Pyrethroids are a class of synthetic insecticides with chemical structure similar to that of natural pyrethrum, but much more stable. They have been subdivided into two classes based on their structural differences, toxicological and neurophysiological actions. Structurally, type I pyrethroids (permethrin and tetramethrin) lack cyano substituent, whereas type II pyrethroids (fenpropathrin and deltamethrin) contain α-cyano group (1).

Pyrethroids have progressively replaced other classes of insecticides, such as organochlorines, organophosphates and carbamate derivatives, mostly because of the notably high ratio between the toxic dose in mammalian species and the effective dose in target species (2). Moreover, pyrethroids have become increasingly popular because of their relatively low costs, ease of application, and length of efficacy (usually lasting several weeks). Pyrethrins and pyrethroids are estimated at 23% of the insecticide world market, with more than 3500 registered formulas. Pyrethroids, as well as tetramethrin, have been widely used in the field of agriculture, household (against flies, mosquitoes, cockroaches, termites, and other harmful insects), forestry, horticulture and public health care (3). Tetramethrin has found its use in the treatment of pediculosis in humans (4). Tetramethrin, among all synthetic pyrethroids, is also extensively used against a wide range of ectoparasites in large and small animals, with various insecticides used in different formulations, including sprays, powders, lotions, shampoos, and aerosols (3).

There are numerous different instrumental techniques used for the determination of tetramethrin. The available methods include voltammetric determination (5), capillary electrophoresis (6), spectrophotometric (7), as well as infrared spectroscopic methods (8). However, the most widely applied techniques are chromatographic methods, such as gas chromatography (GC) with electron capture detection (ECD) (9) or with mass spectrometry (MS) (10) as well as high performance liquid chromatography (HPLC) with diode array (DAD) (11) or with MS (12) detection. The majority of analytical methods was developed for detection of trace levels of the pesticides in environments like: air (13), soil (16), fruit, vegetable, water samples (14) or in human fluids (samples of breast milk, for example) (15). Chromatographic methods for tetramethrin determination in diverse samples implicate some-what complex sample preparation, including solid phase extraction (12) or dispersive liquid microx-
traction (14, 16). There are HPLC methods to determine permethrin in different types of preparation (treated wood, lotion, creams, shampoo, etc.) (3, 17, 18), but HPLC methods to qualify or identify tetramethrin as an active component in these products are less known.

This paper, for the first time, presents an HPLC-DAD method of determination of tetramethrin in shampoo against Pediculus capitis. The aim of this study was to optimize and validate the developed method of tetramethrin being an active component, as well as to evaluate the suitability of a new method for quality control of tetramethrin content in commercial shampoos.

EXPERIMENTAL

Chemicals and materials

The standard of tetramethrin (mixture of cis- and trans-isomers, 99.0%) was obtained from Echrenstrofer GmbH, Germany. Organic solvents methanol and acetonitrile (HPLC gradient grade) were obtained from Merck KGaA, Germany. Deionized water (WP 4100 reagent grade water purifier-SMEG) was used for standard and sample preparations. Supitox shampoo, with declared tetramethrin content of 0.3% against pediculosis, was purchased in a local market in Serbia. Matrix solution that contains all substances present in the product that we analyzed (Supitox), but without active component (tetramethrin) was obtained from the shampoo producer (Veterinary Institute, Subotica).

Chromatographic system

HPLC chromatographic apparatus consisted of an HPLC Dionex UltiMate 3000 Series system with a DAD-3000SD/RS detector, autosampler WPS-3000(T)RS, degasser, binary pump HPG 3200SD/RS and column oven TCC-3000SD (Thermo Scientific, Germany). The system control, data acquisition and data evaluation were performed by Chromeleon®7 software (Thermo Scientific, Germany).

Chromatography procedure

Mobile phase consisted of acetonitrile and deionized water or methanol and deionized water, filtered through a 0.45 µm filter, degassed in an ultrasonic bath, and pumped at a flow rate of 0.8 mL/min. Injection volume was 20 µL. Compared columns were Supelcosil TM LC-18-DB, 4.6 × 250 mm, 5 µm particle size (Sigma-Aldrich Co. LLC) and Hypersil GOLD aQ, 3 × 150 mm, 3 µm particle size (Thermo Scientific) at 30°C. The detection wavelength of the detector was set at 220 nm for all used mobile phases.

Preparation of standard solution and shampoo sample solution

The stock standard solution was prepared by dissolving 5 mg standard tetramethrin in 10 mL of methanol. The stock standard solution was kept in the refrigerator at +4°C in an amber reagent bottle. For preparing the working standard solutions, 100, 150, 200, 250, and 300 µL of the stock standard solution were diluted to 10 mL in volumetric flask with mobile phase to obtain standard solutions in concentrations 4.95, 7.425, 9.90, 12.375 and 14.85 µg/mL of tetramethrin. Such prepared standard solutions were used for method development and validation.

One milliliter of antiparasitic shampoo sample (Supitox®) was accurately weighted into a 50 mL volumetric flask and filled to the mark with methanol. Then, working sample solution of Supitox®, was made by diluting 1.65 mL in 10 mL volumetric flask with mobile phase to obtain 10 µg of tetramethrin per mL of sample solution. This sample solution was filtered through a 0.45 µm PTFE filter to glass vial.

Twenty microliters of freshly prepared and filtered standard or sample solution was injected into the HPLC system.

RESULTS AND DISCUSSION

Since no method for determination of tetramethrin in human antiparasitic shampoo with HPLC-DAD has been reported in literature, method development and optimization were done before validation and sample analysis. Method parameters that were optimized are mobile and stationary phases.

Optimization of HPLC analysis and method development

With the aim to develop fast and effective separation of tetramethrin in short time, simple mobile phases were used for evaluation. In order to determine the most suitable conditions for HPLC-DAD separation and detection of tetramethrin, two different columns and different mobile phase compositions in different ratios of organic and water phase were used. Since acetonitrile provided lower viscosity, faster elution and better peak symmetry of insecticides comparing to methanol on reverse phase column (3), mobile phase consisting of acetonitrile : water (55 : 45, v/v) was first chosen for evaluation.
Figure 1. Separation of cis/trans-tetramethrin in matrix of antiparasitic shampoo; conditions: column 3 × 150 mm, particle size 3 µm, mobile phase acetonitrile : water (55 : 45, v/v), flow rate 0.8 mL/min, temperature 60°C (A); column 3 × 150 mm, particle size 3 µm, mobile phase methanol : water (78 : 22, v/v), flow rate of 0.8 mL/min, temperature 60°C (B); column 4.6 × 250, 5 µm particle size, mobile phase methanol : water (78 : 22, v/v), flow rate of 0.8 mL/min, temperature 30°C (C)
In order to decrease mobile phase viscosity and to lower back-pressure in chromatography system (3), we used the column oven temperature of 60°C. Results showed that the mobile phase acetonitrile : water (55 : 45, v/v) on column Hypersil GOLD aQ (3 × 150 mm, 3 µm), at a column oven temperature of 60°C provides very poor separation of cis- and trans- tetramethrin. As it can be seen from Figure 1A, they have shown complete overlapping (peak 2), while resolution (Rs) with the peak of matrix (peak 1) was 1.07. The maximum absorption peak for tetramethrin was recorded at 220 nm, and that is why this wavelength was used to detect tetramethrin.

According to literature data, methanol is a solvent that gives long retention time for permethrin (3). Because of that, to obtain acceptable resolution for tetramethrin isomers and sample matrix, acetonitrile was replaced with methanol. Mobile phase methanol : water (78 : 22, v/v) gave the complete separation of cis- and trans- isomer peaks of tetramethrin (Fig. 1B). Still, there is a slight overlapping between tetramethrin and the peak of matrix solution (tR 4.203 min), since the resolution was unsatisfactory (Rs = 1.22) according to ICH guideline (2005). To provide better resolution of all peaks and complete separation, the column was replaced with the longer one (4.6 × 250 mm, 5 µm, Supelcosil TM LC-18-DB). Increasing the column length and decreasing the column temperature to 30°C gave the acceptable peak base-separation with Rs values 1.85. Optimal conditions for the separation of tetramethrin and matrix peaks established in this trial were as follows: mobile phase that consisted of methanol : water (78 : 22, v/v), isocratic elution at a flow rate of 0.8 mL/min and temperature of 30°C. According to the requirements of ICH (2005), these conditions were found to be most suitable for separation and quantification of both tetramethrin isomers concerning the resolution and peak symmetry, with relatively short time of analysis (Fig. 1C).

**Validation of the developed method**

In order to evaluate reliability of the results, validation of the method included the evaluation of performance parameters such as specificity, selectivity, linearity, limits of detection and quantitation, accuracy, precision, and robustness. As a standard, a solution of cis- and trans- tetramethrin was prepared at a concentration of 1 mg/mL in methanol. The solution was analyzed using the optimized conditions described above.

Table 1. HPLC system suitability parameters for tetramethrin isomers determination.

<table>
<thead>
<tr>
<th></th>
<th>Cis-tetramethrin</th>
<th>Trans-tetramethrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td>8.09</td>
<td>8.73</td>
</tr>
<tr>
<td>Retention time repeatability RSD (%) a</td>
<td>0.38</td>
<td>0.27</td>
</tr>
<tr>
<td>Peak symmetry</td>
<td>0.97</td>
<td>1.12</td>
</tr>
<tr>
<td>Resolution</td>
<td>2.21</td>
<td>1.76</td>
</tr>
</tbody>
</table>

a Made in six replicates

![Figure 2. Chromatograms of matrix solution (A) and separation of cis/trans- tetramethrin in standard solution (B) and antiparasitic shampoo sample (C)](image-url)
Optimization and validation of HPLC method for tetramethrin determination...

Specificity and selectivity

For chromatographic procedures, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately labeled (19). For the purpose of determining the specificity and selectivity of the tested analytical method the obtained chromatograms for matrix, standard solution and the solution of the examined shampoo sample were compared (Fig. 2). As can be seen from this Figure, chromatograms are showing that there is no peak overlapping. Other substances potentially present in the shampoo, besides the main active ingredient, are of no influence on the analysis, thus, precise determination of active ingredient is possible even in the presence of matrix interferences.

Linearity

Linearity study was performed by testing five test solutions at concentration levels from 50 to 150% of the target analyte concentration, in the range 4.95–14.85 µg/mL. Each concentration of calibration standard was measured in triplicates. Under the tested chromatographic conditions, linear relationships of standard solutions were verified for the sum of both cis- and trans- tetramethrin. The detector response was linear in the whole range of the calibration curve. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method. The peak area was plotted against tetramethrin concentration at each level. By linear regression analysis, good linearity \( r = 0.99985 \) was achieved in the researched range. Linear regression parameters were described by the following equation: \( A = 1.5079c + 0.462 \) for total cis- and trans- tetramethrin.

Precision

In order to determine the precision of the method, standard solution containing 9.90 µg/mL of tetramethrin standard was analyzed. A number of ten measurements were analyzed in one day. Precision was expressed as relative standard deviation (RSD) value, which was 0.26%. Since this result showed a value less than 2%, the intraday precision of the method is in accordance with ICH guidelines.

Accuracy

The accuracy of the method was evaluated by determination of tetramethrin in spiked samples within the same day. Spiked samples were prepared by spiking of the sample matrix with different content of tetramethrin standard solution (added amount 80-120% of tetramethrin content) in triplicates. The accuracy was calculated as deviation of the mean from the nominal concentration within the day (spike recovery). As it can be seen from the results shown in Table 2, recovery value is in an acceptable range of 100 ± 2% (19).

Tetramethrin determination in shampoo sample

An optimized and validated method has been applied to determination of tetramethrin in antiparasitic shampoo Supitox®. Shampoo sample was commercially available in the local market. The established average amount (3 determinations) of tetramethrin was 0.305% of cis/trans-tetramethrin.

CONCLUSION

In this paper, we developed and validated a fast and simple HPLC-DAD method for separation of cis/trans- tetramethrin and determination of total tetramethrin in antiparasitic shampoos. All validation parameters were within an acceptable range according to ICH (2005). The developed method
was successfully applied to estimate the amount of
tetramethrin in the shampoo against pediculosis.
This method can be used for the routine analysis.

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Conflict of interests

The authors declare that they have no conflict
of interests.

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