Development and application of GC-MS method for monitoring of long-term exposure to the pesticide cypermethrin

Matthaios P. Kavvalakis, Manolis N. Tzatzarakis, Athanasios K. Alegakis, Dionyios Vynias, Andreas K. Tsakalof and Aristidis M. Tsatsakis

Cypermethrin (CPMN) is a synthetic pyrethroid used as an insecticide in large-scale commercial agricultural applications as well as for domestic purposes. In the present study a gas chromatography–mass spectrometry (GC–MS) based method was developed and validated for the quantitation of CPMN metabolites, 3-phenoxybenzoic acid (3-PBA) and cis- and trans- 3-(2,2-dichlorovinyl)-2,2-dimethyl-1-cyclopropane (cis- and trans-Cl2CA). The developed method was applied for the monitoring of CPMN metabolites in hair of laboratory animals (rabbits) intentionally exposed per os to CPMN at 40 (low dose) and 80 (high dose) mg/kg weight/day for 16 weeks. The analytical method comprises three main steps: isolation of analytes from hair, analytes derivatization, and subsequent instrumental analysis by GC–MS. The limits of detection ensured by the method are 4.0, 3.9 and 1.0 pg/mg hair for cis-Cl2CA, trans-Cl2CA and 3-PBA, respectively. The instrument response is linear ($r^2 > 0.99$) in the investigated concentrations range from 25 to 1000 pg/mg. With and between-run precision as well as accuracy were estimated and found satisfactory. Analytes were efficiently isolated by solid–liquid extraction from hair with recoveries greater than 84.8% for cis-Cl2CA, 87.2% for trans-Cl2CA and 96.4% for 3-PBA. Rabbit’s hair showed increasing levels for all metabolites (metabolites accumulation in a time and dose dependent manner) over time and in a dose-dependent manner. The developed experimental procedure could be used for biomonitoring of population exposure to CPMN. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: hair; cypermethrin; 3-phenoxybenzoic acid; Cl2CA; in-vivo; rabbit

Introduction

Pyrethrins are natural products extracted from chrysanthemum flowers and have insecticidal properties. Pyrethroids on the other hand, are synthetic compounds with similar structure to pyrethrins. Over the years, pyrethroids have been modified to enhance their insecticidal activity and reduce their persistence in the environment. They tend to replace organophosphate and carbamate pesticides known to be substantially more toxic to humans. Both pyrethrin and synthetic pyrethroid compounds are effective against a wide spectrum of pests and they are used for the protection of both agricultural production and human health. Synthetic pyrethroids are considered an emergency response to outbreaks of mosquito-borne diseases on humans, such as the West Nile Virus.

One of the most commonly used synthetic pyrethroid is cypermethrin (CPMN), which is the active gradient of a large variety of commercial products nowadays supplied on the market, for example Ammo, Arrivo, Cy mbush, Cymperator, Cypercopel, Cyperguard, Cyperkill, Cypermar, Demon, Folcord, Polytrin, Siperin and others.

CPMN is reported to be a moderately toxic compound to mammals by dermal absorption or ingestion. It is not a skin or eye irritant, but it may cause allergic skin reactions. Numbness, tingling, itching, burning sensation, loss of bladder control, incoordination, seizures, and possible death are the possible symptoms of dermal exposure. As all the pyrethroids, CPMN may adversely affect the central nervous system. In insects, CPMN acts on the nervous system by disturbing the neuron functions by interaction with the sodium channel. Moreover, some recent research data revealed that CPMN may have toxic effects on the male reproductive system. Investigators reported that long-term exposure to CPMN can induce impairments of the structure of seminiferous tubules and spermatogenesis in the male rats, attributed to the reduced androgen receptor expression.

In humans, CPMN is rapidly metabolized (Figure 1) and the major metabolic reactions are cleavage of ester linkage and oxidation by microsomal cytochrome P450s (Figure 1). Cis- and trans- 3-(2,2-dichlorovinyl)-2,2-dimethyl-1-cyclopropane carboxylic acid (cis- and trans-Cl2CA) are specific metabolites of cypermethrin, cyfluthrin, and permethrin, while 3-phenoxybenzoic acid is a major metabolite for several synthetic pyrethroids (Table 1). These metabolites have both estrogenic and anti-androgenic activity, with potencies much greater than that of the parent compound.
Hair has a special value concerning the exposure evaluation of chronic exposure to various chemicals (drugs of abuse, medicines, metals, various xenobiotics, environmental pollutants) and for the assessment of exposure to organophosphate pesticides, dialkylphosphates and recently to neonicotinoids.19–23}

One of the aims of our study was to develop a rapid, specific and sensitive GC-MS based method for quantifying CPNM metabolites in hair. To evaluate its performance, the whole analytical procedure was applied to hair samples collected from intensively fed rabbits.

### Experimental

#### In vivo study protocol

Six male rabbits aged three months, weighing approximately 3000 g each were selected for this study. Rabbits were divided into three treatment groups of two animals each and housed in individual metal cages in the Medical School’s test animal facilities (University Hospital of Heraklion, Crete). They kept in a 12-h dark/light cycle with average temperature 25°C and fed with commercial rabbit pellets. Potable (tap) water was also provided. Since CPNM is not soluble in water, corn oil was used for all used formulation of CPNM. Following a one-week acclimation, two of the groups were treated with two different sub-acute doses of CPNM. The sub-acute doses were calculated to represent the 1/30 and the 1/15 of the reported oral LD50 of cypermethrin in rabbits (3000 mg/kg).16 The CPNM low dose (CLD) group was exposed to 40 mg/kg/day and the CPNM high dose (CHD) group to 80 mg/kg/day, diluted in corn oil, during a period of 16 successive weeks (approx. 4 months) with the administration performed three times per week. The total amount of ingested CPNM for each test animal of the CLD group was 12 900 mg and the corresponding amount for the CHD group was 25 800 mg. A control group of rabbits was also used and were also orally administrated the same amount of blank corn oil as for the other treatment groups. Dietary habits concerning water and food consumption were noted during the study. All rabbits were observed regularly and their health condition was recorded as well. There was no clinical evidence of acute poisoning by the ingested amounts of CPNM.

The present study was approved by the Veterinary Administration Office of Heraklion (Crete, Greece), the Animal Investigation Committee of the University of Crete (Heraklion, Crete, Greece) and the University Hospital of Heraklion (Crete, Greece) and conformed to the National and European Union directions for the care and treatment of laboratory animals.

### Hair sampling

Hair sample collection was performed before the first dose administration and at the end of second and fourth month of treatment. In order to assess the bioaccumulation of target metabolites, hair samples were collected from two different anatomical sites (neck and lower back) of each animal. The same anatomical sites were periodically used for hair sampling. Hair of these two anatomical sites were mixed and analyzed as one for each one sampling. At the end of experiment (fourth month) an additional sampling was taken place, collecting total length hair sample representative to all of the four months of exposure, from the same anatomical sites. Hair specimens were labeled and stored in paper envelopes in a dry place, at room temperature until analysis.
Stock and working solutions

Stock solutions of 3-PBA, cis- and trans-Cl2CA at the concentrations of 1 mg/mL were prepared in methanol. Working solutions of mixed analytes were prepared weekly by dilutions in methanol in the concentrations of 0, 0.01, 0.02, 0.04, 0.1, 0.2 and 0.4 μg/mL. All working and stock solutions were stored at -20°C.

Quality controls and spiked samples

Spiked and quality control samples were prepared according to previous studies. Control group rabbits’ hair was used as blank hair samples after analyzed and found negative for all target analytes. Blank hair samples were further used for the preparation of the spiked samples and the quality controls. The blank rabbit hair samples were spiked with known concentrations of 3-PBA, cis- and trans-Cl2CA in a concentration up to 1000 pg/mg (0, 0.01, 0.02, 0.04, 0.1, 0.2 and 0.4 μg/mL) and used for the preparation of the calibration curves and further quantification of in vivo rabbits’ hair samples. Quality control samples were prepared by immersion of blank rabbit hair in 1 μg/mL aquatic solution of 3-PBA, cis- and trans-Cl2CA. In each analysis batch, three quality control samples were included.

Hair sample treatment

Decontamination step

For the removal of the external contaminants, hair samples were washed once in 5 mL of water (for 10 min) and twice in 5 mL of methanol (for 1 min). Washed hair samples were dried in the oven (temperature did not exceed 50°C). The methanol wash was tested for 3-PBA, cis- and trans-Cl2CA to confirm that external contamination was eliminated. Subsequently, 100 mg of hair was cut in small pieces (some mm), weighed and finally transferred in a glass test-tube with screw-top. One hundred (100 ng) of 2-PBA were added as internal standard.

Extraction step

Hair was incubated with 2 mL of methanol at room temperature in an ultrasonic bath for 2 h. The temperature of the bath during the ultrasonic extraction did not exceed 40°C. The methanol extract was filtered through 0.2 μm membrane filter (Filtropur S, Sarstedt, Germany) to a glass test-tube. The same procedure was repeated once more and finally the total 4 mL of methanol were evaporated to dryness under a gentle nitrogen stream at room temperature (Figure 2).

Derivatization process

For the derivatization process the method described by Leng and Gries was followed with slight modifications. Briefly, 250 μL of N,N’-diisopropylcarbodiimide (99%), 1,1,1,3,3,3-hexafluoro-2-propanol (99%) and corn oil were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Cis- and trans-3-(2,2-dichlorovinyl)-2,2-dimethyl(1-cyclopropane)carboxylic acid (99.0%) was obtained from Dr. Ehrenstorfer (Ausborg, Germany). n-Heptane (≥99.5%) was all purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained by a Direct-Q 3 UV water purification system (Merck, Darmstadt, Germany).

Materials

CPMN technical grade was a kind gift of Agriphar (Belgium). 3-phenoxypybenzoic acid, 2-phenoxypybenzoic acid (98%), methanol (≥99.9%), N,N’-diisopropylcarbodiimide (99%), 1,1,1,3,3,3-hexafluoro-2-propanol (≥99%) and corn oil were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Cis- and trans- 3-(2,2-dichlorovinyl)-2,2-dimethyl(1-cyclopropane)carboxylic acid (99.0%) was obtained from Dr. Ehrenstorfer (Ausborg, Germany). n-Heptane (≥99.5%), sodium hydrogen carbonate (≥99.5%) and corn oil were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained by a Direct-Q 3 UV water purification system (Merck, Darmstadt, Germany).

Instrumental conditions

Electron ionization mass spectrometric analysis was performed on a GC-MS QP-2010 Shimadzu system equipped with a SLB-5ms (30 m x 0.25 mm x 0.25 μm) capillary column (Supelco, Bellefonte, PA, USA). Pure helium (99.999%) with a column flow of 1 mL/min was used as a carrier gas. One (1 μL) of the solution was injected into the system in the splitless mode and analyzed under the following conditions: the column temperature was initially held at 60°C for 1 min, raised to 310°C at 15°C/min, where it remained stable for 5 min. The injector temperature was 230°C. The interface and ion source temperatures were set at 220°C and 310°C, respectively. An autotune of the mass spectrometer using perfluorotributylamine (PFTBA, tuning standard) was performed before the analysis of every set of samples. Quantitative analysis was achieved in selected ion monitoring (SIM) mode with an event time of 0.3 s, using one target ion for quantitation and two qualifier ions for the confirmation of each compound. The m/z fragments for cis- and trans-Cl2CA were 323, 163 and 127, for...
2-PBA and 3-PBA were 197, 364 and 141. The column temperature was programmed in this way in order to avoid any interference from the matrix (hair) and the derivatizing agent.

The target (quantitation) ions (m/z) for each analyte derivatives of 1,1,1,3,3,3-HFIP were 323 for cis- and trans-Cl₂CA and 364 for 3-PBA and 2-PBA (IS). The retention times of cis-Cl₂CA, trans-Cl₂CA, 3-PBA, 2-PBA were 7.26, 7.33, 10.96 and 10.79 min, respectively. Typical chromatograms of a standard solution, a blank hair sample and a CLD group hair sample are shown in Figure 3.

Statistical analysis

Concentrations of cis-Cl₂CA, trans-Cl₂CA and 3-PBA were expressed in the form of mean (± standard deviation-SD) for laboratory animal hair samples. Bonferroni post hoc test was applied for two groups comparisons. Two-way analysis of variance (2-way ANOVA) was applied to examine the effect of dosage (low-high) and time of exposure (second and fourth month). Figure 5 depicts the estimated mean cis-Cl₂CA, trans-Cl₂CA and 3-PBA concentrations per dosage. IBM SPSS Statistics 21.0 was used for analysis.

Results

The developed method for CPMN metabolites quantification in hair was comprised of three main steps, analytes extraction from the biological matrix, derivatization process and subsequent instrumental analysis. During method development the conditions and parameters of these steps were optimized for the most efficient analytes extraction, derivatization, detection, and quantification.

The developed method was validated in terms of efficiency of analytes extraction from hair (method recovery), linearity of instrument response, limit of detection (LOD) and limit of quantitation (LOQ) of the method, accuracy and precision of the method. Method was validated according to procedures and guidelines described elsewhere.[24,25]

Optimization of analytes extraction from the hair specimen

For analytes extraction from hair, methanol and acetonitrile were tried in spiked concentration levels of 25, 250, and 1000 pg/mg and methanol proved to be the most efficient solvent with recovery >84.8% for all analytes (Table 2), while with acetonitrile achieved recoveries were below 60%. Besides, methanol confirmed to be a suitable solvent for extraction of pesticides and its metabolites from hair by previous studies.[20,22,23]

After selection of extraction solvent we optimized the sonication time. One hundred mg of randomly chosen rabbit hair (n = 2, one from CLD and one from CHD) with unknown analytes concentrations were sonicated in methanol for 2 h. The methanol was collected, filtered, concentrated and analyzed, while another aliquot of 2 mL of methanol were added in the same samples and the same procedure was followed until the measured levels of both analytes were below LOD. In total, 12 h of extraction were performed for both samples. Results indicated that in 4 h of ultrasonic extraction the achieved recovery was greater than 90.6% for cis-Cl₂CA, 92.0% for trans-Cl₂CA and 92.5% for 3-PBA.

Derivatization parameters optimization

As mentioned before the derivatization process was followed as presented by Leng and Gries,[14] with slight modifications. Moreover, the stability of the products of derivatization was tested. Standard mix solutions of 3-PBA, cis- and trans-Cl₂CA were prepared in two different levels (A: 500 and B: 1000 ng/mL) and followed the derivatization procedure as described before. The final organic phase from both vials was measured in GC-MS immediately (zero min) and every 45 min until completing total time of 1440 min (approx. 24 h). Results were compared to the first measurement and found that no observed degradation of the derivatives occurs in the first 24 hin room temperature for cis- and trans-Cl₂CA-HFIP (Figure 4). For 3-PBA a 15.6% degradation was observed at 1440 min for the level of 500 ng/mL and 22.1% degradation for the level of 1000 ng/mL. Although, samples of this study were prepared and analyzed immediately, data of this test may be useful in case of routine analysis.
Extraction recovery

For the evaluation of the method recovery, decontaminated blank hair samples (100 mg) were spiked (three replicates) with the target compounds prior to ultrasonic bath extraction, in the same way as the spiked samples, and the aforementioned procedure was followed. This was performed at three different concentration levels (25, 250, and 1000 pg/mg). The recovery was determined calculating the measured concentration of the treated spiked sample by using the corresponding standard solution curve. The mean recovery was estimated to 84.8% ± 17.5 (n = 8) for cis-Cl₂CA, 87.2% ± 7.8 (n = 8) for trans-Cl₂CA and 96.4% ± 20.4 (n = 8) for 3-PBA (Table 2).

Linearity

Internal standard method was used for analytes quantification. The instrument response was linear in the investigated concentration range 0 to 0.4 μg/mL with $r^2 > 0.99$. For analytes quantification corresponding matrix calibrators were used. Our results also indicated a good linearity for spiked hair at the concentration levels 25, 50, 100, 250, 500, and 1000 pg/mg. The hair spiked sample curve was linear, $r^2 = 0.998$ for both cis-Cl₂CA and trans-Cl₂CA and $r^2 = 0.995$ for 3-PBA (Table 2).

Limit of quantitation and limit of detection

The LOD of the method for each analyte was evaluated from the chromatogram of the lowest level spiked sample (25 pg/mg) of the calibration curves, by considering a response equivalent to three times the background noise for the selected quantification ions (S/N≥3). The LOQ of the method was determined as the concentration of analyte at which the signal-to-noise ratio of the quantification ion was at least 10 (S/N≥10). The LOD values for hair were calculated 4.0 pg/mg for cis-Cl₂CA, 3.9 pg/mg for trans-Cl₂CA and 1.0 pg/mg for 3-PBA, while the LOQ values were estimated 13.3, 12.9, and 3.3 pg/mg, respectively (Table 2).

Method precision and accuracy

The precision of the method was measured as % relative standard deviation (%RSD) of instrument response for replicate measurements of spiked samples. Inter-days precision of the method was evaluated by a triplicate assay for three different days of hair spiked samples in concentrations of 25, 250, and 1000 pg/mg. Inter-days precision ranged from 6.6 to 10.3% for cis-Cl₂CA (average 7.8%, n = 9), from 2.4 to 13.6% for trans-Cl₂CA (average 8.6%, n = 9) and from 2.6 to 9.5% for 3-PBA (average 6.5%, n = 9). Intra-day precision of the method was evaluated by measuring an assay for hair spiked samples in concentrations of 25, 250, and 1000 pg/mg in the same day. Intra-day precision was calculated from 6.6 to 13.2% for cis-Cl₂CA (average 9.9%, n = 3), from 3.2 to 13.6% for trans-Cl₂CA (average 8.7%, n = 3) and from 2.6 to 12.3% for 3-PBA (average 6.9%, n = 3). In all cases method precision was below 15%. Accuracy of the method was

Table 2. Basic analytical parameters of the validated method

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Rt (min)</th>
<th>m/z</th>
<th>$r^2$ (spiked)</th>
<th>LOD*</th>
<th>LOQ*</th>
<th>% Recovery ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-Cl₂CA</td>
<td>7.26</td>
<td>323, 163, 127</td>
<td>0.998</td>
<td>4.0</td>
<td>13.3</td>
<td>84.8 ± 17.5 (n=8)</td>
</tr>
<tr>
<td>trans-Cl₂CA</td>
<td>7.33</td>
<td>323, 163, 127</td>
<td>0.998</td>
<td>3.9</td>
<td>12.9</td>
<td>87.2 ± 7.8 (n=8)</td>
</tr>
<tr>
<td>3-PBA</td>
<td>10.96</td>
<td>364, 197, 169</td>
<td>0.995</td>
<td>1.0</td>
<td>3.3</td>
<td>96.4 ± 20.4 (n=8)</td>
</tr>
</tbody>
</table>

*: pg/mg, LOD: limit of detection, LOQ: limit of quantitation, SD: standard deviation
also calculated to be 95.3% ± 14.2 (n = 8) for cis-Cl2CA, 99.4% ± 8.2 (n = 8) for trans-Cl2CA and 98.0% ± 23.0 (n = 8) for 3-PBA (Table 3).

Levels of CPMN metabolites in rabbit hair specimen

Methodological standard solutions of cis-Cl2CA, trans-Cl2CA and 3-PBA (at concentrations levels as described earlier) and spiked hair samples (at concentrations as described earlier) followed the same procedure and were included in every batch of samples analyzed. Quality control samples were used to monitor any possible change to analytical parameters.

The detected levels of cis-Cl2CA, trans-Cl2CA and 3-PBA in rabbit hair corresponding to the same anatomical site are shown in Table 4 and graphically presented in Figure 5. We underline once more that hair collected from two different anatomical sites were mixed before analysis for each sampling. Thus, adequate amount of hair was used in order to assess the burden of subjects were exposed to different synthetic pyrethroids, since they are not specific to different synthetic pyrethroids.

For CHD group, the corresponding values were 2134.3 and 5361.4 pg/mg. The mean concentrations of trans-Cl2CA for CLD group were 270.7 and 1367.0 pg/mg. For CHD group, the corresponding mean concentrations were 2615.2 and 5448.2 pg/mg. Finally, the mean concentrations of 3-PBA for CLD group were 63.8 and 146.7 pg/mg, while for CHD group, the corresponding mean concentrations were 5647.4 and 695.9 pg/mg. For control group were also found low concentrations for all analytes during the experiment, with ranging from 18.7 to 112.6 pg/mg for cis-Cl2CA, from 22.5 to 112.0 pg/mg for trans-Cl2CA and from 16.5 to 42.8 pg/mg for 3-PBA (Table 4; Figure 5).

Results regarding the total length hair sample that represented the total burden of the animal of four months of exposure indicated mean concentration levels 1388.5, 1802.8 and 189.4 pg/mg of cis-Cl2CA, trans-Cl2CA and 3-PBA for CLD group. The corresponding values for CHD group were 5320.8, 6111.8 and 854.4 pg/mg (Figure 6). For control group all CPMN metabolites were below LOD.

Discussion

Purpose of this study was to develop an analytical method for the simultaneous detection of cis-Cl2CA, trans-Cl2CA and 3-PBA in rabbit hair. The implemented validation of the method demonstrate its low LOD, LOQ, high accuracy and good precision (Tables 2 and 3) and thus this method could be applied for the evaluation of chronic exposure of human or animals to CPMN via hair analysis and correlate the exposure with possible adverse health effects.

The developed method was applied for biomonitoring of rabbit exposure to CPMN under controlled sub-acute dosing. CPMN metabolites were detected in low background concentrations in hair of some control animals during the experiment (Table 4). This fact may be attributed to dietary exposure of the rabbits before or during their acclimation in the Medical School’s test animal facilities. As has also been previously reported,[26] measurable levels of 3-PBA and other CPMN metabolites may reflect multiple sources from environmental or dietary exposure to different synthetic pyrethroids, since they are not specific metabolites (Table 1).

![Table 3. Intra-day, inter-day precision and accuracy per concentration level of the validated method](image)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration*</th>
<th>Precision (% RSD)</th>
<th>% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intra-day</td>
<td>Inter-days</td>
</tr>
<tr>
<td>cis-Cl2CA</td>
<td>25</td>
<td>6.6</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>9.9</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>13.2</td>
<td>10.3</td>
</tr>
<tr>
<td>trans-Cl2CA</td>
<td>25</td>
<td>3.2</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>9.2</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>13.6</td>
<td>13.6</td>
</tr>
<tr>
<td>3-PBA</td>
<td>25</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>5.8</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>12.3</td>
<td>9.5</td>
</tr>
</tbody>
</table>

*: pg/mg, RSD: relative standard deviation

![Table 4. Mean concentrations of cis-Cl2CA, trans-Cl2CA and 3-PBA in rabbit hair (pg/mg) per treatment group over time of exposure](image)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mean concentration (pg/mg) in rabbit hair samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cis-Cl2CA</td>
</tr>
<tr>
<td>Administration period (months)</td>
<td>0</td>
</tr>
<tr>
<td>Control Group (SD)</td>
<td>&lt; LOD</td>
</tr>
<tr>
<td>Low Dose Group (CLD) (SD)</td>
<td>&lt; LOD</td>
</tr>
<tr>
<td>High Dose Group (CHD) (SD)</td>
<td>&lt; LOD</td>
</tr>
</tbody>
</table>

2-way Anova results

<table>
<thead>
<tr>
<th>Months x Dose</th>
<th>p=0.009</th>
<th>p=0.003</th>
<th>p=0.012</th>
</tr>
</thead>
<tbody>
<tr>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>p=0.064</td>
<td>p=0.023</td>
<td>p=0.025</td>
<td></td>
</tr>
</tbody>
</table>

Ratio (CHD) / (CLD) - 15.75 | 5.25 | - | 9.66 | 3.98 | - | 8.85 | 4.74 |

P: probability of the statistical test, LOD: Limit of Detection, SD: standard deviation
On the contrary, substantially higher concentrations were found in the samples of exposed animals and concentrations found for all the months of exposure are correlated to the total amount of insecticide ingested (Table 4). This testifies for the bioaccumulation of CPMN metabolites in the animal body. Interestingly, results demonstrated the dose dependent accumulation of CPMN metabolites (p < 0.001) in animal hair (Table 4). Statistically significant differences were found in all CPMN metabolites levels measured in CLD and CHD groups’ hair (p = 0.009 for cis-Cl₂CA, p = 0.003 for trans-Cl₂CA, p = 0.012 for 3-PBA) over time (Table 4). Also, examining the dose-time effect all results indicated statistical significant differences (p = 0.064 for cis-Cl₂CA, p = 0.023 for trans-Cl₂CA, p = 0.025 for 3-PBA) (Table 4). Regarding the total hair samples’ results, the dose-dependent accumulation of CPMN metabolites in animal hair was confirmed (Figure 6).

It is also quite interesting that a doubling in the dose administered to animals is associated with a much greater increase in the hair concentration. The difference between the metabolites is however less marked after four months of exposure (x 4.74 for 3-PBA; x 3.98 for trans-Cl₂CA and x 5.25 for cis-Cl₂CA) than after two months of exposure (x 8.85 for 3-PBA, x 9.66 for trans-Cl₂CA and x 15.75 for cis-Cl₂CA). This difference could perhaps be explained by incomplete incorporation at two months, for the CLD. The difference between CLD and CHD might therefore be more reliable at 4 months of exposure. The fact that the ratio CLD to CHD was from 3.98 to 5.25 for the three metabolites at four months whereas it was quite more variable (8.85 to 15.75) at two months tends to support this idea (Table 4).

Summarizing the hair samples corresponding to a specific anatomical site showed a burden to CPMN related to the dose and the time of exposure. Furthermore, the total hair samples confirmed the dose-dependent manner.

**Conclusion**

A rapid, sensitive, precise and accurate GC-MS based method has been developed for the simultaneous quantification of cis-Cl₂CA, trans-Cl₂CA and 3-PBA in hair samples. The method was applied and tested in the study of animal chronic sub-acute long-term exposure. This study demonstrates the dose dependent accumulation of CPMN metabolites in animal hair.

Nowadays CPMN is one of the most used pesticides worldwide. Future studies focusing on biomonitoring of population exposure could be a useful tool for risk assessing of the burden and for the protection of public health.

**Acknowledgements**

Authors deeply thank Mr Papadogiorgakis Manolis for the care and treatment of animals and also Konstantina Vorou for her contribution in the current study.
References


