Journal of Food and Drug Analysis, Vol. 17, No. 3, 2009, Pages 198-208 藥物食品分析 第十七卷 第三期

Simultaneous Determination of Macrolide Pesticides in Fruits and Vegetables by Liquid Chromatography

CESHING SHEU[1]* AND HUI-CHI CHEN[1,2]

[1.] *Department of Applied Chemistry, Chaoyang University of Technology, Taichung, Taiwan (R.O.C.)*

[2.] *Residue Control Department, Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Taichung, Taiwan (R.O.C.)*

(Received: November 11, 2008; Accepted: May 29, 2009)

ABSTRACT

A multiresidue method was developed and validated for the determination of four macrolide pesticides (spinosad, emamectin benzoate, abamectin and milbemectin) and their metabolites in six kinds of vegetables and fruits (Chinese kale, cabbage, eggplant, orange, papaya and strawberry). The pesticide residues were extracted from the samples with acetonitrile/10 mM aqueous ammonium acetate (NH_4OAc), pH 4.0 (90:10, v/v) and the purification was carried out with an aminopropyl (NH_2) solid-phase extraction cartridge. Reversed-phase high-performance liquid chromatograpy (RP-HPLC) using an ODS-AM column 5 μm, where a gradient elution with methanol/acetonitrile/10 mM aqueous NH_4OAc, pH 4.0 mobile phase was employed for the separation and quantification of the pesticides. The method accuracy and precision were determined via recovery experiments and four replicates spiked with three fortification levels (0.15, 1.5, 7.5 μg/g) each. From the overall results of analytes, method LOD values were ranged from 0.01 to 0.03 μg/g, the mean recoveries were in the range of 75-117% and the relative standard deviations (RSD) were lower than 14%. The recovery data from six sample matrices met the acceptability criteria (European Union, SANCO/2007/3131) of recovery (70-120%) and RSD (<20%) at the three levels studied. The simple and efficient method with acceptable performance was thus established for the simultaneous determination of four macrolide pesticides and their metabolites in agricultural products.

Key words: macrolide pesticide, abamectin, emamectin benzoate, milbemectin, spinosad, solid-phase extraction, LC

INTRODUCTION

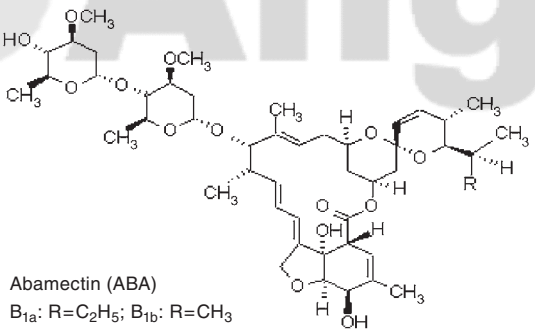
In recent years, some natural products with antiparasitic effects and their synthetic analogues are widely used as pesticides. Abamectin (ABA), emamectin benzoate (EMA), milbemectin (MIL) and spinosad, insect control agents, derived from a naturally occurring actinomycetes bacterium, have become increasingly important due to their broad spectrum of biological activity, low toxicity to non-target organisms and their persistency in the environment. These compounds are a family of macrocyclic lactone compounds, also known as "macrolide" pesticides, and are used as insecticides and acaricides which exhibit contact and stomach actions[(1-7)].

ABA, is a mixture of two homologues containing > 80% of avermectin B_{1a} and < 20% of avermectin B_{1b}. EMA, a semi-synthesis of ABA by replacement of the 4'-hydroxyl constituent with an epi-methylamino group, is a mixture of two homologues containing > 90% of avermectin B_{1a} and < 10% of avermectin B_{1b}[(8)]. MIL is a mixture of two homologues containing 30% of milbemectin A_3 and 70% of milbemectin A_4[(9)]. Spinosad comprises a mixture of spinosyns A (SPA) and spinosyns D (SPD)[(10,11)]. In this study, the metabolites of spinosad including spinosyn K (SPK), spinosyn B (SPB) and *N*-demethylspinosyn D (NDSD) were also investigated (Figure 1). Each of the macrocyclic lactones consists of 2 main components will be assigned as major and minor according to their concentration proportions. Since it is not economical to separate the homologues on a large scale, only the mixtures are commercialized for agricultural use. Except the spinosad, of which spinosyn A, D, K, B and *N*-demethylspinosyn D, were individually separated in this study.

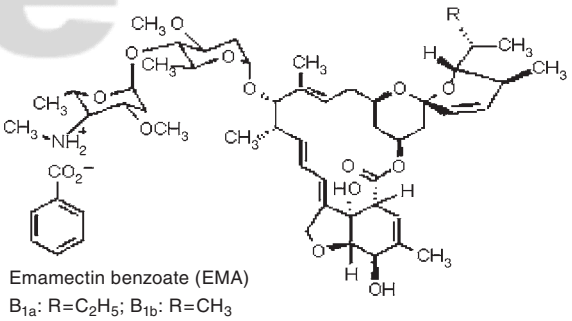
Multi-residue method development for the macrolide pesticides at residual levels is a complicated process, due to the fact that compounds of different polarities, solubilities, and *pKa* values have to be simultaneously extracted and analyzed. Although a number of LC methods have been reported for agricultural products and biological and forest matrices[(12-22)], few multi-residue LC/UV determination methods have been reported for the simultaneous

* Author for correspondence. Tel: +886-4-23323000 ext. 4448; Fax: +886-4-23742341; E-mail: cssheu@cyut.edu.tw

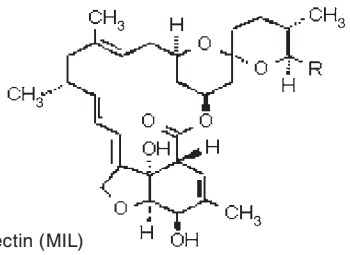
Journal of Food and Drug Analysis, Vol. 17, No. 3, 2009



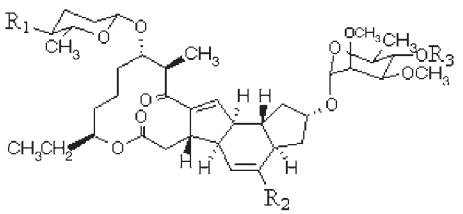
Abamectin (ABA)
B_{1a}: R=C_2H_5; B_{1b}: R=CH_3



Emamectin benzoate (EMA)
B_{1a}: R=C_2H_5; B_{1b}: R=CH_3



Milbemectin (MIL)
A_3: R=CH_3; A_4: R=C_2H_5



Spinosad and its metabolites

	R1	R2	R3
Spinosyn A(SPA)	$N(CH_3)_2$	H	CH_3
Spinosyn D(SPD)	$N(CH_3)_2$	CH_3	CH_3
Spinosyn K(SPK)	$N(CH_3)_2$	H	H
Spinosyn B(SPB)	$NHCH_3$	H	CH_3
N-Demetylspinosyn D(NDSD)	$NHCH_3$	CH_3	CH_3

Figure 1. Chemical structures of the selected macrolide pesticides

analysis of the listed four macrolide pesticides and their metabolites. These previously reported methods demand extensive clean-up of the extracts prior to analysis. Residue extraction and clean-up techniques are required in order to give a high recovery of the target analytes while minimizing mutual interferences. Extensive clean-up of extracts may result in the partial loss of some compounds, while the inadequate clean-up can lead to adverse effects related to the quality of the generated data.

An appropriate method should be capable of separating as many as possible the target analytes from the other substances that might interfere with the analysis. Therefore, in order to exclude these interfering substances, a variety of clean-up techniques may be employed. A clean-up method for lipophilic compounds, such as macrolide antibiotics, that has been used for many years is the liquid-liquid extraction (LLE) method[23,24]. These macrolides are generally extracted from the biological matrix into organic solvents (typically employing dichloromethane or ethyl acetate) at a pH where the ionization of their amino-sugar basic function is suppressed. In addition, solid-phase extraction (SPE) is one of the most often used techniques for sample preparation prior to analysis by the chromatographic procedures. SPE technique offers not only the simple clean-up of the initial extract but also the reduction of solvent consumption. SPE is being increasingly used for environmental, food, pharmaceutical and biological applications, and has many advantages over traditional LLE. SPE method with higher selectivity than LLE method has a priority to being quantitatively analyzed for trace amounts of macrolide antibiotics. A number of SPE procedures have been reported for agricultural products and biological matrices such as cartridges of NH_2[12,13,22,25,26] and C_{18}[14].

A disadvantage of reversed-phase LC analysis of basic substances on silica column is peak tailing due to interaction with residual silanols on the silica backbone[27]. It occurred to the macrolide pesticides selected in this study, EMA, SPA, SPD, SPK, SPB and NDSD, which are basic molecules containing amino-sugar in their structures and are strongly affected by silanol groups remaining in the column packing material. Therefore the LC column of ODS-AM, an end-capped C_{18} column was thus used.

In this paper, the development of a rapid multi-residue method for the quantitative determination of macrolide pesticides and their metabolites in agricultural products was performed. The work was done using acetonitrile-

aqueous NH_4OAc mixture as extraction solution as well as SPE/clean-up procedure. The RP-HPLC with UV detection was chosen for the final analysis and quantification.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

Acetonitrile, methanol, *n*-hexance, dichloromethane, anhydrous sodium sulfate, ammonium acetate, glacial acetic acid (glacial HOAc) and triethylamine (TEA) were of HPLC or analytical reagent grade, and purchased from Mallinckrodt (MO, USA) or Merck (Darmstadt, Germany). Abamectin (ABA, 85.0%) and emamectin benzoate (EMA, 96.6%) were kind gifts from SYNGENTA (Basel, Switzerland); Milbemectin (MIL, 96.6%) was purchased from SUPELCO (St. Louis, MO, USA); Spinosyn A (SPA, 90.9%), spinosyn D (SPD, 94.0%), spinosyn K (SPK, 98.0%), spinosyn B (SPB, 93.5%) and *N*-demethylspinosyn D (NDSD, 97.4%) were kind gifts from Dow AgroSciences (IND, USA).

Around 0.77 g of ammonium acetate was dissolved in 1000 mL water, mixed well and the pH was adjusted to 4.0 with glacial HOAc to make 10 mM aqueous NH_4OAc buffer, pH 4.0. Extraction solution was prepared by mixing acetonitrile with 10 mM aqueous NH_4OAc buffer, pH 4.0 (90:10, v/v).

II. *Samples*

In order to develop a multi-residue method applicable to a variety of vegetable and fruit matrices, six types of food commodities, including Chinese kale, orange, papaya, strawberry, cabbage and eggplant were selected. The samples were purchased and directly cut into small pieces without any pre-treatment, such as washing or peeling.

III. *Standard Solutions*

Individual pesticide stock solutions were prepared at concentrations of about 1000 μg/mL in acetonitrile. From these stock solutions, a mixed standard solution of all pesticides was prepared, of which subsequent dilutions to obtain working standard solutions were made. The mixed standard solutions at appropriate concentrations were used to calibrate the HPLC system or spike samples in fortification experiments.

IV. *Chromatographic Conditions*

Determinations were performed on an Agilent 1100 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany). The HPLC system consisted of a QuatPump, a thermostablilizer, an automatic sampler with a 100 μL loop and a variable wavelength UV/Vis detector. Data acquisition and treatment was performed using HP ChemStation software. An ODS-AM 5 μm column (150 × 4.6 mm i.d.) from YMC-Pack (Separation Technology, Kyoto, Japan) and a similar pre-column (25 × 4.6 mm i.d.) were used for the separation. The mobile phase was composed of methanol (channel A), acetonitrile (channel B) and 10 mM aqueous NH_4OAc, pH 4.0 (channel C) under a gradient elution which was run at initial condition of 60% A-10% B-30% C, then linearly adjusted to a composition of 15% A-60% B-25% C within 25 min at a flow rate of 1.0 mL/min, followed by a flow rate of 1.5 mL/min at the 25.0l min. All measurements were carried out at room temperature with absorbance detection at 250 nm, the injection volume was 20 μL and the whole running time was around 40 min.

V. *Sample Preparation and Clean-up*

A 20 g aliquot of the sub-sample was homogenized with 60 mL of extraction solution for 1 min using a polytron (Kinematica AG, PT 3100, ON, Canada). 30 mL of dichloromethane and 5 g of anhydrous sodium sulfate were then added into the sample mixture solution and mixed well. The extract was filtered through a Buchner funnel (fitted with filter paper) by a gentle vacuum, and adjusted to a volume of 150 mL with acetonitrile. A 7.5 mL of the extract was transferred into a 15 mL centrifuge tube and evaporated to nearly dryness in a water bath at ca 40°C under gentle stream of nitrogen with temperature-controlled nitrogen gas evaporator (N-Evap 112 Analytical Evaporator from Organomation Associates Inc., MA, USA). The residue was reconstituted in 5 mL *n*-hexane, and sonicated for 1 min to prevent the reversible adsorption of analytes from the glass wall.

Sample purification was performed with solid-phase extraction (SPE) (Vacuum manifolds from J&W Scientific, CA, USA). The aminopropyl SPE cartridge (500 mg/6 mL of NH_2 cartridge, AccBond, from Agilent Technologies, CA, USA), was preconditioned with 5 mL *n*-hexane. Five mL of residue solution was transferred into the cartridge without a vacuum and the eluent was discarded. The pesticides was eluted from the SPE cartridge with 10 mL acetonitrile/TEA (99:1, v/v). The eluent was evaporated to dryness under a stream of nitrogen with a water bath at ca 40°C, and re-dissolved in 1.0 mL acetonitrile and sonicated for 1 min. A 20 μL of the solution was injected into HPLC system.

VI. *Linearity Study, LOD and LOQ Determination*

The linearity of calibration curves was evaluated based on injections of the standard mixture solutions, prepared in blank sample extract in acetonitrile, at concentrations of 0.05, 0.25, 1.0, 2.5 and 10.0 μg/mL. Thus the corresponding range of pesticide concentration in sample extract is from 0.05 to 10.0 μg/mL. The calculations were done by using the peak height, calibration curve equations, and the determination of coefficients (R^2) for each analyt-

Journal of Food and Drug Analysis, Vol. 17, No. 3, 2009

es in the six different samples. The calibration curves were constructed without including the origin point.

From the relative standard deviations of repeatability at each lowest concentration level of the 11 target analytes, the instrument LOD and LOQ (LOD_i and LOQ_i) were determined. The LOD_i was calculated as three times of the relative standard deviation from the six replicate injections at the lowest or detectable concentration level, using the formula: LOD_i (μg/mL) = 3 × RSD% × concentration. The LOQ_i was defined as 10× RSD% × concentration, that is, $LOQ_i = 3.3 \times LOD_i$[28]. The estimated method LOD (LOD_m) was calculated as three times of the relative standard deviation from the six replicate injections at the lowest or detectable concentration, in blank sample extract in acetonitrile, using the formula: estimated LOD_m (μg/g) = $LOD_i \times V_{final} \div V_{analyzed} \times V_{total} \div W$, where V_{total} = total volume of 150 mL, $V_{analyzed}$ = analyzed volume of 7.5 mL, V_{final} = final volume of 1.0 mL, and W = sample weight of 20 g.

VII. *Fortification Recovery Experiments*

Homogenized blank samples were spiked with 1.5 mL of a standard solution (three concentrations of 2, 20 and 100 μg/mL) directly to the matrix. The method accuracy and precision were assessed using the residue free samples spiked with all target analytes. Recoveries were determined for four replicates, at the three different spiking levels (0.15, 1.5 and 7.5 μg/g in the samples). The recovery values and the relative standard deviations were calculated for each level.

RESULTS AND DISCUSSION

I. *Optimization of Chromatographic Conditions*

The choice of appropriate conditions for the LC procedure is affected by the ionizable groups, amino sugar of the macrolide pesticides. The chromatography on silica-based C_{18} column was effective by using a mixture solution of acetonitrile-methanol-10 mM aqueous NH_4OAc, pH 4.0 solution as mobile phase. In order to optimize conditions various mobile phases were tested in a series of preliminaries, such as the pH level, the ionic strength of aqueous NH_4OAc solution and the composition of organic modifiers (acetonitrile-methanol). However there were quite different affinities among the analytes in the reversed-phase column. It is difficult to determine all the 11 analytes by an isocratic elution. The gradient elution method was thus adopted in this experiment. From the study of composition of the mobile phase, we found that the retention of capacity factor (*k'*) decreased markedly for the target analytes as the acetonitrile increased. For the optimum chromatographic separation, gradient elution with the initial composition of 60% methanol, 10% acetonitrile and 30% aqueous NH_4OAc solution, was changed to 15% methanol, 60% acetonitrile and 25% aqueous NH_4OAc solution within 25 min, linearly.

The acidic solution could minimize the retaining of basic macrolide compounds from the residual silanol. Effect of aqueous NH_4OAc solution on the chromatographic performance, such as pH level (pH 3.0, 4.0, 5.0 and 6.0) and ionic strength (5, 10, 20, 30, 40 and 50 mM) was also examined. As a result, the aqueous 10 mM NH_4OAc solution, pH 4.0 was found to be the best. Under these optimized conditions, the superposition of the chromatograms for the individual injections of each analyte was shown in Figure 2, and the typical chromatograms of the standard mixture solution in Figure 3. The experimental results demonstrated that the appropriate resolution among the all target analytes was achieved. The resolution between each two target peaks was calculated. All of the each two peaks showed the resolution values (Rs) ranging between 2.3 for $Rs_{8,9}$ and 18.2 for $Rs_{7,8}$, the peak factors of symmetry

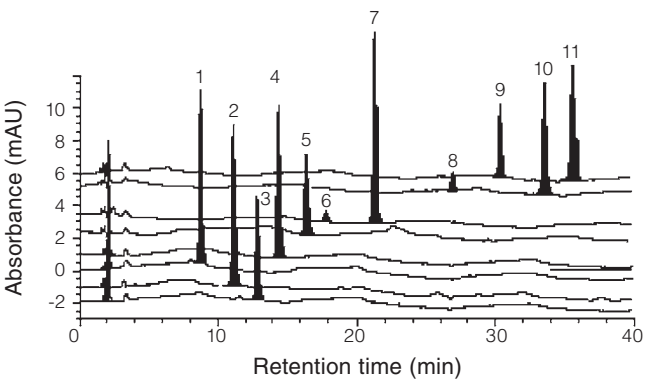


Figure 2. The superposition of the chromatograms from the individual standard injections under the optimum LC conditions, at concentration of 10 μg/mL. Peaks: (1) SPK; (2) SPB; (3) SPA; (4) NDSD; (5) SPD; (6) EMA-B_{1b}; (7) EMA-B_{1a}; (8) ABA-B_{1b}; (9) MIL-A_3; (10) ABA-B_{1a}; (11) MIL-A_4.

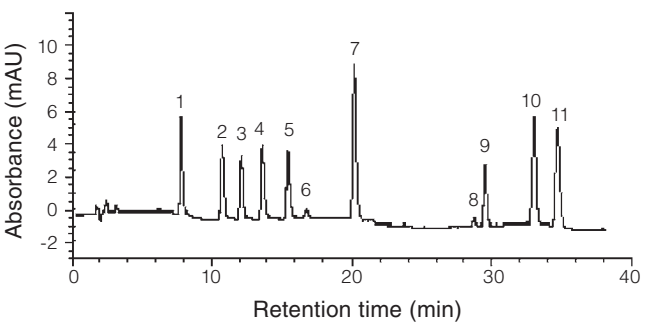


Figure 3. Typical chromatogram of a standard mixture solution under the optimum LC conditions, at concentration of 5 μg/mL of each analyte. Peaks: (1) SPK; (2) SPB; (3) SPA; (4) NDSD; (5) SPD; (6) EMA-B_{1b}; (7) EMA-B_{1a}; (8) ABA-B_{1b}; (9) MIL-A_3; (10) ABA-B_{1a}; (11) MIL-A_4.

ranging between 0.80 and 1.12, and the retention factors ranging between 3.4 for k'_1 and 16.9 for k'_{11}, respectively.

II. *Validation of Chromatographic Method*

(I) *Linearity, Instrument LOD and LOQ*

The linearity of the calibration curves was studied using matrix-matched standard solutions at five concentration level between 0.05 and 10.0 μg/mL (equivalent to 0.05-10.0 μg/g). In Table 1, the response function was found to be linear with a coefficient (R^2) of determination higher than 0.999 in the tested range for all pesticides. It has to be noted that the concentration of the minor compounds, EMA-B_{1b}, ABA-B_{1b}, and MIL-A_3 were calculated by taking into account the total signal given from EMA, ABA and MIL, respectively.

The instrument LOD and LOQ (LOD_i and LOQ_i) showed the values in a range of 0.01-0.03 μg/mL and 0.03-0.11 μg/mL, respectively, for the major compounds. In addition, the values of LOD_i and LOQ_i for the minor compounds, EMA-B_{1b}, ABA-B_{1b} and MIL-A_3 were in a range of 0.02-0.14 μg/mL and 0.07-0.48 μg/mL, respectively.

(II) *Repeatability and Reproducibility*

In general, the application of the gradient elution has displayed a relatively poorer performance than isocratic elution for HPLC determination. Thus, the repeatability and reproducibility were investigated with a 5 μg/mL standard mixture solution. Repeatability study can allow checking of the accuracy of the chromatographic system. Six replicate injections were carried out within a day and relative variations were calculated for retention time, peak height, peak factors of symmetry and resolution. In Table 2, the results showed a good repeatability of the chromatographic system, standard deviation (SD) for retention time lower than 0.03 min, and RSD of peak height in a range of 0.28-1.40%. Thus, it would be suitable to quantify peaks by heights.

Reproducibility study can allow checking of the precision of the chromatographic system between days. In each day of the studies, the same solution was injected three times per day. Between-run variations were calculated from the eighteen injections for retention time, peak height, peak factors of symmetry and resolution. Moreover, the results revealed a good reproducibility of the chromatographic system, standard deviations (SD) for retention time lower than 0.04 min, and RSD of peak resolution lower than 4%. Thus, it would be feasible to inject lots of samples and standard solutions within several days.

III. *Method Validation*

(I) *Extraction*

Mixed solution of acetonitrile/10 mM aqueous NH_4OAc solution, pH 4.0 (90:10, v/v) was used as the extraction solution, due to the acidic extraction solution having two significant advantages over other extraction solutions (e.g. acetonitrile, acetone or acetonitrile-ethyl acetate solution) in trace pesticide residue analysis. One is that acetonitrile exhibits a very strong dissolving ability and is readily miscible with water. The other advantage, an acidic medium offers the good extraction for the basic macrolides from the sample matrices. For the dehydrated process, the lower polarity organic modifier solvent, dichloromethane was added in the extraction to improve the dehydrated course with anhydrous sodium sulfate and to reduce the time-cost of dryness procedure.

(II) *Clean-up*

For the clean-up, we examined the liquid-liquid extraction (LLE) method using dichloromethane. However, LLE method was not suitable for strawberry and eggplant samples, owing to the emulsion occurred, leading to a poor recovery for the EMA and ABA. Subsequently, solid-phase extraction method was employed instead.

In preliminary experiments, we worked on the various SPE eluents and SPE cartridges. Triethylamine (TEA) is an organic base, serving as the substitute of the basic macrolide compounds away from the SPE cartridge. The SPE eluent of acetonitrile/TEA (99:1, v/v) was thus used. We also studied on the SPE eluent of methanol/TEA (99:1, v/v) but the co-extract exhibited the complicated chromatographic background. The volume of SPE eluent, acetonitrile/TEA) (99:1 v/v) solution, on the recovery was also investigated. For the first 5 mL of eluent extraction, the recoveries ranged between 88% and 95% for the analytes, except for EMA and ABA (69% and 62%, respectively). In order to improve the recovery of EMA and ABA, addition of the secondary 5 mL eluent in SPE procedure could boost the recoveries up to 25% for EMA and 31% for ABA. Thus, a 10 mL acetonitrile/TEA (99 : 1, v/v) solution was adopted for the SPE procedure. For the optimum SPE cartridge, six different kinds of SPE cartridge were studied, including C_{18}, C_8, CN, florisil, silica, and NH_2. The results showed that poor recoveries for EMA and ABA were obtained from the florisil and silica SPE cartridges, both below 60%, but satisfactory recoveries, ranged from 80% to 99%, were achieved from the other four cartridges. However, the sorbents of C_{18}, C_8 and CN were not satisfactory for the interference removing, and chromatographic background was complicated as comparing with NH_2 cartridge. Both good recoveries and purification efficiency were important considerations concerning the clean-up conditions. Thus, NH_2 SPE cartridges were chosen to purify the sample extracts. It revealed the clean-up procedure was relatively selective for the tested analytes as shown in Figure 4.

(III) *Accuracy and Precision*

Journal of Food and Drug Analysis, Vol. 17, No. 3, 2009

Table 1. Calibration curves, instrument LODs and LOQs from standard solutions prepared in the sample extract

Vegetable and fruit	Macrolide	Slope	Intercept	Correlation coefficient	LOD_i (μg/mL)	LOQi (μg/mL)
Chinese kale	SPK	2.273	0.053	0.99993	0.02	0.05
	SPB	1.970	0.020	0.99997	0.01	0.05
	SPA	1.852	0.071	0.99995	0.01	0.03
	NDSD	1.574	0.048	0.99995	0.02	0.08
	SPD	1.855	-0.010	0.99997	0.03	0.09
	EMA[a]	2.895	0.102	0.99990	0.01 (B_{1a}), 0.14 (B_{1b})	0.04 (B_{1a}), 0.45 (B_{1b})
	ABA[a]	2.004	0.050	0.99990	0.01 (B_{1a}), 0.14 (B_{1b})	0.03 (B_{1a}), 0.45 (B_{1b})
	MIL[b]	3.842	-0.350	0.99984	0.02 (A_3), 0.02 (A_4)	0.08 (A_3), 0.07 (A_4)
Cabbage	SPK	2.126	0.088	0.99997	0.01	0.03
	SPB	1.906	0.009	0.99999	0.01	0.04
	SPA	1.590	0.052	0.99992	0.01	0.03
	NDSD	1.502	-0.019	0.99998	0.02	0.07
	SPD	1.365	0.050	0.99994	0.02	0.08
	EMA[a]	2.613	0.112	0.99988	0.01 (B_{1a}), 0.12 (B_{1b})	0.04 (B_{1a}), 0.39 (B_{1b})
	ABA[a]	1.927	0.041	0.99990	0.01 (B_{1a}), 0.12 (B_{1b})	0.03 (B_{1a}), 0.40 (B_{1b})
	MIL[b]	3.221	0.094	0.99993	0.02 (A_3), 0.02 (A_4)	0.07 (A_3), 0.06 (A_4)
Eggplant	SPK	2.233	0.047	0.99996	0.01	0.03
	SPB	1.936	-0.011	0.99999	0.01	0.04
	SPA	1.679	0.063	0.99991	0.01	0.03
	NDSD	1.559	0.026	0.99998	0.02	0.07
	SPD	1.482	0.062	0.99991	0.02	0.08
	EMA[a]	2.766	0.079	0.99990	0.01 (B_{1a}), 0.11 (B_{1b})	0.04 (B_{1a}), 0.36 (B_{1b})
	ABA[a]	2.029	0.059	0.99974	0.01 (B_{1a}), 0.11 (B_{1b})	0.03 (B_{1a}), 0.37 (B_{1b})
	MIL[b]	3.507	-0.025	0.99999	0.02 (A_3), 0.02 (A_4)	0.07 (A_3), 0.06 (A_4)
Papaya	SPK	2.133	0.057	0.99993	0.01	0.05
	SPB	1.950	0.023	0.99995	0.01	0.05
	SPA	1.778	0.073	0.99993	0.01	0.03
	NDSD	1.669	0.038	0.99996	0.03	0.09
	SPD	1.568	0.052	0.99992	0.03	0.10
	EMA[a]	2.688	0.067	0.99989	0.01 (B_{1a}), 0.13 (B_{1b})	0.05 (B_{1a}), 0.44 (B_{1b})
	ABA[a]	2.128	0.079	0.99984	0.01 (B_{1a}), 0.13 (B_{1b})	0.04 (B_{1a}), 0.42 (B_{1b})
	MIL[b]	3.671	0.048	0.99996	0.02 (A_3), 0.02 (A_4)	0.07 (A_3), 0.07 (A_4)

Table 1. Continued

Vegetable and fruit	Macrolide	Slope	Intercept	Correlation coefficient	LOD_i (μg/mL)	LOQi (μg/mL)
Citrus	SPK	2.231	0.168	0.99992	0.01	0.05
	SPB	1.941	0.022	0.99997	0.02	0.06
	SPA	1.933	0.036	0.99998	0.01	0.04
	NDSD	1.595	0.054	0.99990	0.03	0.08
	SPD	1.697	0.013	0.99996	0.03	0.11
	EMA[a]	2.840	0.095	0.99988	0.01 (B_{1a}), 0.14 (B_{1b})	0.04 (B_{1a}), 0.48 (B_{1b})
	ABA[a]	1.952	0.089	0.99979	0.01 (B_{1a}), 0.12 (B_{1b})	0.04 (B_{1a}), 0.41 (B_{1b})
	MIL[b]	3.157	0.042	0.99999	0.03 (A_3), 0.02 (A_4)	0.09 (A_3), 0.07 (A_4)
Strawberry	SPK	2.094	0.037	0.99999	0.01	0.05
	SPB	1.635	0.014	0.99999	0.01	0.05
	SPA	1.845	0.059	0.99992	0.01	0.03
	NDSD	1.442	-0.001	0.99997	0.03	0.09
	SPD	1.789	0.071	0.99998	0.03	0.10
	EMA[a]	2.776	0.139	0.99990	0.01 (B_{1a}), 0.14 (B_{1b})	0.04 (B_{1a}), 0.48 (B_{1b})
	ABA[a]	2.008	0.087	0.99971	0.01 (B_{1a}), 0.12 (B_{1b})	0.04 (B_{1a}), 0.41 (B_{1b})
	MIL[b]	3.206	0.014	0.99999	0.03 (A_3), 0.02 (A_4)	0.09 (A_3), 0.07 (A_4)

[a] Calculation taking into account the total signal given by B_{1a} plus B_{1b}.
[b] Calculation taking into account the total signal given by A_3 plus A_4.

Table 2. Repeatibiliy and reproducibility of the chromatographic method

Macrolide	Repeatibility (n = 6)				Reproducibility (n = 18)			
	Retention time, SD (min)	Peak height, RSD (%)	Factor of symmetry, Mean	Rs, RSD (%)	Retention time, SD (min)	Peak height, RSD (%)	Factor of symmetry, Mean	Rs, RSD (%)
SPK	0.01	0.37	0.87	-	0.03	1.4	0.90	-
SPB	0.01	0.89	1.12	1.7	0.02	0.7	1.08	1.6
SPA	0.01	1.40	0.86	2.1	0.02	1.0	0.81	2.8
NDSD	0.01	1.04	0.85	0.9	0.03	0.9	0.95	1.9
SPD	0.01	0.47	0.80	1.9	0.03	1.4	0.79	1.2
EMA-B_{1b}	0.02	0.87	0.98	2.3	0.03	1.2	1.03	1.2
EMA-B_{1a}	0.01	0.92	0.90	1.4	0.02	1.1	0.96	1.1
ABA-B_{1b}	0.01	0.52	1.00	2.4	0.03	2.9	1.07	2.5
ABA-B_{1a}	0.02	0.47	0.81	2.0	0.02	1.4	0.87	1.1
MIL-A_3	0.01	0.32	0.95	0.8	0.02	0.7	0.91	1.7
MIL-A_4	0.01	0.28	0.83	2.2	0.02	1.0	0.87	3.7

Journal of Food and Drug Analysis, Vol. 17, No. 3, 2009

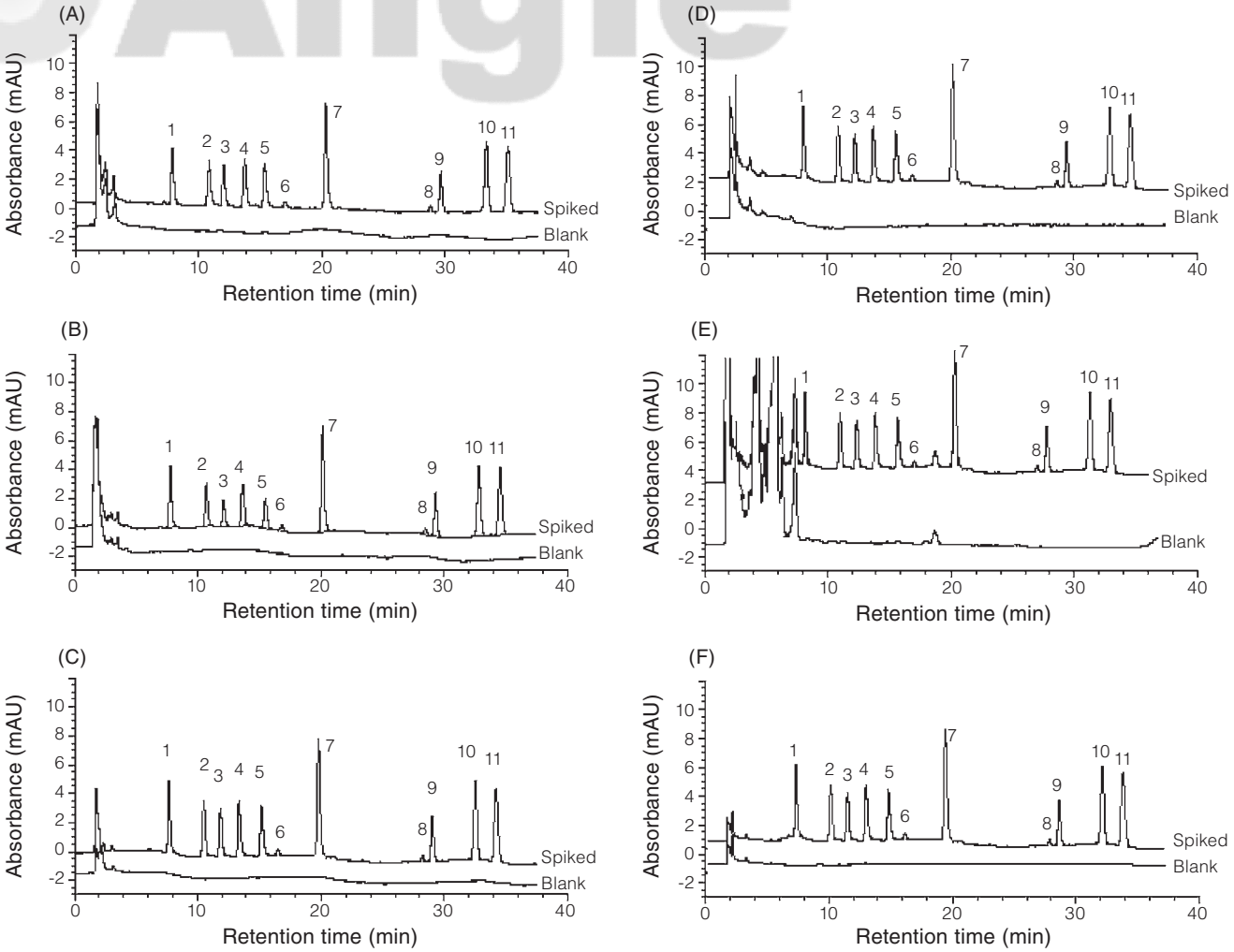


Figure 4. Chromatograms of extracts from (A) Chinese kale, (B) cabbage, (C) eggplant, (D) papaya, (E) citrus and (F) strawberry obtained by a NH_2 SPE cartridge, spiked at 2.0 μg/mL level. Chromatographic conditions: injection volume: 20 μL; column: ODS-AM (150 mm × 4.6 mm, 5 μm) with pre-column (25 mm × 4.6 mm); mobile phase: methanol/acetonitrile/10 mM aqueous NH_4OAc, pH 4.0; detection: at 250 nm; peaks: (1) SPK; (2) SPB; (3) SPA; (4) NDSD; (5) SPD; (6) EMA-B_{1b}; (7) EMA-B_{1a}; (8) ABA-B_{1b}; (9) MIL-A_3; (10) ABA-B_{1a}; (11) MIL-A_4.

Table 3. Mean recovery, RSD and estimated LOD_m obtained for all pesticides from the six kinds of vegetables and fruits determined by LC

Vegetable and fruit	Pesticide	Mean recovery (%)			Estimated LOD_m (μg/g)
		0.15 μg/g[a]	1.5 μg/g	7.5 μg/g	
Chinese kale	SPK	87 (5.8)[b]	91 (2.5)	87 (2.9)	0.02
	SPB	82 (8.3)	106 (3.3)	83 (2.4)	0.01
	SPA	92 (7.4)	96 (4.1)	89 (3.8)	0.01
	NDSD	75 (7.8)	95 (1.1)	84 (2.3)	0.02
	SPD	96 (8.2)	94 (2.0)	88 (3.0)	0.03
	EMA[c]	78 (3.3)	100 (1.4)	89 (1.9)	0.01 (B_{1a}), 0.14 (B_{1b})
	ABA[c]	89 (3.4)	93 (1.1)	90 (0.5)	0.01 (B_{1a}), 0.14 (B_{1b})
	MIL[d]	101 (4.5)	96 (1.5)	94 (0.4)	0.02 (A_3), 0.02 (A_4)
Cabbage	SPK	100 (7.5)	85 (1.7)	92 (1.9)	0.01
	SPB	86 (10.4)	102 (2.9)	90 (1.7)	0.01
	SPA	90 (11.1)	82 (4.1)	95 (2.1)	0.01

Table 3. Continued

Vegetable and fruit	Pesticide	Mean recovery (%)			Estimated LOD_m (μg/g)
		0.15 μg/g[a]	1.5 μg/g	7.5 μg/g	
Cabbage	NDSD	92 (13.3)	96 (2.5)	90 (2.4)	0.02
	SPD	83 (6.9)	82 (2.6)	95 (1.4)	0.02
	EMA[c]	89 (5.5)	93 (2.6)	91 (2.4)	0.01 (B_{1a}), 0.12 (B_{1b})
	ABA[c]	87 (2.5)	91 (0.9)	91 (2.1)	0.01 (B_{1a}), 0.12 (B_{1b})
	MIL[d]	100 (10.2)	95 (3.3)	95 (1.0)	0.02 (A_3), 0.02 (A_4)
Eggplant	SPK	93 (4.7)	97 (1.6)	92 (4.8)	0.01
	SPB	112 (10.2)	85 (3.2)	88 (5.5)	0.01
	SPA	103 (7.0)	94 (2.0)	94 (4.2)	0.01
	NDSD	117 (3.3)	100 (1.1)	90 (4.3)	0.02
	SPD	88 (11.9)	96 (3.4)	95 (5.0)	0.02
	EMA[c]	85 (6.8)	100 (2.0)	89 (5.5)	0.01 (B_{1a}), 0.11 (B_{1b})
	ABA[c]	79 (4.5)	96 (3.4)	90 (8.0)	0.01 (B_{1a}), 0.11 (B_{1b})
	MIL[d]	106 (5.8)	101 (0.9)	95 (5.1)	0.02 (A_3), 0.02 (A_4)
Papaya	SPK	94 (1.9)	103 (1.1)	100 (1.6)	0.01
	SPB	82 (3.6)	84 (3.9)	85 (4.1)	0.01
	SPA	99 (3.9)	101 (1.9)	102 (1.5)	0.01
	NDSD	80 (4.1)	88 (3.1)	84 (4.0)	0.03
	SPD	99 (5.2)	104 (2.1)	101 (1.9)	0.03
	EMA[c]	92 (1.0)	103 (3.1)	88 (2.9)	0.01 (B_{1a}), 0.13 (B_{1b})
	ABA[c]	83 (3.0)	88 (3.6)	86 (1.6)	0.01 (B_{1a}), 0.13 (B_{1b})
	MIL[d]	95 (3.1)	103 (2.3)	101 (2.3)	0.02 (A_3), 0.02 (A_4)
Citrus	SPK	95 (6.1)	89 (2.2)	97 (0.9)	0.01
	SPB	86 (7.8)	89 (2.1)	91 (3.3)	0.02
	SPA	103 (11.1)	96 (0.9)	96 (2.3)	0.01
	NDSD	82 (6.1)	90 (3.9)	92 (3.0)	0.03
	SPD	103 (5.4)	97 (2.1)	96 (1.4)	0.03
	EMA[c]	85 (3.9)	86 (7.0)	97 (4.0)	0.01 (B_{1a}), 0.14 (B_{1b})
	ABA[c]	85 (8.7)	86 (5.1)	100 (3.0)	0.01 (B_{1a}), 0.12 (B_{1b})
	MIL[d]	95 (7.9)	95 (3.6)	103 (2.2)	0.03 (A_3), 0.02 (A_4)
Strawberry	SPK	93 (4.2)	90 (1.9)	89 (2.3)	0.01
	SPB	90 (11.8)	87 (4.0)	88 (1.8)	0.01
	SPA	95 (2.5)	91 (0.9)	92 (1.8)	0.01
	NDSD	104 (11.0)	82 (5.6)	87 (2.9)	0.03
	SPD	89 (4.8)	96 (1.6)	93 (2.0)	0.03
	EMA[c]	86 (5.3)	90 (3.3)	87 (3.7)	0.01 (B_{1a}), 0.14 (B_{1b})
	ABA[c]	91 (1.7)	90 (1.7)	89 (2.9)	0.01 (B_{1a}), 0.12 (B_{1b})
	MIL[d]	99 (3.1)	97 (0.8)	94 (1.3)	0.03 (A_3), 0.02 (A_4)

[a] spiked at three different levels.
[b] RSD (%) measured in four replicates.
[c] Calculation taking into account the total signal given by B_{1a} plus B_{1b}.
[d] Calculation taking into account the total signal given by A_3 plus A_4.

Journal of Food and Drug Analysis, Vol. 17, No. 3, 2009

According to the criteria established by the European Union[29] for analytical method performance, multi-residue methods applied for pesticides residue analysis in foods are validated based on two main factors, the range of recovery percentage and its corresponding RSD value. A recovery from 70% to 120% and RSD value not higher than 20% should be acceptable.

In Table 3, listed were mean recovery, RSD and estimated method LOD (LOD_m) for all pesticides from the six kinds of vegetables and fruits (Chinese kale, cabbage, eggplant, papaya, citrus and strawberry), spiked at 0.15, 1.5 and 7.5 μg/g levels in four replicates. The results were summarized as: (1) mean recovery in a range of 75-117%, 82-106% and 83-103% for three spiked levels, respectively. (2) corresponding RSD in a range of 1.0-13.3%, 0.9-7.0% and 0.4-8.0% for three spiked levels, respectively. (3) LOD_m calculated as three times of relative standard deviation (RSD, %) from the six replicate injections, at 0.05 μg/mL in blank sample extract in acetonitrile, in a range of 0.01-0.04 μg/g for the major compounds and 0.02-0.14 μg/g for the minor compounds (EMA-B_{1b}, ABA-B_{1b}, and MIL-A_3) among the six tested samples.

CONCLUSIONS

A simple, easy, robust and efficient multi-residue assay method, by fast SPE clean-up procedure using aminopropyl (NH_2) bonded silica cartridges and analyzed by RP-HPLC at 250 nm UV detection, has been developed and validated for the six kinds of vegetable and fruit samples. The performance characteristics of the testing method for the studied macrolide pesticides and their metabolites were qualified according to the EU guidelines for method validation. Good linearity of the calibration curves was obtained within the range from 0.05 to 10.0 μg/mL with $R^2 > 0.999$. Instrument LOD values were mostly ≤ 0.03 μg/mL. Method accuracy and precision were satisfactory and recoveries were in the range of 75-117% with RSD < 14%.

REFERENCES

1. Burts, E. C. 1985. SN72129 and avermectin B1, two new pesticides for control of pear psylla, *Psylla pyricola* (Homoptera: Psylllidae). J. Econ. Entomol. 78: 1327-1330.
2. Mitchel, W. C. and Saul, S. H. 1990. Current control methods for the Mediterranean fruit fly *Ceratitis capitata* and their application in USA. Rev. Agric. Entomol. 78: 923-940.
3. Reed, D. K., Tromley, N. J. and Reed, G. L. 1985. Activity of avermectin B1 against codling moth (Lepidoptera: olethreutidae). J. Econ. Entomol. 78: 1067-1071.
4. Schuster, D. J. and Everett, P. H. 1983. Response of *Liriomyza trifolli* (Diptera: agromyzidae) to insecticides on tomato. J. Econ. Entomol. 76: 1170-1174.
5. Wright, J. E. 1984. Biological activity of avermectin B1 against the boll weevil (Coleoptera: curculionidae). J. Econ. Entomol. 77: 1029-1032.
6. Mascarenhas, V. L. and Boethel, D. J. 1997. Responses of field-collected strains of soybean looper (Lepidoptera: noctuidae) to selected insecticides using an artificial diet overlay bioassay. J. Econ. Entomol. 90: 1117-1124.
7. Sparks, T. C., Thompson, G. D., Kirst, H. A., Hertline, M. B., Larson, L. L., Worden, T. V. and Thibault, S. T. 1998. Biological activity of the spinosyns, new fermentation derived insect control agents, on tobacco budworm (Lepidoptera: nocuidae) larvae. J. Econ. Entomol. 91: 1277-1283.
8. Dybas, R. A. and Babi, J. R. 1988. 4”-Deoxy-4”-methylamino-4”-epiavermectin B1 hydrochloride (MK-243): a novel avermectin insecticide for crop protection. Brighton Crop Protection Conference. Pests Diseases 1: 2-6.
9. Takiguchi, Y., Mishima, H., Okuda, M. and Terao, M. J. 1980. Milbemycins, a new family of macrolide antibiotics: fermentation, isolation and physico-chemical properties. J. Antibiot. 33: 1120-1127.
10. Mertz, F. P. and Yao, R. C. 1990. *Saccharopolyspora spinosa* sp. nov. isolated from soil collected in a sugar mill rum still. Inter. J. System. Bacteriol. 40: 34-39.
11. Sparks, T. C., Thompson, G. D., Kirst, H. A., Hertline, M. B., Mynderse, J. S., Worden, T. V. and Tuner, J. R. 1999. Fermentation-derived insect control agents: the spinosyns. Methods Biotechnol. 5: 171-188.
12. Cobin, J. A. and Johnson, N. A. 1996. Liquid chromatographic method for determination of total avermectin B1 and 8,9-Z-avermectin B1 residue in hops. J. Assoc. Off. Anal. Chem. 79: 503-507.
13. Cobin, J. A. and Johnson, N. A. 1996. Determination of total avermectin B1 and 8,9-Z-avermectin B1 residue in wine by liquid chromatography. J. Assoc. Off. Anal. Chem. 79: 1158-1161.
14. Diserens, H. and Henzelin, M. 1999. Determination of abamectin residues in fruits and vegetables by high-performance liquid chromatography. J. Chromatogr. A 833: 13-18.
15. Valenzuela, A. I., Popa, D. S., Radondo, M. J. and Manes, J. 2001. Comparison of various liquid chromatographic methods for the analysis of avermectin residues in citrus fruits. J. Chromatogr. A 918: 59-65.
16. Yoshii, K., Kaihara, A., Tsumura, Y., Ishimitsu, S. and Tonogai, Y. 2000. Liquid chromatographic determination of emamectin, milbemectin, ivermectin and abamectin in crops and confirmation by liquid chromatography-mass spectrometry. J. Chromatogr. A 896: 75-85.
17. West, S. D. 1996. Determination of the naturally derived insect control agent spinosad in cottonseed and processed commodities by high-performance liquid

chromatography with ultraviolet detection. J. Agric. Food Chem. 44: 3170-3177.

18. Yeh, L. T., Schwedler, D. A., Schells, G. B. and Balcer, J. L. 1997. Application of empore disk extraction for trace analysis of spinosad and its metabolites in leafy vegetables, peppers and tomatoes by high-performance liquid chromatography with ultraviolet detection. J. Agric. Food Chem. 45: 1746-1751.
19. West, S. D. and Turner, L. G. 2000. Determination of spinosad and its metabolites in citrus crops and orange processed commodities by HPLC with UV detection. J. Agric. Food Chem. 48: 366-372.
20. West, S. D. and Turner, L. G. 1998. Determination of the naturally derived insect control agent spinosad and its metabolites in meat, milk, cream and egg by high-performance liquid chromatography with ultraviolet detection. J. Agric. Food Chem. 46: 4620-4627.
21. Brooks, M. W. and Uden, P. C. 1995. The determination of abamectin from soil and animal tissue by supercritical fluid extraction and fluorescence detection. Pestic. Sci. 43: 141-146.
22. Chamkasem, N., Papathakis, M. L. and Lee, S. M. 1993. Liquid chromatographic determination of abamectin in fruits and vegetables. J. Assoc. Off. Anal. Chem. 76: 691-694.
23. Moats, W. A., Harris, E. W. and Steele, N. C. 1985. Comparison of liquid chromatographic and bioassay procedures for determination depletion of intramusculayly injected tylosin. J. Assoc. Off. Anal. Chem. 68: 413-416.
24. Nagata, T. and Saeki, M. 1986. Liquid chromatographic determination of spiramycin residues in chicken tissues. J. Assoc. Off. Anal. Chem. 69: 644-646.
25. Cobin, J. A. and Johnson, N. A. 1995. Liquid chromatographic method for rapid determination of total avermectin B1 and 8,9-Z-avermectin B1 residues in apples. J. Assoc. Off. Anal. Chem. 78: 419-423.
26. Prabhu, S. V., Varsolona, R. J., Wehner, T. A., Egan, R. S. and Tway, P. C. 1992. Rapid and sensitive high-performance liquid chromatographic method for the quantitation of abamectin and its delta 8,9-Z-isomer. J. Agric. Food Chem. 40: 622-625.
27. Paesen, J., Claeys, P., Roets, E. and Hoogmartens, J. 1993. Evaluation of silanol-deactived silica-based reversed phases for liquid chromatography of erythromycin. J. Chromatogr. 630: 117-122.
28. Pizzutti, I. R., de Kok, A., Zanella, R., Adaime, M. B., Hiemstra, M., Wickert, C. and Prestes, O. D. 2007. Method validation for the analysis of 169 pesticides in soya grain, without clean up, by liquid chromatography–tandem mass spectrometry using positive and negative electrospray ionization. J. Chromatogr. A 1142: 123-136.
29. European Commission. DG-SANCO, method validation and quality control procedures for pesticide residues analysis in food and feed. Document No. SANCO/2007/3131.