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Article

Simple Method to Determine Residual Cypermethrin and Deltamethrin in Bovine Milk

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Um método simples, validado e econômico está descrito para a determinação de deltametrina e cipermetrina em leite bovino. Baseia-se na extração simultânea e *clean up* da matriz por dispersão em fase sólida (MSPD) com Celite® usando hexano/acetona (7:3 v/v), seguido de análise por cromatografia gasosa com detecção por captura de elétrons (GC-μECD). A análise de confirmação foi feita por GC-espectrometria de massas (MS). O processo simultâneo mostrou-se eficiente para amostras de leite. As recuperações médias em amostras de leite bovino fortificadas ficaram na faixa de 60 a 81%, com um desvio-padrão relativo (RSD) de 9 a 18%. O método apresentou limites de quantificação (LOQ) de 0,010 μg g^{-1} para ambos os piretróides e os limites de detecção (LOD) foram 0,007 e 0,002 μg g^{-1} para cipermetrina e deltametrina, respectivamente. A principal vantagem do método proposto é o número reduzido de etapas envolvidas, além de ser simples, rápido e barato. O método foi aplicado em amostras de leite integral coletadas em propriedades leiteiras do município de Chapada dos Guimarães, MT, Brasil. Deltametrina não foi detectada nas vinte amostras analisadas e cipermetrina foi detectado em quatro amostras (20%) em quantidades inferiores ao limite de quantificação.

A simple, validated and economic method is described for the determination of deltamethrin and cypermethrin in bovine milk. It is based on simultaneous extraction and clean up on matrix solid-phase dispersion (MSPD) with Celite® using hexane/acetone (7:3 v/v), followed by gas chromatography with electron capture detector (GC-μECD) analysis. Confirmatory analysis was carried out by GC-mass spectrometry (MS). The simultaneous process showed to be efficient for milk samples. Average recoveries from fortified bovine milk samples were in the range of 60 to 81%, with relative standard deviation (RSD) from 9 to 18%. Method limits of quantification (LOQ) were 0.010 μg g^{-1} for both pyrethroids and limits of detection (LOD) were 0.007 and 0.002 μg g^{-1} for cypermethrin and deltamethrin, respectively. The main advantage of the proposed method is the reduced number of steps involved, besides being simple, rapid and inexpensive. The method was applied to whole milk samples collected at dairy farms in the municipality of Chapada dos Guimarães, MT, Brazil. Deltamethrin was not detected in the twenty analyzed samples, and cypermethrin was detected in four samples (20%) at trace levels (< LOQ).

Keywords: pyrethroids, cow milk, GC-μECD, matrix solid-phase dispersion

Introduction

Pyrethroids are a major class of insecticides, derived from natural pyrethrin, introduced in the 1970s.[1] These substances are chemical ingredients of many commercial products used for controlling insects in agriculture, ectoparasites in rural animals, such as *Boophilus microplus* and *Haematobia irritans* and domestic insects, as well as vector control in public health.[2-6]

Insect infestation in cows reduces milk production since the animals become agitated with difficulty to feed.[7,8] Pyrethroid insecticides have been extensively used in Brazil for cow ectoparasite control.[2] Brazil is the second bovine producer in the world with a dairy herd of

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23,227,221 animals and a production of 5,686 billions of liters in the first trimester of 2013.[9] Chemical products applied to animals may cross the skin barrier, enter the blood stream and can be excreted in milk.[10,11] Residues of pyrethroids applied to cows were found in milk 28 days after animal exposure (absorbed mainly through the skin). These observed levels were several times higher than the Codex Alimentarius accepted thresholds.[12]

Pyrethroids act *in vitro* on a variety of recognized biochemical and physiological target sites. Voltage-sensitive sodium channels, the sites of insecticidal action, are important target sites in mammals.[13] However, mammals are three orders of magnitude less sensitive to pyrethroids than are insects and these characteristics has led to pyrethroids becoming the major pesticide class for agricultural and public health applications. The widespread use of pyrethroids and the corresponding increase in human exposure have led to sustained toxicological interest and a number of recent publications have suggested that there may be significant aspects that were not considered in the original evaluations of pyrethroid toxicity, such as the possibilities that pyrethroids may directly produce neuronal death in adults and developmental neurotoxicity in neonates or that their mammalian toxicity may be mediated by active metabolites in addition to the parent molecules.[14]

Moreover, some pyrethroids have endocrine disruptor effect: tetramethrin - estrogen-antagonistic effects in females only; sumithrin - increase of estrogen-sensitive cell proliferation, antagonist of the progesterone action; resmethrin - binding to sex hormone; permethrin - inhibition of estrogen-sensitive cell proliferation; deltamethrin - weak estrogenic activity; and cypermethrin - estrogenic effect.[15] The concern regarding these potential effects indicates the need to analyze pyrethroids used in cattle breeding in milk samples to guarantee consumer safety.

Older analytical methods described in the literature for determination of pyrethroid residues in milk involve laborious processes of extraction using large volumes of solvents, clean up steps and quantification by liquid or gas chromatography.[12,16-19] New methodological concepts have been proposed in methods[20,21] with extraction and clean up processes integrated in a single step, improving economy, sustainability and efficiency, with good recovery and limits of quantification, objectives that were aimed in the present study.

Thus, this study aimed to develop a rapid and simple method for the determination of deltamethrin and cypermethrin residues in bovine milk. The proposed method involves simultaneous extraction and clean up steps by liquid-solid dispersion procedure followed by quantification by gas chromatography with electron capture detector (GC-μECD).

Although Brazil has a considerable dairy herd, exports milk to Latin American countries and pyrethroids are largely used to control ectoparasites, few studies have analyzed these substances in milk[20-22] and the present study is the first one carried out in the Central Western region of the country.

Experimental

Reagents

Deltamethrin 99% and cypermethrin 99% primary analytical standards were purchased from Sigma Aldrich Brazil Ltda. Individual stock solutions of the analytes were prepared by diluting ca. 1.0 mg of the standard in 10.0 mL of toluene to obtain a concentration of 100 μg mL^{-1}. The working standard solutions were prepared by diluting the stock solutions as required with toluene while for sample fortification the stock solution was diluted with acetone. Solvents (toluene, hexane and acetone) for organic trace analysis were purchased from Tedia Brazil. Celite® 545 Merck (0.02-0.1 mm) was obtained from Hexis Cientifica.

Apparatus

Analyses were performed using an HP 6890 gas chromatograph with split/splitless injector, and μECD detection system. A DB-5 MS (5% phenyldimethylsiloxane) fused-silica capillary column 30 m, 320 μm i.d., 0.25 μm of film thickness (J & W Scientific, Folsom, CA, USA) was used, with nitrogen (purity 99.999%) as carrier and make up gas at flow-rates of 1.2 and 6 mL min^{-1}, respectively. Injector temperature was set at 250 ºC and detector temperature was 300 ºC. The oven temperature was programmed as follows: 92 ºC for 2 min, increased to 280 ºC at 20 ºC min^{-1} and hold at 280º C for 14 min. Data were acquired and processed by HP Chemstation software. An aliquot (1 μL) of the milk extracts, standards and blanks was injected in splitless mode into the GC-μECD system. For confirmatory analysis an HP 6890 gas chromatograph with split/splitless injector and mass spectrometry (MS) detection system was employed, using helium (purity 99.9999%) as carrier gas, with a similar capillary column (with 250 μm i.d. and carrier gas at flow-rate of 1.0 mL min^{-1}) and the same oven temperature programming as described for the GC-μECD system. The analysis was performed in selected ion monitoring (SIM) mode and the following ions were monitored: *m/z* 163, 165 and 181 for cypermethrin, and

m/z 181, 250 and 253 for deltamethrin. The use of GC-MS in SIM mode was chosen since its limit of detection (LOD) is lower than in scan mode. The selection of three monitored ions for each analyte allowed confirmation of identification by comparison of the relative ratio of qualifiers and target ion abundance in samples with those in standard solutions. Differences of up to 20% in the ratios were accepted.

Extraction procedure

Sample extraction was performed by matrix solid-phase dispersion (MSPD) using Teflon® centrifuge tubes (50 mL) and Celite®, which was pre-treated at 150 ºC for 8 hours. Five grams of the milk sample and 5 mL of acetone were added to the centrifuge tubes, which were shaken for 5 minutes in a mechanical horizontal agitator. In sequence, 10 mL of extraction solution (*n*-hexane:acetone, 7:3 v/v) were added to 2 g of Celite® and poured into the milk sample. The sample was extracted four times with 10 mL extraction solution by horizontal shaking during 15 min at 90 rpm. Phase separation occurred quickly after agitation without needing to centrifuge. The extracts were combined and concentrated under nitrogen stream until near dryness, and made up with toluene (1 mL) for analysis by GC-μECD, and confirmation by GC-MS. For optimization of the extraction process, different adsorbent masses and combinations of extracting solvents were evaluated.

Recovery studies

Recovery studies were carried out with whole milk (3% fat content) samples free of residual pesticides, which were obtained from an organic farm, located in Santo Antonio city, state of Mato Grosso, Brazil. Samples were spiked with the appropriate amount of standard mixture in order to achieve concentration levels of 0.010, 0.02 and 0.100 μg g^{-1}. The lowest concentration level was chosen considering that it should be higher than the equipment limit of detection (0.005 μg g^{-1} corresponding to 0.025 μg mL^{-1} in the extract - concentration factor equal to 5 - determined by visual evaluation of signal/noise ratio) and lower than the limits established by the Codex Alimentarius (0.02 and 0.05 μg g^{-1} for cypermethrin and deltamethrin, respectively). This limit was considered since there is no legislation in Brazil concerning the presence of pyrethroid residues in bovine milk used for human consumption.

At each fortification level, six replicates were analyzed. Quantification was performed by internal calibration using certified standards (aldrin was used as internal standard). The extraction procedure described above was followed. The method limit of detection was calculated as three times the standard deviation of the determined concentrations at the lowest fortification level divided by the angular coefficient of the analytical curve ($y = 0.9839x + 0.016$ and $R^2 = 0.9942$ for deltamethrin and $y = 1.3335x - 0.0331$ and $R^2 = 0.9934$ for cypermethrin). The limit of quantification (LOQ) was considered as the lowest fortification level that gave good recovery and precision.

The method was applied to twenty milk samples collected in small dairy farms in the Municipality of Chapada dos Guimarães, Mato Grosso, Brazil, in August and September 2004. These samples correspond to 50% of the 40 dairy farms that existed in the studied region.

Results and Discussion

GC-μECD conditions

In a first approach, the GC-μECD and GC-MS conditions were optimized to separate the insecticides studied. For that, different temperature programs were tested in order to resolve the pesticides in the standard mixture. Extracts of blank samples were injected and no interfering peaks were present. The representative chromatogram of the standard mixture and blank sample is shown in Figure 1.

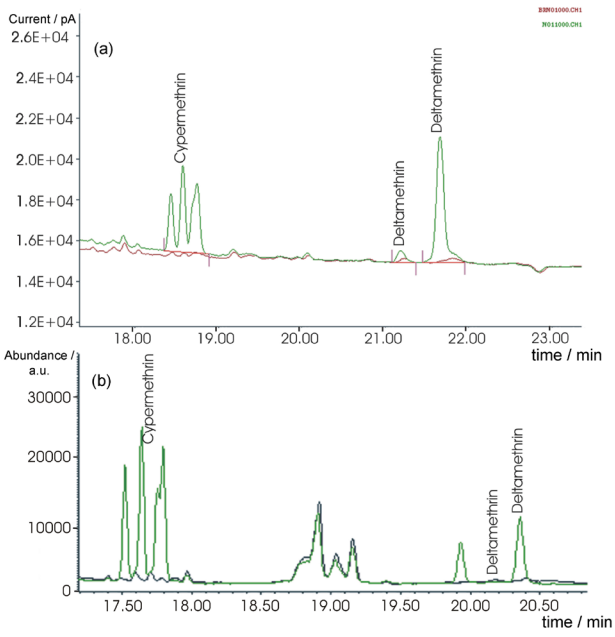

Figure 1. (a) Chromatogram of a 0.100 μg g^{-1} standard mixture and blank sample by GC/μECD; (b) chromatogram of the 0.100 μg g^{-1} standard mixture and blank sample by GC-MS (for operating conditions see text).

Figure 1 shows that the validated method presents good selectivity at the concentration range studied. In these conditions, retention times for each insecticide analyzed

were reproducible with a relative standard deviation never exceeding 0.22% for cypermethrin and 0.28% for deltamethrin and with both high selectivity and resolution.

Good linear chromatographic responses were achieved in the working interval (0.010 to 5.000 μg mL^{-1}) with correlation coefficients higher than 0.991, indicating that the conditions established performed well for quantification of these compounds. This is in agreement with the recommendation of the Brazilian National Agency for Sanitary Vigilance[23] that establishes that the correlation coefficient should be at least 0.990.

Optimization of extraction

The proposed method was based on a previous procedure developed for the determination of organochlorine residues in solid waste compost,[24] which used solid-phase matrix dispersion with Celite®. This method combines the extraction and clean up in a single step by using liquid-solid dispersion with Celite® for removal of interfering substances and to promote the elution of cypermethrin and deltamethrin from the dispersion. Celite® was chosen as adsorbent considering its capacity to retain fat.

Different conditions related to the extraction process were evaluated: Celite® mass, extraction solvents or mixture of solvents, pH change and agitation methods (sonication and mechanical). To promote the extraction, different solvents and mixtures of solvents, such as hexane/acetone, hexane/dichloromethane and hexane/acetone/acetonitrile were tested. The experiments were carried out in sequence until good recovery and precision was obtained. Among these, only the hexane/acetone (7:3 v/v) mixture provided satisfactory recovery results and appropriate extract purification. Methods G and E (see Table 1) differ only in the mass of Celite® used. The lower recovery of method E may be due to the fact that the sample interferents (mostly fatty acids) occupy the active sites of adsorbent leaving the pesticides in solution. A higher mass of Celite®, with a consequently higher number of active sites, could lead to irreversible adsorption of the pesticides. Table 1 shows the experiments carried out for the optimization of the extract procedure in order to improve recovery. Different agitation methods were equally efficient (data not shown).

The main difficulty in pesticide residue analysis in complex matrices, such as fatty samples, as is the case of milk, is the interference of co-extracted substances in chromatographic response. To overcome this problem, most published methods use several clean up steps, which involve liquid-liquid and/or solid phase extraction, among others. However, excessive sample manipulation may also introduce errors affecting method accuracy in addition to being longer and using higher amounts of solvents, with consequently higher costs. Thus, the proposed method presents advantages when compared to other published methods for being simpler, as can be seen in Table 2.

In another study involving fatty matrix the authors also used matrix solid phase-dispersion (MSPD), but only after a liquid-liquid extraction step.[27]

Recovery and precision

Method G (Table 1), which consisted of addition of 2 g of Celite® and extraction using *n*-hexane:acetone (7:3 v/v) was validated using recovery experiments. Satisfactory results were found with recoveries between 60 and 81% considering that the Association of Official Analytical Chemists accepts recoveries ranging from 60 to 115% for analyte concentration in the order of 10^{-6}% (corresponding to 0.010 μg g^{-1}).[28]

The method precision was determined by repeatability studies, expressed by the relative standard deviation. Average recoveries and relative standard deviation are summarized in Table 3. The proposed method gave better precision (RSD < 18%) than other methods presented for milk samples as for example the one reported by Bordet *et al.* with standard deviations from 33 to 50% for pyrethroid analysis in milk in the concentration range of 26 to 45 ng g^{-1}.[25]

Table 1. Influence of different solvents and sorbent mass on pesticide recovery in the extraction procedure. Milk samples fortified at 1.0 μg g^{-1} (n = 3)

Method	Procedure	Average recovery / %	
		Cypermethrin	Deltamethrin
A	Celite® 2.5 g; *n*-hexane:acetone (6:4 v/v)	18	21
B	Celite® 2.5 g; *n*-hexane:acetone (9:1 v/v)	26	24
C	Celite® 3 g; dichloromethane:hexane (1:1 v/v)	28	29
D	Celite® 2.5 g; *n*-hexane:acetone:ACN[a] (70:26.5:3.5 v/v/v)	29	34
E	Celite® 3 g; *n*-hexane:acetone (7:3 v/v)	54	77
F	Celite® 2 g; pH 8-9; *n*-hexane:acetone (7:3 v/v)	54	92
G	Celite® 2 g; *n*-hexane:acetone (7:3 v/v)	80	90

[a]ACN: acetonitrile.

Table 2. Analytical methods for determining pyrethoids in food matrices reported in the literature

Ref.	Extraction method	Analytical method	LOD	Sample	Recovery / %	RSD
16	Sample mass 25 g (*i*) extract with 100 mL acetone, (*ii*) concentration. (*iii*) Partition with hexane, (*iv*) concentration, (*v*) partition three times with acetonitrile, (*vi*) dryness, (*vii*) clean up with (SPE) silica gel.	GC-ECD	0.001 μg g^{-1}	Milk	70-100	± 7%
12	Sample volume 10 mL (*i*) partition twice with acetonitrile, (*ii*) partition three times with hexane. (*iii*) Dry residue (*iv*) clean up with silica gel.	LC-UV	0.001 μg g^{-1}	Milk/blood	78-91	Not informed
19	Sample volume 10 mL (*i*) sample homogenization with acetonitrile and ethanol. (*ii*) Extraction using disposable liquid/liquid extraction cartridges. (*iii*) Size-exclusion chromatography clean up	GC-ECD	Not informed	Milk	60-119	2.5-14.4%
17	(*i*) Dissolved the sample in petroleum ether, (*ii*) partition with acetonitrile/water, (*iii*) clean up on Florisil (SPE).	GC-ECD	Not informed	Milk, fat, oil, fish, cheese,	Not informed	Not informed
25	(*i*) Cryogenic extraction, (*ii*, *iii*) clean up 2 successive SPE cartridges C18 and Florisil.	GC-ECD	Not informed	Milk, fat, egg, fish	Not informed	33-50%
22	Sample mass 10 g (*i*) mixed the sample with silica-gel and placed in the column, (*ii*) elution with dichloromethane and *n*-hexane (50:50 v/v)	GC-MS	0.0033 μg mL^{-1}	Milk	Up to 70	Not informed
20	Sample 4 mL (*i*) liquid-liquid extraction with 8 mL of acetonitrile, agitation at 175 rpm for 20 min. The extracts are frozen for 12 h.	GC-ECD	0.25 μg L^{-1}	Milk	84-93	3.8-8.5%
21	Sample mass 0.25 g (*i*) mixed the sample with 1 g C18 and 1 g $NaSO_4$, placed in the SPE column containing 1 g Florisil with 5 mL MeCN, (*ii*) elution with 5 × 2 mL MeCN.	GC-MS	0.025 μg g^{-1}	Milk	79-92	2-26%
26	Sample mass 2.5 g (*i*) QUECHERS	GC-MS	0.01 μg g^{-1}	Milk	92-105	< 7%
A	Sample mass 5 g (*i*) extract and clean up by liquid-solid dispersion with celite®. (*ii*) Elution four times with *n*-hexane-acetone (7:3 v/v) solution.	GC-μECD	CP 0.007 DT 0.002 μg g^{-1}	Milk	60-81	8-18%

LOD: method limit of detection; RSD: relative standard deviation; A: proposed method; CP: cypermethrin; DT: deltamethrin.

Table 3. Percentage recoveries, relative standard deviations and limits of quantification and detection obtained by MSPD procedure of fortified milk for the pesticides studied (n = 6)

Substance	Fortification level / (μg g^{-1})	Mean recovery / %	Relative standard deviation / %	LOD[a] / (μg g^{-1})	LOQ[b] / (μg g^{-1})
Cypermethrin	0.10	60	9	0.007	0.01
	0.02	74	6		
	0.01	73	15		
Deltamethrin	0.10	66	12	0.002	0.01
	0.02	81	10		
	0.01	77	18		

[a]LOD: limit of detection; [b]LOQ: limit of quantification.

Method limits of detection (LOD) and quantification (LOQ)

The method LOD were 0.007 μg g^{-1} for cypermethrin and 0.002 μg g^{-1} for deltamethrin. LOQ values were 0.010 μg g^{-1} for both pyrethroids. The limits of detection obtained were in good agreement with those previously published by other authors (Table 2), and were sufficiently low to allow comparison to the proposed Codex limits for these substances.

Method application

Among the twenty analyzed milk samples, deltamethrin was not detected (below LOD) while cypermethrin was

detected in four samples (20%) at trace levels (below LOQ). These values are lower than the limits established by the Codex Alimentarius (0.02 and 0.05 μg g^{-1} for cypermethrin and deltamethrin, respectively) indicating no immediate risk to consumers.

Conclusions

A rapid and simple method for determining pyrethroid residues in milk by GC-μECD was described. The method was developed and validated aiming milk monitoring in a region with intense pyrethroid use on livestock activities. The simplicity and applicability of the proposed method allow its use for routine analysis of cypermethrin and deltamethrin in milk matrices, with enough sensitivity to determine concentrations below the limits established by the Codex Alimentarius. It offers the advantages of being simple, rapid and inexpensive, with reduced solvent consumption and demanding shorter time of analysis. Despite being present in low concentrations in the analyzed samples, the occurrence of cypermethrin indicates the need to monitor pesticide residues in milk in order to guarantee its quality. In addition, the producers should be oriented to use good agricultural practices, to prevent contamination.

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