Having that in mind, determination of neonicotinoids in honey has become necessary and requires sensitive, selective and efficient analytical methods. The residue of neonicotinoids may be found in honey, pollen and other bee products (van der Sluijs et al., 2013) and may be linked to their increased disorientation, reduced communication, impaired learning and memory, reduced longevity and disruption of honeybee brood cycles (Farooqui, 2013). 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 µg kg⁻¹; LOQ, 7.5–10.0 µg 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.

**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 µg kg⁻¹; LOQ, 7.5–10.0 µg 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.

**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 interested in 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; Jovanov et al., 2015) or mass spectrometer (MS or MS/MS) (Fidente et al., 2005; Jovanov et al., 2013; Jovanov 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 dinofuran, 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 (Jovanov, 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 were obtained from Sigma-Aldrich (Steinheim, Germany).

**Keywords:** Neonicotinoids, liquid chromatography, DLLME, QuEChERS, honey.
(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 μg 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 μg 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 kit part number ECQUEU750CT and general cleanup kit part number ECMP515CT) 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).

**DLLME**

- 5.0 mL of the honey sample (blank or spiked), 2.0 mL of dichloromethane (DHM) (extraction solvent) and 0.5 mL of acetonitrile (ACN) (dispersive solvent) were added into a 10.0 mL round-bottom tube.
- The tube was shaken for 1 min by vortex (BOECO, Germany) and soaked for 10 min in the ultrasonic bath (LR& ultrasonics LTD, United Kingdom) (37 kHz, 400 W). Shaking was repeated for 1 min.
- The tube was centrifuged for 5 min at 2500 rpm (Thermolyne, Yugoslavia).
- The sediment was removed by a syringe and collected in another test tube.
- The dichloromethane was evaporated under the flow of air using the Reacti-Therm heating module (Thermo-Scientific, USA).
- The final residue was reconstituted with 0.2 mL of mobile phase and shaken by vortex for 2 min.
- Samples were transferred into the vials for further analysis.

**QuEChERS**

- The honey samples (15.0 mL of 50.0 g L$^{-1}$ spiked honey solution corresponding to 10.0 g of honey) were mixed with buffering salts (4.0 g of MgSO$_4$, 1.0 g of NaCl, 0.5 g of sodium citrate dibasic sesquihydrate and 1.0 g of sodium citrate tribasic dehydrate) and acetonitrile (10.0 mL) in a 50 mL extraction vial.
- The mixture was vortexed (1 min) and centrifuged at 3000 rpm (10 min).
- The acetonitrile layer (6.0 mL) was transferred into a sample cleanup vial, which contained 0.9 g MgSO$_4$ and 0.15 g primary-secondary amine (PSA).
- The cleanup vial was once again subject to vortexing and centrifugation.
- The extract (1.5 mL) was transferred into the 2 mL vial and solvent was evaporated under the stream of nitrogen.
- The residue was reconstituted in 1.0 mL (1.5 mL for spiked honey samples) of the mobile phase prior to analysis.

**RESULTS AND DISCUSSION**

Residue-analysis methods (concentration less than μg 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 μg 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 μg kg$^{-1}$ concentration levels calculated with MMC curves. The recovery results showed that both extraction methods provided optimal neonicotinoids recovery (70–120 % at each spiking level with RSD of ≤ 20 %).
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 µg kg⁻¹ and LOQs of 7.5–10.0 µg 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 willow, 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.

ACKNOWLEDGMENT: The authors appreciate funding from the Ministry of Education and Science of the Republic of Serbia (Project No. ON172012 and TR31029) for supporting this effort.

REFERENCES