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ORIGINAL PAPER

Determination of selected neonicotinoid insecticides by liquid chromatography with thermal lens spectrometric detection

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Abstract We studied the analysis of trace amounts of neonicotinoid insecticides by liquid chromatography coupled with a thermal lens spectrometric detector (TLS). This multi-residue analysis method is based on the reversed phase separation on C_{18} column, isocratic elution and collinear dual beam TLS detection. The insecticides thiamethoxam, imidacloprid, acetamiprid and thiacloprid were detected with retention times of 4.4, 5.7, 6.5 and 8.5 min and limits of quantifications of 50, 89, 10, and 25 μg/L, respectively. The retention times agreed well with those obtained by the same chromatographic method but using a diode-array detector (DAD). The limits of quantifications for imidacloprid were identical in both techniques. However, the limits of quantifications for thiamethoxam, acetamiprid and thiacloprid were up to 8.5 times lower using the TLS detector compared to the diode-array detector. The applicability of the developed procedure was tested on spiked river water and potato samples.

Keywords Neonicotinoid insecticides · HPLC/TLS · Quantitation · Potato · River water

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Introduction

Neonicotinoid insecticides are a relatively new group of active ingredients with novel modes of action (Tomlin 2000). They act as antagonists by binding to postsynaptic nicotinic receptors in the insect's central nervous system. This leads to the accumulation of acetylcholine, resulting in the paralysis and death of insects (Iwasa 2004). Due to the growing use of insecticides from the family of neonicotinoids, their increased presence in the environment is evident. Even though the use of four neonicotinoids, specifically imidacloprid, thiamethoxam, acetamiprid and thiacloprid, is allowed in EU member states and other countries, legal regulations concerning their threshold limit values are, however, becoming more restrictive. In fact, reference data indicate negative consequences of their use, as well as the effects of their traces and degradation products to certain species like, for example, honey bees (Iwasa 2004).

For these reasons, it has been necessary to develop a sensitive analytical method for monitoring the low levels of neonicotinoid residues in soil, water, and agricultural products. Recently, various analytical methods have been proposed for the determination of neonicotinoids, including gas chromatography-mass spectrometry, GC/MS (Vilchez et al. 1996). However, because of the thermolability and low volatility of neonicotinoids, the GC/MS procedures involve complex sample preparation. Hence, liquid chromatographic methods such as HPLC/DAD (Obana et al. 2002; Mandić et al. 2005), HPLC/MS (Obana et al. 2003; Fidente et al. 2005; Seccia et al. 2005), and HPLC/post-column photoactivation/electrochemical detection (Rancan et al. 2006) appeared to be advantageous for the determination of neonicotinoids in various matrices.

Recently, highly sensitive photothermal techniques, including thermal lens spectrometry (TLS), have evolved and have been attracting attention for trace-level analysis of different biological and environmental samples (Franko 2001; Luterotti et al. 2002; Pogačnik and Franko 2003). In addition, new laser sources, providing stable continuous wave emission with over 100 mW of single line power in the spectral range of 229–257 nm, have become available recently. Hence, it was interesting to investigate the applicability of TLS detection for the determination of neonicotinoids. The present report deals with the possibility of applying HPLC/TLS as a promising sensitive technique for the determination of neonicotinoids. The applicability of the developed HPLC/TLS method was tested on fortified potato and river water samples.

Experimental

Chemicals and solutions

All chemicals used were of analytical reagent grade. The purity of insecticide standards was 99.4% for thiamethoxam, 99.9% for imidacloprid and thiacloprid (Riedel de Haën, Germany) and 99.0% for acetamiprid (Dr. Ehrenstorfer GmbH, Germany). HPLC-grade solvents (acetonitrile and dichloromethane) were purchased from J. T. Baker (Mailinckrodt Baker B. V., Neederland). LC-grade water was produced using a Milli-Q water purification system (Millipore Corporation, USA).

Individual stock solutions (200 μg/mL) of each analytical standard were prepared in mobile phase. The single compound standard solutions (20 μg/mL) were prepared by diluting each primary standard solution with the mobile phase. Multi-component solutions of appropriate concentrations of neonicotinoids (2.5–2,500 μg/L) were prepared daily by mixing the single compound solutions and diluting the standard multi-component solution with the mobile phase. Spectrophotometric analysis of these solutions showed that the content and properties of the standard did not change over a 2-week period if the solutions were kept in dark at 4°C.

Apparature

The TLS measurements were made on a dual beam (pump/probe configuration) thermal lens spectrometer. A frequency doubled Ar-ion laser (Coherent, Sabre MotoFred) operating at 244 nm (100 mW) was used as excitation source (pump beam). The pump beam was modulated with a variable speed mechanical chopper (Scientific Instruments, Model 300). A He–Ne laser (Uniphase, Model 1103P) provided the probe beam. After focusing the pump beam with a 100 mm focal length quartz lens, collinear propagation of pump and probe beams was obtained by a beam-splitter, which directed the two laser beams through an 8 μL (1 cm path-length) flow-through cell (Hellma, Cat. no. 178.313-QS) connected to the output of an HPLC column. The fluctuation in the probe beam intensity was measured with a silicon photodiode (Laser Components, OSD 5-E) located 2 m from the sample cell. A red filter and a pinhole were located in front of the photodiode. The output of the photodiode was fed to a lock-in amplifier (Stanford Research, Model SR830) connected to a personal computer, where the HPLC/TLS chromatograms, represented as time-dependent changes in the probe beam intensity, were recorded. All measurements were carried out in the dark.

For comparison, the same samples were analyzed using HPLC/DAD technique. Chromatograms were recorded on an Agilent 1100 series liquid chromatograph (Agilent Technologies Inc., USA) coupled with Agilent 1100 DAD detection unit.

Procedures

Chromatography

The chromatographic separation procedure, which was based in principle on modified manufacturer's method (Certificate of Analysis, Riedel-de Haën), was applied in combination with TLS and DAD techniques. The isocratic separation of neonicotinoids was performed on a Pinnacle ODS (250 × 4.6 mm, 5 μm) column (Restek Corporation, Cally 911457). The mobile phase was 7:3 (*v*/*v*) water (0.2% phosphoric acid):acetonitrile. The flow rate of the eluent was 1.0 mL/min and the column temperature was held at 25°C. Aliquots of 20.0 μL were manually injected through an injection loop in the case of HPLC/TLS, while an auto sampler (Agilent 1100) was utilized for HPLC/DAD analysis. Thiamethoxam, imidacloprid, acetamiprid and thiacloprid were detected at 254, 270, 245 and 242 nm, respectively, when determined by HPLC/DAD.

Real sample preparation

Potato samples were obtained from the ''Kupusina'' trial field, located in Vojvodina province (Serbia). The analyzed samples (2 kg) were: sample S-1 (non-sprayed) and sample S-2 (spiked with insecticide mixtures). Each potato was washed with doubly distilled water and dried at room temperature. A representative portion of each sample (40 g) was chopped to small pieces and treated in a conventional food blender for 2 min to obtain thoroughly mixed homogenates. For the recovery test, the amount of 10 g of homogenate was weighed in a beaker and spiked

with an aliquot of the pesticide working standard mixture. Two spiking levels with final concentrations of 0.025 and 0.10 mg thiamethoxam, imidacloprid, acetamiprid and thiacloprid/kg potato were tested. The spiked samples and the remaining unspiked samples were kept in the dark at 4°C for 1 h. Then the unspiked control vegetable samples and the spiked samples were processed by following the same stepwise procedure. Initially, 50 mL of CH_2Cl_2 and 1 g of sodium chloride were added to the sample, the mixture was carefully shaken manually three times in 15 min time intervals during 1 h, filtered, and the liquid phases (aqueous and organic) were collected. The solid phase was washed two times with 10 mL of CH_2Cl_2, and the obtained organic extract was added to the liquid phase. The obtained liquid phases were quantitatively transferred to the extraction funnel. The liquid–liquid extraction was repeated three times by adding 20 mL of CH_2Cl_2 to the aqueous phase each time. The organic extract was evaporated on a rotary vacuum evaporator at 25°C to dryness and the residue was reconstituted in 1.00 mL of mobile phase using sonication, to assist dissolution.

Additionally, the water samples were collected from the Vipava river (Slovenia) and stored in the dark at 4°C for 1 week before further treatment. The aliquot of river water sample was spiked with the standard mixture of four neonicotinoids and kept in the dark at 4°C for 1 h before analysis without any kind of sample pre-treatment.

For all measurements, the solutions were filtered through 0.45-μm membrane filters (Millipore, USA).

Validation of the analytical methods

The linear range of the HPLC/TLS and HPLC/DAD methods was determined on the basis of the calibration graphs**.** The limits of detection (LOD) and quantitation (LOQ) were calculated according to Miller and Miller (1988). All recovery experiments were conducted in triplicate.

Results and discussion

Optimization of the HPLC/TLS method

TLS with single excitation wavelength is a non-specific detection technique, and therefore requires prior separation of analyte species. A mixture of water and acetonitrile is commonly used for the HPLC separation of different neonicotinoids on a reverse phase (Mandić et al. 2005; Rancan et al. 2006). In the procedure of mobile phase optimization it was found that the increase of acetonitrile content, e.g. to 4:6 (*v*/*v*) water (0.2% phosphoric acid):acetonitrile, resulted in a higher sensitivity of the method. This stems from the lower thermal conductivity and higher temperature coefficient of refractive index of organic solvents, which is reflected in increased sensitivity compared to TLS measurements in water, and was for example exploited to improve the sensitivity of TLS detection in ion chromatography (Šikovec et al. 2001). However, the increased acetonitrile content caused the drop of the separation coefficient, which led to the co-elution of certain compounds. This effect was most pronounced in the case of imidacloprid and acetamiprid. Thus, the isocratic system 3:7 (*v*/*v*) water (0.2% phosphoric acid):acetonitrile was found optimal to separate four insecticides as sharp, independent peaks.

An earlier study showed that the presence of phosphoric acid had a favourable effect on the shape of chromatographic peaks (Mandić et al. 2005), making them sharper and thus improving the separation. On the other hand, it is well known that the presence of a higher amount of phosphoric acid can decrease the *S*/*N* ratio. By optimizing the composition of the mobile phase, it was found that the presence of phosphoric acid at $\leq 0.2\%$ had no significant effect on the noise level in TLS chromatograms.

In contrast to TLS measurements in the non-flowing samples, the fluctuation in eluent flow strongly affects the signal stability in HPLC/TLS. Hence, the TLS parameters, such as modulation frequency and lock-in amplifier time constant, must be carefully optimized to increase the *S*/*N* ratio. Our experiments showed that by selecting longer lock-in time constants (up to 3 s), the signal noise was reduced due to averaging of the signal over longer time intervals. However, due to the longer averaging periods, the chromatographic peaks were broadened and also shifted to longer retention times. The optimal time constant was therefore set at 1 s.

Additional improvements in the *S*/*N* were achieved by optimizing the modulation frequency (*f*) of the pump beam. The noise level of HPLC/TLS signals was reduced when the modulation frequency was changed from 12.5 to 120 Hz (Fig. 1). On the other hand, a decrease of the peak height due to shorter excitation periods at higher modulation frequencies could also be observed. These contrary effects were equalized at 80 Hz and this frequency was used as optimal modulation frequency as it provided the optimal *S*/*N* ratio.

Determination of neonicotinoid insecticides

Retention times (t_R) and reproducibility (RSD), determined individually for each compound by HPLC/TLS and HPLC/DAD techniques, are presented in Table 1.

Initial verification of the efficiency of the HPLC/TLS separation and determination procedure was performed under optimized TLS conditions. From the chromatograms

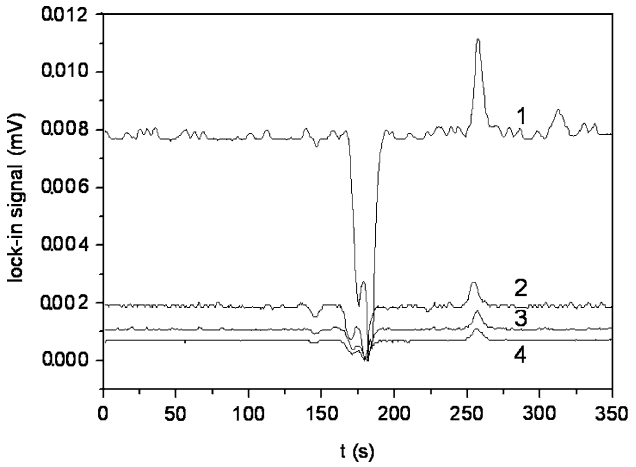

Fig. 1 Effect of modulation frequency on analytical signal and *S/N* ratio. Sample, 200 μg/L imidacloprid; P = 100 mW; time constant 1 s; f (1) = 12.5, (2) 60, (3) 80 and (4) 120 Hz. Mobile phase was 6:4, *v/v* water (containing 0.2% phosphoric acid):acetonitrile

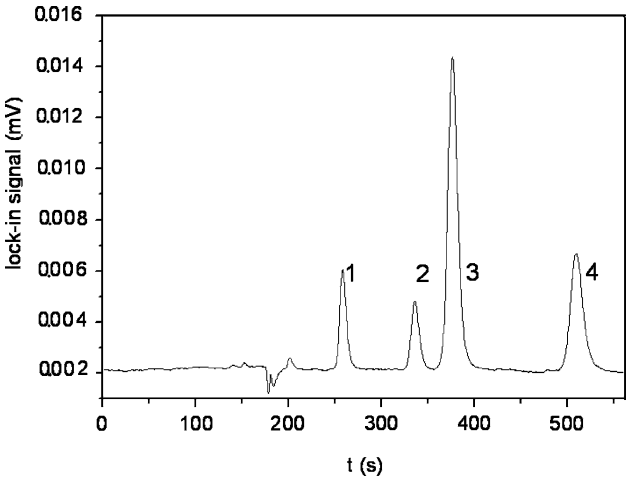

Fig. 2 HPLC/TLS chromatogram of 1 μg/mL level multi-residue analysis: (*1*) thiamethoxam, (*2*) imidacloprid, (*3*) acetamiprid and (*4*) thiacloprid. P = 100 mW, time constant 1 s; f = 80 Hz. Mobile phase: 7:3, *v/v* water (containing 0.2% phosphoric acid):acetonitrile, v = 1.0 mL/min

Table 1 Retention times and reproductibility of individual neonicotinoid signals obtained by HPLC/TLS and HPLC/DAD techniques (n = 6)

Insecticide	Technique			
	HPLC/TLS		HPLC/DAD	
	t_R (s)	RSD (%)	t_R (s)	RSD (%)
Thiamethoxam	261.0	0.12	257.8	0.10
Imidacloprid	342.4	0.66	337.4	0.12
Acetamiprid	391.9	0.33	373.1	0.10
Thiacloprid	510.6	0.57	511.6	0.22

such as the one shown in Fig. 2 separation coefficients higher than 1.5 were calculated for all insecticides.

Quantitative evaluation was based on the linear relationship between the peak area and concentration of neonicotinoids. The analytical parameters are presented in Table 2. Reproducibility of the responses obtained for the HPLC/TLS, assessed by six replicate measurements (100 μg/L) and the corresponding peak areas, were evaluated. A comparison of the reproducibility with that of the comparative HPLC/DAD technique showed that comparable precision was obtained in both cases.

As can be seen from Fig. 2 and Table 2, the TLS technique is sensitive enough for the determination of neonicotinoids at a ppb level. In the case of imidacloprid and thiamethoxam the essential analytical parameters obtained with TLS and DAD detectors are in good agreement. The highest sensitivities found for acetamiprid and thiacloprid determination can be explained by the fact that the broad absorption band with maxima at 245 and 242 nm, respectively, matched well with the excitation laser wavelength of 244 nm. The LOQs for acetamiprid and thiacloprid provided by TLS technique, which is known to enable measurement of absorbances as low as 10^{-7}, are almost ten times lower compared to DAD.

The applicability of the optimized HPLC/TLS was investigated with the objective of using this technique for the determination of the above insecticide mixture in spiked samples of potato and river water.

The procedure was validated by carrying out a recovery test on potato and river water samples fortified at two levels (0.025 and 0.1 mg insecticide-mixture/kg potato or mg/L water samples) as described in ''Experimental'', which is in agreement with the concentrations (0.01–0.3 mg/kg) quoted in the literatures (Di Muccio et al. 2006; Fernandez-Alba et al. 1996; Mandić et al. 2005; Obana et al. 2002, 2003) for the determination of neonicotinoids in different real samples (river water, pepper, potato, cucumber, apple etc.). The insecticides from potato were extracted with dichloromethane. Average recoveries were in the range of 78–93% and RSD ranged from 2.9 to 5.3%, suggesting that the investigated pesticides can be effectively extracted from samples with dichloromethane. Figure 3 shows the HPLC/TLS signals obtained for the blank potato extract (Fig. 3a, curve S-1) and river water (Fig. 3b, curve S-1) and spiked ones (Fig. 3, curves S-2). As can be seen, after the extraction and separation steps there are no co-eluted compounds that could interfere with the determination of the investigated insecticides.

The concentration in mg/kg or mg/L of 0.01 imidacloprid or thiamethoxam, 0.002 acetamiprid and 0.004 thiacloprid can be considered as the LOQ limits of the method; lower concentrations did not always result in well-defined

Table 2 Analytical parameters for the HPLC/TLS and HPLC/DAD determinations

Insecticide	Technique					
	HPLC/TLS			HPLC/DAD		
	LOD (μg/L)	LOQ (μg/L)	RSD (%)	LOD (μg/L)	LOQ (μg/L)	RSD (%)
Thiamethoxam	15	50	0.9	39	130	0.9
Imidacloprid	27	89	1.1	27	89	1.0
Acetamiprid	3.2	10	0.8	26	85	0.8
Thiacloprid	7.5	25	0.7	23	76	0.9

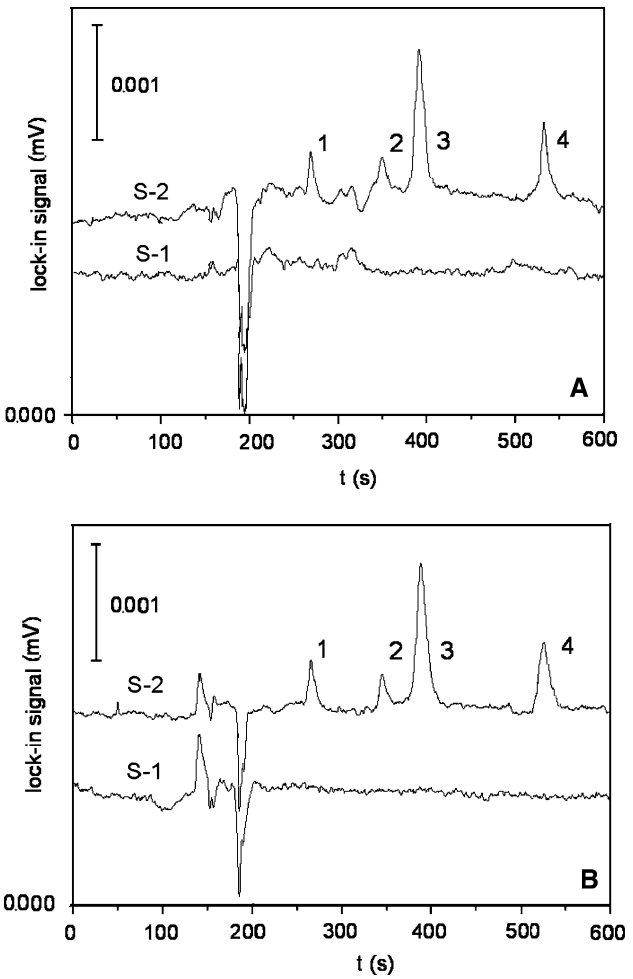

Fig. 3 HPLC/TLS chromatograms of non-treated potato extract (**a** S-1), non-treated river water sample (**b** S-1); 100 μg/L level multi-residue analysis in spiked potato extract (**a** S-2) and in spiked river water sample (**b** S-2): (*1*) thiamethoxam, (*2*) imidacloprid, (*3*) acetamiprid and (*4*) thiacloprid. P = 100 mW, time constant 1 s; f = 80 Hz. Mobile phase: 7:3, *v*/*v* water (containing 0.2% phosphoric acid):acetonitrile, v = 1.0 mL/min. Curve S-2 was evenly shifted in the vertical direction for clarity

chromatograms. These levels are low enough to allow monitoring of the investigated neonicotinoid insecticides in vegetables and surface water samples.

The sensitivity of the developed TLS detection technique surpasses the DAD technique, especially in the determination of acetamiprid and thiacloprid. The advantage is evident in respect of the lower LOD and LOQ values, smaller sample size, and shorter time needed for its preparation. Thanks to the demonstrated advantages this trace-level analytical method may find further applications in the analysis of real environmental samples.

Conclusion

A simple and sensitive HPLC/TLS analytical method was elaborated for a simultaneous determination of four neonicotinoid insecticides, viz. thiamethoxam, imidacloprid, acetamiprid and thiacloprid in potato and river water. The developed HPLC/TLS method allows sensitive, selective, and reproducible trace-level determination of these four neonicotinoid insecticides and in some cases surpasses the comparative HPLC/DAD method. Namely, the LODs and LOQs are lower by 2.5–8.5 times for thiamethoxam, acetamiprid and thiacloprid, compared to HPLC/DAD, with similar RSDs. The method was successfully tested for the determination of insecticides in several real samples and the HPLC/TLS results were comparable to HPLC/DAD data, showing a maximal discrepancy of 15%. Extraction efficiencies from spiked potato samples of 84–93% were achieved. Thus, it can be concluded that the developed HPLC/TLS method represents a useful tool for a sensitive and rapid determination of neonicotinoid insecticides. Hence, the method may find further application in the analysis of real vegetable and water samples contaminated with these insecticides at a ppb level. HPLC/TLS appears to be a promising technique for sensitive determination of neonicotinoids in biological samples as well. This would enable very much needed investigations of the physiological effects of low doses of neonicotinoids on honey bees which are very much affected by the use of new insecticides.

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