Neonicotinoids Detection by new LC-MS/MS Method in Romanian Surface Waters

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Increasing and widespread use of neonicotinoid insecticides in all world, together with their highly toxicity to invertebrates and environmental persistence mean that surface waters need to be monitored for these compounds. In the 2015, neonicotinoid insecticides have been incorporated in the watch list of substances for a European Union monitoring program (495/2015/ EU). A new method using automated solid phase extraction (SPE) with polymeric cartridges (OASIS HLB) followed by LC-MS/MS provided good separation of the most common neonicotindoid compounds. The method was developed for the determination of four neonicotinoid insecticides (nitenpyram, thiamethoxam, clothianidin, acetamiprid) in surface water with low limit of quantification (0.3-0.9 ng/L, nanograms per liter). Recoveries in surface water samples fortified at 200 ng/L for each compound ranged from 71.4 to 109.9 %; relative standard deviation ranged from 4 to 9%. The method was applied to water samples from four streams in Romania, Danube River and its tributaries (Arges River, Jiu River, and Olt River). The surface water samples were found to be contaminated clothianidin (1.08-6.4 ng/L) and by thiamethoxam (1.1-3.8ng/L). The highest concentrations were recorded in Danube River in Oltenita point (6.4ng/L) and in Gura-Vaii point (5.5ng/L). The concentration of acetamiprid and nitenpyram were situated below limit of quantification in all samples.

Keywords: neonicotinoid, LC-MS/MS, priority substances, Danube River

The neonicotinoid insecticides belong to the group of nitroguanidine systemic insecticides frequently applied to crops as soil and seed treatments at planting to protect seedlings from early-season root and leaf-feeding pests, as well as via later season foliar treatments [1]. These insecticides are currently registered for use on more than 140 different crops in 120 countries in over 120 countries [2]. The neonicotinoid class of pesticides was first developed in the early 1990s, partly in response to increasing pest resistance, concerns over cumulative exposure from organophosphorous and carbamate insecticides [3]. Imidacloprid has been the most widely used and is the active ingredient in a high number of commercial formulations [4]. In 2008, neonicotinoids represented 24% of the global market share for insecticides and 80% of the seed-treatment market [2]. Neonicotinoids are receiving increased control since they have been involved in adversely affecting pollinators and related to colony collapse disorder in bees [5]. Thiamethoxam has been linked to decreased survival in honeybees, while imidacloprid has been linked to reduced colony growth in bumble bees and sub lethal affects to flies [6]. Neonicotinoids are generally toxic to insects in minute quantities; for example, the LD50 (dose that kills 50% of individuals) for ingestion of imidacloprid and clothianidin in honeybees is 5 and 4 ng per insect, respectively [7]. Continued exposures to neonicotinoid insecticides may lead to a cumulative effect in insects. Birds are also susceptible to neonicotinoid exposure, including both the direct ingestion of treated seeds and through contamination of the aquatic environment. In 2013, the European Commission adopted a proposal to restrict the use of 3 neonicotinoids (clothianidin, imidacloprid and thiamethoxam) for a period of 2 years, including their use for seed treatment [8]. The Directive on Environmental Quality Standards/Priority Substances Directive -Directive 2008/105/EC (EQSD) [9], set environmental quality standards (EQS) for the substances in surface waters (river, lake, transitional and coastal) and confirmed their designation as priority or priority hazardous substances. In the 2015, neonicotinoid insecticides have been incorporated in the *watch list* of substances for a European Union monitoring program (495/2015/ EU, LOD 9ng/l) using the proposals made by Joint Research Centre, so that more information about environmental occurrence of those substances will be available in the future [10-11]. Monitoring data provide also important information for the prioritization of toxic emerging pollutants and for possible including in the *Water Framework Directive (*WFD).The neonicotinoid insecticides are high soluble in water and they are persistent in soil, for these reason they can contaminate water after storm events that produce runoff pulses and by leaching to the groundwater [12]. Liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS) is a powerful technique for the analysis of organic pollutants like pesticides and emerging contaminants in environmental samples [13-16]. Generally, the research concerning the pollution of the Danube surface water and his tributaries undergone in our country was focused on detecting priority organic compounds, emerging contaminants like pharmaceutical substances, toxic and persistent heavy metals and inorganic substances deriving from chemical fertilizers [17-19]. Therefore, there is a need of data on the presence of these contaminants in the Romanian part of Danube River basin. The lack of environmental data about the occurrence of neonicotinoids in surface water is considered an important research gap for these insecticides. Thus, this research was realized to provide the first national study of neonicotinoids presence in Romanian part of Danube River and in its three major tributaries. Such national investigation provides important baseline concentration data for the assessment of potential environmental effects from exposure to neonicotinoids in rivers. The Danube is the second longest river of Europe

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Table 1
CHARACTERISTICS OF THE FOUR NEONICOTINOID INSECTICIDES INVESTIGATED

Compound	Lipophilicity ($logK_{ow}$)	Water solubility (mg/L) 20°C	Soil affinity ($logK_{oc}$)	Water hydrolysis; pH 9 (DT_{50} in days) [a]	Water photolysis (DT_{50} in days)	Soil degradation half-life (days)
Nitenpyram	-0.66	590000	1.78	Stable 2.9	Not available	1-15
Thiamethoxam	-0.13	4100	1.75	Stable 11.5	2.7-39.5	50
Clothianidin	0.91	340	2.08	Stable 14.4	0.1	545
Acetamiprid	0.8	2950	2.3	Stable 420	34	3

[a] *At pH 4-7 compounds are stable, but at pH 9 hydrolysis can occur*

(after the Volga) and is the only European river that flows from West to East [20].

The objective of the present study was to develop a sensitive and selective SPE-LCMS/MS method with positive electrospray ionization (ESI+) able to determine four neonicotinoid insecticides from surface water at trace level concentration (ng/L). The method was applied to establish the occurrence of selected insecticides along the Romanian part of the Danube River and assess the contribution by its main three tributaries: Jiu, Olt and Arges. Due to low contaminants concentrations determined in surface water samples, and the complexity of these matrices, a purification and pre-concentration step of the samples, prior to analysis is required. For the isolation of neonicotinoid insecticides from environmental samples was used solid-phase extraction (SPE) [12, 13, 17]. From 2014 to 2016, Romania granted emergency authorisations for neonicotinoid use on maize, sunflower seed crops and for rape seed crops. Based on official data, maize crops, were treated with neonicotinoids in 246,195 ha out of a total of 3,500,000 ha. Also, sunflower crops, another highly bee-attractive crop, were treated with neonicotinoids in 151,308 ha out of a total of 1,000,000 ha in 2015 in Romania [21]. The chemical properties (solubility, lipophilicity, K_{oc}) and environmental persistence (DT_{50} for water photolysis and hydrolysis) of neonicotinoid insecticides can be observed in table 1 [1, 12]. All neonicotinoids are high water soluble, and they are stable to hydrolysis at neutral and acidic *p*H. Their water solubility, lipophilicity, and soil affinity suggest that these insecticides are moving through runoff and generate persistence of neonicotinoids.

Experimental part

Chemicals and reagents

Analytical grade standards (purities higher than 99.7) of the four most commonly used neonicotinoids (acetamiprid, nitenpyram , thiametoxan, clotianidin) were purchased from Sigma-Aldrich (Steinheim, Germany). Individual *s*tandard stock solutions of compounds (500mg/L) were prepared by dissolving of the solid standards in methanol. The acetonitrile, methanol, LC-MS grade formic acid (98%), used for preparation of standards, mobile phase were supplied by Sigma-Aldrich. The intermediate standard solutions were prepared by dilution of stock standard solutions in acetonitrile; the working standard mixtures were prepared by diluting the intermediate standard solutions in acetonitrile. High purity water was produced by passing double distilled water through Milli-Q water purification system (Millipore, Bedford, MA, USA). Glass microfibre filters were obtained from Whatman (United Kingdom). SPE cartridges, built of a hydrophilic and a lipophilic monomer (Oasis HLB: 500 mg, 6 mL), were acquired from Waters (Milford, Massachusetts, USA).

S*ample collections*

The collection of the samples was performed in March 2016, from 10 locations along the Romanian part of the Danube River and from 2 locations from each of the main tributaries, one location being close to their confluence with Danube River, as shown in figure 1. The samples were taken from a depth of approximately 0.5 m at 2 m from the river bank. River water samples were collected in 500mL amber glass bottles, previously rinsed with water sample at the sampling site. After collection, the samples were stored at 4°C until arrival to the laboratory and pretreated by solid phase extraction within 48h. Location data, GPS coordinates and description are given in table 2.

Equipment

For the LC analysis, an Agilent 1260 HPLC system equipped with a binary pump was used. The chromatographic separation of compounds was made on a Hypersil Gold analytical column of 100mm × 2.1 mm and 3µm particle size (Thermo Scientific). The mobile phases,

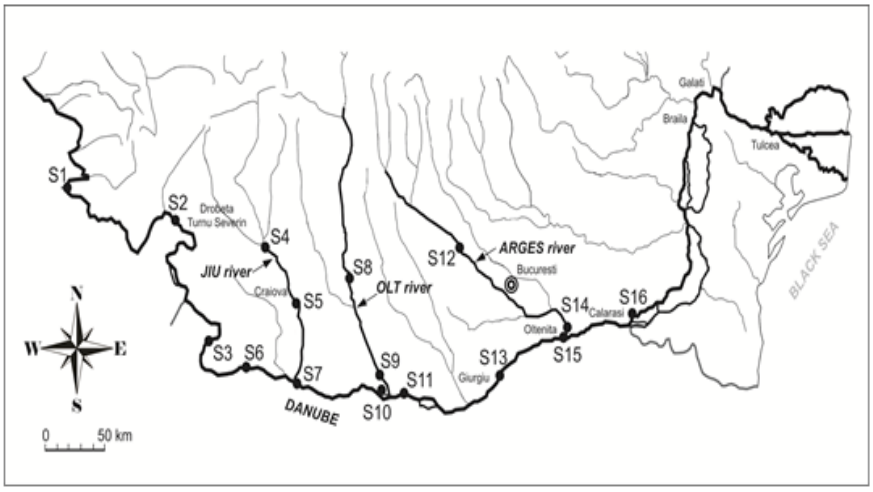

Fig. 1. Map of the study area in the Danube River basin, location and codes of the sampling points

Table 2
LOCATION DATA AND GPS DATA

Sampling points	GPS coordinates	Sampling points	GPS coordinates
S1 – Bazias locality on Danube River	44°47′32.61″N 21°23′20.07″E	S9 – Izbiceni, upstream confluence with Danube River	43°48′41.71″N 24°42′27.71″E
S2 – Gura Vaii locality on Danube River	44°40′7.40″N 22°33′10.74″E	S10 – Islaz (Danube), upstream confluence with Olt river	43°42′22.72″N 24°44′4.01″E
S3 – Calafat locality on Danube River	43°57′50.94″N 22°54′15.72″E	S11 – Turnu Magurele (Danube), downstream confluence with Olt river	43°43′2.69″N 24°48′56.65″E
S4 – Filiasi, Jiu river, upstream Craiova	44°34′8.32″N 23°27′18.14″E	S12 – Km 36 upstream Bucharest on Arges river	44°28′45.25″N 25°40′47.87″E
S5 – Podari, Jiu river downstream Craiova	44°15′18.48″N 23°47′25.08″E	S13 – Giurgiu (Danube), upstream confluence with Arges river	43°52′37.50″N 25°58′49.92″E
S6 – Rast (Danube), upstream confluence with Jiu river	43°51′24.84″N 23°17′18.79″E	S14 – Chirnogi on Arges river	44° 6′38.09″N 26°38′15.33″E
S7 – Bechet (Danube), downstream confluence with Jiu river	43°45′11.32″N 23°56′30.69″E	S15 – Oltenita, downstream confluence with Arges river	44° 3′51.89″N 26°38′45.49″E
S8 – Olt river downstream Slatina / bridge	44°23′29.63″ N 24°21′4.84″	S16 – Calarasi (Danube)	44° 8′15.69″N 27°20′8.26″E

A and B, were ultrapure water with 0.2% formic acid and acetonitrile, respectively. The separation was realized with the following gradient: 0-2 min 10%B, 2-9 min 10-80%B, 9-13 min 80%B, equilibration 6 min with 10%B. The column temperature was kept at 20°C. The flow rate was constant, 0.2mL/min during the whole process and a volume of 10µL of standard solutions and sample extract was injected in every case. All the insecticides were eluted within 12 min. The LC system was connected to a triple quadrupole mass spectrometer Model 6410 Agilent (Agilent Technologies, Waldbronn, Germany) equipped with electro-spray ionization (ESI) source, operating in positive ion mode. The optimal MS parameters were as follows: gas temperature, 300^{0}C; gas flow, 8 L/min; nebulizer gas, 40 psi; capillary voltage, 3500 V. Nitrogen was used as the nebulizer and collision gas. The analyses were done in the positive ion mode for all compounds. For increased sensitivity and selectivity, data acquisition was performed working in Multiple Reaction Monitoring (MRM) mode. For each compound, two signals were monitored, corresponding to the transition between the precursor ion of the protonated molecule $[M+H]^+$ of the two most abundant product ions. The most abundant one was used for quantification while the other one was used for confirmation. Instrument control and data processing were carried out by means of Mass-Hunter software from Agilent Technologies. The cell acceleration voltage (CAV) used was 7 for all substances. Instrumental detection limits were lowered also after a rigorous MS optimization procedure in which all MS parameters were optimized for each analyte. MRM transitions, the optimum collision energies and cone voltages selected for each transition are indicated in table 3. The TIC (total ion chromatogram) MRM Chromatogram of mixture standard solution in acetonitrile (100µg/L) obtained in these conditions is presented in figure 2.

Sample preparation

In order to obtain the sensitivity required in this field, and to be able to determine low neonicotinoids concentration levels, possible present in surface water, a pre-concentration of 1000 times has been applied. The pre-concentration applied to the surface water sample was adapted partially form previous literature [6, 12, 13]. All water samples were filtered using glass fiber filters to remove particles large than 0.45µm and the filter was washed with methanol in order to pass all analytes in filtrate. The samples were extracted with Dionex Autotrace 280 automated solid-phase extraction apparatus (Thermo Scientific). Oasis HLB cartridges (500 mg sorbent/6mL

Table 3
RETENTION TIMES AND MS/MS PARAMETERS FOR THE ANALYSIS OF SELECTED INSTECTICIDES

Compound	Polarity	t_R (min)	MRM transitions (m/z)	Fragmentor voltage (V)	Collision energy (eV)	Dwell time (ms)
Nitenpyram	ESI +	5.638	271→ 224 271→237	100 100	15 15	100 100
Thiamethoxan	ESI +	8.438	291 → 211 291 → 181	80 80	10 20	100 100
Clothianidin	ESI +	10.020	250 → 169 250 → 132	90 90	5 15	100 100
Acetamiprid	ESI +	10.811	223 →126 223→ 56	80 80	15 15	100 100

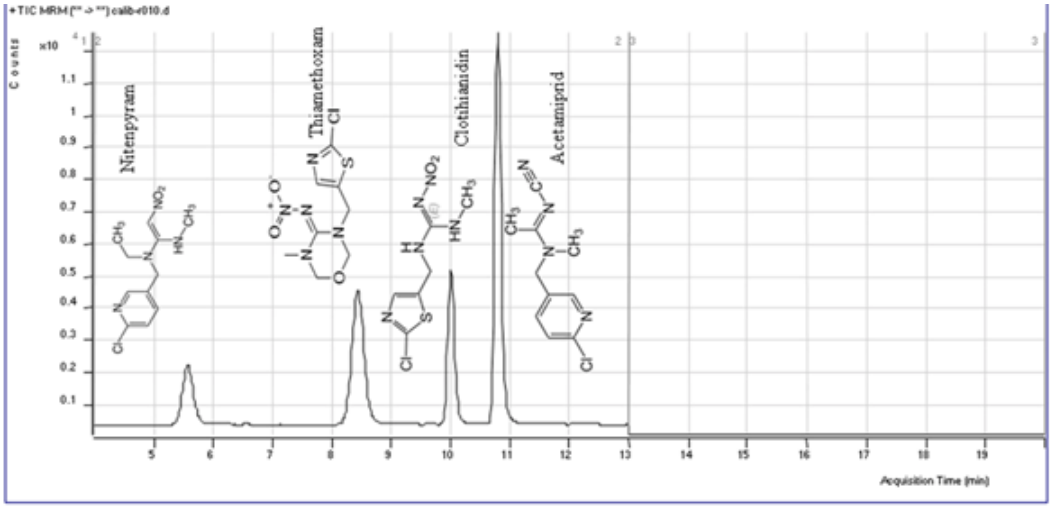

Fig. 2. The TIC MRM Chromatogram of nitenpyram, thiametoxam, clothianidin, acetamiprid mixed standard solution in acetonitrile (100µg/L)

cartridge, Waters, USA) were conditioned with 10mL methanol and 10mL ultrapure water at 10mL/min and then the sample was loaded at a flow rate of 10 mL/min. Cartridges were rinsed with 10mL ultrapure water and then dried for 20 min. under a current of nitrogen at 20mL/min and eluted with 6mL methanol at 5mL/min. The sample extracts were then evaporated to almost dryness in a TurboVap LV (Caliper Life Sciences Inc., Hopkinton, MA, USA) under a current of nitrogen at 35^{0}C. The residue was reconstituted with 1mL mixture acetonitrile:formic acid 0.2% (90/10, v/v) and analyzed by LC-MS/MS.

Validation study

Prior to its application, the method was validated for surface water considering the following parameters: linearity, limits of detection, intra-day and inter-day precision, accuracy and recovery. The calibration curve was obtained by analyzing standard solutions at five concentrations between 1µg/L and 100µg/L. Linearity was assumed if the correlation coefficient value was higher than 0.991. Limits of detection (LOD) and limits of quantification (LOQ) were determined as the minimum detectable amount of analyte, in the sample chromatogram, giving peaks for which the signal-to-noise ratio was 3 and 10, respectively. The estimated values of LODs were in the range from 0.09ng/L to 0.27ng/L, whereas corresponding LOQ values were in the range of 0.3-0.9ng/L. For *intra-day* experiments, four replicates were spiked, extracted and analyzed in the same day, whereas for *inter-day* assays, the extraction and analysis were performed for one sample, in four days. Individual recoveries for overall analytical procedure were determined by spiking surface water with working standard mixture at approximately 200ng/L. Un-spiked surface water samples were previously analyzed to confirm the absence of any significant peak at the selected transitions and positive findings were subtracted from spiked samples. The whole method was considered accurate if recoveries were in the 71.4–109.9% range, and precision was satisfactory if the relative standard deviation RSD was lower than 9%.

Results and discussions

LC separation optimization

The LC method was developed to gain high efficient compound peaks and good separation between them in the shortest possible time. A short retention study was made using acetonitrile (10 ÷ 90%) as organic modifier. The best separation in the shortest time was realized when using a gradient elution program. Mobile phase flow rate was ranged between 0.1, 0.2 and 0.3 mL/min. The final flow rate selected value was 0.2mL/min because it allowed both fast separation and good MS sensitivity of the target neonicotinoids. The optimum column temperature giving the highest resolution and the strongest analyte response was found to be 20°C.

MS parameters optimization

Tuning of the equipment was realized for each compound using standard solution prepared in acetonitrile at 5mg/L. Identification of each precursor ion for each substance was realized in the full scan mode by recording mass spectra from m/z 50 to 500 in the positive ionization mode at a flow rate of 0.2mL/min, using a mixture of acetonitrile and 0.2m formic acid (80/20, v/v) as mobile phase. To optimize performance and sensitivity of MS, the fragmentor voltage was selected to produce the highest intensity for the precursor ion. After that, the collisions energy was adjusted to produce the highest intensity for the precursor ion. To optimize the fragmentor voltages it was varied the fragmentor from 70 to 130V. From total ion chromatogram (TIC), the corresponding $[M+H]^+$ for positive electro-spray ionization (ESI+) were used to produce the extracted ion chromatogram (EIC) for different fragmentor voltages. Using optimized fragmentor was realized the optimization of collision energy by injecting of 5mg/l standard solutions of each substance at 0.2mL/min constant flow of mobile phase. Each acquisition was performed in steps of 5V between 10 and 30V. The optimized collision energies were those that provide the highest signal for the precursor ion. The protonated molecule $[M+H]^+$ which produced the most intense signal for all compounds was selected as the precursor ion. Once the $[M+H]^+$ signal was optimized, different collision energies were tested in order to obtain the best sensitivity with the highest number of product ions. The collision energies and fragmentor voltage were optimized for each compound and ranged from 10eV to 30 eV and from 70V to 130 V, respectively. The following parameters were also optimized and finally nebulizer pressure was set at 40 psig; drying gas was set at 8 L/min and source temperature was set at 300°C. Mass spectrometer parameters for polarity, MRM transitions fragmentor voltage, collision energy, dwell time and retention times are shown in table 3.

The optimum column temperature giving the highest resolution and the strongest analyte response was found to be 20°C. As regard the optimal flow rate, 0.2 mL/min was observed to provide best analyte separation and maximum sensitivity. The MRM spectrum of the two

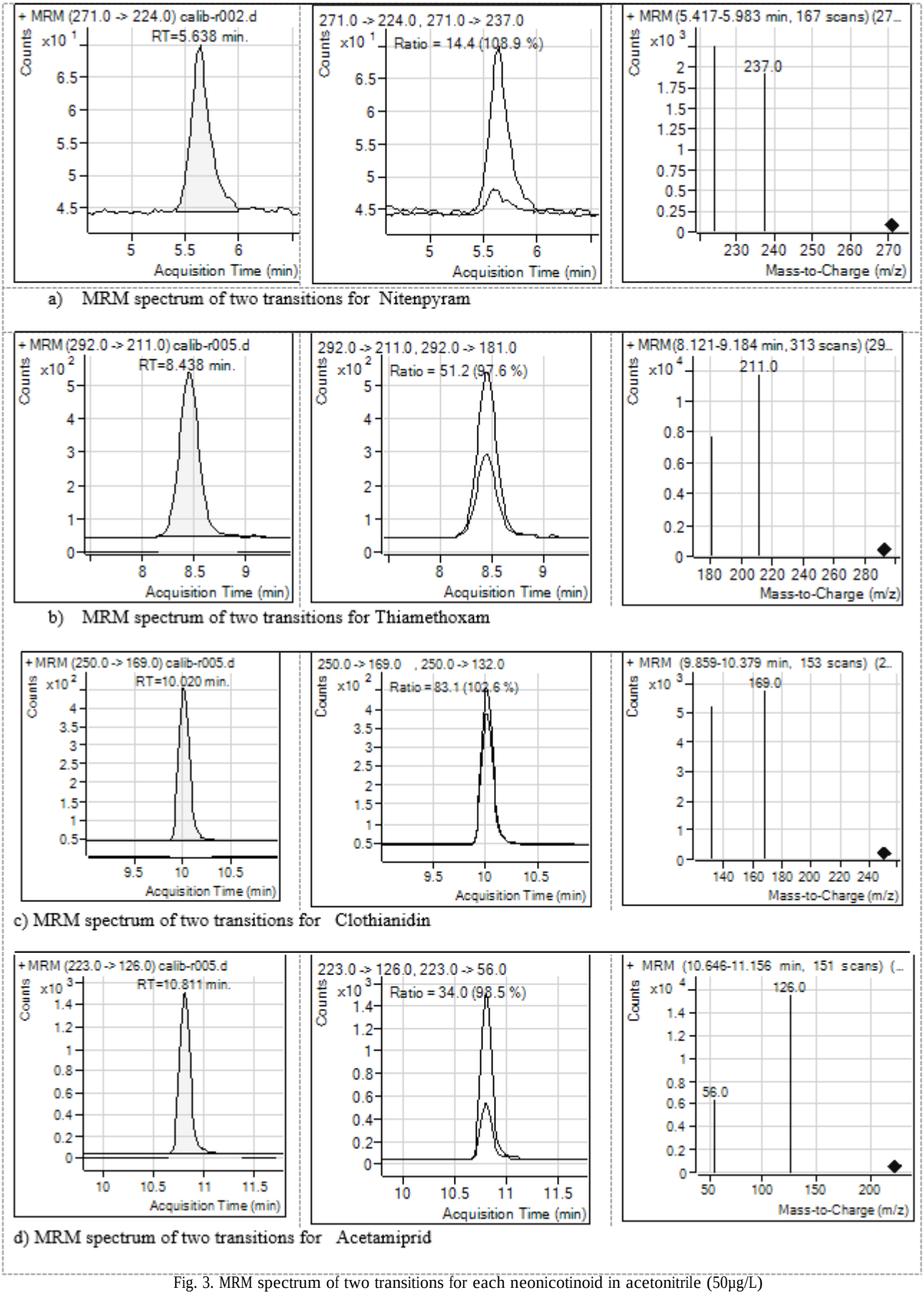

Fig. 3. MRM spectrum of two transitions for each neonicotinoid in acetonitrile (50µg/L)

transitions for each compound (50µg/L), obtained in optimized conditions, is presented in figure 3.

Results of validation study

Good linear responses, in the concentration range from 1µg/L to 100µg/L, with very good correlation coefficients (R^2> 0.991), were obtained. The limits of quantification (LOQ) for all neonicotinoids were situated below 0.9ng/L, ranging from 0.3ng/L to 0.9ng/L. The calculated LOQs are situated under limit of method (9ng/L) imposed by EU 495/2015 and are comparable to those reported in other studies and suitable to quantification of neonicotinoids in surface

Table 4

VALIDATION PARAMETERS OF METHOD: COEFFICIENT OF DETERMINATION (R^2), LOQ, RECOVERY, INTER-DAY AND INTRA-DAY PRECISIONS FOR THE DEVELOPED METHOD

Compound	R^2	LOQ (ng/L)	Recovery (%)	Intra-day precision (% RSD_r)	Inter-day precision (% RSD_R)
Nitenpyram	99.66	0.3	71.4	5.5	6.9
Thiamethoxam	99.11	0.9	109.96	4	5.8
Clothianidin	99.85	0.79	80.24	6.7	9
Acetamiprid	99.71	0.59	82.08	7.2	8.4

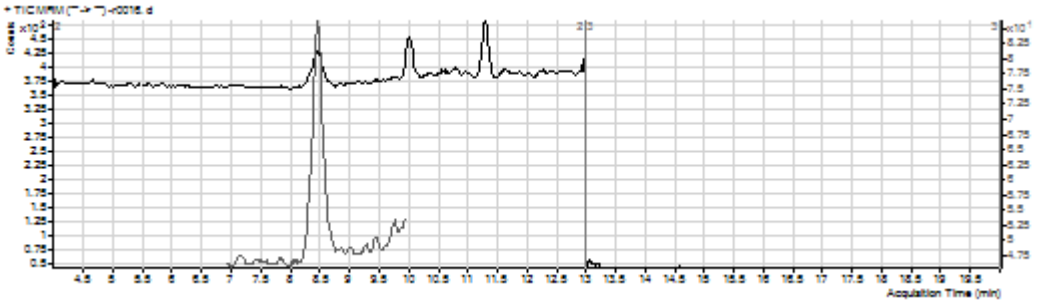

Fig. 4 LC–MS/MS chromatogram of Danube River sample in Oltenita point showing the detection of neonicotinoids

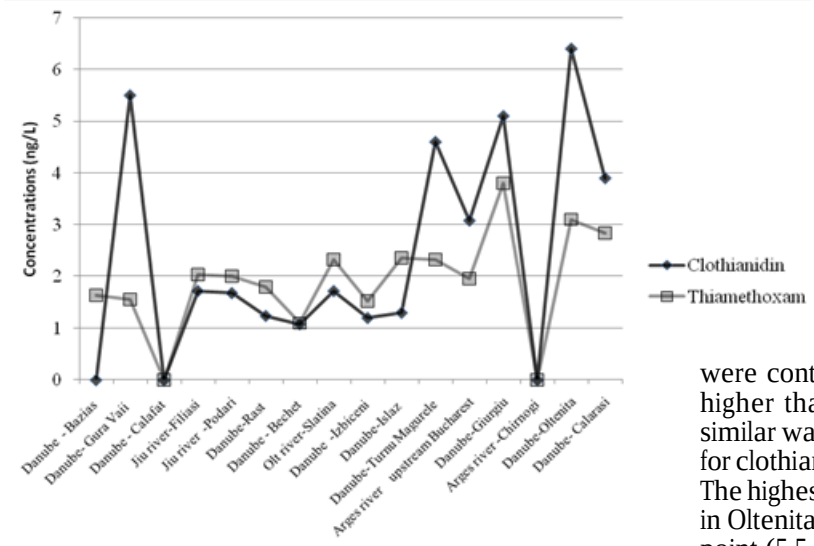

Fig. 5 The variation of thiamethoxam and clothianidin concentrations in Danube River and its three tributaries (Arge river, Olt river, Jiu river)

water [1,2]. Recoveries achieved for all target compounds were higher than 71.4%. Precision was expressed as a relative standard deviation (RSD) with values from 4% to 7.2% and from 5.8% to 9% for inter-day and intraday test, respectively. The validation parameters of the method and data obtained by the external standard calibration methodology are presented in table 4.

Determination of neonicotinoid insecticides from surface water samples

Sixteen real surface water samples influenced by agriculture were analysed, using the SPE-LC-MS/MS method for the quantification of neonicotinoid insecticides. Positive detection of analytes is shown in figure 4. The variation of analyte concentrations with sampling points is presented in figure 5. The optimized analytical method was applied to characterize the presence and concentrations of target neonicotinoids in the surface water of Danube River and three of its tributaries. The study has shown that 15 investigated samples contained neonicotinoids compounds.

Acetamiprid and nitenpyram were situated under the limits of quantification (LOQs) in all river samples. Our study revealed that 87% (14/16) of surface water samples were contaminated by neonicotinoids in concentrations higher than LOQ. Two neonicotinoids are detected at similar water concentrations ranging from 1.08 to 8.6 ng/L for clothianidin, and from 1.1 to 3.8ng/L for thiamethoxam. The highest concentrations were recorded in Danube River in Oltenita point (6.4ng/L thiamethoxam) and in Gura Vaii point (5.5 ng/L thiamethoxam).

Thiamethoxam was the most frequently detected insecticide in 87% of water samples, followed by clothianidin (81.2%). The co-occurrence of clothianidin and thiametoxam can be explained by their use in the same watersheds and because clothianidin is a transformation product of thiametoxam[6]. The frequent detection of thiamethoxam and clothianidin can be due to their intense agricultural and to long soil degradation life (table 1). The levels of thiamethoxam and clothianidin found in Romanian rivers are lower or similar than levels reported for surface water in USA (2-185ng/L thiamethoxam, 8.2-257ng/L clothianidin) [6], in Canada (1.6 -4300 ng/L clothianidin, 2.7-1490ng/L thiamethoxam), in Australia (60-420 clothianidin, 100-170 ng/L thiamethoxam) [22]. Hladik et al. observed that neonicotinoids are mobile and persistent in the environment, their chemical use and precipitation are the important factors for their transport to streams [6]. Neonicotinoids insecticides are a major danger to surface waters because of their water solubility, intense use, environmental persistence and high toxicity.

Conclusions

A high sensitive analytic LC-MS/MS method for neonicotinoids detection in surface water samples was

developed. The LOQs obtained with this method are situated below the recommendation of the European regulation (0.3-0.9 <9ng/L**).** The applicability of the method was demonstrated by analysis of 16 surface water samples from Danube River and its three major tributaries Arges river, Jiu River, Olt River. The surface water samples were found to be contaminated by clothianidin and by thiamethoxam. Thiamethoxam was the most frequently detected insecticide in 87% of water samples, followed by clothianidin (81.2%). The levels of the two most frequently detected neonicotinoids had correlations with each other, suggesting that that may coming from similar source (i.e. used for sunflower, maize). Frequent detection of neonicotinoids in surface water samples is probably a property of this group of substance which is highly water soluble and stable to hydrolysis. Environmental monitoring data suggests that some neonicotinoids are transported into river.

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