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LC-MS/MS Analysis of Glyphosate, Aminomethylphosphonic acid and Glufosinate in Honey

A.V. SOROKIN*, and V.V. OVCHARENKO

Federal State Budgetary Institution "The Russian State Center for Animal Feed and Drug Standardization and Quality" (FGBU "VGNKI"), 5 Zvenigorodskoe Highway, 123022, Moscow, Russia

*Corresponding author: Fax: +7 499 2531491; Tel: +7 495 9825084; E-mail: alex_sorokin@list.ru

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A confirmatory method was developed and validated for determination of glyphosate (Gly), glufosinate (Glu) and aminomethylphosphonic acid (AMPA) in honey using reversed-phase high performance liquid chromatography (RP-HPLC) coupled with tandem mass-spectrometry (MS/MS). The sample preparation approach consists of extraction by acidified solutions followed by SPE on Oasis HLB, derivatization by FMOC-Cl, concentration and another SPE stage on Oasis WCX. The limit of quantification (LOQ) of 0.05 ppm was reached for Gly, AMPA and Glu. All accuracy values ranged from 85% to 110% for all analytes using both primary and secondary quantitative ion transitions (RSD ≤ 10%). Correlation coefficients were higher than 0.99. The method was applied in 2018-19 for monitoring of honey samples, which revealed agricultural territories with intensive use of glyphosate.

Keywords: Glyphosate, Aminomethylphosphonic acid, Glufosinate, Mass-spectrometry, Herbicides, RP-HPLC, Honey.

INTRODUCTION

Controlling weeds is a major problem of the modern agriculture. Uncontrolled spread of weeds can lead to a decrease in the world yield of most agricultural crops by 34% [1]. One of the effective ways to control weeds is the use of herbicides. Glyphosate (Gly) and glufosinate (Glu) are broad-spectrum herbicides widely used for weed control on crops fields [2]. At present time, the International Database of Herbicide-Resistant Weeds contains about 300 records, including *ca*. 50 plant species with resistance to Gly [3]. The applicant countries are USA, Brazil, Argentina, Canada, Paraguay and Bolivia (by decreasing the number of entries per country and genetically modified crops production). The ranked range of weeds, according to the decrease in the precentage contribution of each species to the total number of records, is as follows: *Amaranthus palmeri* (13.3) > *Conyza canadensis* (12.7) > *Amaranthus tuberculatus* (8.8) > *Lolium perenne* ssp. *Multiflorum* (7.6) > *Ambrosia artemisiifolia* (6.2) > *Ambrosia trifida* (5.0) > *Kochia scoparia* (5.0) > *Lolium rigidum* (5.0), the rest contribute less than 5%. Over time, it became clear that Gly is able to accumulate in plants. Genetically modified crops (GM crops) have the highest capacity and ability to transfer Gly up the food chain [4,5]. Intensive use of the herbicides leads to crops contamination not only by original agents, but also by their degradation products. It is known that Gly can be metabolized down to sarcosine under the influence of *Arthrobacter atrocyaneus*, *Enterobacter aerogenes*, *Lysinibacillus sphaericus*. Also, Gly can be metabolized down to methylamine and formaldehyde under the influence of *Geobacillus caldoxylosilyticus* T20 and *Flavobacterium* sp. [6,7]. Aminomethylphosphonic acid (AMPA) is the major Gly metabolite [8] (Fig. 1).

Due to their main role in the pollination process, honey bees may be contaminated by the herbicides applied to flowering plants. This leads to the contamination of apiculture products by glyphosate (Gly), glufosinate (Glu) and aminomethylphosphonic acid (AMPA) residues [9]. There are maximum residue levels (MRLs) established in the European Union (EU) for Gly and Glu residues control in honey at 0.05 ppm (Reg. (EU) No. 293/2013 Applicable from: 06/04/2013 and Reg. (EU) 2016/1002 Applicable from: 14/01/2017). In Russia, the herbicide residues in products of animal origin are forbidden, according to the Technical Regulation of the Customs Union TR CU 021/2011.

It is considered that Gly has moderate toxicity. The lethal concentration (LC_{50}) for amphibian species *Crinia insignifera*,

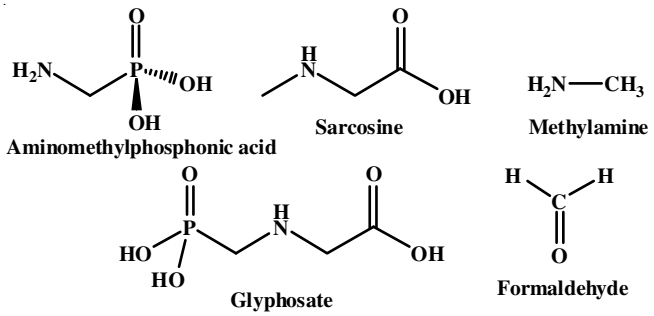

Fig. 1. Structures of glyphosate and it metabolites

Heleioporus eyrei, *Limnodynastes dorsalis*, *Litoria moorei*, ranged from 81.2 to 121 mg/L, when exposed to technical Gly. Touchdown (a Gly-based formulation) applied to these species within 48 h, had LC_{50} from 27.3 to 48.7 mg/L [10]. The LC_{50} for *Prochilodus lineatus* exposed by Roundup was 13.7 mg/L for 96 h [11]. The LC_{50} for *Cyprinus carpio* under the influence of technical Gly was 620-645 mg/L within 48 h [12]. In humans, roundup has been reported to cause problems during pregnancy, as evidenced by its effect on a human placental cell line [13]. A cytotoxic effect, which, in the long term, can lead to cancer, was observed during the treatment of the buccal epithelium cell line [14]. The International Agency for Research on Cancer (IARC) classified Gly as a "potentially carcinogenic to humans" (Group 2A).

Glyphosate (Gly), glufosinate (Glu) and aminomethylphosphonic acid (AMPA) are "difficult analytes" due to their structures and properties. There are several ways for their determination *e.g*. HPLC methodology with derivatization approach and fluorimetric detection, GC and GC-MS methodology with derivatization and HPLC-MS. The use of HPLC-MS technique gives a choice between derivatization approach or direct analysis, on ionic or HILIC chromatography columns. In this article, the selective confirmatory analysis of Gly, Glu and AMPA by derivatization approach technique is described.

EXPERIMENTAL

All chemicals used in this study *e.g*. methanol (CAS 67-56-1), acetonitrile (CAS 75-05-8), formic acid (CAS 64-18-6), acetone (CAS 67-64-1), ammonium acetate (CAS 631-61-8), 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-$^{13}C2$ ^{15}N (CAS 1185107-63-4), hydrochloric acid 37% (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 MCX solid-phase extraction cartridges (60 mg, 3 mL) were purchased from Waters (USA).

Detection method: HPLC separation was carried out on Eclipse Plus C_{18} RRHD column (50 × 2.1 mm, 1.8 µm) from Agilent (USA). Phase A consists of 10 mM ammonium acetate in water, while phase B consists of 10 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. Flow rate 0.3 mL/min, temperature 30 ºC. Detection was performed on QTRAP 6500 (Sciex, CIIIA) in negative ionization mode (Table-1).

Sample preparation: Honey sample (1 g) mixed with standards and glyphosate (Gly) internal standard was dissolved in 4 mL of warm water, acidified with 0.026 mL of HCl. The sample was then treated for about 10 min on ultrasonic bath and centrifuged at 4750 rpm and 20 ºC. Clear extract was applied for the first SPE step, as described below. The extract was precleaned before derivatization on Oasis HLB (60 mg, 3 mL) cartridge as follows: 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 and collected into derivatization tube. For derivatization, FMOC-Cl and borate buffer (pH 10-10.5) were added in to tube with extract in relation 1/1/1. Derivatization carried out at 50 ºC for 30 min. Liquid-liquid extraction by diethyl ether was set for FMOC-Cl and organic phase excess elimination. Extract was concentrated to 1 mL at 40-50 ºC and diluted to 3 mL by water for next SPE step on Oasis WCX (60 mg, 3 mL) cartridge. 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 mL of 30% methanol and 5% formic acid in water; elution by 3 mL of 0.5% NH_4OH in 90% methanol. Clean extract was concentrated down to 0.3 mL and reconstituted with 1% acetic acid in water to 1 mL. Final extract was centrifuged at 4750 rpm and 10 ºC, during 20 min and used for analysis. The mass-chromatograms of glyphosate (Gly), glufosinate (Glu) and aminomethylphosphonic acid (AMPA) are demonstrated on Fig. 2.

RESULTS AND DISCUSSION

Sample preparation: Correct selection of an extraction solution is a very important step. Into honey blank samples, an aliquots mixture of standards were added at 0.5 ppm level of each analyte. The extraction solutions (4 mL) were added after several minutes of waiting, for aliquots absorbing by sample surface. The extraction solutions (ES) were as follows: ES1 – 0.1% of formic acid in methanol, ES2 – water, ES3 – water acidified by 26 mkL of hydrochloric acid, ES4 – water acidified

TABLE-1
MS/MS DETECTION PARAMETERS

Analyte	Parent ion (*m/z*)	Daughter ions (*m/z*)	RT (min)	DP (V)	CE (V)	CXP (V)
Gly-FMOC	390.1	150.2/124.2	4.7	-95/-40	-30/-32	-10/-8
AMPA-FMOC	332.1	136.2/110.2	6.3	-107/-111	-11/-20	-12/-17
Glu-FMOC	402.1	180.2/206.2	5.5	-55/-73	-17/-20	-4/-12
Gly IS-FMOC	393.1	153/126.2	4.7	-95/-114	-30/-41	-10/-12

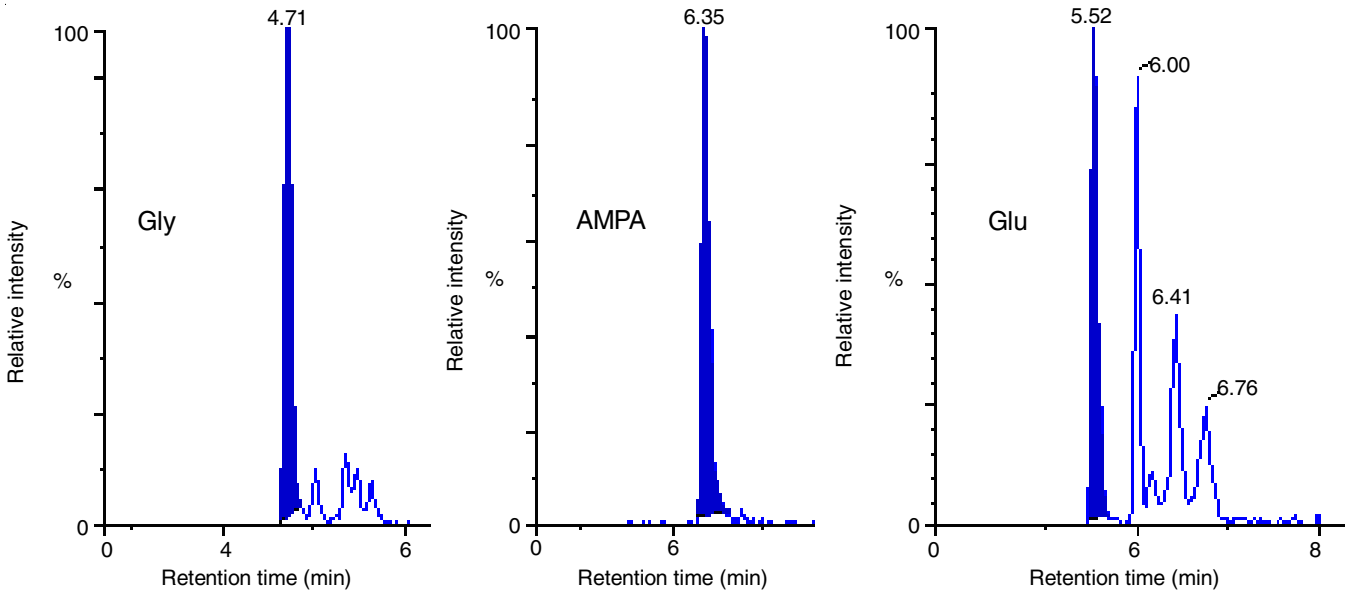

Fig. 2. Mass-chromatogram of Gly, AMPA and Glu at 0.05 ppm level in honey

by 50 mkL of hydrochloric acid. All experiments produced clean and transparent colour, there were no precipitate found during centrifugation and evaporation steps. It was found that water acidified by 26 mkL of hydrochloric acid (ES3) allows to achieve the best extraction efficiency in comparison to other experiments (Fig. 3).

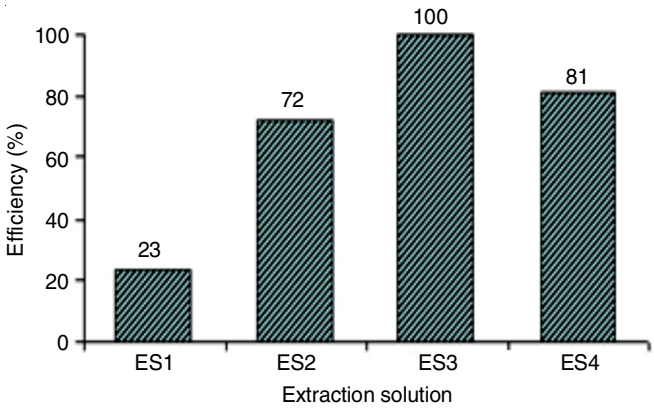

Fig. 3. Comparison of relative extraction efficiency for honey. As a sum of Gly, AMPA, Glu Areas per ES

Different SPE sorbents were analyzed in this work. Oasis HLB was tried as a derivatization precleaning step (SPE1), in comparison with null cleaning approach (SPE0). For final clean-up stage, Oasis HLB (SPE2), MCX (SPE3) and WCX (SPE4) were tested. For final clean up stage the procedure was as follows: activation - 2 mL of methanol, 2 mL of 5% formic acid in water; extract load; priming by 3 mL of water; elution by 3 mL of 0.5% NH_4OH in methanol. The sum of Gly, Glu and AMPA areas, converted in to percents, were compared between experiments as their efficiency (Fig. 4). The use of null cleaning approach (SPE0) before derivatization and Oasis HLB (SPE2) as a second cleanup step allows to determine Gly, Glu and AMPA, but with lower accuracy of calibration curves and probably, LOQ will be higher.

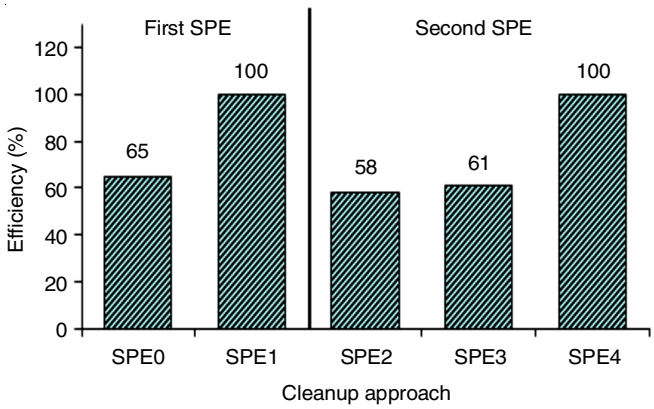

Fig. 4. Comparison of different cleanup approaches

HPLC separation: For Gly first transition, a matrix effect was observed as overlapping peak, if gradient starts after sample injection. This hindrance was solved by adding isocratic part from 0 to 3 min at 30% phase B (Fig. 5). Usage of smooth and long LC program gave no better result. There were no matrix effects for other transitions and other analytes observed.

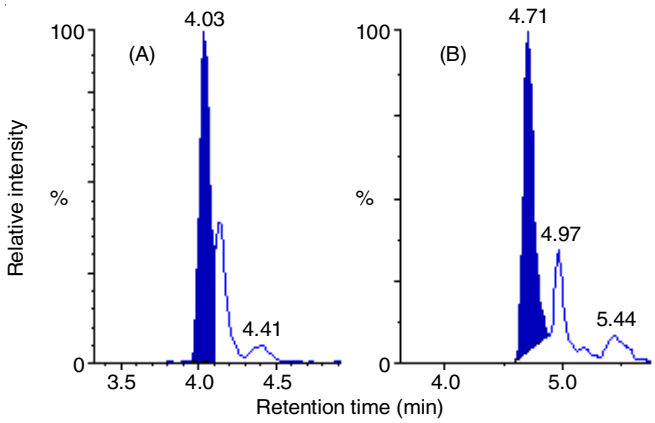

Fig. 5. Mass-chromatogram of Gly. (A) fast gradient; (B) with 3 min of waiting

TABLE-2
COEFFICIENT OF VARIATION (CV) AND RECOVERY (MR)

Analyte	0.05 ppm		0.1 ppm		0.5 ppm		1.0 ppm		2.0 ppm	
	CV (%)	MR (%)	CV (%)	MR (%)	CV (%)	MR (%)	CV (%)	MR (%)	CV (%)	MR (%)
Gly-FMOC	15.1	82	12.8	85	10.0	96	7.5	90	6.2	102
Glu-FMOC	17.6	85	11.3	80	13.2	88	10.1	76	8.6	95
AMPA-FMOC	19.1	79	18.7	83	16.1	82	11.4	94	9.5	108

Validation: The method was validated. The following variable parameters were chosen: matrix (2 per type), analysts (at the sample preparation stage) and storage time (immediate injection *vs.* overnight storage). The calibration curves and experimental samples were in duplicate with two blank samples analyzed in one batch. Recoveries of the herbicides in spiked samples ranged from 90 to 117% depending on concentration level. Specificity of the method was confirmed by analysis of 20 blanks for each matrix where no interferences were observed. Correlation coefficients of calibration curves were above 0.99 during validation experiments (Table-2). Stability of the analytes was confirmed for overnight storage. All the data are listed in Table-2. The typical equation ($y = ax + b$) of calibration curves, depending on their correlation coefficients (R) are shown in Table-3. The S/N for Gly at 0.05 ppm is shown on Fig. 6.

TABLE-3
PARAMETERS OF THE CALIBRATION CURVES

Analyte	Equation	R	LOD/LOQ
Gly-FMOC	Y = 0.111x + 8.28e-005	0.999	0.01/0.05
Glu-FMOC	Y = 1.62x + -0.0116	0.998	0.01/0.05
AMPA-FMOC	Y = 0.117x + -0.00238	0.997	0.02/0.05

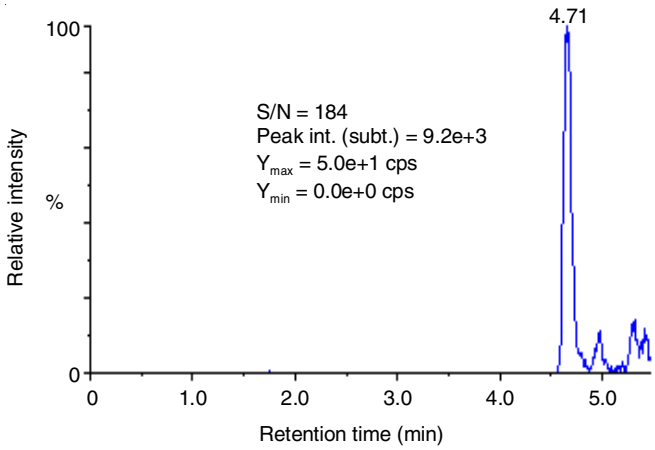

Fig. 6. Mass-chromatogram of Gly with calculated value of S/N

Method approbation: The method was applied for analysis of 100 honey samples from several regions of Russia, of which six samples were found to be positive. In two samples from Kaliningrad region and Republic of Mordovia, Gly was found below LOQ, approx. at 0.01-0.02 ppm level. In four samples from Krasnodar, Samara, Altai regions and Republic of Mordovia, Gly was found at levels above the EU MRL for honey (0.05 ppm): 0.11-0.07-0.17 ppm and 0.13 ppm, respectively. The study could help to control the Gly usage on agricultural territories, by using honey samples as a contamination indicator.

The problem of honey contamination by Gly exists in many countries. The similar levels were found in US honey [14]. Several samples contained Gly concentration below 0.016 ppm and other positive samples contained Gly from 0.023 to 0.12 ppm with average value 0.054 ppm. One sample contained 0.65 ppm of Gly. Gly was also found in 41 samples from 69 collected in Philadelphia, USA [15]. Gly was found by ELISA on 0.017-0.16 ppm levels.

Regarding the recent study [16], 200 samples of honey from western Canada were tested for Gly baseline determination and 197 of them were positive. The low level was found as 0.001 ppm and high was 0.05 ppm. AMPA (up to 0.05 ppm) and Glu (up to 0.033 ppm) were also found in this study.

Conclusion

The developed analytical method can be used as the confirmatory analysis of glyphosate (Gly), glufosinate (Glu) and aminomethylphosphonic acid (AMPA) at reasonable levels in honey. The monitoring results showed the actual occurrence of Gly in honey samples of several regions of Russia. The average value is 0.12 ppm and 0.085 ppm including results below LOQ. The comparison of existing data about Gly concentrations in honey samples and data obtained in this study, shown an average value is 0.069 ppm. Taking into account minimum and maximum of the values, the existing EU MRL for Gly in honey seems to be established on adequate level and should not require changes upward.

CONFLICT OF INTEREST

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

REFERENCES

1. S. Singh, V. Kumar, S. Datta, A.B. Wani, D.S. Dhanjal, R. Romero and J. Singh, *Environ. Chem. Lett.*, **18**, 663 (2020); https://doi.org/10.1007/s10311-020-00969-z
2. C.M. Benbrook, *Environ. Sci. Eur.*, **28**, 3 (2016); https://doi.org/10.1186/s12302-016-0070-0
3. http://www.weedscience.org/ (Accessed on 22.01.2022).
4. M.C. Arregui, A. Lenardón, D. Sanchez, M.I. Maitre, R. Scotta and S. Enrique, *Pest Manag. Sci.*, **60**, 163 (2004); https://doi.org/10.1002/ps.775
5. S.H. Bai and S.M. Ogbourne, *Environ. Sci. Pollut. Res. Int.*, **23**, 18988 (2016); https://doi.org/10.1007/s11356-016-7425-3
6. T. Shushkova, I. Ermakova and A. Leontievsky, *Biodegradation*, **21**, 403 (2010); https://doi.org/10.1007/s10532-009-9310-y
7. M. Pérez Rodríguez, C. Melo, E. Jiménez and J. Dussán, *Agriculture*, **9**, 217 (2019); https://doi.org/10.3390/agriculture9100217

8. L. Simonsen, I.S. Fomsgaard, B. Svensmark and N.H. Spliid, *J. Environ. Sci. Health B*, **43**, 365 (2008); https://doi.org/10.1080/03601230802062000
9. W.M. Farina, M.S. Balbuena, L.T. Herbert, C. Mengoni Goñalons and D.E. Vázquez, *Insects*, **10**, 354 (2019); https://doi.org/10.3390/insects10100354
10. R.M. Mann and J.R. Bidwell, *Arch. Environ. Contam. Toxicol.*, **36**, 193 (1999); https://doi.org/10.1007/s002449900460
11. V. Langiano and C.B.R. Martinez, *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, **147**, 222 (2008); https://doi.org/10.1016/j.cbpc.2007.09.009
12. N.K. Neskovic, V. Poleksi, I. Elezovi, V. Karan and M. Budimir, *Bull. Environ. Contam. Toxicol.*, **56**, 295 (1996); https://doi.org/10.1007/s001289900044
13. S. Richard, S. Moslemi, H. Sipahutar, N. Benachour and G.-E. Seralini, *Environ. Health Perspect.*, **113**, 716 (2005); https://doi.org/10.1289/ehp.7728
14. V.J. Koller, M. Fürhacker, A. Nersesyan, M. Mišík, M. Eisenbauer and S. Knasmueller, *Arch. Toxicol.*, **86**, 805 (2012); https://doi.org/10.1007/s00204-012-0804-8
15. N. Chamkasem, C. Morris and T. Harmon, *J. Reg. Sci.*, **5**, 1 (2017).
16. F.R. Emily Guo, *J. Environ. Anal. Toxicol.*, **5**, 1 (2014); https://doi.org/10.4172/2161-0525.1000249