 95.68%, respectively, and the corresponding RSDs ranged between 2.02% and 3.86% and 2.17% and 3.17%, respectively. These results suggested that the method was highly accurate and was appropriate for the quantification of multi-class pesticide residues in Wuyi Rock tea samples.

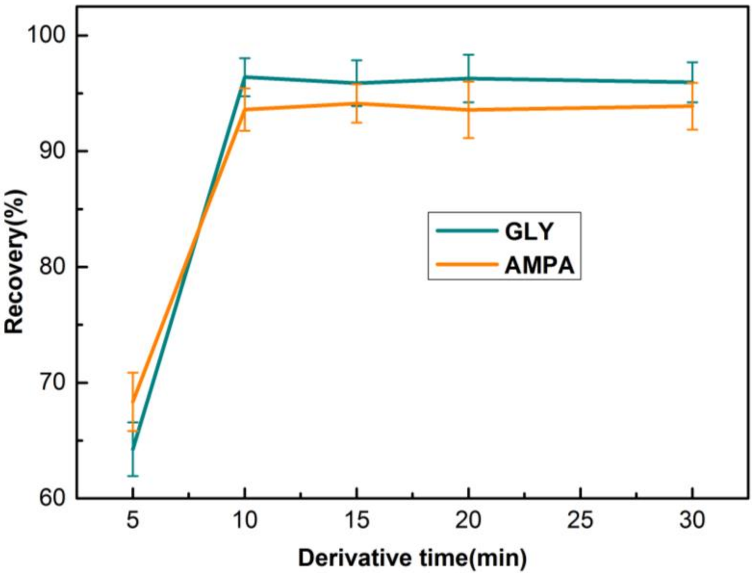

Figure 4. *Effect of derivatisation time on the recoveries of GLY and AMPA from Wuyi Rock tea samples at concentration of 80 µg/kg*

The LOQ of the HPLC–MS/MS method was estimated using spiked samples and calculated considering a signal-to-noise ratio of 10:1. The LOQs of GLY and AMPA in Wuyi Rock tea samples were 0.03 mg/kg and 0.05 µg/kg, respectively. These results indicated that the LOQ of GLY in Wuyi Rock tea samples was high. Moreover, the developed method satisfied the newly proposed EU policy, which suggested that the MRLs of GLY in many agricultural products, including tea, should be 0.05 mg/kg. Therefore, this method is suitable for determining trace amounts of GLY in Wuyi Rock tea samples.

Sample analysis

To evaluate the effectiveness of the developed method for real-life samples, seven Wuyi Rock tea samples were analysed. We used five pesticide-free samples (Dahongpao 1, Dahongpao 2, Shuixian 1, Shuixian 1, and Huangguanyin) and two pesticide-containing samples (Rougui 2 and Shuixian 2). The presence or absence of GLY and AMPA was evaluated using the Chinese industry standard ‘Determination of glyphosate residues in food for import and export HPLC–MS/MS method’ (SN/T 1923-2007) and the method developed in this study. The results are summarised in *Table 3*.

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Table 2. *Linear equations, correlation coefficients (R^2), recoveries, RSDs, and LOQs of GLY and AMPA*

Compound	**Linear equation**	**R^2**	**40 μg/kg**		**80 μg/kg**		**160 μg/kg**		**LOQ (mg/kg)**
			Recovery (%)	**RSD (%)**	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	
GLY	Y = 287.071X + 644.84	0.999	89.32	3.86	96.56	2.54	97.62	2.02	0.03
AMPA	Y = 162.54X + 537.692	0.996	90.24	3.17	94.87	2.19	95.68	2.79	0.05

Table 3. *Test results of GLY (including GLY and AMPA) in several Wuyi Rock tea samples*

Sample type	**Sample name**	**Test result (mg/kg)**	
		SN/T 1923-2007	**The method in this study**
Negative sample	Dahongpao 1	ND	ND
	Dahongpao 2	ND	ND
	Shuixian 1	ND	ND
	Shuixian 1	ND	ND
	Huangguanyin	ND	ND
Positive samples	Rougui 2	0.65 (0.05 mg/kg of GLY and 0.60 mg/kg of AMPA)	0.73 (0.65 mg/kg of GLY and 0.08 mg/kg of AMPA)
	Shuixian 2	0.23 (only AMPA)	0.22 (0.16 mg/kg of GLY and 0.06 mg/kg of AMPA)

ND denotes not detected; the LOQ was not reached

According to the tea evaluation rules (Zhang et al., 2019), the GLY content of each tea sample was calculated as the sums of the GLY and AMPA contents. The presence of GLY was not detected in any of the pesticide-free samples using either method. In contrast, the GLY content of the Rougui 2 sample was 0.65 mg/kg (0.05 mg/kg of GLY and 0.60 mg/kg of AMPA) according to SN/T 1923-2007 and 0.73 mg/kg (0.05 mg/kg of GLY and 0.60 mg/kg of AMPA) according to the newly developed method. The GLY content of the Shuixian 2 sample was 0.23 mg/kg (only AMPA) according to the SN/T 1923-2007 method and 0.22 mg/kg (0.16 mg/kg of GLY and 0.06 mg/kg of AMPA) according to the newly developed method. Although the total amounts of GLY in the pesticide-containing samples detected using the two methods were comparable, the standard before import and export processing operation was that a large amount of water was evaporated to a nearly dry state, resulting in the complete degradation of GLY by methyl phosphate and ammonia nitrogen. Moreover, owing to long pretreatment times that decreased the content of aminomethyl phosphate, the experimental results were slightly lower than those obtained using the proposed method. We believe that the method developed herein can be used to quantify GLY in tea samples. The method presents convenient pretreatment and short derivatisation times, which can effectively improve detection efficiency and lower the detection limit.

Conclusions

In this study, a new method was developed for quantifying GLY and AMPA in Wuyi Rock tea samples using triple quadrupole MS. For this method, a 0.05 mol/L NaOH

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solution was used to extract residual GLY and AMPA from tea samples, an HCl solution was used to adjust the pH to 7–9, and then an SPE column was used to purify the samples. After derivatisation, the amounts of positive ions were measured using the MS instrument in the MRM and quantified using an external standard method.

The repeatabilities and recovery rates of GLY and AMPA from the Wuyi Rock tea samples indicated that the method presented good analytical performance. Furthermore, the method was precise, and its LOQ was lower than the MRL of 0.05 mg/kg. This demonstrated that the proposed method was suitable for accurate quantification of GLY and AMPA multi-class residue pesticides in tea samples. The optimised method presented facile operation, remarkable purification performance, high work efficiency, and low cost. In future studies, we plan to optimise a similar method for the quantification of pesticides in other tea commodities (e.g. black, white, red, and green tea) collected from various locations in China.

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Conflict of interests. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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