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Multi-residue method for determination of selected neonicotinoid insecticides in honey using optimized dispersive liquid–liquid microextraction combined with liquid chromatography-tandem mass spectrometry

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ABSTRACT

The objective of this study was to develop analytical method based on optimized dispersive liquid–liquid microextraction (DLLME) as a pretreatment procedure combined with reversed phase liquid chromatographic separation on C18 column and isocratic elution for simultaneous MS/MS determination of selected neonicotinoid insecticides in honey. The LC–MS/MS parameters were optimized to unequivocally provide good chromatographic separation, low detection (LOD, 0.5–1.0 μg kg^{-1}) and quantification (LOQ, 1.5–2.5 μg kg^{-1}) limits for acetamiprid, clothianidin, thiamethoxam, imidacloprid, dinotefuran, thiacloprid and nitenpyram in honey samples. Using different types (chloroform, dichloromethane) and volumes of extraction (0.5–3.0 mL) and dispersive (acetonitrile; 0.0–1.0 mL) solvent and by mathematical modeling it was possible to establish the optimal sample preparation procedure. Matrix-matched calibration and blank honey sample spiked in the concentration range of LOQ-100.0 μg kg^{-1} were used to compensate the matrix effect and to fulfill the requirements of SANCO/12495/2011 for the accuracy (*R* 74.3–113.9%) and precision (expressed in terms of repeatability (RSD 2.74–11.8%) and within-laboratory reproducibility (RSDs 6.64–16.2%)) of the proposed method. The rapid (retention times 1.5–9.9 min), sensitive and low solvent consumption procedure described in this work provides reliable, simultaneous, and quantitative method applicable for the routine laboratory analysis of seven neonicotinoid residues in real honey samples.

1. Introduction

Honey is produced by *Apis mellifera* bees, and its composition depends mainly on the floral origin of the nectar [1]. It is a natural food product with world-wide consumption, and in terms of food safety various chemical contaminants, especially pesticides residues and their metabolites must be regularly monitored [2]. Taking into consideration the fact that honey bees frequently pasture on agricultural crops and other plants contaminated by pesticides, there is a need for accurate and reliable determination of pesticides residues in honey.

Neonicotinoid insecticides, as one of the fastest growing new generation of insecticides, have contributed to a significant reduction of toxicity for the environment; however, there is a concern that upon their use, as a measure of pest management, beneficial insects such as honeybees may also be affected. Neonicotinoids act as agonists at the insect nicotinic acetylcholine receptors, and they are active against many sucking and biting insects, including aphides and some *Lepidoptera* species [3]. This class of pesticides is commonly used on rice, maize, sunflowers, rape, potatoes, sugar beets, vegetables, and fruits crops [4]. Commercialized neonicotinoids include imidacloprid, acetamiprid, nitenpyram, thiacloprid, thiamethoxam, clothianidin and dinotefuran. Depending on the application form of neonicotinoid insecticides there are different routes of exposure of honeybees to these pesticides [5]. When honeybees come into contact with neonicotinoids, they may be taken along into the beehive, and their residues may be found in bee products such as honey. For the different neonicotinoids, maximum residue limits (MRLs), including some neonicotinoid metabolites (acetamiprid metabolite IM-2-1, flonicamid metabolite TFNA-AM, thiamethoxam metabolite clothianidin) in honey, set by the European Union (EU) regulations, ranging from 10 to 200 μg kg^{-1}, refer to the sum of the parent compound and its metabolites [6].

Neonicotinoids are usually determined by liquid chromatography (HPLC) coupled with diode array (DAD) detection [7,8], because direct analysis by gas chromatography [9–11] is unsuitable

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due to their low volatility and high polarity. Moreover, they are determined by HPLC coupled to either electrochemical detector or post-column photochemical reactor [12,13] or mass spectrometer [14–17]. Enzyme-linked immunosorbent assays has also been explored as simple screening method for these compounds [18–20]. Recently, highly sensitive thermal lens spectrometric (TLS) detection, has been successfully coupled to HPLC and has attracted attention for use in the neonicotinoid and other target analysis of different biological and environmental samples [21–24].

Because of the complex composition of honey, sample pretreatment plays a key role in the pesticide residue analysis [25,26]. In literature, several sample pretreatment techniques have been reported for the extraction of pesticides from honey including: the traditional liquid–liquid extraction (LLE) [27], solid phase extraction (SPE) [28], supercritical fluid extraction [29], accelerated solvent extraction (ASE) [30], solid phase microextraction [31] and QuEChERS extraction [32]. For the analysis of neonicotinoid insecticides residues in various food matrices like milk, vegetables, honey, etc., LLE [20,33], SPE [15,17,34] and combinations of LLE and SPE [15,35,36] are the most commonly used techniques as pretreatment procedures. On the other hand, there is a few literature data specifically targeted to the analysis of neonicotinoid residues in honey [5,14,37,38]. In terms of sample preparation, different approaches were employed like modified QuEChERS method [38,39] or other methods using LLE with dichloromethane [14] or cyclohexane/ethyl acetate [36].

In the last decade, a novel liquid phase extraction technique known as dispersive liquid–liquid microextraction (DLLME) has been introduced [40,41]. DLLME is based on a ternary component solvent scheme when a cloudy mixture (microdroplets) is formed upon injection of a mixture of an extractant (typical non-miscible organic solvent used in classical LLE or ionic liquids [42]) and disperser solvents (miscible organic solvents e.g. methanol, acetone, acetonitrile etc) into an aqueous sample. Due to the large contact surface area of the two immiscible phases, high extraction efficiency is achieved in a relatively short time. After extraction and phase separation [43,44], the organic solvent is collected and further analyzed. The main advantages of the DLLME include simple operation, low cost, rapidity, high preconcentration factors, and the use of small volumes of solvents. On the other hand, the disadvantages of the DLLME are its relatively low selectivity towards target analytes and the use of chlorinated solvents which poses a potential threat to the environment. Since its introduction, DLLME has been used for determinations of many compounds such as phenols [45], trihalomethanes [46], carbamate pesticides [47] and organophosphorus pesticides [48]. Based on recent literature findings there are only few publications on the use of DLLME as a sample pretreatment procedure in honey analysis [30,42,49].

The present study reports the development and validation of a DLLME-based LC–MS/MS analytical method for neonicotinoid analysis and its application toward the analysis of various honey samples. To the best of our knowledge, this is the first time that DLLME is applied as a pretreatment procedure for the determination of selected neonicotinoids in honey samples using liquid chromatography coupled to tandem mass spectrometry.

2. Experimental procedure

2.1. Chemicals and reagents

Certified standards of neonicotinoids (purity > 98%) were purchased from Sigma-Aldrich, (Steinheim, Germany). Common names and structures of the seven neonicotinoids evaluated in this study are shown in Fig. 1. HPLC grade acetonitrile and dichloromethane were obtained from Merck (Darmstadt, Germany). Formic acid (purity 98%, w/w) was purchased from Sigma-Aldrich (Steinheim, Germany). The water used was purified with a Simplicity UV water purification system from Millipore (Bedford, MA, USA). Individual standard pesticide stock solutions (100.0 mg L^{-1}) were prepared in water and stored at −10 °C in the dark. They were stable over a period of at least three months. Standard multi component solution (100.0 μg L^{-1}) was prepared by mixing appropriate volumes of each neonicotinoids standard solutions diluted with water, and such solution was used for spiking honey samples, to prepare the matrix-matched calibration standards and for studying the linear dynamic range of the LC–MS/MS analysis. Matrix-matched calibration standards were prepared by adding appropriate volumes of standard multicomponent working solution to blank honey samples at the final reconstitution step, over the range the limit of quantification (LOQ) to 100.0 μg kg^{-1} for all analyzed neonicotinoids. The standard solutions were stored under refrigerator conditions (4 °C) and protected from light; under these conditions the standard solutions were stable for at least 1 month.

2.2. Sample preparation

Honey samples of different floral origins were purchased from local markets (Novi Sad, R. Serbia). All samples were kept in their original packaging at ambient temperature as in everyday use. For the optimization and validation of the extraction and chromatographic method, a representative sample ($n=5$) was prepared by weighing 10.0 g of multifloral honey from a known location with no neonicotinoid contaminations, and it was used as a blank sample. A 50.0 g L^{-1} honey solution was prepared in water, spiked with different concentration of standard neonicotinoid multicomponent solutions and used throughout this study. All samples were left to "equilibrate" for at least 15 min prior to further analysis.

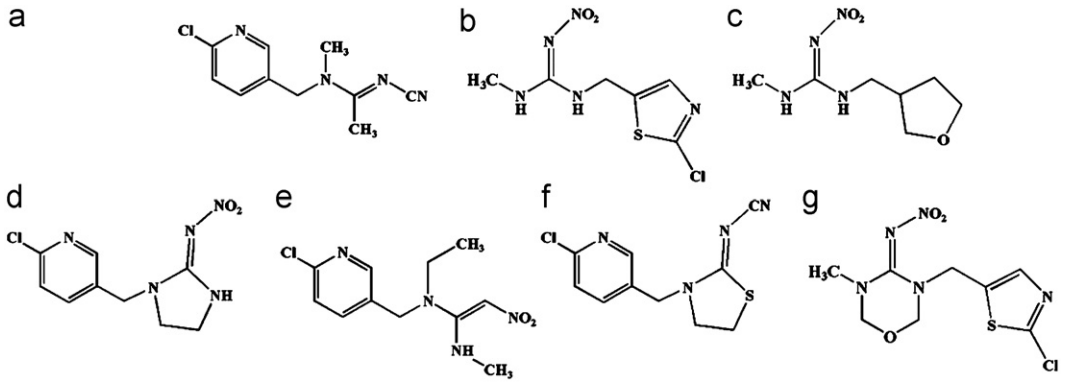

Fig. 1. Structures of neonicotinoids: (a) acetamiprid, (b) clothianidin, (c) dinotefuran, (d) imidacloprid, (e) nitenpyram, (f) thiacloprid and (g) thiamethoxam.

2.3. The DLLME protocol

The DLLME was performed as follows: an aliquot of 5.0 mL of the honey samples (blank or spiked), 0.5 mL acetonitrile (ACN) as a dispersive solvent and 2.0 mL dichloromethane (DHM) as extraction solvent, were added into the 10.0 mL round-bottom tube. The extraction tube was shaken for 1 min by vortex (BOECO, Germany) and soaked for 10 min in the ultrasonic bath (L&R ultrasonics LTD, United Kingdom) (37 kHz, 400 W), and once more shaken for 1 min by vortex. Then, the extraction tube was centrifuged for 5 min at 2500 rpm (Tehtnica, Yugoslavia). The sediment phase was removed using a syringe and collected in another test tube. The dichloromethane was evaporated under the flow of air by 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.

2.4. Statistical analysis for optimization of the DLLME method

To ensure that experimental conditions of the proposed DLLME protocol were optimal for given purpose, the mathematical response surface methodology (RSM) was used. A second-order central composite design (CCD) with two factors (in our study extraction solvent DHM and dispersive solvent ACN volumes) was used at five levels, each to take into account the individual and interaction effects of the factors. The experimental design included 14 experiments (divided into two blocks) with six replications of the center point [50]. For the description of the response sample extraction recovery based on matrix-matched calibration curves of each neonicotinoid (R, %), a second-degree polynomial model was fitted to the data:

$$R = b_0 + \sum_{i=1}^{n} b_i \times X_i + \sum_{i=1}^{n} b_{ii} \times X_{ii}^{\,2} + \sum_{i<j=2}^{n} b_{ij} \times X_i \times X_j \quad (1)$$

where b_0 represents intercept, b_i represents the linear, b_{ii} quadratic and b_{ij} interaction effect of the factors. The factor variables and their ranges were: X_1 ACN volume (0.0–1.0 mL) and X_2 DHM volume (1.0–3.0 mL). Since the final goal of RSM is process optimization, the developed model (equation) can be used for this purpose.

The desirability function is one of the most widely used methods for optimization of response processes in science and engineering [51]. Each of the estimated responses is transformed to an individual desirability value ranging from 0 to 1. The value of individual desirability increases as the desirability of the corresponding response increases. The overall desirability of the process is computed as a geometric mean of the individual desirability functions [52].

Statistical analyses and optimization were performed using the software StatSoft Statistica 10 and Stat-Ease Design-Expert 7. Response surface plots were generated using the same software and drawn by using the function of two factors.

2.5. The LC–MS/MS system and operating conditions

An Agilent 1200 Series LC system (Agilent Technologies Inc., USA) consisting of a solvent degassing unit, a quaternary pump, an autosampler and a thermostated column compartment was used in the LC–MS/MS system. Separation of the analytes was achieved on a ZORBAX Eclipse XDB-C18 column (50 mm × 4.6 mm i.d., 1.8 μm) with a column temperature of 30 °C. The mobile phase consisted of two eluents, ultrapure water with 0.1% formic acid and ACN, delivered at a flow rate of 0.5 mL min^{-1}. The isocratic elution was employed with the ratio of ACN/water (0.1% formic acid) 20:80, v/v. An Agilent 6410 Triple Quad LC/MS mass spectrometer (Agilent Technologies Inc., USA) was operated with a multimode interface in positive ion mode. MassHunter workstations software version B.03.01 (Agilent Technologies Inc., USA) was used for the control of equipment, data acquisition and analysis. The instrument was operated with the heater gas temperature of 325 °C and vaporization temperature of 200 °C. Additionally, nitrogen was used as a nebulizer gas at 50 psi, and a flow rate of 5 L min^{-1}, capillary voltage of 2500 V and charging voltage of 2000 V. For all the investigated compounds total ion chromatograms (TIC) were recorded. The multiple reaction monitoring (MRM) transitions used a dwell time of 20 ms (ms). For the proposed method, the most intense characteristic MRM transitions were chosen for each analyte.

2.6. Method performance

The accuracy and precision of the proposed method were evaluated by spiking blank honey samples to fulfill all the necessary requirements of SANCO/12495/2011-method validation and quality control procedures for pesticides residue analysis in food and feed [53]. The calibration curves for all of the compounds in pure solvent and in matrix were obtained by plotting the peak areas against the concentrations of the corresponding calibration standards at nine calibration levels ranging from the LOQ to 100.0 μg L^{-1}.The linearity of calibration curves was expressed by the correlation coefficient (r^2). The limit of detection (LOD) was determined at signal-to-noise ratio of three, whereas the LOQ was determined by considering a signal-to-noise ratio of 10 by using matrix matched calibration curves. For the matrix-matched calibration curves, the blank honey samples were enriched with working standard solutions at the final reconstitution step providing linearity over the range from the LOQ to 100.0 μg kg^{-1} for all seven analyzed neonicotinoids (Table 4). The accuracy of the method was determined with the percentage recovery (R, %) using spiked blank honey samples prior to analysis and matrix-matched calibration curves by comparing the mean measured concentration with the spiked concentration of the analyzed neonicotinoids. The precision of the method was expressed in terms of repeatability and within-laboratory reproducibility.

The repeatability was determined by spiking samples at two concentration levels (10.0 and 100.0 μg kg^{-1}) with the analyzed neonicotinoids. Samples were analyzed on the same day ($n=5$) with the same instrument and the same operator, and repeatability was calculated as relative standard deviation (RSD).

The within-laboratory reproducibility was determined by spiking blank honey samples at the same concentration levels of analyzed neonicotinoids as for the repeatability, and by analyzing them on three different days with the same instrument and by the different operators. The within-laboratory reproducibility was calculated as the overall RSD.

3. Results and discussion

For the highest efficiency of the developed method it was necessary to obtain optimal sample weight and experimental parameters for the DLLME-based extraction, liquid chromatographic separation and MS/MS quantification.

3.1. Optimization of sample weight

The complex composition of the honey matrix (monosaccharides, carbohydrates, organic acids) depends on its type, origin, etc. This issue would potentially lead to uncertainty in terms of the accuracy, selectivity and the precision of the DLLME method. The experiments showed that for sample concentrations higher than 50.0 g L^{-1} the reproducibility and accuracy of the method was poor (RSD > 20%, R < 60%). This may be attributed to the higher

solution viscosity at elevated honey concentrations that seem to affect the sedimentation of the organic phase microdroplets. For this reason, a fixed honey concentration of 50.0 g L^{-1} was selected for the subsequent experiments.

3.2. Optimization of DLLME

Different experimental conditions were optimized in order to select the appropriate parameters for the DLLME sample pretreatment procedure. In order to accomplish this, a series of parameters that affected DLLME efficiency (e.g. type and volume of extraction and dispersive solvents, time of vortex mixing, centrifugation and soaking in the ultrasonic bath, etc) were carefully investigated using the univariate approach and also simultaneously changing the investigated parameters for building an optimized statistical model system of the DLLME procedure.

3.2.1. Sample aliquot volume

In the optimization process for the proposed DLLME pretreatment procedure use was made of spiked honey samples, reaching the final concentration level of 100 μg kg^{-1} of each selected neonicotinoid. After varying the aliquot volume from 5.0 to 10.0 mL (in steps of 1.0 mL), 5.0 mL was chosen as a sample aliquot volume for further investigations because there were no significant ($p < 0.05$) differences between the final recoveries of the tested spiked honey samples (from 5.0 to 10.0 mL of aqueous phase), and because it was easier to manipulate smaller volumes in the test tubes throughout the sample pretreatment procedure.

3.2.2. Choice of extraction and dispersive solvents

Selection of an appropriate extracting solvent, which is the major parameter for the DLLME process, is of primary importance. Within the solvents with density higher than water, two extraction solvents were investigated, chloroform and dichloromethane, and dichloromethane showed better overall extraction recovery values (with the lowest $R = 74.3\%$) than chloroform ($R < 70.0\%$ for the thiacloprid and acetamiprid), and it was used as an extraction solvent in the further optimization tests.

The miscibility of the dispersive solvent in both extracting solvent and aqueous phase is a connection between two immiscible phases, and selection of an appropriate dispersive solvent is one of the essential points in the DLLME pretreatment procedure. The type of the dispersive solvent influences the interfacial tension of the extraction solvent, determining the microdroplet size and, consequently, the efficiency of the extraction. Throughout this study ACN was used as a dispersive solvent based on the works of Zacharis et al. [25].

3.2.3. Optimization of extraction and dispersive solvent volume

The extracting solvent volume has an important impact on the sample recovery. By its increase, the volume of sedimented phase obtained by centrifugation increased too, thus influencing the sample recovery. Therefore, the optimal extracting solvent volume should ensure both high recovery and sufficient volume of the sedimented phase for the subsequent analysis after centrifugation. The extraction solvent volume was varied from 0.5 up to 3.0 mL (0.5, 1.0, 2.0, 3.0 mL) to provide the best sample recovery while keeping the other parameters as described in the DLLME protocol. For all investigated neonicotinoids, the extraction recovery increased with the increase in the DHM volume up to 2.0 mL ($R > 70\%$), while its further increase to 3.0 mL did not significantly affect the neonicotinoid extraction recovery. The use 2.0 mL of DHM also satisfied the low solvent consumption prerequisite for the environmental benignity.

The dispersive solvent volume influences the formation of a cloudy solution system (water/ACN/DHM), the dispersion degree of the extracting solvent in the aqueous phase and, subsequently, the extraction efficiency through the test sample extraction recovery. For the optimization of the dispersive solvent volume, different volumes of ACN were used (0.0, 0.5, 1.0 mL). The extraction recoveries of the investigated neonicotinoids indicated that without the dispersive solvent (there is no clear formation of two phases, which is one of the problems accounted in traditional liquid–liquid extraction procedures) the extraction recoveries were below 75% for all tested neonicotinoids. However, with increased volume of ACN the extraction efficiency increased first with 0.5 mL of ACN ($R > 70\%$) for all the analytes, but with the further increase of ACN the extraction recovery decreased ($R < 65\%$). One of the possible reasons for this could be that higher volumes of ACN lead to a higher solubility of the neonicotinoid insecticides in water, causing a decrease in the distribution coefficient and extraction efficiency.

3.2.4. Optimization of the extraction time and centrifugation speed

A main characteristic of the DLLME pretreatment procedure is the formation of microdroplets of the extraction solvent that is dispersed in the aqueous phase. Thus, the large surface area of the contact between the two phases results in a fast mass transfer process, providing fast extraction. The performance of the DLLME was examined at the vortex mixing time intervals between 1–5 min. As expected, the extraction time had a negligible influence on the peak areas of the analytes and therefore the mixing times of 1 min before and after the ultrasonic extraction were employed. The effect of ultrasonic extraction time was tested in the time interval from 5 to 15 min (in 1 min steps), giving an increase in the sample extraction recovery to 10 min (R 76.1–114.0%), after which it had no effect on the extraction recovery. The influence of the centrifugation speed on the extractability of neonicotinoids was examined in the range of 2000–4000 rpm. The extraction recoveries were slightly improved up to 2500 rpm and were unaffected at higher rates. Therefore, a centrifuge speed of 2500 rpm was chosen for further analyses.

3.3. Mathematical model system for DLLME

In order to find the optimal parameters for the DLLME procedure a statistical data evaluation based mathematical model (equation) was created using all experimental data of investigated extraction method parameters described above. The RSM is a useful model for studying the effect of several factors influencing the system by varying them simultaneously and carrying out limited number of experiments. The obtained regression equation

Table 1
Regression equation coefficients for recovery (based on matrix-matched calibration curves R, %) during the process of extraction of clothianidin.

Coefficients	Value	Sequence of significance
Intercept		
b_0	96.12	–
Linear		
b_i	0.35	5
b_j	−1.22	4
Quadratic		
b_{ii}	−7.75	2
b_{jj}	−19.65	1
Interaction		
b_{ij}	−2.80	3
R^2	0.9642	

coefficients are given in Table 1. A relatively high value of r^2, obtained for the R (%) indicates good fit of the experimental data to Eq. 1. The significance of each coefficient was determined by Student's t-test and p-values. The larger the magnitude of the t-value and the smaller the p-value, the more significant is the corresponding coefficient in the proposed model system.

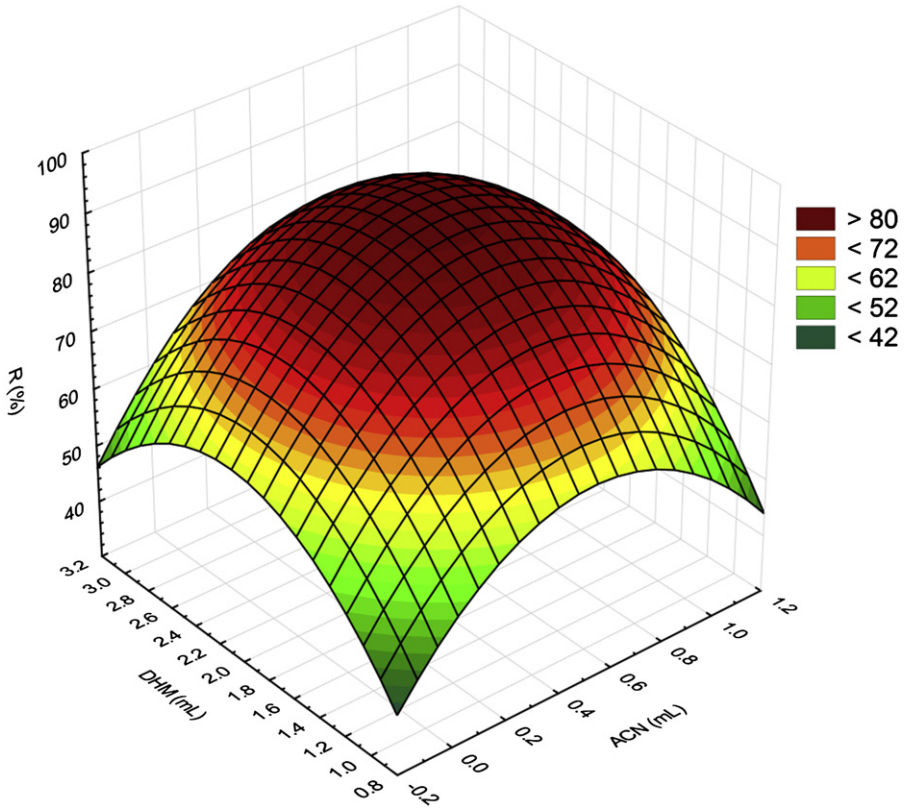

Fig. 2. Extraction recovery (based on matrix-matched calibration curve R, %) for clothianidin as a function of the volumes of ACN and DHM. The inserted legend shows different ranges of R values.

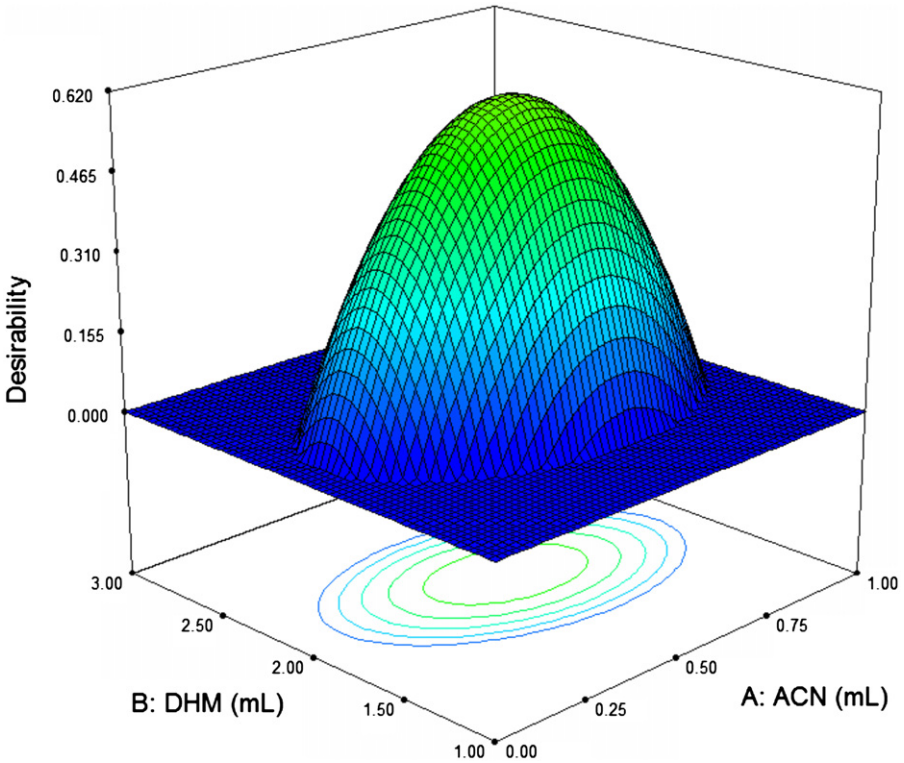

Fig. 3. The overall desirability function of the clothianidin extraction process in 3D surface plot.

The results given in Table 1 show that the recovery was linearly influenced more by the volume of DHM than of ACN. The quadratic effect of the DHM volume was the most significant, while the significance of ACN and DHM volumes interaction was somewhere between the linear and quadratic effect.

The effect of varying the ACN and DHM volumes on the extraction recovery (*R*, %) of clothianidin as a representative of the investigated neonicotinoid is presented in Fig. 2. As it can be seen, there is a peak (recovery maximum) for the examined range of the extraction and dispersive solvent volumes, but the effect of the volume of the solvents on the increase in the extraction recovery was not unlimited. Therefore, it was necessary to optimize the extraction process in terms of the used volumes of ACN and DHM. All the investigated neonicotinoids showed also similar extraction properties as clothianidin.

3.4. Optimization of DLLME by desirability function

The results of the optimization by desirability function approach for maximization of the extraction recovery of clothianidin as a representative of the investigated neonicotinoid insecticides are shown in Fig. 3. The criteria for the optimization were selected so that the volumes of ACN and DHM remained in the examined experimental range while trying to obtain a maximum recovery with a lower limit of 90% (0 desirability) and the upper limit of 100% (1 desirability). As it can be seen from Fig. 3, the highest values of desirability function can be obtained in the region of the center point. The optimal values of ACN and DHM volume were thus 0.51 mL and 1.97 mL, respectively, for the highest value of desirability function (0.614), i.e., the highest extraction recovery (96.14%). The same optimization by the desirability function approach was made for all investigated neonicotinoids, giving the same optimal extraction parameters. Because of that, 0.5 mL of ACN and 2.0 mL of DHM were used in further extraction experiments, showing an agreement between the experimental results and desirability function based mathematical model.

Table 2
The *m*/*z* of precursor ion (*Q*1), *m*/*z* of monitored product ion (*Q*3), fragmentor voltage (*FV*) and collision energy (*CE*) of each pesticide.

Neonicotinoid	*Q*1	*Q*3	*FV* (V)	*CE* (V)
Thiamethoxam	292.2	211.1	82	8
		181.1	82	20
Thiacloprid	253.1	126.0	102	20
		90.0	102	30
Nitenpyram	271.2	225.1	102	8
		56.1	102	30
Imidacloprid	256.1	209.0	92	12
		175.1	92	16
Dinotefuran	203.1	129.1	82	8
		113.1	82	8
Clothianidin	250.0	169.1	92	8
		131.9	92	16
Acetamiprid	223.1	126.0	124	12
		56.1	124	20

3.5. Optimization of MS/MS parameters

In order to achieve good separation with high sensitivity and unambiguous identification of seven neonicotinoid insecticides in honey samples at trace levels, different experiments were conducted with the purpose of finding the best instrumental conditions.

To choose the optimum transitions and associated acquisition parameters in the MRM modes, different parameters were studied (MassHunter optimizer software, Agilent Technologies Inc., USA). The initial study consisted of two parts. First part was to optimize the fragmentor voltage for each of the seven compounds in order to produce the greatest signal for the precursor ion. For the positive electrospray ionization mode, formic acid substantially promoted the formation of $[M+H]^+$ precursor ion, leading to better sensitivity, as well as better resolution and peak shape. Each compound was analyzed using fragmentor voltages in range 80–150 V (in 10 V increments). For each compound, optimal values for fragmentor voltage (corresponding to the highest precursor ion abundance) were used for all subsequent analyses and each compound was injected in a programmed run at a concentration of 10 μg mL^{-1} to determine collision energies for both the quantifying and qualifying ions. Also, various collision energies (5, 10, 15, 20, 25, and 30 V) were applied. The energies were then optimized for each of the ions, and the voltages that gave the best sensitivity were selected. The MRM transitions used for this study are shown in Table 2.

As next, honey samples were spiked with the mixture of all seven neonicotinoids (LOQ-100.0 μg kg^{-1}), and after the extraction using DLLME the optimized LC–MS/MS method was performed. The representative TIC for 75.0 μg kg^{-1} was presented in Fig. 4, showing a good separation of neonicotinoids. The typical MRM chromatograms (Fig. 5) of quantifier and qualifier ions and composite spectrum for both transitions of spiked honey sample at the neonicotinoid concentration level of 75.0 μg kg^{-1} were acquired under the conditions described above.

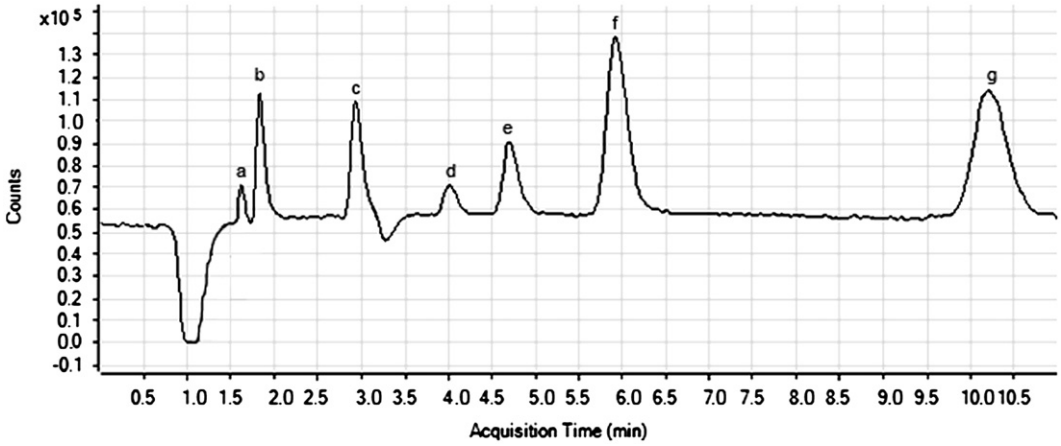

Fig. 4. Total ion chromatogram (TIC) of: (a) dinotefuran, (b) nitenpyram, (c) thiamethoxam, (d) clothianidin, (e) imidacloprid, (f) acetamiprid and (g) thiacloprid ions monitored at 75.0 μg kg^{-1} spiked honey sample.

3.6. Matrix effects

For the proposed method development, the use of matrix-matched calibration standards was done to compensate for the matrix effect, i.e., signal suppression or enhancement of the studied neonicotinoids in the honey matrix. The matrix effect, expressed as the signal from the pesticide in the matrix compared to the signal in the solvent was tested at two spiked concentration levels (50.0 and 75.0 μg kg^{-1}). The recoveries calculated by solvent calibration and matrix-matched calibration curves of all investigated neonicotinoids are shown in Table 3.

Imidacloprid and thiacloprid displayed the enhancement of the signal, while thiamethoxam, clothianidin, acetamiprid, dinotefuran and nitenpyram displayed the suppression of the analytical signal due to the matrix effects. The coeluting substances may cause problems in the quantification by compound-specific suppression or enhancement, with the signal suppression being most significant for early eluting compounds. Considering this, it can be noted that nitenpyram, dinotefuran and clothianidin were significantly affected by the matrix components with the extraction recoveries less than 70%. However, the extraction recoveries calculated by matrix-matched calibration curves were all satisfactory (76.1–114.0%). Based on these comparisons, an accurate quantification required the use of matrix-matched standards.

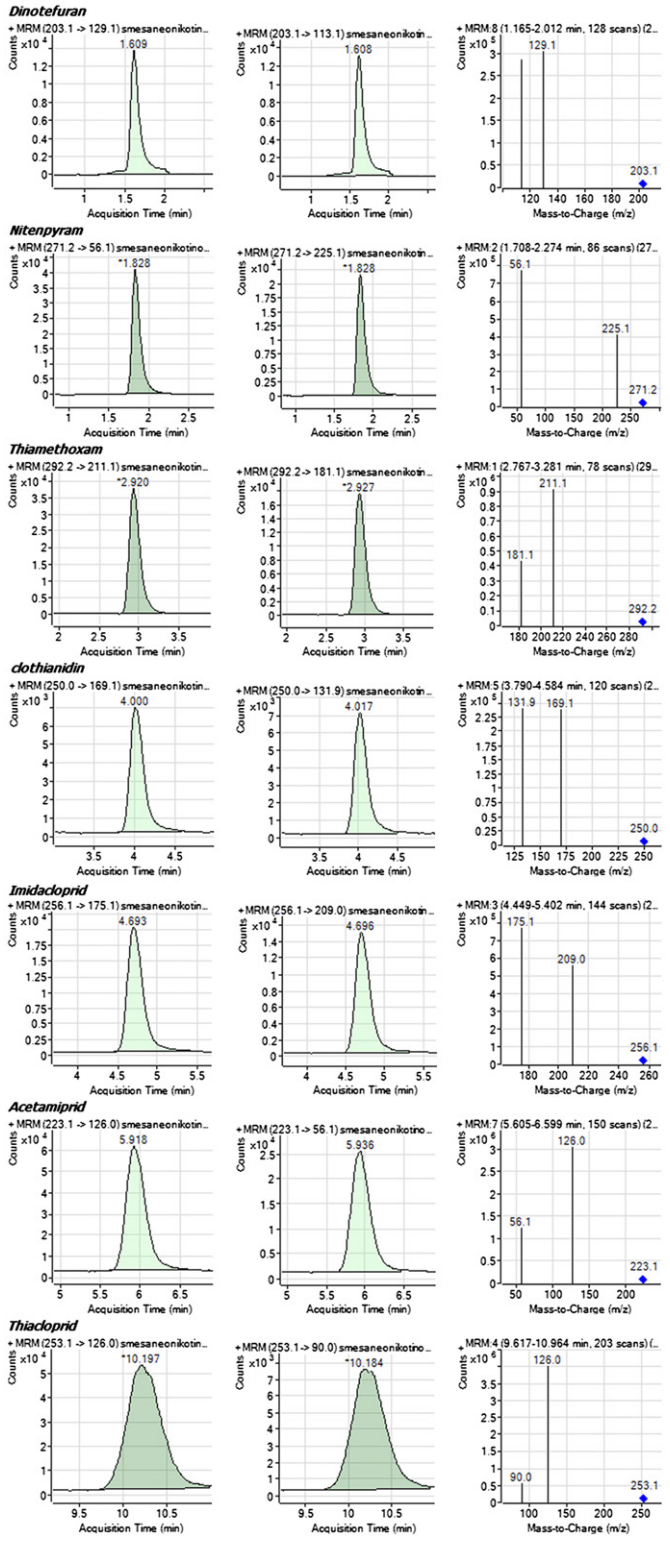

Fig. 5. MRM chromatograms of quantifying and qualifying ions and composite spectrum (dinotefuran, nitenpyram, thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid) for both transitions at neonicotinoid concentration level of 75.0 μg kg^{-1}.

3.7. Method validation

3.7.1. Linearity of calibration standards

The matrix-matched calibration curves were linear over the range LOQ-100.0 μg kg^{-1} for the analyzed compounds. Linearity, expressed as the correlation coefficients (r^2), gave the values all above 0.9906 for the linear range as shown in Table 4.

3.7.2. Assay selectivity

The selectivity was evaluated by the analysis of five blank honey samples, and no interfering peaks from endogenous

Table 3
Recoveries of neonicotinoids based on solvent (R_s) and matrix-matched (R) calibration curves.

Neonicotinoid	Spiking level μg kg^{-1}	R_s (%), Solvent calibration curve	R (%), Matrix-match calibration curve
Acetamiprid	75.0	77.8	88.8
	50.0	81.1	92.2
Clothianidin	75.0	53.1	78.4
	50.0	56.8	85.9
Dinotefuran	75.0	67.0	98.8
	50.0	63.3	100.0
Imidacloprid	75.0	77.4	76.1
	50.0	80.3	80.0
Nitenpyram	75.0	30.9	98.0
	50.0	33.9	90.0
Thiacloprid	75.0	131.0	114.0
	50.0	131.0	100.0
Thiamethoxam	75.0	71.9	84.0
	50.0	76.9	90.0

Table 4
Retention times (RT), matrix-matched calibration curves and correlation coefficients (r^2) for each neonicotinoid.

Neonicotinoid	Mean RT ($n=15$) (min)	RSD (%) of RT	Linear range (μg kg^{-1})	Linear equation	Correlation coefficients (r^2)
Dinotefuran	1.58	2.20	2.5–100.0	$y=91.9x-146.4$	0.9993
Nitenpyram	1.79	3.80	1.8–100.0	$y=241.9x+1250.1$	0.9956
Thiamethoxam	2.84	3.70	1.5–100.0	$y=420.1x-30.9$	0.9968
Clothianidin	3.89	2.50	2.5–100.0	$y=100.1x-322.3$	0.9967
Imidacloprid	4.59	0.60	1.5–100.0	$y=352.4x-1271.1$	0.9915
Acetamiprid	5.76	1.26	1.5–100.0	$y=1259.3x+3675.4$	0.9906
Thiacloprid	9.94	1.20	1.5–100.0	$y=1147.7x+2150.1$	0.9980

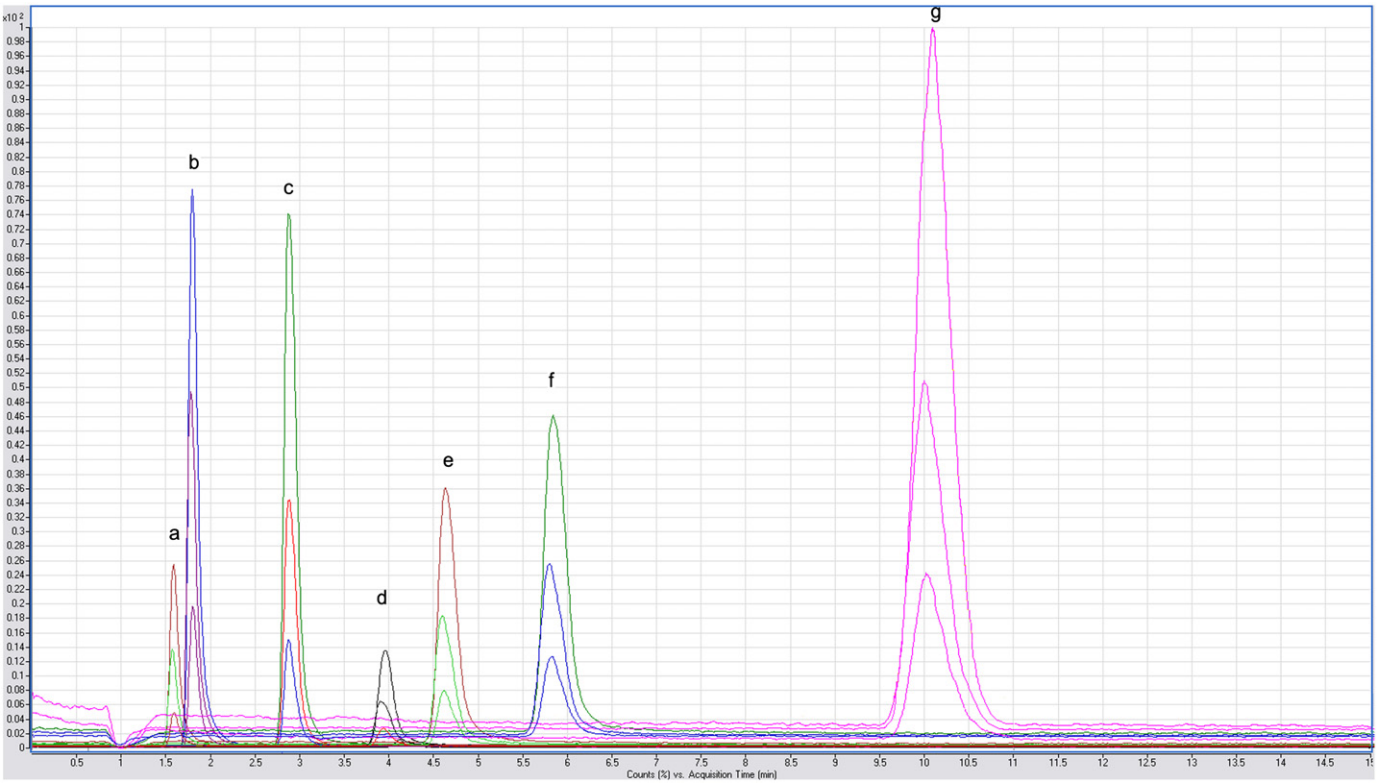

Fig. 6. Extracted MRM ion chromatograms of honey samples with spiked neonicotionoids mixture ((a) dinotefuran, (b) nitenpyram, (c) thiamethoxam, (d) clothianidin, (e) imidacloprid, (f) acetamiprid and (g) thiacloprid) at 10.0, 25.0 and 50.0 μg kg^{-1} concentration levels.

Table 5
Accuracy and precision parameters of selected neonicotinoids.

Neonicotinoid	Spiking level (μg kg^{-1})	Accuracy	Repeatability ($n=5$)	Within-laboratory reproducibility ($n=3\times5$)
		Mean R (%)	RSD (%)	RSDs (%)
Acetamiprid	100.0	90.7	4.26	15.30
	10.0	82.4	4.16	14.70
Clothianidin	100.0	96.1	8.16	13.02
	10.0	74.3	5.12	8.87
Dinotefuran	100.0	95.4	9.58	11.70
	10.0	96.2	8.09	16.20
Imidacloprid	100.0	90.6	4.07	6.70
	10.0	97.5	10.80	13.98
Nitenpyram	100.0	89.2	11.00	15.70
	10.0	104.4	6.66	13.00
Thiacloprid	100.0	99.4	2.74	6.64
	10.0	113.9	10.30	15.60
Thiamethoxam	100.0	88.2	11.80	13.10
	10.0	92.1	7.23	12.20

compounds were found at the retention time of the analyzed neonicotinoids. The extracted MRM ion chromatograms of the honey sample with spiked neonicotinoids mixture at 10.0, 25.0 and 50.0 μg kg^{-1} concentration levels are shown in Fig. 6 with selected recoveries included in Table 5. The chromatogram shows the selectivity and good chromatographic characteristics of the proposed method at different concentration levels.

3.7.3. Accuracy and precision

The accuracy of the method was evaluated by recovery studies: recoveries and RSDs were determined for 15 replicates at two concentration levels (10.0 and 100.0 μg kg^{-1}) using the spiked blank honey samples prior to analysis and matrix-matched calibration curve. According to the EU validation guideline for pesticide residues, mean recovery values should be within the range of 70–120% at each spiking level with RSDs of ≤20%. The recovery results presented in Table 5 confirmed that the optimal recovery was obtained for all selected pesticides.

Precision, expressed as the repeatability, was examined by analysis of the same samples ($n=5$) at two different concentrations on the same day. The RSD values were within the range of 2.74–11.8% for all selected pesticides, fulfilling the mentioned criteria of RSD ≤20%.

The within-laboratory reproducibility was determined by analyzing the fortifying blank honey samples at the same concentration levels of analyzed pesticides as for the repeatability, during three different days with the same instrument and the different operators. The calculated RSDs are showed in Table 5, and the values within the range of 6.64–16.2% indicate a good precision of the developed method.

3.7.4. Limit of detection and quantification

For the identification and confirmation of the presence of a target compound, at least two ion transitions must give signals distinguishable from the background signal when MS/MS detection is performed. The calculated LODs determined as signal-to-noise of three and LOQs determined by considering a signal-to-noise ratio of 10 by using matrix-matched calibration curves are shown in Table 6. These parameters were determined by analyzing a series of decreasing concentrations of the spiked honey samples. The presented LOQs are lower than the MRLs for neonicotinoids, valid for honey, established by the European Commission. However, certain MRLs consist of residue definitions encompassing a metabolite besides the respective neonicotinoid. These

Table 6
Limits of detection and quantification with maximum residue levels regulated in EU.

Neonicotinoid	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)	MRLs of EU ($\mu g\ kg^{-1}$) [6]
Acetamiprid	0.5	1.5	50
Clothianidin	1.0	2.5	10
Dinotefuran	0.9	2.5	10
Imidacloprid	0.5	1.5	50
Nitenpyram	0.8	1.8	10
Thiacloprid	0.5	1.5	200
Thiamethoxam	0.7	1.5	10

metabolites were not included in the developed method, and further work should focus on the inclusion of those compounds.

3.8. Application to real samples

The developed method was applied to the analysis of 15 commercial honey samples with different floral origin, purchased from local markets (Novi Sad, R. Serbia). All honey samples were analyzed following the proposed method. The results indicated that the all investigated honey samples were free from the target neonicotinoid insecticides. The applicability of the proposed method on the honey samples was demonstrated by spiking the samples at different concentrations. The average recoveries and RSD of analyzed samples fulfilled the SANCO criteria, and were also used for the method validation (Table 5).

4. Conclusion

A rapid, sensitive and low solvent consumption method described in this paper provides reliable, simultaneous quantitative analysis of seven neonicotinoid residues in honey samples. The optimized DLLME procedure provides significant advantages including simplicity, low operation cost and volumes of solvents, employment of usual laboratory equipment, high extraction efficiency, and short extraction time. Reverse phase and isocratic elution based liquid chromatographic conditions are set to provide efficient separation of investigated neonicotinoids. The MS/MS parameters were optimized to unequivocally provide quantification of low concentrations of selected neonicotinoids. External matrix-matched calibration method was used to eliminate variable matrix effect and ensure precise quantification. The obtained calibration curves displayed good linearity. The proposed method has been validated with good recoveries and low LODs and LOQs, lower than MRLs set by the EU, proving its successful usage for the intended application and routine laboratory analyses. Further experiments will be performed to extend the developed rapid and sensitive analytical method for the analysis of neonicotinoids in some other bee products.

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