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Fast supercritical fluid extraction and high-resolution gas chromatography with electron-capture and flame photometric detection for multiresidue screening of organochlorine and organophosphorus pesticides in Brazil's medicinal plants

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Abstract

A description is given of a rapid and environmentally friendly method to determine organochlorine and organophosphorus pesticide multiresidues—malathion, methidathion, fenitrothion, fenthion, parathion-ethyl, parathion-methyl, lindane, hexachlorobenzene, chlorothalonil, tetradifon, α-endosulfan, β-endosulfan and dieldrin—in *Passiflora alata* Dryander and *Passiflora edulis* Sims. f. *flavicarpa* Deg. leaves by supercritical fluid extraction and high-resolution gas chromatography with electron-capture and flame photometric detection (HRGC–ECD/FPD). The mild extraction conditions [pure CO_2; 100 bar (1 bar=10^5 Pa) and 40 °C (ρ=0.62 g/ml); 5 min static+10 min dynamic extraction time; ODS trap and elution with 1 ml *n*-hexane at 2 ml/min] allow for direct analysis by HRGC–ECD/FPD with no prior cleaning procedure. The method provides, in accordance with the validation criteria of the European Pharmacopoeia, analytical results that are similar or even better than the official procedures, and is simpler, faster and cheaper. Mean recoveries of 69.8–107.1% were obtained, with 1.4–14.7% reproducibility (RSD). The method was applied to analyse commercial samples of *Passiflora* L. from Brazil. Twenty-three percent of the samples showed the presence of the organochlorine or organophosphorus pesticide residue investigated.

Keywords: *Passiflora* spp.; Plant materials; Supercritical fluid extraction; Pharmaceutical analysis; Pesticides; Organochlorine compounds; Organophosphorus compounds

1. Introduction

In recent decades, public interest in therapies using natural products, namely herbal medicine, has grown dramatically in both industrialised and developing countries [1]. However, compared with synthetic preparations, herbal products display a number of unique quality-related problems. These problems originate from the complexity of these remedies, which may vary greatly in chemical composition due to a variety of factors and compounds (such as pesticides) to which plants are exposed during their growth, storage and different stages of manipulation [2–4]. So, reference codes such as the British, American and European Pharmacopoeias, have also included methods for the analysis of pesticides in medicinal products of plant origin [5–7]. The European Pharmacopoeia (EP) sample preparation method [7], which is essentially similar to

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other mentioned codes, is carried out by organic solvent extraction followed by gel permeation chromatography (GPC) and solid-phase extraction (SPE) cleanup. Considering Brazil's distinctive herbal drug market [8]—and the fact that Brazil lacks official methods for the analysis of common contaminants in herbal matrices, even though it is the largest worldwide pesticide consumer [9]—a conventional methodology for pesticide analysis in *Passiflora* L. species based on the EP recommendations was recently evaluated and found to be highly effective [10].

Many analytical methods for pesticide analysis in herbal medicines involve labour-intensive cleanup procedures, are time-consuming, and require large amounts of toxic solvents, as is the norm in traditional liquid solvent extractions [9]. An attractive alternative to overcome these unfavourable analytical characteristics may be the supercritical fluid extraction (SFE)-based methods since, in comparison with liquid solvents, the high diffusivity and low viscosity of supercritical fluids offer more effective contact or diffusion in plant matrices and faster molecular diffusion of analytes [11,12]. Despite the potential of SFE, few reports have been published on pesticide analyses of medicinal plant material using this technique (see Refs. [9,13] and Refs. cited therein). The advantages of SFE over classic extraction methods are evident and may justify its use in the official methodologies of laboratories or regulatory agencies [14–17]. The main advantages of SFE are its high concentration capability, cleanliness and safety, quantitativeness, expeditiousness, simplicity and above all, selectivity. Moreover, it is an environmentally friendly analytical methodology that can be automated easily and completely [18,19]. Furthermore, commercial instrumentation is readily available and once the proper conditions are set up, routine extractions are straightforward and labour costs are low compared to conventional methodologies [20].

Several species of *Passiflora* L., popularly known as passionflower, are widely employed due to their anxiolytic, hypnotic, antispasmodic and anodynic properties [21]. *Passiflora incarnata* L. is the most widely used species in Europe and in parts of South America, while *P. alata* Dryander is the plant used officially in Brazil [22]. Although the pharmacological effects of *P. alata* Dryander leaves are reported in the Brazilian Pharmacopoeia, this species is often replaced by *P. edulis* Sims. f. *flavicarpa* Deg., which is more commonly available owing to its extensive use in the fruit juice industry and the fresh fruit market [23]. While some pesticides are registered and used only for fruit production in Brazil, in practice such compounds are widely applied in the cultivation of *Passiflora* L. for medicinal purposes, even those that have long been forbidden elsewhere, e.g., some organochlorine pesticides (OCPs) (lindane, hexachlorobenzene, chlorothalonil, tetradifon, α-endosulfan, β-endosulfan and dieldrin) and organophosphorus pesticides (OPPs) (malathion, methidathion, fenitrothion, fenthion, parathion-ethyl, parathion-methyl) pesticides.

The objective of this study was to develop and apply a simple SFE method using non-modified supercritical CO_2 in the analysis of 13 relevant OCPs and OPPs in complex herbal Brazilian matrices such as *P. alata* Dryander and *P. edulis* Sims. f. *flavicarpa* Deg. leaves, which are species extensively utilised in herbal remedies. The proposed SFE method was also applied to the analysis of pesticide residues in commercially available *Passiflora* L. samples from several Brazilian states (São Paulo, Rio de Janeiro and Amazonas).

2. Experimental

2.1. Material

The nanograde *n*-hexane used in the analysis of the pesticide residue was supplied by Riedel-de Häen (Seelze, Germany). The extraction fluid was SFC/SFE-grade carbon dioxide from SIAD (Rosta, Italy). The ODS trap (C_{18}, octadecylsilyl derivatized silica, 1 ml, 30 μm) was supplied by Hewlett-Packard (Waldbronn, Germany). The solvent used for rinsing the trap was nanograde *n*-hexane. The pesticide standards malathion, methidathion, fenitrothion, fenthion, parathion-ethyl, parathion-methyl, lindane, hexachlorobenzene, chlorothalonil, tetradifon, α-endosulfan, β-endosulfan, dieldrin and the internal standard carbophenothion were obtained from Riedel-de Häen and S.&I. Erhenstorfer (Augsburg, Germany), all above 93% purity. The herbal samples

were spiked with *n*-hexane solutions of analytical standard at different concentrations, namely 0.3 μg/ml OPPs+0.1 μg/ml OCPs; 0.15 μg/ml OPPs+0.05 μg/ml OCPs; 0.03 μg/ml OPPs+0.01 μg OCPs. Samples of *P. alata* Dryander and *P. edulis* Sims. f. *flavicarpa* Deg. (*Passiflora* L.) leaves were obtained from cultivated specimens grown in Ribeirão Preto, state of São Paulo, Brazil. The plant material was dried at 35 °C for 24 h, powdered, sieved (1–2 mm) and stored under dry and dark conditions.

2.2. Sample preparation

The extractions were performed using a Hewlett-Packard 7680 supercritical fluid extractor. For validation and commercial sample analysis, a 10-g portion of dried and powdered *Passiflora* L. leaves was fortified and 1-g subsamples were used for each analysis. Each subsample was placed between two filter paper disks and loaded into a 7-ml extraction thimble. The extraction conditions were 100 bar and 40 °C (pure CO_2 ρ=0.62 g/ml), 5 min equilibration time, 10 min dynamic extraction time at 1 ml/min and a restrictor temperature of 45 °C. Collection was done with an ODS trap at 10 °C, followed by elution with 1 ml of *n*-hexane at 2 ml/min and 20 °C. The trap was rinsed with 3 ml of *n*-hexane at 30 °C and with 2 ml/min between each extraction. The volume of extracts was checked and, whenever necessary, adjusted to 1 ml with *n*-hexane. The fraction containing pesticides was analysed by high-resolution gas chromatography (HRGC) with electron-capture detection (ECD) and flame photometric detection (FPD) and/or HRGC with mass spectrometric (MS) detection.

2.3. Chromatographic analysis

All the experiments were performed using a Carlo Erba Mega 5300 gas chromatograph (Carlo Erba, Milan, Italy) equipped with Ni^{63} ECD and FPD systems, operating in the phosphorus mode. Dual detection in parallel was achieved by using two-way press-fit connectors. A Mega 13 column (Mega, Legnano, Italy), 50 m×0.25 mm I.D., coated with a 0.15-μm film of a cross-linked 13% phenyl–methylpolysiloxane stationary phase was used. Two μl of each standard solution or sample extract were injected under the following conditions: injector temperature, 280 °C; ECD and FPD temperatures were 350 and 160 °C, respectively. Injection: split mode, split ratio was 1:20. Temperature programme: from 140 to 220 °C at 8 °C/min (3 min), then to 280 °C at 15 °C/min (5 min). Hydrogen (UP grade) was used as carrier gas at a flow-rate of 3.0 ml/min. Chromatographic data were collected using Hewlett-Packard (HP) HP 3396-II integrators and transferred to an HP Chem Station Data system for data elaboration. To confirm the results obtained for real-world samples, an Agilent 6890 GC-5973N MS system (Agilent, Little Falls, USA) applying the same column and conditions as for the HRGC–ECD/FPD analysis were employed. The pesticide were identified by comparison of their mass spectra with those of authentic samples or with data from the literature.

3. Results and discussion

The SFE conditions were optimised so as to obtain a highly selective extraction resulting in a ready-to-analyse extract. Selectivity was first explored by changing the CO_2 pressure to 100, 150 and 200 bar (ρ=0.62, 0.78 and 0.84 g/ml) at a fixed temperature (40 °C). The *Passiflora* L. extracts obtained with higher pressures showed a number of endogenous matrix components, thus calling for cleanup steps. At 100 bar the extracts were directly analysable without additional cleanup, displaying recovery and reproducibility results within the intervals established by the EP (Table 1). The reproducibility results, expressed as RSD at 100 bar were lower than 10.2%, whereas at 150 and 200 bar these values were lower than 18.7 and 37.9%, respectively. These higher values are those found for tetradifon, due to coelution of interferents: in general, it was observed that the RSD increased with the increasing pressure for several pesticides because a greater amount of endogenous sample compounds (e.g., waxes, hydrocarbons, etc.) were extracted at these conditions, some of them co-eluting with the pesticide's peaks.

Preliminary recoveries showed that an organic reagent (modifier) to increase the analytes' solubility in supercritical fluid was unnecessary. However, as recommended by the EP for pesticide analysis, the

Table 1
Effect of pressure on the SFE recoveries of OCPs and OPPs from the fortified *Passiflora edulis* Sims f. *flavicarpa* Deg. at 1 μg OCP and 3 μg OPP/g level (fixed temperature at 40 °C, $n=3$)

Compound	Recovery (%)					
	100 bar	RSD (%)	150 bar	RSD (%)	200 bar	RSD (%)
(1) Hexachlorobenzene	89.9	2.2	86.9	5.8	85.7	10.3
(2) Lindane	86.7	3.9	87.4	5.6	77.4	8.9
(3) Chlorothalonil	84.9	2.5	85.4	2.2	86.3	5.7
(4) Parathion-methyl	90.6	3.1	78	4.9	107.8	13.5
(5) Fenitrothion	92.6	5.0	79.5	7.2	97.1	9.3
(6) Malathion	86.5	3.8	66.8	8.4	77.9	16.7
(7) Parathion-ethyl	88.6	4.7	71	5.8	86.2	9.2
(9) α-Endosulfan	96.8	2.8	77.5	3.9	82	5.1
(11) Dieldrin	88.8	2.3	87.3	3.4	93.9	4.6
(12) β-Endosulfan	84.9	4.3	89.5	7.2	94.4	8.8
(13) Tetradifon	75.2	10.2	77.3	18.7	81	37.9

See Section 2.2 for additional experimental conditions employed.

Passiflora L. samples contained less than 15% water, all ranging from 7.5 to 8.5% [24]. This amount of water, though quite low, may have acted as a ''natural'' modifier, improving the extraction of relatively polar pesticides.

Another parameter investigated was the extraction time, after 5 min had been established as the equilibration time. Because the analytes in *Passiflora* L. leaf samples are mostly nonsystemic pesticides, since they are located on the surface or in easily extractable sites, the kinetics of extraction was linearly related to recovery and extraction time, i.e., to the volume of supercritical CO_2. After 10 min (2.1× thimble volume), recovery values remained practically constant for all pesticides, except for α-endosulfan. So, an extraction time of 10 min was chosen as the optimum extraction time, as also confirmed by confronting recoveries versus dynamic extraction times (data not shown herein).

According to Lang and Wai [13], an advantage of solid trapping is that the selectivity can be further improved by selective trapping coupled with selective eluting. Based on the successful use of ODS traps to collect OCPs and OPPs from plant matrices [25], methanol and *n*-hexane were investigated and 1 ml of *n*-hexane proved to be the most suitable eluting solvent to recover trapped pesticides completely, with the cleanest extracts. Different solid-phase traps were not tested because trapping efficiency was believed to be almost 100% with the ODS trap used, as discussed in previous studies [26].

After establishing the optimal SFE experimental conditions, we have observed that *Passiflora* extracts obtained by SFE contained less matrix peaks than those obtained by the EP method, so two pesticides were also included in this SFE study due to their importance for *Passiflora* L. cultivation, i.e., fenthion and methidathion. Thus, the procedure was validated according to the current EP regulations for a total of 13 pesticides, using external standard method. Standard pesticide solutions were prepared in the hexanic plant extracts, for avoiding matrix effect (in this case, enhanced GC responses were observed in comparison to those from solutions of the same pesticides on organic solvents) and this strategy proved to be suitable with the external standard method. The calibration graphs, plotted using both detectors at concentrations between 0.01 and 0.45 μg of each compound per ml of plant extract, presented good linearity (r from 0.9973 to 0.9999). The results that were obtained through the detector and that provided the highest area responses were considered for analytical purposes. The pesticides' limits of detection (LODs) determined by ECD varied from 1 ng/ml (hexachlorobenzene) to 46 ng/ml (fenthion) and those by FPD from 7 ng/ml (fenthion) to 14.5 ng/ml (methidathion). The use of an internal standard as recommended by the EP

method (in this case, carbophenothion from Riedel-de Häen was used) did not improve quantitative analysis' figures (results not shown herein). The *Passiflora* L. species studied here revealed similar GC profiles, i.e., no characteristic interference for either matrix was observed under the analytical conditions applied (Fig. 1a,b).

Table 2 shows recovery and reproducibility data obtained from nine assays of each *Passiflora* L. species: the average recovery and reproducibility values of nearly all the pesticides were in agreement with the EP validation recommendations. Table 3 shows LODs for the analysed pesticides on each *Passiflora* L. species, these values being very close due to similarity of both matrixes. The general results indicate that the proposed SFE method offers a satisfactory route for the extraction and determination of 13 OCPs and OPPs in *Passiflora* L. samples.

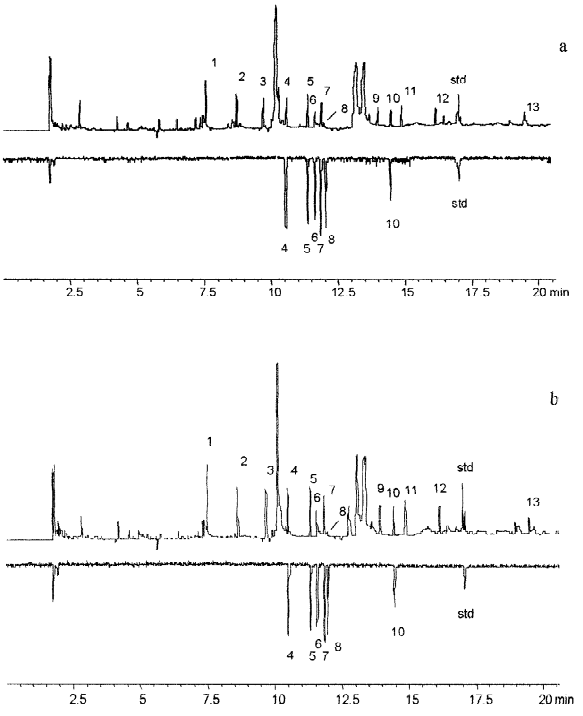

Fig. 1. HRGC–ECD and HRGC–FPD chromatograms of (a) *P. edulis* Sims f. *flavicarpa* Deg. and (b) *P. alata* Dryander extracts spiked with 0.05 μg OCP and 0.15 μg OPP/g of sample. (1) Hexachlorobenzene, (2) lindane, (3) chlorothalonil, (4) parathion-methyl, (5) fenitrothion, (6) malathion, (7) parathion-ethyl, (8) fenthion, (9) α-endosulfan, (10) methidathion, (11) dieldrin, (12) β-endosulfan, (13) tetradifon. Std: carbophenothion (internal standard; 0.10 μg/ml). See Section 2.3 for experimental conditions.

3.1. Analysis of real-world samples

An extensive study to develop procedures for the analysis of pesticide residues in Brazilian medicinal plants revealed that, out of several methodologies (including a method modified from the EP method which was applied for analysis of reference samples of *Passiflora*, see Ref. [10]), the SFE method provided the best overall results [27] and it was therefore selected to analyse commercial samples. No report on the analysis of pesticides in medicinal plants sold in Brazil's domestic market has been published prior to this study. For this reason, 26 samples of Brazilian *Passiflora* L. were obtained from different herbal drug suppliers and processed according to the EP guidelines. Table 4 shows the results obtained for the samples containing the pesticides under study. With the exception of one sample containing malathion (Fig. 2a), most of the commercial samples analysed here showed the presence of OCPs (dieldrin, lindane, tetradifon, chlorothalonil, and α-endosulfan at 21–71.4 ng/g level) (Fig. 2b). HRGC–MSD data (for full data, see Ref. [28]) confirmed that 23% of real *Passiflora* L. samples showed OCP or OPP residues, but the levels of contamination found were lower than the maximum residue limit (MRL) stipulated by the EP. Nonetheless, due to their high persistence in body tissues, the presence of even small amounts of OCPs may represent a serious risk to human health. The OPPs were found in low amounts in the herbal samples, probably owing to their lower persistence in the environment compared to that of OCPs. Bicchi et al. [28] recently determined the pesticide levels in herbal teas, showing that from 30 to 90% of all OCPs and OPPs present in *Passiflora* L. samples were transferred from the vegetable matrix to the herbal tea during the infusion process, depending mainly on their water solubility.

4. Conclusion

SFE was shown to be a good alternative technique

Table 2
Statistical data on pesticide recovery of samples of *P. edulis* Sims. f. *flavicarpa* Deg. and *P. alata* Dryander leaves

Compound	Level (μg/g)	*P. edulis* Sims. f. *flavicarpa* Deg.[a]			*P. alata* Dryander[a]		
		Average recovery (%)	RSD (%)	CL (%)	Average recovery (%)	RSD (%)	CL (%)
(1) Hexachlorobenzene	0.10	78.7	2.4	4.7	90.9	3.4	7.6
	0.05	74.1	7.2	12.9	82.3	5.1	10.3
	0.01	71.6	10.3	18.1	79.7	11.2	21.8
(2) Lindane	0.10	72.4	3.9	6.9	94.0	2.2	4.9
	0.05	73.9	4.9	8.8	81.8	4.2	8.3
	0.01	73.2	7.4	13.2	81.9	2.4	4.9
(3) Chlorothalonil	0.10	80.5	3.6	7.1	87.4	2.7	5.9
	0.05	72.6	3.2	5.6	77.4	4.3	8.1
	0.01	70.4	7.1	12.3	74.9	6.4	11.8
(4) Parathion-methyl	0.30	84.8	3.1	6.4	100.1	3.9	9.6
	0.15	86.0	2.9	6.1	93.7	7.7	17.6
	0.03	82.1	5.5	11.0	99.8	6.1	14.9
(5) Fenitrothion	0.30	86.6	5.3	11.3	105.3	2.8	7.4
	0.15	83.1	7.5	15.2	99.5	5.3	13.0
	0.03	80.3	3.4	6.6	96.2	7.3	17.2
(6) Malathion	0.30	100.8	3.7	9.1	105.2	5.0	13.0
	0.15	87.2	2.1	4.4	102.9	4.9	12.3
	0.03	86.6	5.4	11.5	103.4	9.4	23.8
(7) Parathion-ethyl	0.30	91.9	5.6	12.7	101.4	3.8	9.6
	0.15	80.2	3.6	7.1	101.3	3.3	8.1
	0.03	78.5	14.3	27.4	93.8	3.7	8.6
(8) Fenthion[b]	0.30	79.7	7.9	15.4	81.4	11.1	*22.1*
	0.15	89.2	9.3	20.3	91.7	7.9	17.6
	0.03	80.6	9.8	19.4	87.2	3.4	7.4
(9) α-Endosulfan	0.10	80.7	2.4	4.7	91.8	3.7	8.3
	0.05	86.1	3.4	7.1	89.3	5.4	11.8
	0.01	85.2	10.8	22.5	82.8	9.7	19.6
(10) Methidathion	0.30	75.6	3.2	5.9	97.6	2.2	5.1
	0.15	90.1	4.2	9.3	87.5	4.1	8.8
	0.03	86.3	6.6	14.0	95.1	8.1	18.9
(11) Dieldrin	0.10	82.9	2.1	4.2	94.6	1.6	2.7
	0.05	79.4	2.9	5.6	79.8	1.4	2.7
	0.01	76.2	10.6	19.8	77.9	3.2	6.1
(12) β-Endosulfan	0.10	80.7	5.8	11.5	81.4	3.7	7.4
	0.05	73.4	14.7	26.5	76.0	6.7	12.5
	0.01	71.8	11.7	20.6	*69.8*	8.0	13.7
(13) Tetradifon	0.10	96.8	6.4	15.2	107.1	8.1	17.4
	0.05	92.5	6.6	14.9	87.4	10.2	24.7
	0.01	93.3	12.9	29.4	99.4	8.2	20.3

RSD, relative standard deviation; CL, confidence limit (95%).
[a] In italics, results exceeding the recovery or reproducibility limits (EP).
[b] Except for fenthion, all the analytical results were obtained through HRGC–ECD determination.

Table 3
LOD values (ng/ml) for the studied pesticides on *Passiflora* L. extracts

Compound	LOD (ng/ml)	
	P. edulis Sims. f. *flavicarpa* Deg.	*P. alata* Dryander
(1) Hexachlorobenzene	1.0	1.0
(2) Lindane	1.4	1.3
(3) Chlorothalonil	1.6	1.7
(4) Parathion-methyl	5.6	5.6
(5) Fenitrothion	4.6	4.5
(6) Malathion	9.5	10.0
(7) Parathion-ethyl	4.8	4.6
(8) Fenthion[a]	7.0	7.5
(9) α-Endosulfan	2.2	1.9
(10) Methidathion	14.3	10.9
(11) Dieldrin	1.7	1.7
(12) β-Endosulfan	1.8	1.8
(13) Tetradifon	2.8	2.7

[a] Except for fenthion, all the analytical results were obtained through HRGC–ECD determination.

for the extraction of selected OCPs and OPPs in *Passiflora* L. samples, being faster than traditional solvent-based approaches and with the additional advantage of applicability of SFE systems (including

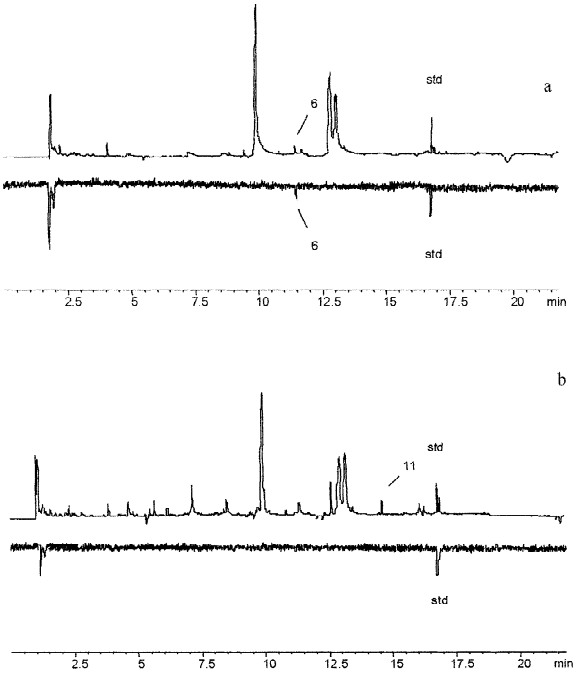

Fig. 2. HRGC–ECD and HRGC–FPD chromatograms of *Passiflora* L. commercial samples containing (a) 60 ng/g of malathion (peak 6) and (b) 23 ng/g of dieldrin (peak 11). Std: carbophenothion (internal standard; 0.10 μg/ml). See Section 2.3 for experimental conditions.

Table 4
Data of *Passiflora* L. samples containing pesticide residues

Sample number	Origin	Macroscopic aspects	Identification data[a]	Humidity[b] (%)	Pesticide residues[c] (ng/g)
1	São Paulo	Dried and chopped leaves and branches	''Maracujá'' lot 08/00	7.9	Dieldrin 23 (6.3)
3	São Paulo	Dried and chopped leaves and branches	''Maracujá tea'' lot 08/00	7.5	Lindane 39 (4.8)
7	São Paulo	Dried and chopped branches and several leaves	''Maracujá tea, *Passiflora edulis*'' lot 01/00	7.5	Tetradifon 68.9 (7.0)
11	Mato Grosso do Sul	Small branches, dried and chopped, and several leaves	''Maracujá'' lot 08/00	7.9	Malathion 60 (5.1)
20	Rio Grande do Sul	Dried and chopped leaves and branches	''Maracujá tea, *Passiflora edulis*'' lot 06/00	7.8	Chlorothalonil 21 (5.9)
26	São Paulo	Dried and chopped leaves and branches	''Maracujá leaves'' lot 12/00	8.5	α-Endosulfan 71.4 (4.6)

[a] Data obtained from the commercial sample's label. The lot number represents the month and the year of herbal drug manufacture.
[b] Water content.
[c] In brackets, the RSD (%) from analysis of real-world samples ($n=3$).

on-line SFE–GC coupling, and automation, etc.) for routine analyses of these residues in medicinal plant material. The proposed method proved to be simple and effective, allowing us to determine the analytes in the samples according to the validation parameters and below the maximum residue limits established by the European Pharmacopoeia and related codes.

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