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Determination of coumaphos, chlorpyrifos and ethion residues in propolis tinctures by matrix solid-phase dispersion and gas chromatography coupled to flame photometric and mass spectrometric detection

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ABSTRACT

A new analytical method has been developed and successfully evaluated in routine application for the quantitative analysis of a selected group of organophosphate pesticides (coumaphos, chlorpyrifos and ethion) which can be found at trace levels in propolis tinctures (ethanolic propolis extracts); a valuable commodity used as raw material in the food and pharmaceutical industries for which there have been few attempts for pesticide residue analysis reported in the literature. The proposed methodology is based on matrix solid phase dispersion (MSPD) using aluminum sulfate anh. a novel dispersant material and subsequent column chromatography clean-up in silica gel prior to gas chromatography (GC) with both flame photometric detector (FPD) and mass spectrometry (MS) detection used for the routine quantification and identification of the residues, respectively. The limits of detection, for coumaphos, chlorpyrifos and ethion were below 26.0 μg/kg in FPD and 1.43 μg/kg for MS detection. Mean recoveries were in the range of 85–123% with RSD values below 13%, which suggests that the proposed method is fit for the purpose of analyzing pesticides in propolis tinctures containing high concentration of polyphenolics. The method has been successfully applied in our laboratory for the last 2 year in the analysis of real propolis tinctures samples.

1. Introduction

Propolis is a valuable by-product from bees. Due to its well known biological activities such as antibacterial, antiviral, fungicidal, anti-inflammatory and anticancer properties, it is widely used in pharmaceutical and food industries. Propolis has been extensively used in dermal pharmaceutical preparations and nowadays it is increasingly being used as food or dietary supplement [1]. Pesticide residues in propolis arise from two main sources; either from contamination from agricultural practices or due to pesticide application in hives, to prevent parasitic acaroids like *Varroa destructor* [2–5]. Nowadays, few data are available on the influence of contaminants in propolis quality. Acaricides that are widely employed in apiculture are common propolis contaminants although neither published multiresidue method for residue determination nor residue regulation is given for this product and apicultural practices. Coumaphos is one of the preferred worldwide used acaricide for Varroasis control [2,6,7].

Since propolis consumption increased, contaminants are now considered of interest as described in recent publications for trace analysis of tetracyclines [8], chloramphenicol [9], polycyclic aromatic hydrocarbons [10] and pesticide residues [11,12] in raw propolis and processed propolis [13]. However, propolis tinctures are the actual commodity used, that is obtained after raw propolis processing which involves water washing and the removal of waxes through resin dissolution in ethyl alcohol with further filtration to separate insoluble waxes and remaining material [1]. Processed propolis gives a highly pigmented sticky gum with different physicochemical properties and composition from the raw material. Residue determination in propolis presents an analytical challenge because of the high polyphenolic composition of this matrix and the chemical variability of the samples depending on different geographical or botanical origins [12,14]. In this sense, clean-up steps must be exhaustive to yield purified extracts for proper routine gas chromatographic (GC) analysis. To the best of our knowledge, attempts for GC residue determination of pesticides in propolis are performed using general methods in products from animal or

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botanical origin, which typically involves the use of gel permeation chromatography (GPC) as also described in most important Pharmacopeias [15–17]. To date, two recent methods were developed for trace determination of pesticide residues in raw propolis. Santana dos Santos et al. [11] applied MSPD (matrix solid-phase dispersion) to analyze residues of bifenthrin, buprofezin, tetradifon and vinclozolin by GC–MS (gas chromatography–mass spectrometry), whereas Chen and co-workers [12] developed a method for residue determination of 17 organochlorine (OC) residues in raw propolis using a combination of tandem graphitized carbon and florisil cartridges and analyzing by GC coupled to an electron capture detector (ECD). Recently, Acosta-Tejada et al. [17] applied MSPD with C_{18} as dispersive material and ethyl acetate as elution solvent, analyzing 5 organophosphates pesticides in propolis tinctures by GC–MS.

The aim of this study was to develop a methodology for routine analysis of coumaphos in propolis tinctures, which was further extended to ethion and chlorpyrifos. In this article, we present validation and analytical features of a new method based on MSPD using aluminum sulfate anh. as sorbent with subsequent clean-up step by column chromatography followed by GC–FPD (flame photometric detector) determination and GC–MS confirmation. Moreover, the method was widely tested in real propolis tinctures samples.

2. Experimental

2.1. Chemicals and materials

Solid phase used for dispersion, $Al_2(SO_4)_3{\cdot}xH_2O$ 98% purity, was provided from Sigma–Aldrich (Milwaukee, USA) and dried 24 h at 350 °C to yield an anhydrous powder. Florisil as bulk powder 0.15–0.25 mm/60–100 mesh and Silica gel 60, 0.063–0.2 mm/70–230 mesh for column chromatography were obtained from Macherey-Nagel GmbH & Co. KG (Düren, Germany). Acetone, dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc) of HPLC grade were purchased from Mallinckrodt Baker Inc. (Phillipsburg, USA). Technical 95% ethyl alcohol (EtOH) was purchased from ANCAP (Montevideo, Uruguay). Coumaphos, chlorpyrifos, ethion, triphenylphosphate (TPP) and bromophos-methyl analytical standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Individual pesticide stock solution of 2000 μg/mL were prepared in pure EtOAc and stored at −18 °C. Mixed pesticide standards were prepared at 100 μg/mL by diluting stock solutions in EtOAc. Appropriate levels of working standard solutions were prepared by diluting the mixed standard solutions in acetone. TPP was selected as surrogate compound (SC) and bromophos methyl as an internal standard (IS) for quality assurance and quantification purposes, respectively. Stock solution of 2000 μg/mL in EtOAc, working standard solution of 4 μg/mL (SC) in acetone and 1 μg/mL (IS) in EtOAc were prepared and stored at −18 °C until analysis.

2.2. Propolis samples

All raw propolis samples used in this study were supplied by Laboratorio APITER Ltda. (Montevideo, Uruguay). Samples were collected in the southwest part of Uruguay in the Colonia Department (34°28′11″S; 57°50′48″O). Chemical characterization of propolis samples from this area was previously described by Kumazawa et al. [14].

All tinctures samples were prepared as defined in current United States (USP 30-NF25) and European Pharmacopeia (6th EP) monographs for extracts giving an standardized tincture of 20% (w/v) of soluble animal matter in EtOH [16,17].

2.3. Instrumentation

Pesticides residue analysis was conducted with a double-channel Shimadzu GC-17A GC equipped with programmable temperature vaporizer (PTV) injector, Shimadzu AOC-20i Autosampler and FPD operating in phosphorus mode. Injector temperature program was from 60 to 270 °C at a rate of 500 °C/min and then held for 15 min. Helium was used as carrier gas at a constant flow rate of 1.7 mL/min. Injection volume 1.0 μL. Separation was performed with a MEGA 68 (cyanophenyl-methylpolysiloxane) fused silica capillary column (25 m × 0.32 mm i.d. × 0.25 μm film thickness). The column oven was temperature programmed from an initial value of 150 °C (2 min hold) to 230 °C at a rate of 10 °C/min, then to 300 °C at 30 °C/min (10 min hold). In this study, MS detection was used for confirmatory purposes of residues. GC–MS analyses were performed using an HP 6890 GC coupled with an HP 5973 MS supported by reference libraries, equipped with an HP-5 (5% diphenyl 95% dimethylsiloxane) bonded fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). Electron impact (EI) mass spectra were obtained at 70 eV and monitored from 50 to 550 m/z for full scan mode analysis. MS system was programmed in selected ion monitoring (SIM) mode for confirmation. The working parameters were: injector temperature 290 °C; interface temperature 300 °C; carrier gas He at 38 cm/s, oven conditions; from 150 °C initial (3 min hold), increased to 230 °C at a rate of 10° C/min, then to 295 at 30 °C min (10 min hold), injection mode: splitless; injection volume: 1.0 μL. The identification of the compounds was confirmed by injection of matrix matched standards and comparison of their retention index and relevant MS ratios in accordance to DG-SANCO guidelines [18].

2.4. Sample preparation

4.0 g of ethanolic propolis tincture (20%, w/v) were accurately weighted into a 10 mL volumetric flask. 1.00 mL of SC solution at 4.0 μg/mL was added and the final volume was adjusted to 10 mL with acetone to yield 8% (w/v) tincture.

2.4.1. Spiking procedure

4.0 g of propolis tincture (20%, w/v) were accurately weighted into a 10 mL volumetric flask. This sample was spiked by the addition of 1.00 mL of appropriate mix of standard solution and 1.00 mL of SC at 4.0 μg/mL prepared in acetone. Volume was adjusted to 10 mL with acetone in all cases. Four levels of spiking were assayed (0.1; 0.5; 1.0 and 5.0 μg/g). The 5.0 μg/g level was assayed because of the existence of real samples containing high levels of coumaphos.

2.4.2. MSPD based clean-up

2 g $Al_2(SO_4)_3$ anh. were weighed and blended in a mortar with 1.00 mL of a fortified propolis solution. The mixture was placed into a glass column (300 mm × 12 mm i.d.) packed with 2 g of water-deactivated florisil at the bottom. A first aliquot (2 mL) of the solvent was used to backwash both the mortar and the pestle. Elution was performed with total 30 mL of CH_2Cl_2:EtOAc (9:1; v/v) mixture at a flow rate of 1–2 drops/s under gravity. Solvent was collected and evaporated to dryness with rotary evaporator at 40 °C. Extract was redissolved in 2 mL of CH_2Cl_2.

2.4.3. Column chromatography clean-up

Column chromatography was performed in a glass column (300 mm × 12 mm i.d.) packed with 7 g of wet silica gel in CH_2Cl_2 as further purification process. The redissolved extract was poured over the column while solvent was flushed to the solid phase, a total of 40 mL of CH_2Cl_2 were eluted and collected. The extract was evaporated until near dryness in a rotary evaporator at 40 °C and finally

to dryness with N_2 stream. In each case, 1.00 mL of IS 1 μg/mL in EtOAc was added and the final extracts were transferred to amber autosampler vials for GC analysis.

2.4.4. Calibration curves

Quantitation was performed in both neat solvent and matrix-matched calibration. Matrix-matched standards were prepared by adding 1.00 mL of appropriate working standard solutions to yield the concentrations assayed including IS at 1 μg/mL, to blank sample extracts. Blank samples were previously analyzed in duplicate to evaluate for not containing any of these OP pesticides with the proposed method.

3. Results and discussion

3.1. Preliminary studies

Sample clean-up is the most important step in residue analysis of propolis samples [12]. In contrast to published reports dealing with raw propolis [11,12], propolis tincture is a homogeneous substance, without debris or waxes which would represent a major problem for sample preparation. MSPD showed very useful performance when working with propolis thanks to the ability of increased interactions between the sample and the dispersant phase by mechanical blending. Due to the complexity of propolis tinctures, preliminary studies using MSPD were performed to exhaustively remove polyphenolic compounds. Different sorbent materials were evaluated with this purpose such as neutral alumina, silica gel and florisil for normal phase MSPD by eluting with non-polar mixtures of CH_2Cl_2:EtOAc as previously described [11]. Reversed phase MSPD in C_{18} was also tested eluting with EtOAc and acetonitrile but the procedure recently described in the literature [13] was not suitable for the high polyphenolic content of Uruguayan propolis. As stated above, MSPD allows the use of combinations of matrix/dispersant that ensures minimal coelution of interfering compounds. Since tinctures are prepared in EtOH we experienced problems when blending the mixture with typical sorbents because of the water content and affinity to the dispersant surface. The selected dispersant phase, $Al_2(SO_4)_3$ anh. not only has high affinity for phenols, but also due to its high hygroscopic properties eliminates the residual water present in the tincture, allowing a better interaction between the sample and the solid phase for fast dispersion step. Complexation of flavonoids with Al (III) ion is a well known property [19] which was used to selectively remove polyphenols and aromatic compounds even in wastewater [20]. Al (III) has great affinity for hydroxyls, particularly the phenolic ones, changing dramatically the sample polarity. Also aluminum salts of carboxylic acids either aromatic or aliphatic are formed. Illustration of complexation of a typical flavonoid such as quercetin with Al (III) is depicted in Fig. S1. The complexation of phenols and acids with Al (III) does not allow their solubilization with common organic solvents like CH_2Cl_2 and EtOAc. During the elution step, most complexed polyphenols are not eluted by the relatively low polarity of the organic solvents employed. On the other hand, acetone was selected as diluting solvent for being a good solvent for propolis tincture and its increased volatility in the dispersion step when performing MSPD with this sorbent giving a homogeneous dry powder.

Investigations were performed to choose the extraction solvent and co-sorbent material. In agreement to Santana dos Santos et al. [11] florisil co-sorbent with subsequent CH_2Cl_2 and CH_2Cl_2:EtOAc eluting mixtures provides extraction of pesticides and reduction of polyphenolic co-extractives. Different solvent mixtures were tested but finally CH_2Cl_2:EtOAc (9:1) was selected as a compromise between quantitatively eluting pesticides and matrix co-extractives. However, a subsequent clean-up step was mandatory for adequate performance in routine analysis, ensuring further removal of co-eluted compounds by adding the commonly used silica gel column chromatography [15].

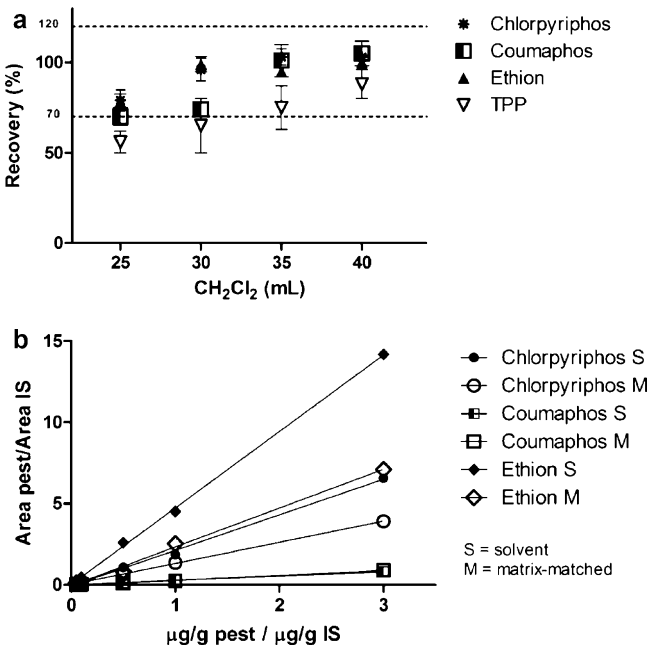

Fig. 1. (a) Optimization of CH_2Cl_2 volume on preliminary silica gel column chromatography at 500 μg/kg; (b) calibration curves evidencing the matrix effect for the selected pesticides.

Fig. 1(a) shows a preliminary investigation performed at a level of 500 μg/kg to determine the optimum CH_2Cl_2 volume in the quantitative extraction of pesticides from the column chromatography step. In accordance to widely accepted requirements (recoveries between 70 and 120%) [18], 40 mL of CH_2Cl_2 was employed for quantitative determination. Note that the dead volume at the selected conditions was approximately 10 mL.

3.2. Method development

The optimization of GC parameters (initial oven temperature, temperature gradient programme, PTV programme, etc.) was done to achieve high sample throughput but maintaining resolution between chlorpyrifos and bromophos methyl. GC–FPD was preferred to GC–MS for routine screening and quantification because of its higher robustness, wider dynamic range and lower maintenance when working in the phosphorous mode. Although the obtained extracts can be analyzed routinely in GC–MS, this technique was used for confirmatory presence or absence of pesticides since increased sensitivity was obtained compared to FPD. Table 1 shows the limits of detection and quantification (LODs and LOQs) of the analyzed pesticides along with their dynamic range and determination coefficient under the selected conditions.

Three SIM ions were used for the identification of the positive findings. The confirmation criteria of positives consider the retention time matching in GC–FPD and GC–MS analysis plus 3 SIM ions with their corresponding relative ion abundance matching. However, when analyzing real samples the matrix usually presented some co-eluting isobaric interferences for the most intensive ions of chlorpyrifos (m/z 197) and ethion (m/z 231). The selected ions along with their relative abundances are summarized in Table 2 which also reports both retention times for FPD and MS analysis. Response factors were calculated for FPD analysis using matrix-matched and solvent-only calibration. LODs values

Table 1
Detection and quantitation limits (LODs and LOQs) and linear regression parameters in matrix-matched calibration.

Compound	GC–FPD					GC–MS
	LOD (μg/kg)	LOQ (μg/kg)	Dynamic range (μg/g)	Calibration curve	Determination coefficient r^2	LOD (μg/kg)
Chlorpyrifos	9.1	30.0	0.03–5.00	$y = 1.0606x + 0.0672$	0.994	0.56
Coumaphos	26.0	88.0	0.09–20.00	$y = 0.7862x - 0.1452$	0.999	0.43
Ethion	4.7	15.0	0.05–5.00	$y = 1.6073x - 0.1152$	0.999	1.43

Table 2
Retention times t_R (min) for GC–FPD and MS analysis. Recoveries, average overall recoveries and RSDs (%), matrix effect (ME), and inter-day reproducibility (%) for each analyte under study ($n = 5$) for GC–FPD and selected quantifier and qualifier ions used for confirmation. Ion ratios obtained in matrix matched standards ($n = 3$).

Compound	t_R (min)	GC–FPD							GC–MS		
		Recovery ± RSD (%) Spiking level (μg/g)				Avg. overall recovery ± RSD (%)	ME (%)	Inter-day precision (RSD, %)	t_R (min)	Selected ions	Ion ratio (%)
		0.1	0.5	1.0	5.0						
Chlorpyrifos	8.6	88 ± 10	87 ± 1	123 ± 4	112 ± 3	102 ± 17	−41	6.8	9.2	316	64
										314	86
										258	52
										197	100
Coumaphos	13.5	102 ± 13	85 ± 3	106 ± 12	111 ± 4	101 ± 11	+14	7.2	12.2	364	42
										362	100
										334	12
Ethion	11.2	98 ± 6	92 ± 1	115 ± 6	110 ± 3	104 ± 11	−49	3.8	11.8	384	16
										231	100
										233	15
										153	39
Bromophos methyl	9.0	–	–	–	–	–	–	2.3	9.5	333	68
										331	100
										329	31
TPP	11.8	92 ± 12	88 ± 9	92 ± 8	89 ± 10	90 ± 2	+22	5.2	12.6	327	81
										326	100
										325	18

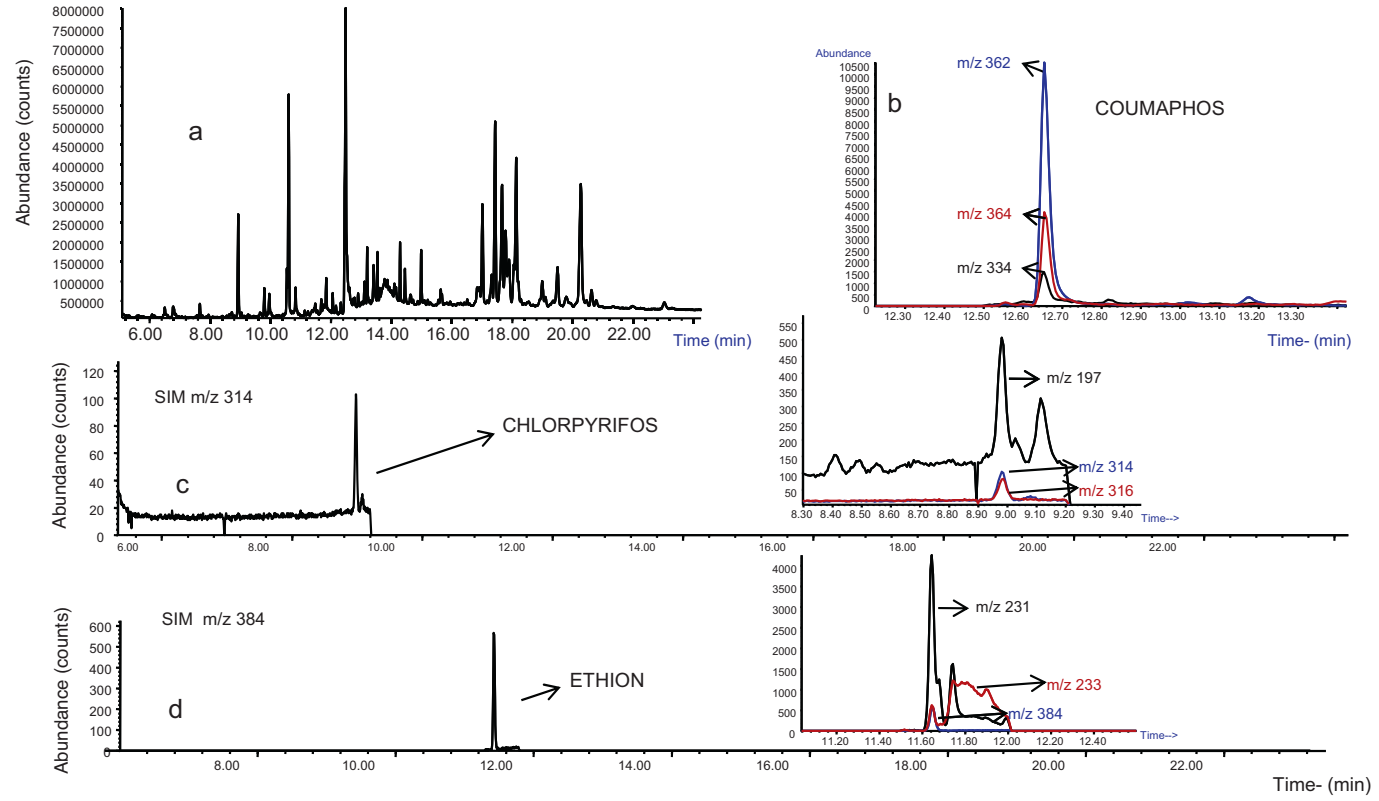

Fig. 2. (a) Total ion chromatogram of final extract; (b) SIM chromatogram of coumaphos in a fortified propolis sample at 100 μg/kg. (c) SIM chromatograms for the confirmation of chlorpyrifos and (d) ethion in a real propolis tincture sample.

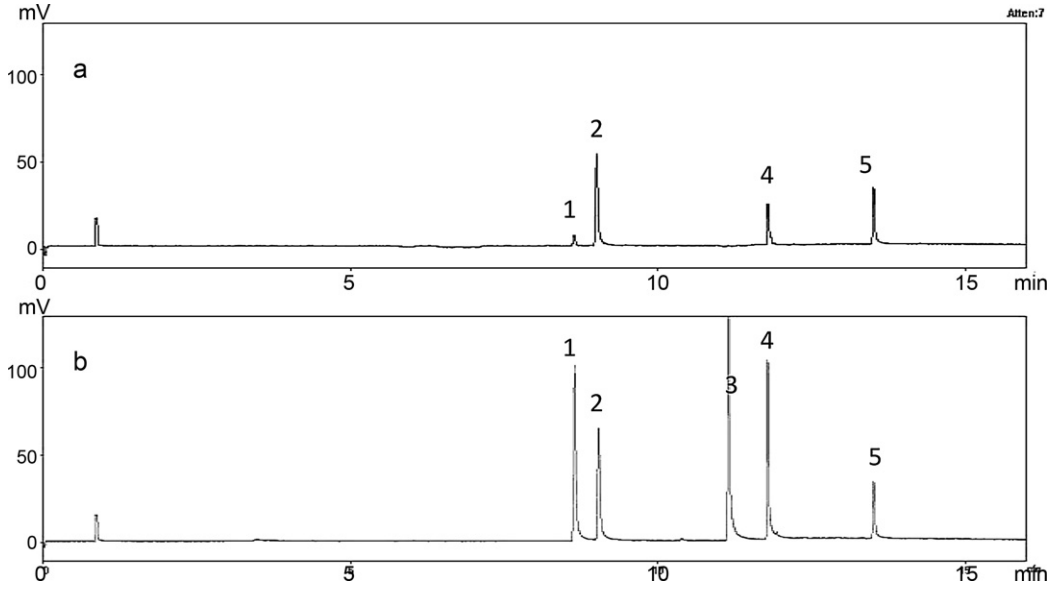

Fig. 3. Typical GC–FPD chromatograms of (a) real sample containing 86 μg/kg of chlorpyrifos and 960 μg/kg of coumaphos and (b) matrix-matched standards; 1 chlorpyrifos; 2 bromophos methyl (IS); 3 ethion; 4 TPP (SC); and 5 coumaphos.

were determined using the graphic approach at an S/N of 3 for each pesticide in spiked samples, whereas the LOQs values were obtained with S/N of 10. LOD for coumaphos in FPD analysis was 26 μg/kg while in MS it was 0.43 μg/kg referred to *m*/*z* 334. As seen in Fig. S2, an exhaustive clean-up is performed as evidenced by liquid chromatography–diode array detector (LC–DAD) monitoring of polyphenols during each step (see Supplementary Information for conditions).

Matrix effect (ME) was evaluated in the dynamic range stated in Table 1. Comparison of the calibration slopes for solvent standards and standards prepared in matrix indicated large matrix effects for these pesticides, as seen in Fig. 1(b). The presence of matrix co-extractives suppressed the response of chlorpyrifos by −49% and coumaphos by −41%. By contrast the response for coumaphos was enhanced by +14% and for TPP by +22%. Therefore, matrix-matched calibration solutions were employed for quantification. As reported in Table 2, recovery study showed recoveries between 70 and 120% and relative standard deviation (RSD) lower than 13%, showing good accuracy and repeatability complying with the analytical requirements stated in DG-SANCO [18]. In agreement with recent reports showing high levels of acaricides in bees by-products due to inadequate beekeeping practices and *Varroa* resistance [21,22], the 5000 μg/kg level was evaluated for a wide extent of the method performance due to findings in real samples contaminated with high levels of coumaphos.

The total ion chromatogram (TIC) depicted in Fig. 2(a) illustrates the complex volatile profile obtained in the injected extract even if exhaustive clean-up is employed to remove most polyphenols. In Fig. 2(b) is shown the SIM confirmation of incurred coumaphos (*m*/*z* 334, 362, 364) at a level of 100 μg/kg. The inter-day RSD ($n = 5$) values were between 3.8 and 7.2% as can be seen in Table 2 which has been a major drawback reported in the literature for GC analysis of pesticides in propolis [11] demonstrating its effectiveness for routine quantitative purposes.

Another key point is the SiO_2 column chromatography clean-up. This step is limiting for multiresidue analysis of more polar pesticides since recoveries are affected by the CH_2Cl_2 eluting volume as seen in Fig. 1(a) and need to be optimized. Moreover, as propolis tinctures showed very variable composition, the amount of co-eluted material was different for each sample. The selected CH_2Cl_2 volume was the best compromise between recoveries and an overloading of ME, ensuring good recoveries and reproducibility for the pesticides under study. The removal of this step or the use of increased volumes of eluting solvent is related with increased co-extractives elution, GC maintenance, ME and lower sensitivity. It should be emphasized that, since propolis extracts are commercialized in a variety of concentrations the method can be easily applied to the referred total solids content in the sample by performing dilution of solid extracts.

3.3. Application to real samples

The proposed method was applied to the analysis of over 1800 real samples from diverse apiaries in the last 2 years. As an example, Fig. 3 shows a typical GC–FPD chromatogram showing the pesticide residue findings in a real sample and comparing retention time of standards by the proposed approach.

Coumaphos and chlorpyrifos residues were identified in the vast majority of samples analyzed, whereas in few cases ethion residues were also identified. Detection rates and levels found for such pesticides in Uruguayan propolis tinctures are in agreement to those

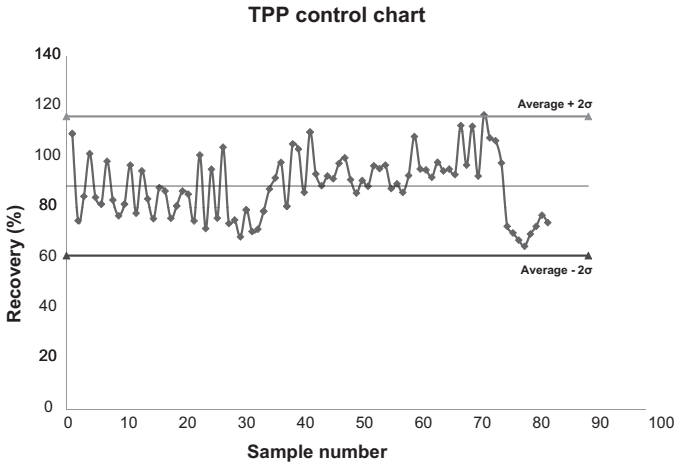

Fig. 4. Quality control chart for TPP surrogate compound at 1000 μg/kg in 83 real samples tested during 10 days period.

recently reported in beeswax in the USA [22]. Actually, quantitative capability was found to be well adjusted in the dynamic range assayed. Examples of SIM confirmation of pesticides in real samples are shown in Fig. 2(c and d). Of great concern is that chlorpyrifos is not used in beekeeping due to its high toxicity to bees (0.36 μg/bee) [23] and therefore its presence in the analyzed samples could be associated to environmental pollution.

Under the proposed analytical strategy, the need for GC maintenance is evidenced after ∼300 injected samples through broadening effect and loss of sensitivity for coumaphos where liner replacement and column shortening (50 cm from the injector port) is recommended. To track method performance, a quality control chart for TPP in 83 real samples during ten days period is shown in Fig. 4. Only one outlier sample was found using the proposed technique by two different analysts if considering 95% of confidence.

4. Conclusions

In this study, we presented a novel analytical method for trace quantitative determination of coumaphos, chlorpyrifos and ethion residues in propolis tinctures. Aluminum sulfate anh. employed as dispersant phase in a MSPD based scheme showed to be efficient in removing high amount of polyphenols in this troublesome matrix. However, subsequent clean-up was performed for routine application using GC–FPD/MS determination. The proposed method was sensitive, reliable and was successfully applied to the analysis of real samples, and it is actually employed in routine analysis in our laboratory. Validation of the method was carried out following EU guidelines. Moreover, as not only the EU banned coumaphos was detected, but also chlorpyrifos was frequently found in the analyzed real samples, more knowledge on the contamination of propolis tinctures by pesticide residues is needed. The future settling of maximum residue limits (MRLs) for this commodity seems to be mandatory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.06.097.

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