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Article

Development of a Supercritical Fluid Extraction Method for Simultaneous Determination of Organophosphorus, Organohalogen, Organonitrogen and Pyretroids Pesticides in Fruit and Vegetables and its Comparison with a Conventional Method by GC-ECD and GC-MS

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O presente trabalho tem como objetivo aplicar uma metodologia multiresíduo visando a determinação de pesticidas em frutas e vegetais, utilizando extração com fluido supercrítico e análise por cromatografia gasosa e detector por captura de elétrons e espectrometria de massas. Um método convencional por extração sólido-líquido baseado na literatura e um método com fluido supercrítico desenvolvido, foram aplicados na determinação simultânea de 32 pesticidas de 4 classes diferentes (organoclorados, organonitrogenados, organofosforados e piretróides) em amostras de alface, batata, maçã e tomate. As recuperações obtidas para a maioria dos pesticidas foram de 74% a 96% para ambos os métodos em níveis entre 0,04 – 0,10 mg kg^{-1}, os limites de quantificação (dependendo da matriz e do pesticida) foram menores que 0,01 mg kg^{-1}. SFE mostrou-se vantajosa quando comparada à extração sólido-líquido como economia de solventes, tempo e custos, podendo ser aplicada no monitoramento de pesticidas em alimentos.

The aim of this paper was to apply a multiresidue method using Supercritical Fluid Extraction (SFE) and capillary gas chromatography with electron capture and mass spectrometry detections in the analysis of the levels of pesticide residues in fruits and vegetables. Single laboratory validation of both solid-liquid and supercritical fluid extraction methods was carried out for 32 compounds selected from four pesticide classes (organochlorine, organonitrogen, organophosphorus and pyretroid) in blank and fortified samples of fresh lettuce, potato, apple and tomato. Recoveries for the majority of pesticides from fortified samples at fortification level of 0.04-0.10 mg kg^{-1} ranged 74-96% for both methods and confirmation of pesticide identity was performed by gas-chromatography-mass spectrometry in a selected-ion monitoring mode. Both methods showed good limits of detection (less 0.01 mg kg^{-1}, depending on the pesticide and matrix) and the SFE method minimized environmental concerns, time, and laboratory work.

Keywords: pesticides multiresidue analysis, supercritical fluid extraction, fruits, vegetables

Introduction

Pesticides are necessary and essential in agricultural production. Increasing public concern, in recent years, about health risks from pesticide residues in the diet, has led to strict regulation of maximum residue limits (MRL) and total dietary intake of pesticide residues in foodstuffs.[1] In an effort to monitor the levels of these residues, many governmental and industrial programs have been implemented for the regulatory analysis of pesticide residues in food through multiresidue methods. Standard multiresidue procedures for fruit and vegetables are described by many monitoring agencies, in their screening programs[2] and are officially accepted in many countries.

The analysis of trace levels of pesticides in food, frequently requires the removal of high molecular weight interferences such as lipids and natural resins before the analysis by gas chromatography or high performance liquid chromatography.[3] The extraction process is the first and a major limiting step in the pesticide residue analysis, often involving sample preparation such as chopping and maceration, followed by solvent extraction. The

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conventional methods often involve laborious blending and vigorous processes, which are time consuming and involve large volumes of hazardous solvents. In recent years, the analysis of pesticide residues in food has incorporated new technologies to develop and use procedures, which minimize environmental concern, time, labor, and exposure of laboratory technicians to toxic chemicals.[3,4]

Sample preparation methods, generally used by analytical chemists, are both time and solvent consuming. According to a recent survey, two thirds of the analysis time is devoted to sample preparation and this step accounts for at least one third of the error generated during the performance of an analytical method.[1] Recent concerns about the hazards associated with most of the solvents used, and the costs and environmental dangers of solvent waste disposal, have led to the development of alternative sample extraction methods such as solid-phase extraction (SPE) and supercritical fluid extraction (SFE).[5]

SFE has gained increased attention as a potential replacement for conventional liquid solvent extraction (sonification or Soxhlet) owing to the properties of supercritical fluids: high diffusivity and low viscosity. The greatest advantage of supercritical fluids, however, is the fact that they have densities (and solvating powers) comparable to the density of liquids, which can be continuously varied by one order of magnitude by varying the temperature and pressure of the extraction vessel.[6]

Supercritical fluid extraction (SFE) is an alternative to the solvent-intensive isolation procedures, especially for environmental samples. In the area of agrochemicals, SFE has been used for selective extractions of organophosphorus and organochlorine pesticides,[7-9] carbamates[10,11] and different herbicides,[12,13] from different matrices.

CO_2 is frequently used as a supercritical fluid due to its suitable critical temperature (31.2 °C) and pressure (72.8 atm), since it can be easily removed by reducing its pressure. A CO_2 density of 0.8-0.9 g mL^{-1} appears to be adequate for most pesticides.[14]

Satisfactory extraction efficiencies were reported for non-polar to low polar pesticides such as organochlorine[15] and organophosphorus.[16] For pesticides of high polarity and metabolites of pesticides, the addition of polar modifiers such as methanol or water to CO_2, enhances its dissolving power. For meat and fatty material, the separation of lipids from lipophilic pesticides is essential for accurate analysis.[17,18]

Multiresidue methods were firstly developed to improve the cost-effectiveness without sacrificing the reliability of the results. The presence of matrix interferences in extracts of fresh fruit and vegetables adversely affect analyte quantification and identification. Clean-up is necessary in order to reduce the detection limits of methods and/or to avoid interferences from the matrix. Sample clean-up techniques include gel permeation chromatography,[19] liquid-liquid partitioning using various solvents,[20] solid phase extraction (SPE), adsorption chromatography (on silica, Florisil, active carbon, alumina) and membrane technologies.[21,22]

Chromatographic methods are the most suitable ones for residue analysis, in particular, gas chromatography (GC) using long, narrow-bore capillary columns equipped with selective and sensitive detection methods such as electron-capture detection (ECD), nitrogen-phosphorus detection (NPD) and flame-photometric detection (FPD) according to different classes of pesticides. The identification and separation by GC can be increased when combined with confirmation capabilities of mass spectrometry (MS). Mass spectrometry is a very sensitive and selective technique for both multiresidue determination and trace level identification over a wide range of pesticides.[23, 24]

The aim of the present work was to apply a rapid and accurate multiresidue method, to determine organohalogen, organonitrogen, organophosphorus and pyretroid pesticides in routine testing of fruit and vegetables entering local markets.

This study describes two different methods of extraction: one, using acetone by solid-liquid extraction and another by SFE (testing many extraction conditions) for multiresidue analysis of pesticides. Clean-up was based on aminopropyl cartridge extraction, followed by GC/ECD for determination, and the confirmatory analysis was carried out by GC/MS in the selected-ion monitoring (SIM) mode. The extraction efficiencies were directly compared to those achieved using Solid Liquid Extraction (SLE). After this point, the methods were applied to the analysis of real samples and the results were discussed.

Experimental

Samples

The studied samples, potatoes, tomatoes, apples and lettuce were purchased at a local supermarket. The pesticides chosen were those most used in our region. For fortification studies, pesticides free matrices, deriving from a special field cultivated with no pesticides (organic) were used. Fruit and vegetables were processed as specified in the European Union Legislation.[25] Circa 500 g of crop sample was washed with water and chopped, blended for 3-5 min using a blender equipped with a stainless steel

cutting unit, a glass jar, weighed and submitted to extraction.

Reagents, solvents and reference pesticide standards

Pesticide reference standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany) with purity ranging from 95-100%. The pesticides investigated are listed in Table 1. Pesticide stock solutions (approximately 500 mg L^{-1}) of individual pesticide standards were prepared by dissolving approximately 0.050 g of the pesticide in 100 mL of acetone:n-hexane (50:50, v/v) and stored in freezer under –18 °C in glass bottles with PTFE- faced screw caps. Pesticide working solutions were prepared for recovery tests by solid-liquid and SFE methods by appropriate dilution of acetone:n-hexane (50:50, v/v).

Acetone, acetonitrile, n-hexane, dichloromethane, ethyl acetate and methanol, of a special grade for pesticide residue analysis were purchased from Mallinkrodt, Merck. Sep-Pak Vac aminopropyl cartridges (3 mL, 500 mg) were purchased from Waters (Milford, MA). A special siphonated CO_2 from White Martins was also used in SFE.

Extraction procedure

The solid-liquid extraction method used in the determination of multiresidue was based on the literature with some modifications.[26] A 25 g portion of the homogenated crop sample was weighed in a 250 mL beaker and fortified when required with the pesticides standard solution. After a 15 min equilibrium period, 40 g of hydromatrix were added to the beaker and the sample was

Table 1. Retention times, recoveries (RSD%, n=5) and detection limits of pesticides in the matrices studied, obtained by solid-liquid extraction and GC/ECD

Pesticides	t_R (min)	Fortification	Recovery (RSD%)				LOD (mg kg^{-1})
		Level (mg kg^{-1})	Apple	Lettuce	Potato	Tomato	
1- Dichlorvos	7.27	0.05	66 (4.9)	71 (6.5)	69 (5.6)	66 (6.4)	0.007
2- Linuron	8.84	0.07	75 (5.8)	76 (4.7)	80 (8.1)	77 (5.9)	0.005
3- Trifluralin	17.30	0.05	83 (6.3)	82 (6.6)	81 (5.0)	81 (5.1)	0.002
4- Hexachlorobenzene	21.11	0.07	85 (6.8)	80 (8.7)	82 (6.1)	83 (4.6)	0.002
5- Dicloran	23.50	0.05	74 (4.9)	76 (6.1)	78 (7.3)	79 (6.5)	0.002
6- Diazinon	24.08	0.07	85 (8.3)	83 (8.1)	82 (7.4)	83 (7.6)	0.005
7- Dimethoate	25.16	0.05	69 (5.3)	65 (6.8)	64 (5.9)	66 (6.0)	0.006
8- Chlorothalonil	26.47	0.05	65 (6.6.)	70 (5.8)	66 (6.8)	68 (7.1)	0.001
9- Vinclozolin	26.57	0.07	74 (6.9)	77 (6.4)	76 (7.2)	79 (5.5)	0.002
10- Aldrin	27.99	0.10	77 (7.1)	73 (6.8)	71 (5.9)	73 (7.4)	0.002
11- Metolachlor	28.87	0.05	78 (5.5)	75 (7.2)	79 (4.6)	81(6.3)	0.005
12- Triadimefon	29.17	0.06	80 (5.8)	79 (6.9)	82 (6.9)	83 (5.4)	0.002
13- Chlorpyrifos	29.53	0.06	81 (5.2)	77 (5.6)	82 (6.9)	83 (7.1)	0.001
14- Dicofol	30.03	0.05	80 (5.3)	76 (6.4)	82 (5.6)	81 (3.8)	0.005
15- Triadimenol	31.28	0.06	77 (7.7)	75 (8.2)	79 (5.3)	80 (6.7)	0.008
16- Endosulfan Alfa	32.50	0.05	83 (3.8)	74 (5.9)	81 (6.6)	84 (7.3)	0.001
17- Hexaconazole	33.27	0.05	85 (5.4)	82 (6.3)	83 (4.8)	81 (4.5)	0.003
18- Imazalil	33.90	0.05	81 (3.6)	80 (4.2)	84 (5.8)	82 (3.3)	0.004
19- Buprofezin	34.96	0.05	81 (8.3)	79 (6.7)	80 (5.5)	83 (7.0)	0.005
20- Endosulfan Beta	36.41	0.06	77 (5.0)	75 (4.6)	79 (6.2)	81 (6.2)	0.001
21- Etaconazole	36.55	0.06	80 (5.7)	77 (7.0)	79 (3.8)	78 (5.6)	0.002
22- Propiconazole	37.73	0.05	78 (7.3)	77 (7.1)	80 (6.8)	81 (6.9)	0.002
23- Tebuconazole	38.41	0.05	83 (8.2)	80 (5.9)	82 (7.2)	84 (6.6)	0.006
24- Diclofop-methyl	38.65	0.08	86 (5.7)	79 (4.8)	84 (6.1)	83 (6.7)	0.005
25- Bromopropylate	39.71	0.10	75 (4.3)	77 (5.1)	81 (4.4)	80 (3.9)	0.002
26- Metoxychlor	41.29	0.06	81 (4.6)	77 (7.3)	82 (6.2)	83 (4.9)	0.003
27- Tetradifon	42.20	0.05	82 (5.1)	79 (5.8)	84 (4.8)	82 (5.9)	0.001
28- Prochloraz	45.64	0.06	82 (5.7)	83 (6.9)	84 (6.6)	82 (5.3)	0.005
29- Cyfluthrin (I)	46.08	0.06	83 (6.5)	81 (7.3)	84 (5.8)	83 (6.1)	0.005
30- Cyfluthrin (II)	46.34						
31- Cyfluthrin (III)	46.68						
32- Cypermethrin (I)	47.67	0.05	79 (5.9)	75 (6.9)	76 (5.5)	76 (7.4)	0.003
33- Cypermethrin (II)	48.20						
34- Cypermethrin (III)	48.36						
35- Quizalofop-ethyl	49.40	0.08	80 (5.3)	76 (8.8)	81 (6.1)	81 (8.4)	0.007
36- Fenvalerate (I)	52.29	0.06	77 (6.8)	78 (7.1)	82 (7.3)	81 (6.5)	0.003
37- Fenvalerate (II)	53.51	0.06	75 (6.7)	76 (8.2)	80 (7.5)	79 (7.7)	0.003

stirred with a glass rod until a dry, free flowing mixture was obtained. The sample was extracted with 80 mL of acetone for 30 min under constant stirring. The mixture was filtered under vacuum, through a Buchner funnel, fitted with Whatman # 1 filter paper and the residue washed with 2 portions of 30 mL acetone. The extracted phases were combined, dehydrated by passing through a filter containing a bed of anhydrous Na_2SO_4 and concentrated in a rotary evaporator under reduced pressure at 65 °C, and the sample was dried under a gentle stream of pure nitrogen. The residue was dissolved in 5 mL of acetone and submitted to clean-up using SPE. SFE was performed by using the SFX-220 extraction system (ISCO, Lincoln, NE, USA), which consists of a SFX-220 extractor, a SFX-200 controller, 100 DX syringe pump, and a siphonated Carbon Dioxide cylinder.

Samples of 2 grams of potato, tomato, apple and lettuce were weighed in a 150 mL beaker and fortified when required with the pesticides standard solution. After a 15 min equilibrium period, 6 g of the hydromatrix were added to the beaker and the sample was stirred with a glass rod until a dry, free flowing mixture was obtained. The sample was placed in a stainless steel extraction cell (5.6 cm x 1.6 mm i.d.) in a sandwich fashion, using silanized glass wool at both the bottom and the top of the cell to protect cell seals. Before extraction, when necessary, a modifier (acetone or methanol) was added to the samples, by pipeting a calculated volume in relation to the total volume of the SFE cell, so as to obtain a 10% v/v supercritical fluid volume. The extractions were carried out at a temperature of 70 °C and tested at different extraction pressures: 19971, 44935 and 69898 kPa, using a flow rate of expanded gas of 1.5 mL min^{-1} for 25 min, for CO_2 or CO_2 modified with 10% of acetone and methanol. A piece of fused silica capillary tube (30 cm x 100 μ i.d.) was attached to the outlet of the extractor as a restrictor and the pesticides were directly collected in an aminopropyl cartridge at 10 °C (clean-up SPE).

Clean-up SPE

A 12-manifold Supelco VISIPREP was used for the sample clean-up, performed in aminopropyl cartridges.

The aminopropyl cartridges were attached to the vacuum manifold and prepared by loading magnesium sulfate to fill approximately one third of the cartridge volume. The clean-up cartridge (magnesium sulfate-aminopropyl cartridge) was conditioned with approximately 5 mL of 50:50 ethyl acetate/n-hexane. The extract was added to the column and eluted under gravity with two portions of 5 mL each of acetone/n-hexane, 80:20, 50:50, and ethyl acetate/n-hexane 20:80. Once elution was completed, the collected extracts were concentrated under a gentle N_2 stream. The residue was quantitatively dissolved in 1 mL of acetone and submitted to analysis by GC/ECD and GC/MS.

Recovery study

The chopped and blended crop sample was fortified in triplicate with each working solution and processed after 15 min (equilibrium time) as described in the extraction procedure. Fortification levels for each pesticide, ranging from 0.04 to 0.10 mg kg^{-1} are reported in Table 1.

GC/ECD

A Hewlett Packard Model 5890 Series II gas chromatograph equipped with a ^{63}Ni electron-capture detector and a fused silica capillary column HP-608 (30 m x 0.25 mm i.d., film thickness 0.25 μm) was used. The operating conditions were as follows: initial temperature 45°C (1 min), increased at 20°C min^{-1} to 150 °C, held for 5 min, then increased at 4 °C min^{-1} to 280 °C for 20 min; injector temperature 250 °C; carrier gas H_2; column linear velocity (μ = 45 cm s^{-1}); detector temperature 300 °C; make-up gas N_2; operated in the splitless mode; purge off time 1 min; injection volume 1 μL.

GC/MS

A confirmatory analysis run was done on a Hewlett Packard Model 5890 Series II gas chromatograph with a HP 5972 mass selective detector (quadrupole) and a fused -silica capillary column LM-5- 5% phenyl 95% dimethylpolysiloxane (35 m x 0.25 mm i.d., film thickness 0.25 μm). GC operated under the following conditions: initial temperature 45 °C (1 min), increased at 21 °C min^{-1} to 150 °C, held for 5 min, then increased at 4 °C min^{-1} to 280 °C, and final temperature being held for 30 min; injector temperature 250 °C; carrier gas He; GC-MS transfer line 280 °C; operated in the splitless mode; purge off time 1 min; injection size 1 μL. MS conditions: delay solvent 2.9 min; electron impact ionization voltage 70 eV; scan rate 1.5 scan s^{-1}; scanned-mass range 40-600 *m/z*.

Results and Discussion

Solid-liquid extraction

In multiresidue monitoring, an important consideration in extraction is the solubility range of the different pesticide

families as well as the character of the food matrix. An inherent problem with a multiresidue approach is that matrix co-extractives increase as the method includes a wider polarity range of analytes, and no current method is suitable for extracting all pesticides from all matrices.[3]

Owing to is effectiveness for polar and nonpolar pesticides, acetone has been selected as the solvent for the extraction of pesticides from fruit and vegetables. Additional advantages include low toxicity and cost, miscibility in plant materials and ease of evaporation due to its low vapor pressure value. However, the sample extracted with acetone may present a high content of co-extractives which can damage the GC capillary column as well as result in a matrix enhancement effect.[27] Samples which contain more sugars or pigments need further clean-up owing to the relatively large amount of sample injected. Previous experiments showed that weak anion exchangers such as NH_2 remove many co-extractives interfering with the GC analysis of pesticides, and are also very efficient in lowering the matrix effects. [28, 29]

In a previous work, a multiresidue pesticide analysis method was applied to honey samples demonstrating a good chromatographic separation efficiency for 32 compounds selected from four pesticide classes.[30] In this work, that analytical methodology was used as a basic method, been adopted to avoid matrix effects due to fruit and vegetables complexity, in order to determine pesticides in such samples. Although no interfering peaks were observed on the chromatograms of the blank extracts obtained under the selected conditions (Figure 1), some matrix peaks were observed when analyzed with ECD, nevertheless, these peaks did not interfere with the selected pesticide analysis, showing to be satisfactory for the studied samples.

High-resolution GC using capillary columns allows us to achieve good separation performances in an adequate time of analysis. Selective and sensitive detectors, such as ECD, provided good responses, even in very low concentrations. Optimal chromatographic conditions for the multiresidue analysis of different families of pesticides were studied by comparing the crop sample chromatograms with those obtained from standard mixtures. The concentrations were calculated on the base of peak areas by the external standard method. Retention times of pesticides are listed in Table 1. The results showed that the pesticides eluted separately with the selected column, as well as cyfluthrin, cypermethrin and fenvalerate isomers.

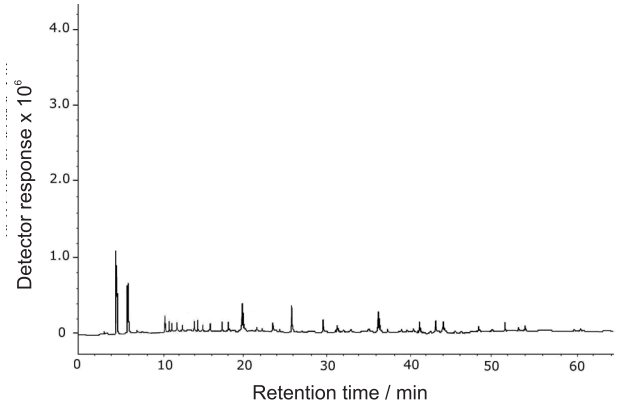

Figure 1. GC-ECD chromatogram of tomato blank extract obtained by the solid-liquid extraction method.

All standard curves were within the acceptable limits of the linearity criterion, with the exception of chlorothalonil, which showed a correlation coefficient of 0.996.

The evaluated method demonstrated an acceptable selectivity for most pesticides analyzed in all the selected matrices.

From the 32 pesticides tested, 29 presented recoveries between 73-85%, which is considered an optimal basis for method validation. Compounds such as dichlorvos, dimethoate and chlorothalonil presented recoveries in the 64-71% range, which could still be considered acceptable.

Moreover, some compounds presented better recoveries in certain matrices than in others (Table 1). This could probably be due to the phenomenon known as "matrix-induced chromatographic response enhancement".

The detection limits (LOD) were less than 0.01 mg kg^{-1} for ECD, which is consistent with the legislation minimum detectable quantity.[1,25] The Relative Standard Deviation (RSD) from 5 to 9%, and the recovery results suggest that the extraction and the clean-up procedure could be considered reliable enough for routine multiresidue screening.

Supercritical Fluid Extraction (SFE)

Many analytical methods for pesticide analysis in fruit and vegetables involve intensive clean-up procedures which are time-consuming, and require large amounts of toxic solvents, as a norm in traditional liquid solvent.[31] SFE has become an alternative to traditional organic-solvent based methods, for the removal of analytes from solid matrices.

The high content of water present in samples such as fruit and vegetables, gives rise to restrictor plugging by ice, which carries water into the collection trap. In this work, hydromatrix was used as a drying agent so as to control water.

An optimization study has been carried out using fortified matrices aiming at determining the conditions that would provide maximum recovery in SFE.

Modifier test

As shown in Table 2, the solvating power of pure CO_2 was too low for exhaustive extraction of the pesticides investigated. Pesticides such as tetradifon, etaconazole, hexaconazole, imazalil, metolachlor, prochloraz, propiconazole, triadimenol, chlorpyrifos, diazinon, dichlorvos and dimethoate were not detected in some matrices or their recovery results ranged from 8 to 26%. However, the best results were obtained for organohalogens, some organonitrogens and pyrethroids with recoveries between 36 and 65%, according to the literature, due to the physicochemical properties of these compounds.[31]

From the results in Table 2, it seems clear that some extraction modification is needed to increase the recovery. To enhance SFE extraction efficiency, CO_2 was modified with 10% acetone and methanol, using samples fortified with target compounds (1 mg kg^{-1}), and clean-up cartridge directly.

As expected, the increase in the addition of the modifier, and consequently the fluid strength, was beneficial for the extraction of most pesticides (Table 2). However, as shown in Table 2, the effect of the modifier in natural matrices is not clear, which means that some pesticides such as bromopropylate, chlorothalonil, endosulfan, tetradifon, buprofezin, metolachlor, dicloran chlorpyrifos, dichlorvos and dimethoate, present maximum recoveries while using acetone, whereas under the same conditions, aldrin, dicofol, hexachlorobenzene, imazalil, trifluralin and diazinon show the presence of co-extractives (recoveries above 140%). The best result, for the studied samples, was accomplished using CO_2 modified with 10% methanol, presenting recoveries above 50% and no result of co-extractive was observed. This result shows that the addition of 10% methanol increases the extraction efficiency, improving the solubility of the target analyte or interacting with active sites on the sample matrix, which can help CO_2 to efficiently extract the analyte and improve the selectivity

Table 2. Recoveries of pesticides in the matrices studied, using CO_2 and CO_2 modified with 10% acetone and 10% methanol of fortified samples, using direct clean-up with aminopropyl cartridges

Pesticides	CO_2				CO_2 10% acetone				CO_2 10% methanol			
	Apple	Lettuce	Potato	Tomato	Apple	Lettuce	Potato	Tomato	Apple	Lettuce	Potato	Tomato
Dichlorvos	7	nd	nd	9	68	70	72	69	62	63	68	65
Linuron	33	35	31	33	66	68	56	59	70	71	66	67
Trifluralin	55	58	51	57	260	158	161	251	66	61	65	63
Hexachlorobenzene	64	61	69	59	63	65	187	169	63	61	71	60
Dicloran	56	54	58	49	72	75	73	72	64	67	65	69
Diazinon	nd	nd	13	nd	55	159	58	57	60	58	65	59
Dimethoate	12	nd	11	nd	71	75	77	70	67	64	68	69
Chlorothalonil	51	58	52	47	66	67	70	65	60	58	59	56
Vinclozolin	32	30	27	29	51	52	58	50	58	63	67	69
Aldrin	55	56	51	58	58	146	59	55	65	69	71	70
Metolachlor	15	18	20	nd	73	69	71	74	59	64	69	68
Triadimefon	nd	11	nd	nd	49	45	42	43	69	65	66	59
Chlorpyrifos	22	11	14	18	72	69	73	68	62	65	60	66
Dicofol	25	29	30	31	154	261	63	158	59	61	62	60
Triadimenol	25	22	28	23	36	39	33	34	53	58	57	60
Endosulfan Alfa	53	59	58	64	59	58	60	61	60	61	58	63
Hexaconazole	26	29	28	25	55	58	59	56	63	68	69	66
Imazalil	10	nd	nd	11	258	158	143	131	71	75	73	72
Buprofezin	39	42	41	38	66	69	69	70	62	63	67	59
Endosulfan Beta	55	52	49	59	58	51	49	60	56	52	53	59
Etaconazole	24	nd	17	29	56	55	59	61	66	59	69	72
Propiconazole	25	26	21	18	55	56	60	62	64	63	69	72
Tebuconazole	48	52	53	47	55	58	54	53	63	64	62	61
Diclofop-methyl	33	35	36	38	53	55	49	44	60	52	53	49
Bromopropylate	41	52	47	53	69	71	68	67	61	59	58	52
Metoxychlor	53	51	50	52	52	63	61	59	55	63	64	60
Tetradifon	43	52	53	59	63	64	61	62	56	52	54	58
Prochloraz	9	nd	10	nd	45	44	68	67	59	62	73	74
Cyfluthrin[b]	63	61	59	57	77	78	69	58	79	75	73	68
Cypermethrin[b]	55	59	54	56	68	65	70	56	69	63	66	69
Quizalofop-ethyl	52	53	55	56	62	60	61	59	67	66	65	64
Fenvalerate[b]	61	56	55	58	65	66	61	64	64	71	63	65

[a]Relative standard deviations 3-7%; [b]Quantification done by the sum of peak areas of isomers forms.

of this method. Based on these results, methanol-modified CO_2 was applied in further experiments.

Pressure test

The choice of pressure and temperature in SFE directly affect selectivity, which is the main advantage over solvents used in solid-liquid extraction methods. The pressure (density) of the extracting fluid is normally of great importance in the determination of the solubility of the analytes in many matrices.[32] Control of density in SFE has enabled unique applications to separate classes of pesticides from common matrix interference that can plague traditional methods. Nemoto et al. showed the effect of CO_2 density for 88 pesticides fortified on celite.[33] The pesticides were separated into groups (non polar and polar) based on the density required to achieve recoveries above 80%.

Thus, a set of experiments was carried out to check the pressure behavior. The combination of three different pressures (19971, 44935 and 69898 kPa) using 10% of methanol as a modifier, was evaluated. These results are summarized in Table 3.

As it can be seen, pressure up to 44935 kPa resulted in increasing recovery results in relation to 19971 kPa (Table 3). The recoveries obtained were higher than 81% for all matrices studied and for 69898 kPa no significant increase in recovery was observed. It is widely believed, by analytical application chemists, that if an analyte is very soluble in supercritical fluid at a low density, this solubility will increase or remain the same at higher densities, or pressures. The combination of both effects of modifiers and pressures lead to the best results in the multiresidue pesticide analysis in fruit and vegetables, as shown in Figures 2 and 3. Accuracy was determined by consecutively analyzing five replicate samples, the standard deviations ranging from 3 to 9%. The recovery results suggest that the SFE procedure could be considered reliable enough for routine multiresidue screening of 32 pesticides in fruit and vegetables samples.

Table 3. Recoveries of pesticides in matrices studied using CO_2 modified with 10% methanol of fortified samples using direct clean-up with aminopropyl cartridges in different pressures and temperature of 70 °C

Pesticides	19971 kPa[a]				44935 kPa[a]				69898 kPa[a]			
	Apple	Lettuce	Potato	Tomato	Apple	Lettuce	Potato	Tomato	Apple	Lettuce	Potato	Tomato
Dichlorvos	62	63	68	65	89	88	87	85	91	89	86	85
Linuron	70	71	66	67	89	91	90	93	85	89	92	94
Trifluralin	66	61	65	63	91	90	90	93	90	92	89	91
Hexachlorobenzene	63	61	71	60	93	91	89	92	92	89	90	91
Dicloran	64	67	65	69	86	89	83	87	83	88	85	86
Diazinon	60	58	65	59	95	92	91	96	94	93	94	93
Dimethoate	67	64	68	69	86	82	84	84	89	84	87	82
Chlorothalonil	60	58	59	56	94	92	90	95	93	90	88	89
Vinclozolin	58	63	67	69	93	92	89	95	91	89	90	96
Aldrin	65	69	71	70	91	89	88	90	92	89	93	88
Metolachlor	59	64	69	68	96	93	91	94	91	90	91	89
Triadimefon	69	65	66	59	85	86	83	89	91	88	92	90
Chlorpyrifos	62	65	60	66	89	87	93	94	90	88	92	95
Dicofol	59	61	62	60	87	82	84	88	88	85	89	92
Triadimenol	53	58	57	60	88	90	91	89	89	87	88	88
Endosulfan Alfa	60	61	58	63	92	91	89	90	91	89	88	94
Hexaconazole	63	68	69	66	86	91	90	93	90	90	88	91
Imazalil	71	75	73	72	82	83	88	86	86	82	87	84
Buprofezin	62	63	67	59	88	86	87	85	89	85	84	88
Endosulfan Beta	56	52	53	59	90	88	85	91	91	87	86	89
Etaconazole	66	59	69	72	92	88	89	91	91	89	85	92
Propiconazole	64	63	69	72	88	85	82	86	90	86	87	88
Tebuconazole	63	64	62	61	85	82	85	93	84	81	86	89
Diclofop-methyl	60	52	53	49	85	88	91	93	83	92	89	93
Bromopropylate	61	59	58	52	93	88	93	92	90	93	89	91
Metoxychlor	55	63	64	60	87	88	86	89	85	90	87	89
Tetradifon	56	52	54	58	91	92	93	95	93	94	90	96
Prochloraz	59	62	73	74	89	91	87	84	88	89	91	87
Cyfluthrin[b]	79	75	73	68	89	87	89	91	91	86	85	90
Cypermethrin[b]	69	63	66	69	90	85	86	92	89	87	85	90
Quizalofop-ethyl	67	66	65	64	87	91	85	90	85	89	86	91
Fenvalerate[b]	64	71	63	65	86	89	90	89	88	84	87	91

[a]Relative standard deviations are 3-9%; [b]Quantification done by the sum of peak areas of isomers forms.

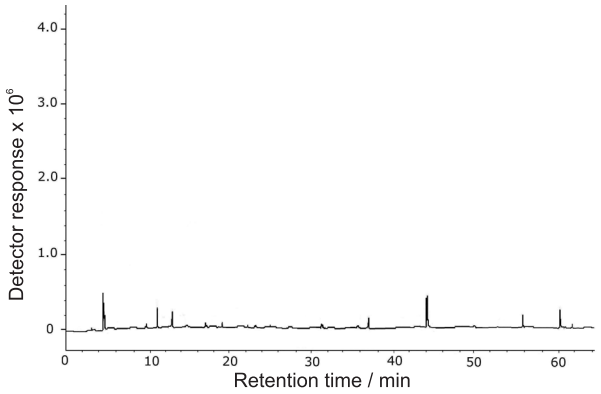

Figure 2. GC-ECD chromatogram of apple blank extract obtained by the supercritical fluid extraction (SFE) method.

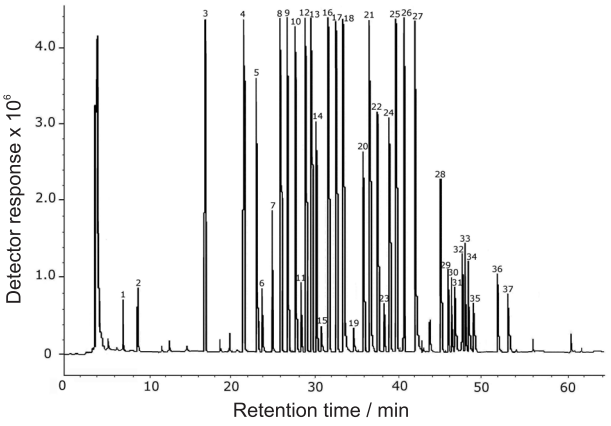

Figure 3. GC-ECD chromatogram of apple fortified extract obtained by the supercritical fluid extraction (SFE) method; pesticides identification correspond to Table 1.

Application of the developed methods

Firstly, the identification of the compounds was performed by ECD, comparing the retention times of the standards and the peaks. Peak confirmation is necessary because the chromatograms of real samples can present peaks corresponding to other contaminants or endogenous compounds which elute at the same retention times as the compounds studied. In order to confirm the pesticide identification, MS, in the SIM fashion was used, as reported in Table 4.

The screening results for multiresidue determination by both methods (solid-liquid extraction and SFE) were used to analyze fruit and vegetables from local supermarkets, which are summarized in Table 5. The results showed that 40% of the analyzed samples gave positive values (higher than routine quantification limits). Only 10% of them were above the levels established by the Anvisa.[34] Diazinon, chlorothalonil, tebuconazole and prochloraz were the pesticides most frequently found above MRL (maximum residue limit). This relatively low number of samples rejected by their consumption owes to the proper usage of pesticides in agricultural matrices, in the studied area. The application of multiresidue methodology using SFE for one real apple sample, for example, is shown in Figure 4.

Table 4. Main ions and relative abundance of pesticides detected by GC/MS

Pesticides	Main ions, *m/z* (relative abundance%)
Dichlorvos	109 (100); 185 (35); 220 (9)
Linuron	61(100); 160 (18); 248 (15)
Trifluralin	263 (74); 306 (100); 335 (10)
Hexachlorobenzene	214 (22); 249 (24); 284 (100)
Dicloran	124 (100), 176 (90), 206 (80)
Diazinon	88 (100); 179 (71); 304 (38)
Dimethoate	87 (100); 125 (55); 229 (12)
Chlorothalonil	263 (70), 293 (28), 329 (9)
Vinclozolin	187 (100); 212 (99); 285 (75)
Aldrin	263 (71); 293 (25); 329 (9)
Metolachlor	162 (100); 211 (12); 238 (52)
Triadimefon	57 (100); 208 (44); 293 (5)
Chlorpyrifos	97 (100); 197 (78); 314 (46)
Dicofol	111 (41); 139 (12); 251 (72)
Triadimenol	112 (100); 128 (45); 168 (59)
Endosulfan alfa	237 (100); 265 (63); 339 (28)
Hexaconazole	83 (100); 214 (45), 231 (20)
Imazalil	173 (96); 215 (100); 296 (10)
Buprofezin	105 (100); 172 (35); 305 (18)
Endosulfan beta	237 (100); 265 (63); 339 (28)
Etaconazole	173 (100); 191 (35); 245 (63)
Propiconazole	173 (100); 221 (58); 259 (58)
Tebuconazole	125 (84); 250 (100); 307 (10)
Diclofop-methyl	253 (100); 281 (44); 340 (80)
Bromopropylate	149 (100); 167 (25); 279 (18)
Metoxychlor	227 (100); 274 (8); 374 (3)
Tetradifon	159 (100); 229 (55); 356 (38)
Prochloraz	180 (100); 266 (26); 308 (91)
Cyfluthrin (I, II, III)	163 (100); 206 (80); 226 (51)
Cypermethrin (I, II, III)	163 (100), 181 (86); 209 (27)
Quizalofop-ethyl	243 (39); 299 (100); 372 (96)
Fenvalerate (I, II)	125 (100), 167 (84), 419 (19)

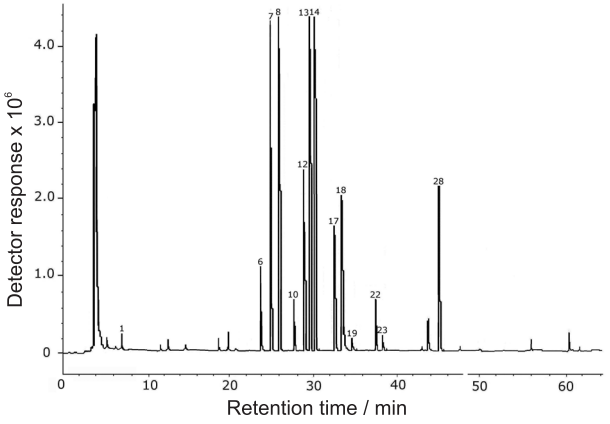

Figure 4. GC-ECD chromatogram of real apple sample obtained by the supercritical fluid extraction (SFE) method, pesticides identification correspond to Table 1.

Table 5. Pesticide residue (mg kg^{-1}) determined in real samples by the solid-liquid and SFE methods

Pesticides	Solid liquid extraction [a]				Supercritical fluid extraction[a]			
	Apple	Lettuce	Potato	Tomato	Apple	Lettuce	Potato	Tomato
Dichlorvos	nd	nd	nd	nd	0.098	nd	nd	nd
Linuron	nd	0.651	0.570	nd	nd	0.773	0.582	nd
Trifluralin	nd	0.045	nd	0.038	nd	0.061	nd	0.059
Hexachlorobenzene	nd	nd	nd	nd	nd	nd	nd	nd
Dicloran	nd	nd	0.129	nd	nd	nd	0.151	nd
Diazinon	0.508	nd	nd	nd	0.534	nd	nd	nd
Dimethoate	0.734	nd	nd	0.896	0.911	nd	nd	0.887
Chlorothalonil	nd	0.009	0.092	0.884	nd	0.012	0.098	0.925
Vinclozolin	nd	nd	nd	nd	nd	nd	nd	nd
Aldrin	nd	0.003	nd	0.003	0.002	0.003	nd	0.004
Metolachlor	nd	nd	nd	nd	nd	nd	nd	nd
Triadimefon	0.144	nd	nd	0.193	0.158	nd	nd	0.336
Chlorpyrifos	1.137	0.921	0.840	0.665	1.249	1.337	1.506	0.949
Dicofol	3.604	nd	nd	nd	3.755	nd	nd	nd
Triadimenol	nd	nd	nd	0.058	nd	nd	nd	0.066
Endosulfan alfa	nd	nd	0.002	nd	nd	nd	0.002	nd
Hexaconazole	0.096	nd	nd	0.087	0.144	nd	nd	0.082
Imazalil	nd	nd	0.084	nd	0.097	nd	0.102	nd
Buprofezin	0.092	nd	nd	0.378	0.088	nd	nd	0.090
Endosulfan beta	nd	nd	nd	nd	nd	nd	nd	0.002
Etaconazole	nd	nd	nd	nd	nd	nd	0.024	nd
Propiconazole	nd	nd	nd	0.024	nd	nd	nd	0.027
Tebuconazole	0.083	0.094	0.013	0.011	0.122	0.097	0.012	0.013
Diclofop-methyl	nd	nd	nd	0.007	nd	nd	nd	0.008
Bromopropylate	nd	nd	nd	0.005	0.002	nd	nd	0.005
Metoxychlor	nd	nd	0.005	nd	nd	nd	0.004	nd
Tetradifon	nd	0.432	nd	0.533	nd	0.489	nd	0.579
Prochloraz	0.355	0.128	nd	0.472	0.373	0.129	nd	0.488
Cyfluthrin[b]	nd	nd	nd	nd	nd	nd	nd	nd
Cypermethrin[b]	nd	0.038	0.022	nd	nd	0.039	0.046	0.091
Quizalofop-ethyl	nd	0.025	0.031	0.029	nd	0.024	0.030	0.033
Fenvalerate[b]	nd	nd	nd	nd	nd	nd	nd	nd

[a]Relative standard deviations are 3-7%; [b]Quantification done by the sum of peak areas of isomers forms.

Fungicide residues were those most frequently found and this fact may be explained by their large application for post-harvest protection. An organohalogen, two organonitrogens and an organophosphorus: chlorothalonil, prochloraz, tebuconazole and chlorpyrifos, respectively, were most abundant and, in many instances, the latter exceeded the legislative limits. In some cases, residues such as aldrin were found in illegally sprayed crops of tomato and lettuce.

As one can see, the main differences within the methods were found for imazalil, tebuconazole, triadimefon, chlorpyrifos and cypermethrin, which at times were not determined in some matrices by the solid-liquid method, but determined through SFE. In addition, in some cases, the residues found by SFE were higher than those obtained by the solid-liquid method. Some works have demonstrated that classical or conventional methods of extraction for certain pesticides and or metabolites often do not always remove all of the residues from soil, plants, and food products.[35, 36] Therefore, it is likely that in routine pesticide residue monitoring programs, the total pesticide residues in various matrices have been underestimated. Hence, SFE may prove useful in the extraction of these non-extractable residues, often referred to as "bound residues". SFE presents the greatest ability to change solvent conditions by controlling simple parameters (polarity, temperature and pressure) and may be most potential for selectively extracting mobile or bound residue from fruit and vegetable.

Furthermore, SFE simplifies the sample preparation step for the analysis of pesticide multiresidues, providing advantages such as a reduced use of organic solvents, shorter extraction time, smaller sample size and providing a higher extraction power, that is, cutting costs.

Conclusions

SFE showed to be a successful analytical technique in the multiresidue pesticide analysis, in the extraction of organohalogen, organonitrogen, organophosophorus

pyrethroid in fruit and vegetables. This method presented advantages over the conventional solvent extraction methods, including reduction in organic solvent consumption, faster analysis time, being potentially more efficient and selective in complex matrices extractions.

SFE is an alternative to solvent-intensive isolation procedures, due to its ability to change solvent conditions and by controlling polarity, temperature and pressure it may present the most potential for extracting mobile or bound residues

With the development of multiresidue methods, 32 pesticides were analyzed in fruit and vegetables. The SFE methodology showed to be efficient and sensitive for all matrices studied, and it can be easily modified to accommodate more compounds. The utilization of mass spectrometric detection provided both information and confirmation of pesticide residues in crops. This proposed method could be incorporated to the routine analysis of fruit and vegetables obtained from local markets in Brazil or any other country.

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