DE GRUYTER
OPEN

Hellenic Plant Protection Journal **11:** 40-46, 2018
DOI 10.2478/hppj-2018-0005

SHORT COMMUNICATION

Direct determination of glyphosate and aminomethyl phosphonic acid in honeybees

K.M. Kasiotis*, Z.D. Tzouganaki and K. Machera*

Summary A straightforward LC-ESI-MS/MS method was developed and validated for the detection and quantitation of the herbicide glyphosate (GLY) and its metabolite aminomethyl phosphonic acid (AMPA) in honeybees. The method was validated, fulfilling the SANTE 11945/2015 guideline criteria, demonstrating acceptable mean recoveries at LOQ and 10×LOQ varying from 75-87% for both compounds. LOQ was determined at 0.2 and 0.5 μg/g $_{\text{bee body weight (bw)}}$ for GLY and AMPA respectively. Analysis of 14 honeybee samples displayed only one positive sample, containing GLY marginally above LOQ and traces of AMPA.

*Additional Keywords***:** AMPA, glyphosate, honeybees, LC-ESI-MS/MS, validation

Introduction

Glyphosate (GLY) is a non-selective herbicide with a broad range of applications against weeds that place it among the most important active substances of plant protection products (PPPs) in the market and the world's top-selling herbicide (Benbrook, 2016). Its broad use has augmented the interest of research community on its possible impact on non-target organisms such as bees. Although glyphosate exhibits moderate to low toxicity in honeybees (LD_{50}≥1000 μg/g $_{\text{bee bw}}$)(Glyphosate-LD50) its frequent applications should not be underestimated. Therefore, possible sublethal effects, and the potential synergistic effects of co-present compounds and other factors on bees, such as the parasitic mite *Varroa*, the microsporidian *Nosema apis*, or other pollutants, should be considered with caution.

With this in view, field-realistic doses of GLY were implicated in reducing sensitivity to nectar reward, and impair associative learning in honeybees (Herbert *et al*., 2014). In addition, sublethal doses of GLY have been reported to impact cognitive abilities of bees, hence their navigation (Balbuena *et al*., 2015). Metabolism of GLY in the environmental compartments and *in vivo* is extensively reported. AMPA is a major metabolite that in the environment is more persistent than GLY (Mamy *et al*., 2008).

GLY is a very polar and amphoteric compound, therefore can easily be engaged in hydrophilic or hydrophobic ion pairing reactions in the context of ionic equilibria. If these interactions are not favorable, poor chromatographic performance is anticipated and obtained. Due to glyphosate's chemical structure, its chemical analysis (incorporating its main metabolite AMPA) usually occurs by derivatization using appropriate agents, such as 9-fluorenylmethyl chloroformate (FMOC-Cl) and subsequent analysis using LC-ESI-MS/MS. The latter has been applied by several researchers with success and is considered as a routine procedure to quantitate GLY and AMPA in several commodities [indicatively see (Hanke *et al.,* 2008; Schrubbers *et al*., 2016)]. However, its main downside is that is laborious and time intensive.

Benaki Phytopathological Institute, Department of Pesticides Control and Phytopharmacy, Laboratory of Pesticides' Toxicology, 8 St. Delta Street, GR-145 61 Kifissia, Athens, Greece.
* Corresponding authors: k.kasiotis@bpi.gr; k. machera@bpi.gr

One of the first reports on GLY and AMPA direct determination in soybean employed the use of a reversed phase C8 column, using LC-MS/MS (Martins *et al*., 2009). In the same matrix, Chamkasem and Harmon reported GLY and AMPA detection using cation-anion mixed mode chromatographic column (Chamkasem and Harmon, 2016). Yoshioka *et al.* (2011) published related analytical work in serum utilizing hydrophilic interaction chromatography (HILIC). Another application of HILIC was reported in rice, maize, and soybean (Botero-Coy *et al*., 2013), whereas in plant-derived food similar analytical approach exhibited low-matrix effect (Ding *et al*., 2016). Recently, Jensen *et al.* (2016) published a pioneering analytical method for the direct determination of both molecules in milk and urine using a Cation-H guard column. This method proved sufficient in determining both analytes with substantial sensitivity in the respective matrices.

In this short communication, and to our knowledge, we present the first report on the direct analysis of glyphosate and AMPA residues in honeybees. Separation of analytes was grounded on the work by Jensen *et al.* (2016). Its importance is reinforced by the circumvention of the derivatization step, used in a previous work for bee larvae (Thompson *et al*., 2014) and the unambiguous prevalence of GLY and AMPA in foraging environments.

Materials and Methods

Certified chemical standards of GLY and AMPA were obtained from Dr Ehrenstorfer (Germany). Standard solutions were prepared in ultrapure water (SG Ultra-pure water system) inside polypropylene volumetric flasks and kept at 4°C away from light. Acetonitrile and formic acid of LC-MS grade were obtained from Fisher Scientific. Bees used as control sample were collected from Agricultural University of Athens experimental apiaries that are not subjected to chemical treatments.

Sample preparation

In 1g of honeybees, water was added (5mL) and homogenized for 5 min (Ultra-Turrax). Consequently, the mixture was magnetically stirred for 1 h, and subjected to ultrasonication for 30 min. Then, the mixture was centrifuged at 4500 rpm for 15 min (10°C), the supernatant was decanted, and 50 μL of buffering agent (1000mM of NH_4-COOH/HCOOH) were added and the mixture shaken for 10 seconds. Additional centrifugation (same conditions) and filtering of the supernatant provided the final extract that was injected into the LC-ESI-MS/MS system.

Chromatographic conditions

Chromatographic separation was performed following the conditions described by Jensen et al. (2016), incorporating the use of the Bio-Rad Cation-H guard column. The only modification was the addition of formic acid (0.05%) in acetonitrile phase (channel B). Retention times obtained were similar to this publication with AMPA eluting slightly earlier (6.7 min instead of 7.0 min).

Analytical Method Validation

Validation of the analytical method was based on the SANTE guideline (SANTE/11945/2015, 2015). Validation parameters considered were linearity, matrix effect, LOQ, specificity, trueness, precision, and robustness. Linear range varied from 0.1-5 and 0.2-5 μg/mL for GLY and AMPA respectively, containing at least five calibration levels. Up to three MRM transitions were monitored for the active substances (Table 1 and Figure 1a).

Results and Discussion

Analytical method validation criteria fulfilled requirements of the SANTE/11945/2015 document. More specifically, linearity was acceptable with residuals < ±20% (and correlation coefficients values, r^2=0.996 for both GLY and AMPA). LOQ for GLY and AMPA was established at 0.2 and 0.5 μg/g $_{\text{bee bw}}$, respec-

tively. Trueness was assessed by the recovery study of both analytes at two concentration levels (LOQ and 10×LOQ), using 5 replicates per level. The results verified sufficient extraction of analytes from bees (recoveries ranging from 75-87%, for GLY and AMPA at both LOQ and 10×LOQ, RSD% 6-9.2%). Negligible matrix effect was evidenced for GLY and AMPA, varying from -1.1 to 1.6% respectively.

Consequently, analysis of 14 honeybees' samples after death incidents (year 2017) using the Cation-H column did not reveal residues of GLY and AMPA above the LOQs, except one sample that contained GLY slightly above LOQ (0.21±0.03 μg/g $_{\text{bee bw}}$) and traces of AMPA (Figure 1b). In this context, it should be pointed out that there is room for further improvement of the LOQs of the method. The latter might assist the disclosure of more positive samples if honeybees' colonies are nearby areas growing attractive crops to bees, where applications of GLY have taken place.

Even though field studies have not yet revealed synergistic effects of GLY with other classes of pesticides [such as the neonicotinoids, for imidacloprid see (Zhu *et al*., 2017)], GLY should be incorporated in monitoring schemes due to its wide and frequent use. The latter is reinforced by the ubiquity of pollutants that can interplay with GLY. In this context, a combination of GLY with cadmium, promoted lipid peroxidation in bees (Jumarie *et al*., 2017). Last but not least, Liao *et al*. (2017) reported that bees display a contradictory preference for floral tissues that contain GLY in sugar water at 10ppb. Therefore, GLY is not an obstacle for bees to visit floral nectar that contains it. Overall, GLY is an abundant active substance that should be screened and quantified in apiculture commodities. The presented methodology is a step forward in this direction and can be a starting point to build upon incorporating highly polar and cumbersome molecules such as GLY in monitoring schemes.

Conclusions

A direct LC-ESI-MS/MS analytical method was developed and validated for the detection and quantitation of GLY and AMPA in honeybees. The method does not require derivatization of GLY and AMPA and was applied in a limited number of honeybee samples collected after death incidents. In one sample GLY was quantified slightly above LOQ and traces of AMPA were detected. Next steps, including further fostering of sensitivity and the addition of internal standards, are ongoing.

Authors would like to thank Professor Harizanis of the Agricultural University of Athens for the provision of honeybees control samples.

Table 1. Chromatographic parameters and MRM transitions for the herbicide glyphosate GLY and its metabolite aminomethyl phosphonic acid (AMPA) in honeybees.

Active substances	Retention time (min)	Q1 (amu)	Q2 (amu)	Dwell time	Fragmentor voltage	CE (eV)
GLY	1.5	168	150[q]	30	100	5
			124[c]	30	100	8
			63[c]	15	150	30
AMPA	6.7	110	63[q]	15	150	26
			79[c]	15	150	36

q: quantitation, c: confirmation

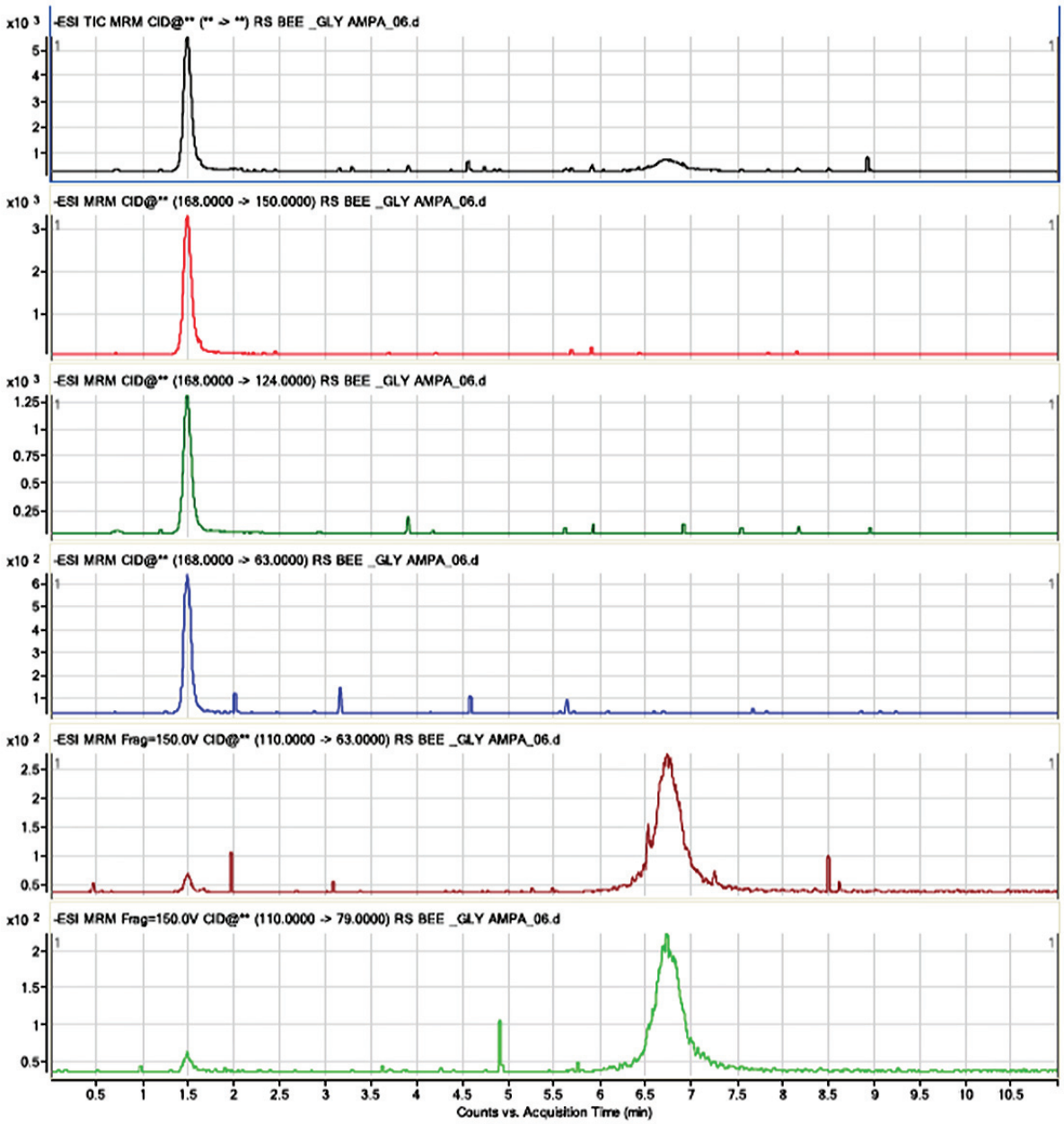

Figure 1a. MRM chromatograms of spiked at 400 ng/g $_{\text{bee bw}}$ control honeybee sample.

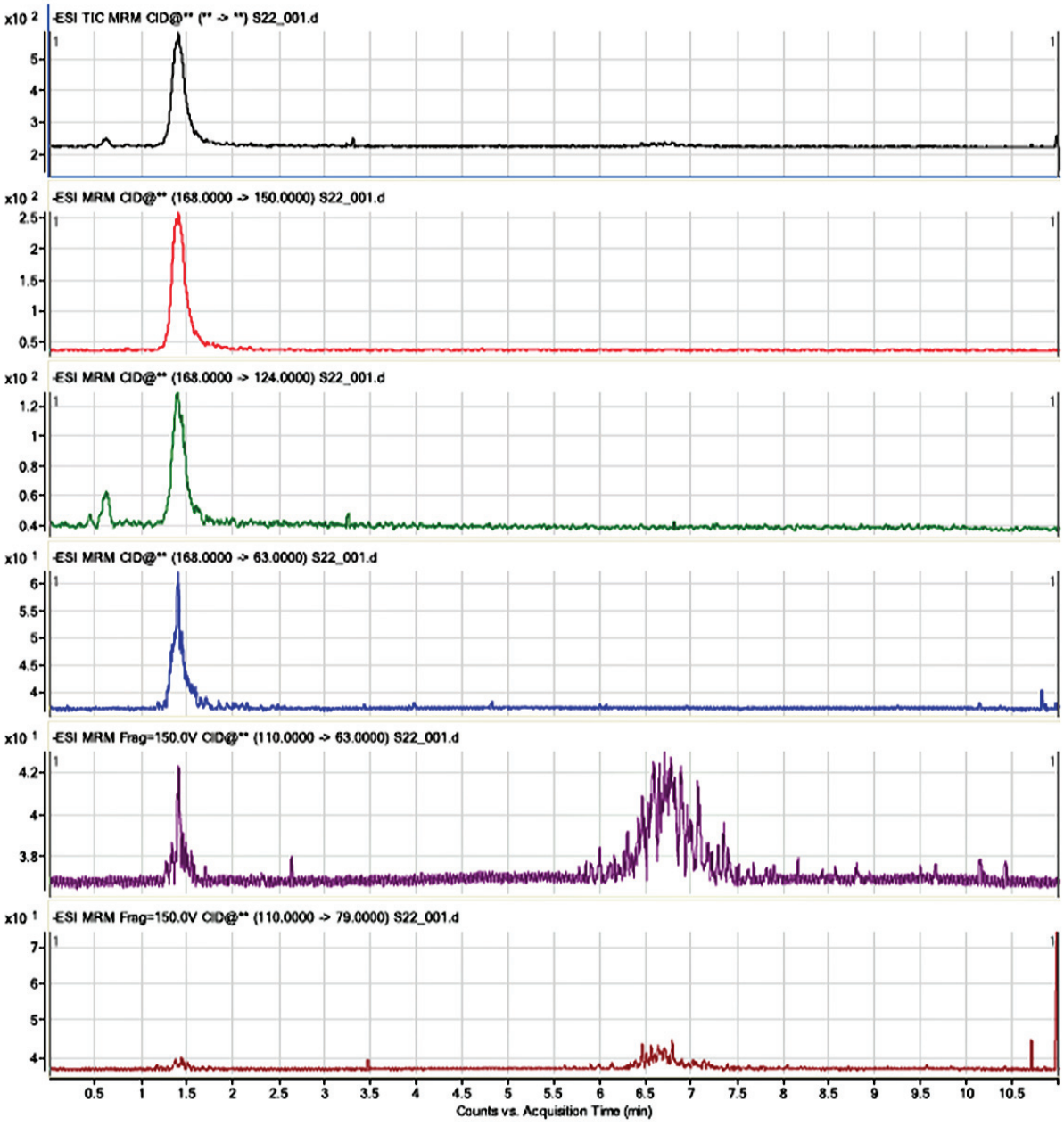

Figure 1b. MRM chromatograms of a honeybee sample containing the herbicide glyphosate (GLY) and traces of its metabolite aminomethyl phosphonic acid (AMPA)*.

*quantitation and confirmation transitions for both GLY and AMPA are presented

Literature cited

Balbuena, M.S., Tison, L., Hahn, M.L., Greggers, U., Menzel, R. and Farina, W.M. 2015. Effects of sublethal doses of glyphosate on honeybee navigation. *Journal of Experimental Biology*, 218: 2799-805.

Benbrook, C.M. 2016. Trends in glyphosate herbicide use in the United States and globally. *Environmental Sciences Europe*, 28: 3.

Botero-Coy, A.M., Ibanez, M., Sancho, J.V. and Hernandez, F. 2013. Direct liquid chromatography-tandem mass spectrometry determination of underivatized glyphosate in rice, maize and soybean. *Journal of Chromatography A*, 1313: 157-165.

Chamkasem, N. and Harmon, T. 2016. Direct determination of glyphosate, glufosinate, and AMPA in soybean and corn by liquid chromatography/tandem mass spectrometry. *Analytical Bioanalytical Chemistry*, 408: 4995-5004.

Ding, J.J., Jin, G.H., Jin, G.W., Shen, A.J., Guo, Z.M., Yu, B., Jiao, Y., Yan, J. and Liang, X. 2016. Determination of Underivatized Glyphosate Residues in Plant-Derived Food with Low Matrix Effect by Solid Phase Extraction-Liquid Chromatography-Tandem Mass Spectrometry. *Food Analytical Methods*, 9: 2856-2863.

Glyphosate-LD50. https://sitem.herts.ac.uk/aeru/ppdb/en/Reports/373.htm.

Hanke, I., Singer, H. and Hollender, J. Ultratrace-level determination of glyphosate, aminomethylphosphonic acid and glufosinate in natural waters by solid-phase extraction followed by liquid chromatography-tandem mass spectrometry: performance tuning of derivatization, enrichment and detection. *Analytical Bioanalytical Chemistry* 2008, 391: 2265-2276.

Herbert, L.T., Vazquez, D.E., Arenas, A. and Farina, W.M. 2014. Effects of field-realistic doses of glyphosate on honeybee appetitive behaviour. *Journal of Experimental Biology*, 217: 3457-3464.

Jensen, P.K., Wujcik, C.E., McGuire, M.K. and McGuire, M.A. 2016. Validation of reliable and selective methods for direct determination of glyphosate and aminomethylphosphonic acid in milk and urine using LC-MS/MS. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes*, 51: 254-259.

Jumarie, C., Aras, P. and Boily, M. 2017. Mixtures of herbicides and metals affect the redox system of honey bees. *Chemosphere*, 168: 163-170.

Liao, L.H., Wu, W.Y. and Berenbaum, M.R. 2017. Behavioral responses of honey bees (Apis mellifera) to natural and synthetic xenobiotics in food. *Scientific Reports*, 7.

Mamy, L., Gabrielle, B. and Barriuso, E. 2008. Measurement and modelling of glyphosate fate compared with that of herbicides replaced as a result of the introduction of glyphosate-resistant oilseed rape. *Pest Management Science*, 64: 262-275.

Martins, H.A., Lebre, D.T., Wang, A.Y., Pires, M.A.F. and Bustillos, O.V. 2009. An alternative and fast method for determination of glyphosate and aminomethylphosphonic acid (AMPA) residues in soybean using liquid chromatography coupled with tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, 23: 1029-1034.

SANTE/11945/2015. 2015. Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed.

Schrubbers, L.C., Masis-Mora, M., Rojas, E.C., Valverde, B.E., Christensen, J.H. and Cedergreen, N. 2016. Analysis of glyphosate and aminomethylphosphonic acid in leaves from Coffea arabica using high performance liquid chromatography with quadrupole mass spectrometry detection. *Talanta*, 146: 609-20.

Thompson, H.M., Levine, S.L., Doering, J., Norman, S,, Manson, P., Sutton, P. and von Mérey, G. 2014. Evaluating Exposure and Potential Effects on Honeybee Brood (Apis mellifera) Development Using Glyphosate as an Example. *Integrated Environmental Assessment and Management*, 10: 463-470.

Yoshioka, N., Asano, M., Kuse, A., Mitsuhashi, T., Nagasaki, Y. and Ueno, Y. 2011. Rapid determination of glyphosate, glufosinate, bialaphos, and their major metabolites in serum by liquid chromatography-tandem mass spectrometry using hydrophilic interaction chromatography. *Journal of Chromatography A*, 1218: 3675-3680.

Zhu, Y.C., Yao, J., Adamczyk, J. and Luttrell, R. 2017. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*). *PLoS One*, 12: e0176837.

Received: 22 December 2017; Accepted: 9 January 2018

ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Άμεση LC-ESI-MS/MS μέθοδος για τον προσδιορισμό του ζιζανιοκτόνου glyphosate και του μεταβολίτη του αμινομεθυλφωσφονικού οξέος στις μέλισσες

Κ.Μ. Κασιώτης, Ζ.Δ. Τζουγανάκη και Κ. Μαχαίρα

Περίληψη Μια απλή και άμεση LC-ESI-MS/MS μέθοδος αναπτύχθηκε και επικυρώθηκε για την ανίχνευση και ποσοτικοποίηση του ευρέως χρησιμοποιούμενου ζιζανιοκτόνου glyphosate (GLY) και του μεταβολίτη του αμινομεθυλφωσφονικού οξέος (AMPA) στις μέλισσες. Η μέθοδος επικυρώθηκε ικανοποιώντας τα κριτήρια της κατευθυντήριας οδηγίας SANTE 11945/2015, επιδεικνύοντας αποδεκτή μέση ανάκτηση σε δύο επίπεδα συγκέντρωσης, στο όριο ποσοτικοποίησης (LOQ) και στο 10×LOQ, που κυμαίνονται από 75-87% και για τους δύο αναλύτες με αποδεκτές τιμές % σχετικής τυπικής απόκλισης (RSD%). Το όριο ποσοτικοποίησης LOQ προσδιορίστηκε στα 0,2 και 0,5 μg/g $_{\text{σωματικού βάρους μέλισσας}}$ για το GLY και το AMPA αντίστοιχα. Η ανάλυση 14 δειγμάτων μελισσών εμφάνισε μόνο ένα θετικό δείγμα, που περιείχε GLY σε συγκέντρωση ελάχιστα ανώτερη του LOQ και ίχνη AMPA.

Hellenic Plant Protection Journal **11:** 40-46, 2018