



Determination of Glyphosate, Aminomethylphosphonic Acid and Glufosinate in Tea Samples of Russia

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A method for the determination of glyphosate (Gly), glufosinate (Glu) and aminomethylphosphonic acid (AMPA) was developed and validated in tea samples (loose leaf and tea-bag tea types) using reversed-phase high performance liquid chromatography (RP-HPLC) coupled with tandem mass-spectrometry. The sample preparation method consists of: extraction by acidified water followed by SPE on Oasis HLB, derivatization by FMOC-Cl, concentration and usage of another SPE stage on Oasis WCX. The limit of quantification (LOQ) of 0.05 mg/kg was reached for Gly and Glu; 0.1 mg/kg for AMPA. Recovery values ranged from 94% to 115% in depending of analyte and concentration level. The RSD values were ranged from 3 to 15%. The correlation coefficients were higher than 0.99. The method was applied in 2021-22 for analysis of tea samples, which revealed about 82% of positives above LOQ level and about 38% of positives with concentrations more than 0.2 mg/kg. Three samples with concentrations of glyphosate between 1-1.07 mg/kg were found. The glyphosate content in green tea was less in 3 times, in comparison with black tea.

Keywords: Glyphosate, Mass-spectrometry, Sample preparation, Tea.

INTRODUCTION

The Russian Federation was on the 4th place in the world in terms of tea consumption per person (1.38 kg per year) as per 2016 data [1]. Russia relies heavily on imports from Asia and Africa as its primary supply of tea. A tea import volume for the period May 2019 to February 2021 amounted up to 271.92 thousand tons [2], 37 thousand tons of tea was exported from Russia in the same period. The weeds are significant problem of tea plantations. Competition of weeds with tea for nutrients, water and sun, leads to decrease in yield, which can reach up to 40% and affect the taste [3]. One of the effective ways to control weeds is use of herbicides. The glyphosate, glufosinate residues can accumulate in tea leaves and to become a food chain contaminants. Glyphosate can be metabolized to methylamine, formaldehyde, aminomethylphosphonic acid (AMPA). The debate over glyphosate toxicity is still ongoing and opinions are radically different from each other. On the one hand, hepatorenal, teratogenic, tumorigenic properties are attributed to glyphosate. On the other hand, relatively high

LD₅₀ and LC₅₀ values for amphibians, hydrobionts, rodents and insects are attributed to this compound, which indicates its moderate toxicity [4-6].

The International Agency for Research on Cancer (IARC) classified glyphosate (Gly) as a potentially carcinogenic to humans (Group 2A). In accordance of a many recommendations, usage of 5%, 41% and 71% solutions of glyphosate salt diluted in water is suitable for spraying against weeds on tea plantations. The most optimal is the use of 71% ammonium salt of glyphosate in the amount of 3 kg of the active ingredient per hectare [7]. Regardless of the applied dose, the concentration of glyphosate in the soil decreases by more than 60% in 30 days [8]. A half-life of glyphosate in tea leaves is 5-8 days after treatment and residues in the leaves were found to be below 1 mg/kg. The maximum residue levels (MRL) has been established for glyphosate in tea: in the EU (Reg. (EU) No. 293/2013) is 2 mg/kg, in India (FSSAI, Compendium Contaminants Regulations 01.28.2022) is 1 mg/kg, in China, it is 1 mg/kg (GB2763-2021), in Malaysia: 0.2 mg/kg (PU(A) 437/85 Food Regulations 1985, PU (A) 200/2017), in Japan: 1 mg/kg

(Ag2006), in USA: 1 in dry tea and 7 mg/kg in instant tea (40 CFR 180.364(a)(1)), *etc.* There are no MRL for glyphosate in the Russia Federation and Kenya for this kind of product. Glyphosate was banned from 2015 till 2022 in Sri Lanka. In this work, it is described the confirmatory analysis of glyphosate (Gly), glufosinate (Glu) and aminomethylphosphonic acid (AMPA) in tea, by the derivatization technique and RP-HPLC-MS/MS methodology.

EXPERIMENTAL

Methanol (CAS 67-56-1), formic acid (CAS 64-18-6), acetone (CAS 67-64-1), acetate ammonium (CAS 631-61-8), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA- Na_2 , CAS 6381-92-6), FMOC-Cl (CAS 28920-43-6), glyphosate (CAS 1071-83-6), glufosinate ammonium (CAS 77182-82-2), aminomethylphosphonic acid (AMPA, CAS 1066-51-9), glyphosate 1,2- ^{13}C ^{15}N (CAS 1185107-63-4), 37% HCl (CAS 7647-01-0), sodium tetraborate decahydrate (CAS 1303-96-4), sodium hydroxide (CAS 1310-73-2), diethyl ether (CAS 60-29-7), ammonium hydroxide (CAS 1336-21-6), acetic acid (CAS 64-19-7) were purchased from Merck (Germany). Oasis HLB, WCX and MAX solid-phase extraction cartridges (60 mg, 3 mL), were purchased from Waters (USA). All of working standard mixtures were prepared in deionized water and stored in polypropylene tubes at 10 °C. Borate buffer was prepared as a mixture of 0.1 M NaOH and 0.05 M sodium tetraborate decahydrate (4.99/5.01 v/v).

HPLC separation was carried out on Eclipse Plus C_{18} RRHD column (50 × 2.1 mm, 1.8 μm , Agilent, USA) operated at 30 °C. Phase A: 20 mM ammonium acetate in water; Phase B: 20 mM ammonium acetate in methanol. The separation program was as follows: from 0 to 3 min – 30% B, up to 8.5

min gradient to 5% A, from 8.5 to 9.5 min – 5% A, to 10 min gradient to 30% B, column equilibration up to 14 min. The flow rate was set at 0.3 mL/min. The retention times (RT) were as follows: Gly (Gly IS) – 4.9 min, Glu – 5.7 min, AMPA – 6.6 min. The detection was performed in negative ionization mode (Table-1) using Shimadzu 8060 - Nexera X2 (Japan).

Sample preparation: Tea sample (2 g) with aliquots of standards and internal standard was mixed with 25 mL of deionized water, acidified by 0.1 mL of 37% HCl. The sample was treated on Heidolph Reax 2 shaker for about 60 min and centrifuged at 4750 rpm and 20 °C. Extract was applied for the first SPE step (Oasis HLB), as described below: cartridge was first pre-conditioned with 2 mL of methanol, 2 mL of water and 0.8 mL of extract (all to waste); then 1 mL of extract was applied on cartridge and collected into derivatization tube. For derivatization, FMOC-Cl (3 mg/mL in acetone) and borate buffer were added in to the tube with extract in relation 1/1/1. The derivatization carried out at 50 °C for 30 min. A liquid-liquid extraction by 2 mL of diethyl ether was set for FMOC-Cl and organic phase excess elimination. The extract was concentrated to 1 mL at 50-55 °C and diluted to 3.0-3.5 mL by water for next SPE step on Oasis WCX cartridge. The clean up procedure was as follows: activation - 2 mL of methanol, 2 mL of 5% formic acid in water; extract load; priming by 2 mL of 5% formic acid in water and 1.5-2.0 mL of 30% methanol and 5% formic acid in water; elution by 3 mL of 1% NH_4OH in 90% methanol. The final extract was concentrated down to 0.3 mL and diluted with 1% acetic acid in 20% methanol to 1 mL. The extract was centrifuged at 10000 rpm and 5 °C, during 20 min and used for analysis. The mass chromatograms of glyphosate (Gly), glufosinate (Glu) and aminomethylphosphonic acid (AMPA) are demonstrated on Fig. 1.

TABLE-1
DETECTION PARAMETERS

Analyte	Parent ion (m/z)	Daughter ions (m/z)	Q1 (V)	CE (V)	Q3 (V)
Gly-FMOC	390.1	150.2/124.2	17/160	23/28	14/24
AMPA-FMOC	332.1	136.2/110.2	28/15	14/10	24/21
Glu-FMOC	402.1	180.2/206.2	17/18	14/17	12/14
Gly IS-FMOC	393.1	153/126.2	11/19	23/28	16/17

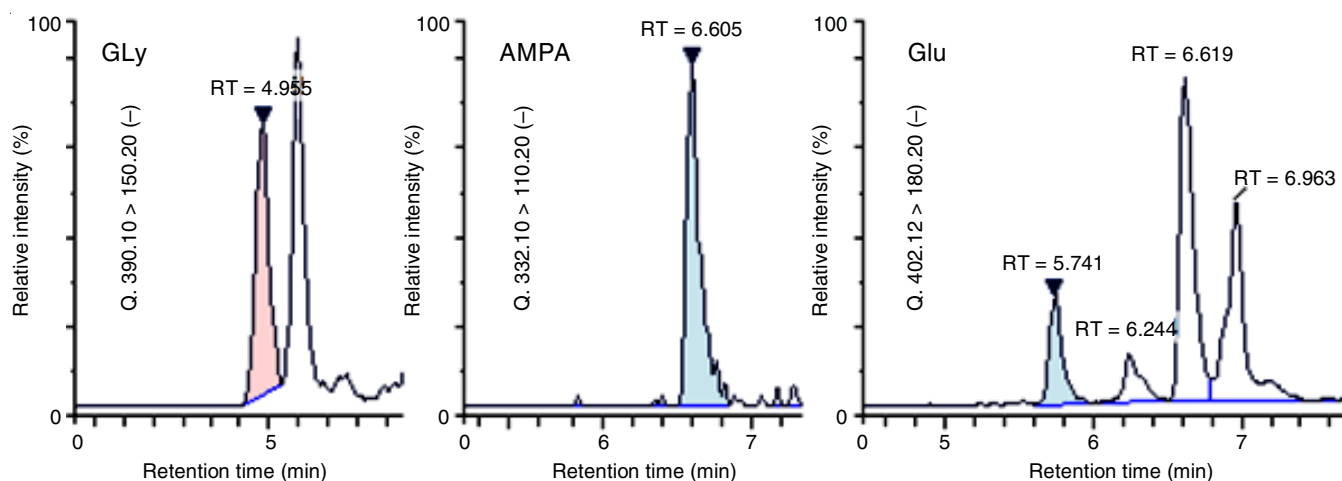


Fig. 1. Mass-chromatograms of Gly and Glu at 0.05 mg/kg, AMPA at 0.1 mg/kg level in tea

RESULTS AND DISCUSSION

Extraction stage: The dried tea is a complicated matrix, which saturate an extract with polyphenols, proteins, lignin, carbohydrates, organic and amino acids *etc.* It is very important to decrease matrix interference on the analytical signal and increase recovery of target substances. Two issues were studied for to find out the best extraction way of the analytes: extraction solvent type and extraction temperature. In case of extraction solvent, there are three ways were tested: extraction with deionized water (25 mL) – “A”, extraction with low concentrated hydrochloric acid (0.1 mL of acid per 25 mL of water) – “B”, extraction with mixture of EDTA-Na₂ and acetic acid (0.1 mL of acetic acid, 1 mL of 200 mM EDTA-Na₂ and 24 mL of water) – “C”. For each of the extraction solvents there are three calibrations point (0.05, 0.1, 0.4 mg/kg) were prepared. To 2 g of dried tea, the mixtures of standards and IS were added followed 25 mL of the extraction solvent (A, B, C). The sample preparation procedure was as described above. Solvent A gave the most coloured extracts; reproducibility of the result was very poor and relative intensity was unstable from sample to sample. The accuracy for Gly was about 56% on the first point of the calibration curve (lack of intensity), 125% on the second point, 99% on third point. There is no any linearity observed for Glu and AMPA calibration curves. Solvent C gave the colourless extracts with high relative intensity of the analytes. The accuracy for Gly was 135% on the first point the calibration curve, 79% on the second point and 100% on the third. Accuracy for Glu and AMPA was in the range of 97-104% range. The “B” solvent relative intensity of calibration points was lower than “C”, but the accuracy was better for Gly and Glu (Gly: 88-115%; Glu: 99-100%; AMPA: 88-119%). Comparison of Gly signal relative intensity is shown on Fig. 2.

The influence of temperature on analytes recovery was studied for positive samples of tea-bag and loose leaf teas. Tea sample (2 g) with aliquot of internal standard was mixed with 25 mL of extraction solvent B (65-70 °C) and the sample preparation procedure was done as described above. It was found what increase of temperature is not useful for analytes recovery in present conditions. The results for extraction at room temperature were: 0.13, 0.11 mg/kg (tea-bag tea) and 0.08, 0.074 mg/kg (loose leaf tea); for 65-70 °C: 0.10, 0.09 mg/kg (tea-bag tea) and 0.06, 0.084 mg/kg (loose leaf tea).

Cleanup stage: A general objective was to obtain a clean 1 mL aliquot of tea extract for derivatization step. For this, there are next sorbents were tested: Oasis HLB, Oasis WAX, Oasis MAX. Solvent B based tea extract was mixed with an aliquot of standards and loaded on the sorbents. The Oasis HLB cleanup protocol was as described above. Oasis WAX and MAX protocols were as follows: 2 mL of methanol, 2 mL of 25 mM ammonium formate, 1 mL of sample extract, 2 mL of 50% methanol in water, elution of the analytes with 2 mL of methanol/NH₄OH (95/5 v/v) [9]. The cleaned extracts were evaporated till 0.05 mL and reconstituted in water for derivatization step, as described above. Oasis HLB was found as the most suitable for cleanup to obtain a good derivatization results for all analytes. Oasis WAX shown the next percentages of loses: 34, 73 and 90 for Gly, AMPA and Glu, respectively. The maximum of loses were registered on the loading step. Oasis MAX shown the next percentage of loses: 100, 97 and 99 for Gly, AMPA and Glu, respectively.

Validation: The next variable parameters were chosen: matrix (2 per type: tea-bags/loose leaf tea), analysts (at the sample preparation stage) and storage time (immediate injection and overnight storage at 10-15 °C). The correlation coefficients of calibration curves were above 0.99 during validation experiments. The typical equation of calibration curves, their correlation coefficients (R), relative standard deviation and recovery of the analytes are shown in Table-2.

Method application: The method was applied for analysis of 57 tea samples available in mass-market of low- and mid-price segments. The tea samples were collected in 2021-2022 period and consisted of 31 brands of 18 manufacturers; 35 samples were tea-bags tea (29 black, 6 green tea) and 22 samples were loose leaf tea (17 black, 5 green tea). Some brands were represented by several samples: different lots, types, classes (tea-bags tea/loose leaf tea/black/green) or taste varieties (for example: tea-bags of Golden Ceylon, Kenian Sunrise, *etc.*). All of numerical values above LOD were taken into account to compile a general picture of tea contamination. The traces or residual amounts of Gly were found in all tea samples. The average Gly content in tea is 0.228 mg/kg, with maximum value of 1.07 mg/kg. The maximum measured value among loose leaf tea samples is 1 mg/kg (black tea), minimum value is 0.01 mg/kg (LOD) and average content is 0.14 mg/kg. The maximum measured value of Gly among tea-bag tea samples

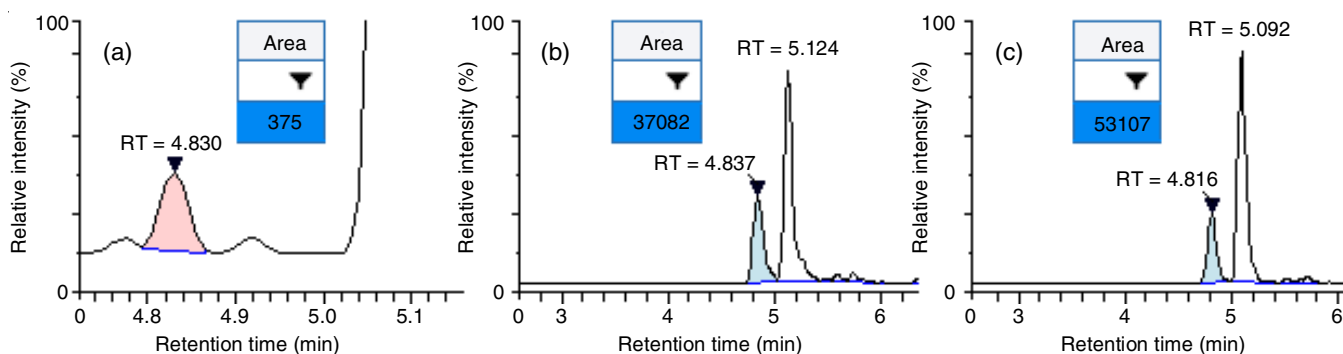


Fig. 2. Mass-chromatograms of Gly (0.05 mg/kg) extracted with different extraction solutions: (a) deionized water, (b) acidified deionized water, (c) EDTA-Na₂ with acidified deionized water

TABLE-2
CALIBRATION CURVES PARAMETERS AND ANALYTES RECOVERY DATA

Analyte	Equation	R	R ²	LOD/LOQ
Gly-FMOC	$Y = 0.2299750x + 0.01268244$	0.997	0.994	0.01/0.05
Glu-FMOC	$Y = 0.9108719x - 0.005294416$	0.998	0.996	0.01/0.05
AMPA-FMOC	$Y = 0.06350909x - 0.0005044757$	0.998	0.996	0.05/0.1

Concentration (mg/kg)	Analyte		
	Gly-FMOC (RSD/MR, %)	Glu-FMOC (RSD/MR, %)	AMPA-FMOC (RSD/MR, %)
0.05	4.90/115.5	13.41/108.7	Not tested
0.1	8.20/101.0	6.40/97.50	15.63/108.12
0.4	5.98/101.5	8.03/101.8	9.27/98.78
1.0	4.50/102.8	5.05/96.72	12.45/94.65
2.0	5.92/102.7	4.83/98.75	12.55/100.0
5.0	3.30/101.5	5.19/102.47	8.87/106.4

is 1.07 mg/kg (black tea), minimum value is 0.038 mg/kg (less than LOQ) and average is 0.28 mg/kg. AMPA was not detected; Glu was detected in tea-bag tea sample (black tea) - 0.33 mg/kg, together with Gly value - 0.69 mg/kg.

It was found that the average content of Gly in green tea was less than in black tea 0.083 against 0.26 mg/kg. The maximum value of Gly content in green tea (tea-bag tea) is 0.215 mg/kg. Detection rate of Gly in the concentration range 0.01-0.05 mg/kg were about 17%. Gly was detected in 82.45% of cases above 0.05 mg/kg level. 22 samples were found did not compliant Malaysian standards (> 0.2 mg/kg), inside of this 3 samples with Gly concentrations above or equal to 1 mg/kg. It was found a high results convergence between tea batches: 0.37 and 0.39 mg/kg for tea-bag tea, 0.6 and 0.66 mg/kg for loose leaf tea (Table-3).

Conclusion

A sensitive analytical method for the estimation of glyphosate (Gly), glufosinate (Glu) and aminomethylphosphonic acid (AMPA) in tea was developed. The validation results showed that the method was accurate enough, and that the working range was

within the limits set by most countries for Gly MRLs. The results of tea market screening showed a moderate low level of contamination. In comparison with data from China (8.9%), Gly was found in 5.26% of cases (> 1 mg/kg); the percentage of tea contamination with Glu was almost the same (1.75 in this work, *versus* 1.8) [10]. The all of maximum and average values also were lower. Nevertheless, using loose leaf tea at least 5 times a day, the consumer's body in Russia can to receive about 1.4 µg of Gly, if 2 g of tea was brewed per cup (the average weight of 1 tea-bag tea). At the similar consumption of tea-bag tea - 2.8 mkg per day. However, these concentrations will be lower when drinking green tea.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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TABLE-3
RAW DATA OF MASS-MARKET TEA RESEARCH

Packaging type	Tea type	Measured value (mg/kg)	Packaging type	Tea type	Measured value (mg/kg)	Packaging type	Tea type	Measured value (mg/kg)
Loose leaf	Black	*0.010	Loose leaf	Black	0.212	Tea-bag	Black	0.186
Loose leaf	Black	*0.015	Loose leaf	Black	0.788	Tea-bag	Black	0.190
Loose leaf	Black	*0.020	Loose leaf	Black	1.000	Tea-bag	Black	0.210
Loose leaf	Black	*0.023	Tea-bag	Black	*0.038	Tea-bag	Black	0.210
Loose leaf	Black	*0.045	Tea-bag	Green tea	*0.045	Tea-bag	Green tea	0.215
Loose leaf	Green tea	*0.048	Tea-bag	Black	0.050	Tea-bag	Black	0.220
Loose leaf	Green tea	*0.049	Tea-bag	Green tea	0.050	Tea-bag	Black	0.230
Loose leaf	Green tea	*0.0495	Tea-bag	Black	0.053	Tea-bag	Black	0.248
Loose leaf	Black	0.050	Tea-bag	Black	0.070	Tea-bag	Black	0.260
Loose leaf	Green tea	0.0508	Tea-bag	Black	1.070	Tea-bag	Black	0.300
Loose leaf	Black	0.054	Tea-bag	Black	0.080	Tea-bag	Black	0.374
Loose leaf	Green tea	0.056	Tea-bag	Green tea	0.0848	Tea-bag	Black	0.376
Loose leaf	Black	0.057	Tea-bag	Green tea	0.0857	Tea-bag	Black	0.382
Loose leaf	Black	0.060	Tea-bag	Black	0.106	Tea-bag	Black	0.430
Loose leaf	Black	0.060	Tea-bag	Black	0.120	Tea-bag	Black	0.478
Loose leaf	Black	**0.069	Tea-bag	Black	0.138	Tea-bag	Black	0.600
Loose leaf	Black	0.0806	Tea-bag	Black	0.160	Tea-bag	Black	0.690
Loose leaf	Black	0.138	Tea-bag	Black	0.180	Tea-bag	Black	0.690
Loose leaf	Black	0.209	Tea-bag	Green tea	0.185	Tea-bag	Black	1.050

* - below LOQ; ** - Glu 0.33 mg/kg

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