REVISED PAPER: NEONICOTINOIDS AS A POTENTIAL THREAT TO HONEY – DEVELOPMENT OF LIQUID CHROMATOGRAPHY METHODS FOR THEIR DETERMINATION

REVIDIRANI RAD: NEONIKOTINOIDI KAO POTENCIJALNA OPASNOST ZA MED – RAZVOJ TEČNO-HROMATOGRAFSKIH METODA ZA NJIHOVO ODREĐIVANJE

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ABSTRACT

There is an increasing interest on neonicotinoid residues determination in honey samples because they are banned by European Union. Their determination is based on liquid chromatography analytical methods and, therefore, the aim of this study was to develop the analytical method for quantifying these compounds using different sample preparation techniques. The sample preparation was conducted by two different techniques and the new HPLC-DAD method was developed and validated assuring reliable accuracy (R, 70–120 %), repeatability (RSD, < 20 %) and within-laboratory reproducibility (RSD, < 20 %). Also, low limits of detection and quantification (LOD, 2.5–5.0 $\mu\text{g kg}^{-1}$; LOQ, 7.5–10.0 $\mu\text{g kg}^{-1}$) for the mentioned method were achieved. To address the importance of honey safety control more than 100 samples from Vojvodina region were analyzed showing residues of neonicotinoids. The HPLC-MS/MS method was used to confirm the presence of neonicotinoids quantified using validated HPLC-DAD method.

Keywords: Neonicotinoids, liquid chromatography, DLLME, QuEChERS, honey.

REZIME

Nakon što je Evropska unija ograničila korišćenje neonikotinoida na cvetnim usevima koje pčele posećuju, došlo je do povećanog interesovanja za utvrđivanje ostataka neonikotinoida u pčeljinim proizvodima poput meda. Stoga je cilj ovog istraživanja bio razvoj analitičkih metoda, zasnovanih na tečnoj hromatografiji, korišćenjem dve tehnike za pripremu uzoraka, za određivanje odabranih neonikotinoida u uzorcima meda. Priprema uzoraka meda za analizu odabranih neonikotinoida ostvarena je optimizacijom dve tehnike pripreme čime je potvrđeno da one ispunjavaju uslove propisane u SANCO/12571/2013 standardu za validaciju, obezbeđujući rezultate za tačnost (R, 70–120%), ponovljivost (RSD, < 20%) i unutar-laboratorijsku reproduktivnost (RSD, < 20%), kao i granicu detekcije (LOD, 2.5–5.0 $\mu\text{g kg}^{-1}$) i kvantifikacije (LOQ, 7.5–10.0 $\mu\text{g kg}^{-1}$) korišćenjem DAD detektora. Aplikativnost metoda je potvrđena na više od 100 uzoraka meda različitog cvetnog porekla (suncokretovog, lipinog, bagremovog i livadskog meda), prikupljenih iz 7 okruga Autonomne Pokrajine Vojvodine, čijom analizom je ustanovljeno prisustvo određenih neonikotinoida, što je ukazalo na neophodnost permanentne kontrole ove vrste namirnica na ostatke pesticida.

Ključne reči: neonikotinoidi, tečna hromatografija, DLLME, QuEChERS, med.

INTRODUCTION

The most widely used class of the insecticides in last 20 years are neonicotinoids, which act as a receptor agonists of nicotinic acetylcholine (Decourtye and Devillers, 2010; Tomizawa and Casida, 2005). Systemic translocation to the whole plant (flowers, pollen and nectar) through xylemic and phloemic transport results in their presence on the leaves through guttation (van der Sluijs et al., 2013), allowing pathways by which honey bees among other beneficial pollinators can be relived to these insecticides. Different studies in Europe and USA demonstrated that sublethal amounts of neonicotinoids cause disorientation, reduced communication, impaired learning and memory, reduced longevity and disruption of honeybee brood cycles (Farooqui, 2013). The residues of neonicotinoids may be found in honey, pollen and other bee product (Kasiotis et al., 2014; Tanner and Czerwenka, 2011). Due to their evidenced negative impact, the European Commission issued two-year ban for the use of thiamethoxam, imidacloprid and clothianidin in crops attractive to bees (Commission, 2013; EFSA, 2013). Having that in mind, determination of neonicotinoids in honey has become necessary and requires sensitive, selective and efficient analytical methods. The residue of neonicotinoids in

honey samples can be measured by liquid chromatography (LC) coupled to different detectors such as diode array detector (DAD) (Campillo et al., 2013; Jovanovic et al., 2015) or mass spectrometer (MS or MS/MS) (Fidente et al., 2005; Jovanovic et al., 2013; Jovanovic et al., 2014; Lazić et al., 2014). The MS/MS detector assures high sensitivity and selectivity compared to DAD, but it is an expensive and high maintenance instrument. In addition, the optimized sample preparation procedures are needed to achieve good performance characteristics. Therefore, the main goal of this research was development of rapid, sensitive, optimized and reliable liquid chromatography method with DAD detector in alliance with chosen extraction techniques for determination of dinotefuran, thiamethoxam, clothianidin, acetamiprid, imidacloprid, nitenpyram, and thiacloprid in honey. The proposed method was validated and applied to around hundred honey samples for the presence of the neonicotinoids (Jovanovic, 2014).

MATERIAL AND METHOD

Chemicals and reagents

Neonicotinoid standards (certified purity > 99 %) and formic acid (purity 98 %, w/w) were obtained from Sigma-Aldrich (Steinheim, Germany), while acetonitrile and dichloromethane

(HPLC grade) were bought from Merck (Darmstadt, Germany). Simplicity UV Millipore system (Bedford, MA, USA) was used to obtain purified water. Stock solutions (100.0 mg L^{-1}) were prepared using purified water and be stable over a three-month period when stored at -10°C . Multicomponent standard solution ($100.0 \mu\text{g L}^{-1}$) was used for matrix-matched and solvent based calibration (MMC and SC, respectively) and for spiking honey samples. The MMC standards were obtained by using blank honey samples spiked with multicomponent stock solution over the range from the limit of quantification (LOQ) to $100.0 \mu\text{g kg}^{-1}$. All standard solutions could be stable over a one-month period when stored at 4°C , protected from light. United Chemical Technologies QuEChERS sample preparation kits (buffered extraction kits part number ECQUEU750CT and general cleanup kits part number ECMPS15CT) were used for the extraction protocols.

Sample collection and preparation

Sunflower, wildflower, linden, and acacia honey samples, collected from 7 different regions of the Autonomous Province of Vojvodina, Republic of Serbia were subjected for the analysis. This study was conducted using 50.0 g L^{-1} of honey solution prepared in water and spiked with neonicotinoid multicomponent solutions on different concentration levels. Prior to analysis all spiked samples were left to “equilibrate” overnight.

Extraction protocols

Before the chromatographic separation two extraction methods were used for sample preparation – DLLME and QuEChERS methods (Fig. 1).

Fig.1. Extraction procedures – dispersive liquid-liquid microextraction (DLLME) and QuEChERS method

HPLC-DAD analysis

Liquid chromatograph (Agilent 1200 Series, Agilent Technologies Inc., USA), equipped with DAD detector and ZORBAX Eclipse XDB-C18 column ($50 \text{ mm} \times 4.6 \text{ mm i.d.}$, $1.8 \mu\text{m}$), was used for separation and quantification of neonicotinoids in the obtained extracts. The column temperature was 30°C and the sample injection volume of $10 \mu\text{L}$ was applied. The mobile phase consisted of two eluents, ACN (A) and ultrapure water with 0.2% formic acid (B). A flow rate was 0.7 mL min^{-1} . The neonicotinoids were separated applying the

gradient program: at start 90% of solvent B linearly reached 60% after 6 min and then went back to 90% at 10 min at the end of the run. The retention times were used to identify the investigated neonicotinoids, while quantification was based on peak areas. The DAD wavelengths were set at 244 and 266 nm.

LC-MS/MS confirmation analysis

The same liquid chromatograph (Agilent 1200 Series, Agilent Technologies Inc., USA) was used as for the HPLC-DAD analysis. The isocratic elution at a flow rate of 0.5 mL min^{-1} was performed using ultrapure water with 0.1% formic acid and ACN with the ratio 20:80 (v/v). An Agilent 6410 Triple Quad LC/MS mass spectrometer (Agilent Technologies Inc., USA) supplied with MassHunter software version B.03.01 (Agilent Technologies Inc., USA) was used with a multimode interface in positive ion mode to conduct the analysis. The heater gas temperature of 325°C and vaporization temperature of 200°C was applied. As a nebulizer gas nitrogen was used (50 psi , a flow rate – of 5 L min^{-1} ; capillary voltage – 2500 V ; charging voltage – 2000 V). Total ion chromatograms (TIC) were recorded for all investigated neonicotinoids in the multiple reaction monitoring (MRM) mode using a dwell time of 20 milliseconds (msec) and the most intense transitions for each investigated compound.

Method validation

The developed method was tested to find out if it satisfied all requirements of method validation procedures for pesticides residue analysis in foods (SANCO/12571/2013). The calibration curves were constructed for all neonicotinoids dissolved in pure solvent and in honey matrix (LOQ to $100.0 \mu\text{g kg}^{-1}$ for both) by plotting the peak areas against the concentrations. The square correlation coefficient (r^2) was used to express the linearity of calibration curves. The limit of detection (LOD) and limit of quantification (LOQ) were also estimated using matrix-matched standards, while the accuracy of the applied method was determined as recovery (R, %) and the precision of developed method was expressed as repeatability and within-laboratory reproducibility.

RESULTS AND DISCUSSION

Residue-analysis methods (concentration less than $\mu\text{g L}^{-1}$) require the special attention when the pretreatment procedure is chosen. Therefore, two pretreatment procedures were tested (DLLME and QuEChERS) as the neonicotinoid extraction methods from honey samples. The QuEChERS procedure was followed by higher matrix influence compared to the DLLME pretreatment procedure due to higher polarity of the extraction solvent applied in the QuEChERS procedure, which ensured the higher amount of coextracted neonicotinoids. The observed

matrix effect required the using the matrix-matched standards and matrix-matched calibration curves, which were linear over the range LOQ- $100.0 \mu\text{g kg}^{-1}$.

The accuracy of the proposed method was expressed as the mean recovery (R, %) where R and RSD values were determined using a spiked blank honey samples in 5 replicates at 10.0 , 50.0 and $100.0 \mu\text{g kg}^{-1}$ concentration levels calculated with MMC curves. The recovery results showed that both extraction methods provided optimal neonicotinoids recovery ($70\text{--}120\%$ at each spiking level with RSD of $\leq 20\%$). Precision, expressed as

the repeatability and within-laboratory reproducibility with RSD values less than 20% assured a good precision of both developed extraction methods. Limits of detection and limits of quantification are the indicators of method sensitivity in residue analysis, and with the achieved LODs of 2.5–5.0 $\mu\text{g kg}^{-1}$ and LOQs of 7.5–10.0 $\mu\text{g kg}^{-1}$ we can assume that this method is applicable for the neonicotinoid residue analysis. Furthermore, the obtained LOQs were much lower than the European Commission MRLs for all neonicotinoids in honey. Applicability of the developed method was tested on the analysis of 104 honey samples from different plant origin (51 sunflower, 26 wildflower, 22 acacia and 5 lindens) for neonicotinoid residues. After preparing honey samples using QuEChERS procedure, the obtained results showed the presence of the thiacloprid in 5 honey samples in quantities below EU MRL. The extraction of the neonicotinoids was repeated using the DLLME procedure on thiacloprid contaminated honey samples, and the obtained quantification results showed excellent matching of two extraction methods ($p < 0.05$), indicating that depending of resources both sample preparation methods can be used for neonicotinoid analysis with HPLC-DAD system. Results confirmation was performed using previously developed LC-MS/MS method (Jovanov et al., 2013).

CONCLUSION

Developed HPLC-DAD method with the DLLME and QuEChERS procedures was applied for the analysis of the selected neonicotinoids in honey samples collected from the Autonomous Province of Vojvodina. The validation results made possible low concentration level quantification of selected neonicotinoids. Neonicotinoid thiacloprid, mostly used for the crops protection was found in residue levels below MRL implicating the importance of permanent honey samples control.

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