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Comparison of various liquid chromatographic methods for the analysis of avermectin residues in citrus fruits

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Abstract

Various liquid chromatographic (LC) techniques for analyzing avermectin (Abamectin) were compared after extraction of residues from citrus fruit samples by matrix solid-phase dispersion (MSPD). LC with UV and fluorescence detection were used as also was LC coupled to the mass spectrometer by an electrospray interface. The results obtained by the three methods were compared in terms of sensitivity and selectivity. The combination of MSPD extraction and LC with fluorescence detection have made it possible to quantify 0.5 $\mu g\,kg^{-1}$ of Abamectin in 0.5 g of orange sample, with an overall average recovery of 94%. The procedure provides a simple and sensitive method for monitoring Abamectin residues in citrus fruit at the levels required by legislation.

Keywords: Fruits; Food analysis; Avermectin; Pesticides

1. Introduction

Avermectin B_1 is a natural macrocyclic lactone from the family of avermectins, the compounds produced by a soil actinomycete, *Streptomyces avermitilis*. It is a mixture of two homologs containing more than 80% avermectin B_{1a} and less than 20% avermectin B_{1b}. These homologs differ by a single methylene group, B_{1a} contains a secondary butyl substituent at the C-25 position, whereas B_{1b} has an isopropyl substituent at that carbon [1].

Avermectin B_1 is a neurotoxin, frequently used in agriculture for its miticide, insecticide, and acaricide activities. It has a broad spectrum of activity at low dosage levels [2,3]. It is not economical to separate the two homologs on a large scale. The mixture is commercialized as Abamectin. In the Valencian Community, Abamectin is widely used for *Phyllocnistic citrella* control in citrus crops. This has been one of the most serious pests in recent years.

Avermectin residues rapidly degrade. The only residues of toxicological significance are avermectin B_1 and its Δ-8,9-isomer [4–6]. The maximum residue limits (MRLs) are established by the regulatory authorities as the sum of avermectin and Δ-8,9-avermectin residues. The Codex Committee on Pesticide Residues under the Joint FAO/WHO Food Standards Program has established avermectin MRLs at 0.01–0.02 $mg\,kg^{-1}$ for fruits and tomatoes [7].

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The MRL set by the Spanish Government for citrus samples is 0.01 mg kg^{-1} [8].

Analysis of avermectin B_1 at residue levels is a complicated process. A number of liquid chromatographic (LC) methods have been reported for agricultural products and biological and forest matrices. These methods require extensive clean-up of the extracts and purification prior to analysis. Residue isolation and clean-up techniques must be such that they give a high percentage recovery of the target analyte while simultaneously minimising interferences. This may contribute to a high background in the analysis. For this reason, the analytical methods generally include complicated schemes for Abamectin extraction, based on liquid–liquid extraction (LLE) and clean-up on solid-phase extraction (SPE) columns of Florisil [2], aminopropyl [4–6,9,10], silica [11] or C_{18} [12–14].

Additional clean-up steps were carried out with cation-exchange cartridges to determine residues in fruits and vegetables [15], and an antibody-mediated clean-up procedure was also described for extracting avermectin from cattle plasma, cattle meat and pear samples [16]. Others methods of sample preparation by adsorption chromatography on alumina and SPE with Sep-Pak C_{18} [17] or supercritical fluid extraction [18] have been employed to extract these macrocyclic lactones from different matrices, like feed, soil and animal tissue.

Matrix solid-phase dispersion (MSPD) has been proposed for isolating and quantifying ivermectin (22,23-dihydroavermectin) from bovine milk with C_{18} columns and elution with ethyl acetate [19]. Solvent consumption can be reduced considerably by miniaturizing the scale of sample extraction. Also, a MSPD multiresidue method has been proposed for isolating pesticides from citrus samples. This allows screening many samples and applying the method to routine analysis [20,21].

Several LC methods have been reported for the determination of Abamectin, based on UV, fluorescence (FL) and mass spectrometry (MS) detection. The most frequently used system until now has been LC–FL. It is known that LC methods based on UV detection lack the required sensitivity for the detection of avermectin-related compounds [11]. However, the use of immunoaffinity chromatography for clean-up and concentration, and analysis by LC–UV has been proposed as adequate, due to the high selectivity of the inmunosorbent [16]. Moreover, a LC–UV method has been proposed for determining ivermectin, a compound synthesized from avermectin having antihelmintic and insecticidal activities, in medicated feed at concentrations ranging from 1.0 to 3.0 mg kg^{-1} [17].

The current methods for monitoring Abamectin residues are based on LC–FL. The Abamectin molecule is very amenable to derivatization to a fluorescent product by dehydration with trifluoroacetic anhydride (TFAA) in the presence of catalytic amounts of *N*-methylimidazole (NMIM) in acetonitrile as the solvent. This LC method with fluorescence detection is rapid and offers both selectivity and sensitivity with detection limits at ppb levels for fruits, vegetables and wine [4–6,9,11,13,15].

LC–MS has recently been accepted for use in trace analysis of pesticide residues in the regulatory area [22]. The sensitivity and specificity of MS detection have been long recognized, and some LC–MS techniques, such as atmospheric-pressure chemical ionization (APCI) with negative-ion detection [23] and LC–ESI (electrospray ionisation)-MS with positive-ion detection [24] were used to determine Abamectin residues in food matrices.

Although the use of Abamectin is continually increasing, owing to its attractive properties, there are only a few analytical methods for measuring it in fruits and vegetables. The main purpose of this study was to develop a method for the routine determination of Abamectin in citrus fruit samples. For this purpose, a multiresidue method, based on MSPD, was applied and different determination techniques (LC–UV, LC–FL and LC–ESI-MS) were compared in order to establish the most suitable technique for quantifying this pesticide in orange samples.

2. Experimental

2.1. Chemicals and reagents

Acetone, acetonitrile, methanol and dichloromethane, all LC grade (LiChrosolv), were supplied by Merck (Darmstadt, Germany). Ultra-pure water was prepared by ultrafiltration of distilled water with a Milli-Q system (Millipore, Bedford, MA, USA).

The standard Abamectin (92.5%) was supplied by Promochem (Wesel, Germany). A stock solution (978 μg ml^{-1}) and working solutions of Abamectin were prepared in acetonitrile or methanol and stored at 4°C. The solid phase used in MSPD was C_{18} bonded silica (40–60 μm) from Análisis Vínicos (Tomelloso, Spain).

Derivatization reagents: TFAA, NMIM and triethylamine were supplied by Sigma (Steinheim, Germany). Silylation reagent: acetone (LiChrosolv) was obtained from Merck and Sylon-CT was supplied by Supelco (Bellefonte, PA, USA).

2.2. MSPD extraction procedure

Citrus samples (200 g of whole fruit) were prepared, using a food processor, and mixed thoroughly. An aliquot (0.5 g) of the samples was placed into a mortar (50 ml capacity) and 0.5 g of the C_{18} sorbent was added and gently blended for 5 min using a pestle, to obtain a homogeneous mixture. This mixture was introduced into a 100×9 mm I.D. glass column with a coarse frit (No. 2) and covered with a plug of silanized glass wool at the top. Abamectin residues were eluted with 15 ml of dichloromethane. The eluate was evaporated to dryness with an air-flow at 50°C.

The residue was dissolved in 0.5 ml of acetonitrile for LC–UV and LC–FL analysis or in 0.5 ml of methanol in the case of LC–ESI-MS analysis, and the solution was thoroughly mixed in an ultrasonic bath for 5 min. Finally, the extract was filtered through an Acrodisk (0.2-μm of nylon).

Recovery studies were carried out by spiking 0.5 g of fresh orange samples with the Abamectin fortification solution at different levels, ranging from 0.01 to 10 mg kg^{-1} for LC with UV or MS detection and from 0.001 to 0.01 mg kg^{-1} for LC–FL.

2.3. Derivatization of samples and standards

This step is only carried out when Abamectin is analyzed by LC with fluorescence detection. The residue obtained by MSPD was removed in silylated tubes, evaporated to dryness under air-flow at 50°C, and reconstituted in 160 μl of acetonitrile. Care must be taken to ensure that no moisture is left in the tube. The standard solutions are also prepared in acetonitrile. The sample, standards, and a freshly prepared derivatization reagent tubes are placed in an ice bath for 3 min.

A 100-μl volume of acetonitrile–triethylamine (95:5) solution, 10 μl NMIM and 10 μl TFAA were added to 160 μl of each sample and working standard. The tubes were gently vortexed. Afterwards each sample and standard was diluted to a final volume of 0.5 ml with mobile phase. These solutions were kept in the dark at 4°C until used for LC–FL analysis. The derivative formed from a standard of B_{1a} Δ8,9-isomer has been shown to be structurally identical to that formed from avermectin B_{1a} by TFAA reaction [9,10].

2.4. LC instrument

2.4.1. LC–UV

A Shimadzu (Kyoto, Japan) SCL-6A LC system equipped with two LC 6A pumps, a Rheodyne Model 7125 injector (20-μl loop), a Merck Hitachi 4250 UV vis detector, and a Shimadzu C-R4A Chromatopac data processor were used. The UV–Vis detector was operated at 245 nm. Quantitation was performed by comparing sample peak areas with those obtained for standard solutions.

2.4.2. LC–FL

A Waters LC system model (Milford, USA), equipped with a 600 E Multisolvent delivery system, a 47/4 scanning fluorescence detector, a Rheodyne model 7125 injector (20-μl loop), in-line degasser, a fraction collector II, and MILLENNIUM software were used.

The Abamectin concentrations in the final extract were calculated by comparing the peak areas with those obtained for standard solutions at λ_{ex} of 365 nm and λ_{em} of 480 nm.

2.4.3. LC–ESI-MS

A Hewlett-Packard (Palo Alto, CA, USA) HP-1100 Series LC–MS system, equipped with a binary solvent pump, an autosampler, a photodiode-array detection (DAD) system and an MS system, coupled with an analytical work station, were used. The MS system consisted of a standard atmospheric-pressure ionisation (API) source configured as ESI. Separations were carried out at room temperature, and

5-μl were injected (as standard volume) into the LC–MS system.

The ESI-MS interface in positive mode was operated under the conditions of 350°C gas temperature, 13.0 l min^{-1} drying-gas flow, 30 p.s.i. (1 p.s.i. = 6894.76 Pa) nebulizer-gas pressure and 4000 V of capillary voltage. Full-scan LC–MS chromatograms were obtained by scanning from m/z 50 to 800. Selected-ion monitoring (SIM) of the most abundant ion was used for quantitation.

2.4.4. LC conditions

The characteristics of the columns and the LC conditions are given in Table 1. These conditions were suitable for routine analysis.

2.5. Results and discussion

The quantitation procedure and conditions reported in this paper for LC–UV and LC–ESI-MS were selected on the basis of earlier publications [21,24]. However, for LC–FL a method was developed and validated for the determination of Abamectin in oranges. In the required derivatization step TFAA and NMIM in acetonitrile are used as reagents. This procedure is instantaneous at room temperature and no further sample clean-up is required following derivatization. The method has been used to determine Abamectin and its Δ8,9-isomer in wine [4], hops [5] and apples [6]. When the derivatization products are kept in the dark at 4°C until analysis by LC, the Abamectin derivative is stable for 24 h. Moreover, the presence of triethylamine in the reaction mixture stabilises the derivative [6].

Once the factors that affect the selectivity of LC for Abamectin had been optimized, three detection systems (UV, FL and MS) were compared. The limits of detection (LODs) for the standard Abamectin solution, computed on the basis of three times the noise level, were 50 pg with LC–UV, 0.5 pg with LC–FL and 12 pg for LC–ESI-MS. The LOD for LC–FL was lower than that obtained with LC–MS, partially because of the difference in the injection volume (LC conditions in Table 1).

All of the detectors studied provided a linear response for a wide range of amounts injected and good correlation coefficients. Calibration graphs were plotted (six points) for standard solutions between 0.01 and 10 μg ml^{-1} for LC–UV and LC–ESI-MS, and between 0.001 and 0.050 μg ml^{-1} for LC–FL. For each calibration point three injections into the liquid chromatograph were performed. To assess the reproducibility, five replicate determinations on the same day of a standard solution were carried out under optimal conditions (precision run-to-run). Moreover, five injections of this solution on three different days allowed the day-to-day precision to be established. All of the LC detection systems showed suitable reproducibility, with relative standard deviation (RSD) values lower than 1.7% for run-to-run and 4.3% for day-to-day precision (Table 2).

In order to demonstrate the suitability of these analytical techniques for determining Abamectin residues in orange samples, recovery experiments were carried out. The proposed extraction method is based on MSPD isolation technology. The most suitable extraction conditions were selected to achieve the highest recovery for Abamectin while eliminating most of the interfering matrix components. Good results were obtained employing C_{18} as

Table 1
LC conditions

	LC–UV	LC–FL	LC–ESI-MS
Column	Kromasil C_{18} (150×4.6 mm I.D., 5 μm)	Nova-Pak^{R} C_{18} (150×3.9 mm I.D., 5 μm)	Kromasil C_{18} (150×4.6 mm I.D., 5 μm)
Precolumn	Kromasil C_{8} (30×4.6 mm I.D., 5 μm)	Kromasil C_{8} (30×4.6 mm I.D., 5 μm)	Kromasil C_{8} (30×4.6 mm I.D., 5 μm)
Mobile phase	Methanol–water (90:10)	Acetonitrile–water (94:6)	Methanol–water (90:10)
Flow-rate (ml min^{-1})	0.5	1	0.5
Injection volume (μl)	20	20	5

Table 2
Linearity and reproducibility of the LC methods ($n=5$)

	LC–UV[a]	LC–FL[b]	LC–ESI-MS[a]
Regression coefficient (r)	0.9985	0.9998	0.9973
RSD (%) (run-to-run)	1.7	0.9	0.5
RSD (%) (day-to-day)	2.8	3.4	4.3

[a] Standard solution, 1 μg ml^{-1}.
[b] Standard solution, 0.01 μg ml^{-1}.

the sorbent and dichloromethane as the eluent, without the need for an additional purification step. The described microextraction procedure is very simple, rapid and requires only small sample sizes and solvent volumes. It constitutes a general method for extraction of pesticides from fruit and vegetables and allows screening of many samples in a short period of time. Chamkasen et al. [10] developed a rapid and sensitive method for determining Abamectin in oranges by combining extraction of 50 g of orange sample with 100 ml of acetonitrile, partition against hexane, and clean-up with an aminopropyl solid-phase system that compared with the proposed method employs larger quantities of sample and organic solvents.

The extracts obtained were injected directly into the LC apparatus with UV or MS detectors, and after a derivatization step in the case of fluorescence detection. This additional step does not constitute a disadvantage in this determination technique, because the reaction is instantaneous and the derivatization process requires no more than 10 min.

The accuracy and reproducibility for Abamectin in oranges, spiked at different levels were evaluated with the three detectors. The accuracy calculated as a percentage of recovery when using the fluorescence detector ranged from 91 to 97%, with RSD values lower than 5%. It was verified in triplicate at four spiked levels, ranging from 0.001 to 0.01 mg kg^{-1}. Using the MS detector, the recovery percentages ranged from 94 to 99%, with RSD values also lower than 5%. However, when an UV detector was employed, the mean recovery values at concentration levels, between 0.03 to 0.1 mg kg^{-1} were found to range from 70 to 81%. At higher spiked levels between 1 and 10 mg kg^{-1}, the recovery values were above 93%. The RSD values were $\leq$7% with this detector. Detection and identification of the analyte were not possible at levels of 0.01 mg kg^{-1}, the MRL set by the Spanish Government for citrus fruits.

Fig. 1A illustrates the chromatogram of a spiked orange (0.03 mg kg^{-1}), extracted by the proposed MSPD method and analysed by LC–UV, while Fig. 1B and C shows the chromatograms of extracts obtained from 0.01 mg kg^{-1} spiked samples (LMR) but analysed using LC–FL and LC–ESI-MS. Differences in sensitivity between the three detectors can clearly be observed in this figure. UV detection is not a good method for low-level determinations, because at 245 nm (wavelength of maximum adsorption of Abamectin) interference from residual matrix components is problematic. In the last two instances, there were no interfering peaks in the Abamectin elution area. Fig. 1A shows that with UV detection the number and height of interfering peaks are much larger than with the other detection systems. Fig. 1B shows that substantially fewer reagent by-products were formed by the derivatization, and this eliminates the need for further clean-up of the derivatized standards. A chromatogram free of interfering peaks is also generated with LC–ESI-MS (Fig. 1C).

The limits of quantitation (LOQs), evaluated as a signal-to-noise (S/N) ratio of 10 in the selected chromatographic conditions, were compared. The LOQ was found to be 0.03 mg kg^{-1} when abamectin was analysed by LC–UV, 0.0005 mg kg^{-1} by LC–FL and 0.0025 mg kg^{-1} by LC–ESI-MS. For quantitation oranges, FL and MS detection offered greater sensitivity and, more importantly, were much more selective than UV detection. Moreover, if lower concentrations need to be determined with LC–ESI-MS, this may be done by injecting a larger volume (up 5 μl).

Of the three techniques studied, LC–FL was preferred for measurement of Abamectin in citrus

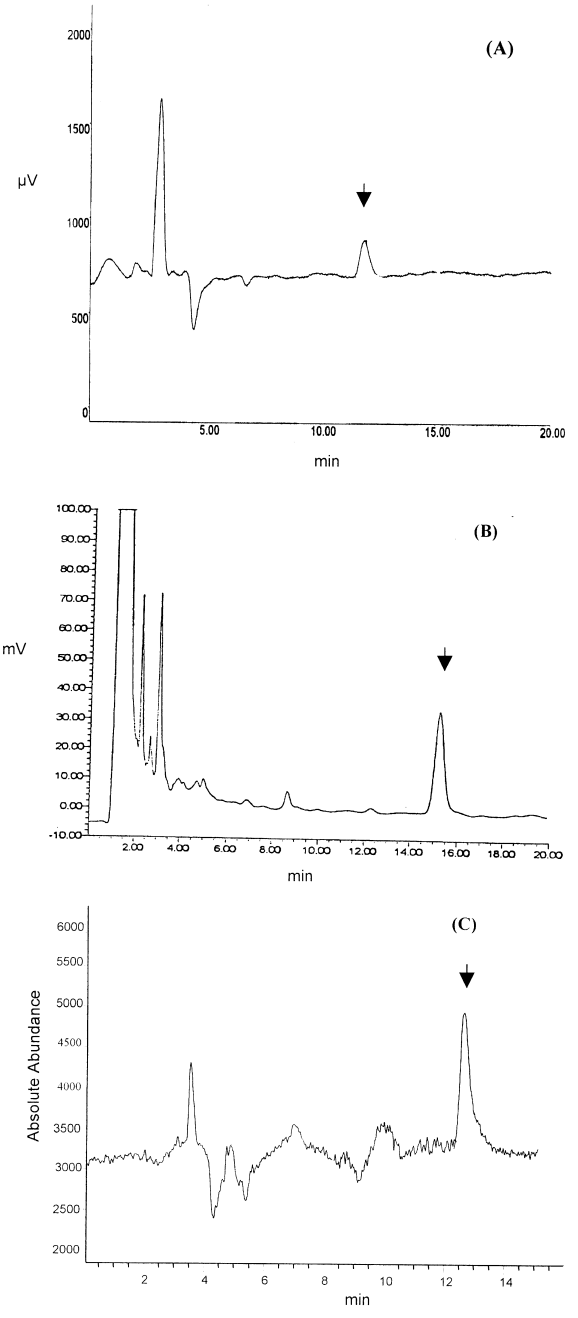

Fig. 1. Chromatogram of spiked orange samples. (A) LC–UV with 0.03 mg kg^{-1} of Abamectin, (B) LC–FL 0.01 mg kg^{-1} of Abamectin, (C) LC–ESI-MS with 0.01 mg kg^{-1} of Abamectin.

fruit samples. The results obtained by using LC–FL are comparable to those obtained with LC–ESI-MS, but the former has the advantage of being economical for use in routine analysis, which is not a characteristic of MS methods

The use of LC–FL combines a high degree of specificity due to the combination of chromogenic and chromatographic selectivity and good sensitivity, arising from the strong fluorescence of the derivative. Moreover, it can be used to quantify Abamectin and its geometric Δ8,9-isomer at the same time [4–6,9,13].

The proposed MSPD extraction method offers some advantages over traditional methods (simplicity, less solvent consumption, fewer interferences, improved precision) and associated with LC–FL, it is an appropriate methodology for routine Abamectin analysis in citrus fruits at concentrations below MRLs.

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