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Determination of abamectin and azadirachtin residues in orange samples by liquid chromatography–electrospray tandem mass spectrometry

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Abstract

A rapid and sensitive LC–ESI-MS–MS method has been developed for the determination of azadirachtin and abamectin residues in orange samples. Samples were extracted with acetonitrile, in a high-speed blender. After the addition of sodium acetate, an aliquot of extract was directly injected into the LC–ESI-MS–MS system. The highest sensitivity of the method was achieved under MS–MS conditions using $[M+Na]^+$ adducts as precursor ions. Recoveries for both compounds from spiked orange samples at 0.01 and 0.1 mg/kg were above 80%, with good repeatability (<10%). Method detection limits achieved (<0.007 mg/kg) were adequate for the determination of these pesticides in this kind of sample from the regulatory point of view. The importance of the solvent used for extraction, as well as the addition of sodium acetate to the extracts and the selection of adequate chromatographic conditions are discussed.

Keywords: Food analysis; Fruits; Abamectin; Azadirachtin; Pesticides

1. Introduction

Nowadays, some natural products with antiparasitic effect, and their synthetic analogues, are widely used as pesticides, due to their low toxicity to non-target organisms and their low persistence in the environment.

Azadirachtin (Fig. 1a) is a tetranortriterpenoid pesticide, isolated from the Neem tree, which acts as a strong anti-feedant and causes growth disruption in many insect species [1]. Abamectin (Fig. 1b) belongs to the family of avermectins, a class of macrocyclic lactones produced by a soil actinomycete, *Streptomices avermitilis*. Abamectin is a mixture of two homologues containing >80% of avermectin B_{1a} and <20% of avermectin B_{1b}. Azadirachtin, and mainly abamectin, are used in the Valencian area (Mediterranean coast, Spain) for leafminer (*Phillocnistis citrella*) control in citrus crops, that has been one of the most serious pests in recent years.

From the regulatory point of view, the maximum concentrations allowed in food and vegetables are given by Maximum Residue Levels (MRL). The MRL for abamectin in citric crops is 0.01–0.02 mg/kg and for azadirachtin is 0.01 mg/kg, in most European countries. In some cases, the absence of

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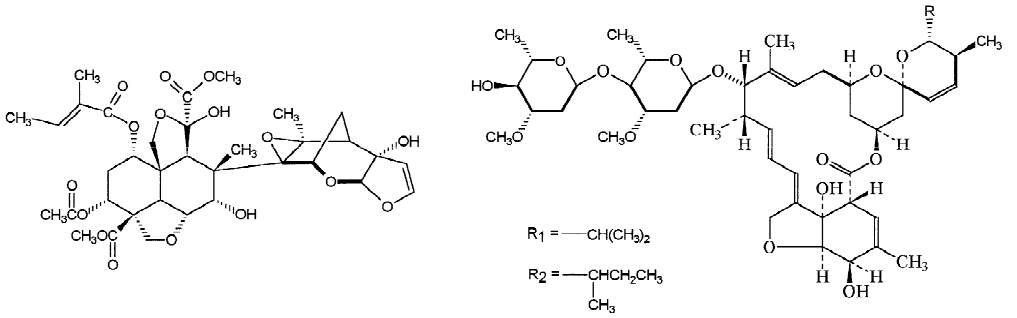

Fig. 1. Chemical structures of azadirachtin (a) and abamectin (b). $R=R_1$ (abamectin B_{1b}); $R=R_2$ (abamectin B_{1a}).

data from field residue trials leads to MRLs based on the limit of quantification of the best analytical method. Given the complex nature of the sample matrix along with the low residues expected as a consequence of the rapid degradation of these ''natural'' insecticides, sensitivity and selectivity are key issues in the development of methods that would produce adequate field residue trials data. Thus, liquid chromatography (LC) seems to be the technique of choice in combination with mass spectrometry (MS), due to their physico-chemical characteristics, high boiling point, presence of hydroxyl groups and high molecular mass.

Azadirachtin residues have been determined at the mg/kg level in environmental matrices making use of LC–UV [2], and one LC–MS method has been published for the determination of azadirachtin in neem seed extracts [3], where azadirachtin is found at concentration levels in the range of 800–1000 μg/ml.

Most of the methods reported for abamectin residues require extensive sample treatment, and are based on fluorescence detection after derivatization with 1-methylimidazole. In recent years, some methods based on LC–MS have been proposed although the complexity of the matrix along with the use of single-quadrupole MS detectors make an extensive clean-up of the extracts necessary in order to obtain accurate quantitation [4–8]. Howells et al. [9] have recently applied this technique for the residue determination of abamectin in liver samples using ion trap technology, but even applying this selective technique, a previous clean-up step was required in order to achieve an acceptable quantitation, due to the complexity of the matrix.

LC in combination with the use of tandem mass spectrometry (MS–MS) is becoming one of the most powerful techniques for the residue analysis of pesticides and metabolites in different environmental matrices [10–12]. Recently, several applications have described the use of LC–MS–MS with triple quadrupole analyzer in the target analysis of pesticide residues by direct injection of a vegetable extract [13–16], making this no sample-handling approach very attractive.

In this study, we investigate the possibility of carrying out direct injection of orange extracts using LC–MS–MS with electrospray interface (ESI) without any type of sample pre-treatment except for extraction, and using triple quadrupole technology. The aim was the rapid residue analysis of two ''difficult'' analytes, the natural pesticides azadirachtin and abamectin, in orange samples at the low mg/kg level required by regulatory agencies.

2. Experimental

2.1. Reagents and chemicals

Azadirachtin and abamectin analytical reference standards were purchased from Sigma (St Louis, MO, USA) and Dr Ehrenstorfer (Augsburg, Germany), respectively. HPLC-grade acetonitrile and

methanol were purchased from ScharLab (Barcelona, Spain). LC-grade water was obtained by purifying demineralized water in a Nanopure II system (Barnstead, Newton, MA, USA). Analytical-grade sodium acetate and ammonium acetate were supplied by Panreac (Barcelona, Spain).

Standard stock solutions were prepared by dissolving abamectin and azadirachtin in acetonitrile to a final concentration of 500 μg/ml. For the LC–MS analysis or for fortification of samples, the stock solutions were mixed and diluted with acetonitrile.

Table 1
HPLC gradients optimized for the multi-residual and individual determination of azadirachtin and abamectin in orange samples

Multi-residual determination		Abamectin determination		Azadirachtin determination	
t (min)	% MeOH	*t* (min)	% MeOH	*t* (min)	% MeOH
0	35	0	75	0	35
2	35	6	90	6	60
10	90	8	90	8	60
15	90	9	75	9	35
16	35	12	75	12	35
20	35				

2.2. Instrumentation

A Waters Alliance 2690 (Waters, Milford, MA, USA) HPLC system was coupled to a triple quadrupole Quattro LC mass spectrometer using an orthogonal Z-spray-electrospray interface (Micromass, Manchester, UK).

Drying gas as well as nebulising gas was nitrogen generated from pressurized air in a NG-7 nitrogen generator (Aquilo, Etten-Leur, Netherlands). The nebuliser gas flow was set to approximately 80 l/h and the desolvation gas flow to 800–900 l/h. Infusion experiments were carried out using a Model 11 Single Syringe Pump (Harvard Apparatus, Holliston, MA, USA), directly connected to the interface.

For operation in MS–MS mode, the collision gas was argon 99.995% (Carburos Metalicos, Valencia, Spain) with a pressure of 5×10^{-4} mbar in the collision cell. Capillary voltages of 3.5 kV were used in positive ionization mode. The interface temperature was set to 350 °C and the source temperature to 120 °C. Dwell times of 0.2 s/scan were chosen and the mass spectrometer was tuned to obtain a half height peakwidth about 0.7 Da. A solvent delay of 5 min was chosen in order to obtain an additional clean-up using the built-in divert valve controlled by Masslynx NT v 3.5 software. This software was also used to process the quantitative data obtained from calibration standards and from orange samples.

The LC separation was carried out on a Nucleosil C_{18} 5 μm, 80×2 mm (Scharlab, Barcelona, Spain) column, at a flow-rate of 300 μl/min, using methanol–water gradient as mobile phase.

2.3. Analytical procedure

Orange samples were cut into small pieces without any pre-treatment, such as washing or removing their skin, and were triturated. Homogenised orange sample (25 g) was accurately weighed (precision 0.1 mg) and mixed with 80 ml of acetonitrile. After extraction for 2 min with a high-speed blender Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany) at 8000 rpm, the entire extract was filtered through a filter paper and washed with 15 ml of acetonitrile. Finally, the volume was adjusted to 100 ml with acetonitrile.

In order to obtain a sodium concentration of 60 m*M*, 200 μl of sodium acetate 500 m*M* were added to a 2-ml vial containing 1.5 ml of the orange extract (or standard solution).

Fortification of homogenised orange samples in recovery experiments was carried out delivering appropriate volumes of mixed standards in acetonitrile (between 0.5 and 2 ml), in order to give fortification levels of 0.01–0.1 mg/kg. These samples were equilibrated, under dark conditions, for 2 h prior to extraction.

Analyses were carried out using the LC–ESI-MS–MS system described above. The mobile phases used for the multi-residual and individual analysis were methanol–water gradients where the percentage of methanol was changed linearly as shown in Table 1. Quantification of samples was carried out by external calibration, using standards solutions in acetonitrile.

3. Results and discussion

3.1. MS optimisation

Preliminary experiments showed that neither

azadirachtin or abamectin were efficiently deprotonated, as the acidic centers in these molecules, the hydroxyl groups, are not strong enough to lose a proton in the ESI interface (Fig. 1). However, adduct formation with sodium and ammonium was observed, in agreement with other studies [3,6,7,17]. The full-scan (bottom) and the MS–MS spectra (top) of abamectin and azadirachtin adducts are shown in Fig. 2 (ammonium) and Fig. 3 (sodium). After addition of 10 m*M* ammonium acetate, the full scan spectra also showed peaks at *m*/*z* 743.6 (azadirachtin) and 895.8 (abamectin) corresponding to sodium adducts that were formed, possibly due to traces of sodium as impurities in the reagents used (Fig. 2).

Fig. 3 shows the fragmentation pattern obtained for $[M+Na]^+$ precursor ions. As expected, low fragmentation was observed in both pesticide MS–MS spectra; azadirachtin fragmentation (Fig. 3a) showed only an abundant fragment at $[M\text{-}18]^+$ due to a water loss reaction, meanwhile abamectin (Fig. 3b) presented a more specific transition $[M\text{-}144]^+$ corresponding to the loss of one monosaccharide residue.

The optimized selected reaction monitoring (SRM) transitions for both analytes were as follows: azadirachtin, 743.6 as precursor ion and 725.3 as product ion, selecting a cone voltage of 50 V and a collision energy of 35 eV; and abamectin, 895.5 as precursor ion and 751.5 as product ion, selecting a cone voltage of 70 V and a collision energy of 50 eV.

3.2. Sample pretreatment optimization

As ammonium adducts normally present better fragmentation and reproducibility than sodium adducts, different attempts were assayed in this paper in order to enhance the formation of ammonium adducts, such as adding ammonium acetate to the mobile phase [7] or to the sample extract. However, only chromatographic peaks for the sodium adduct transitions were observed, possibly favoured by the presence of a high concentration of this element in

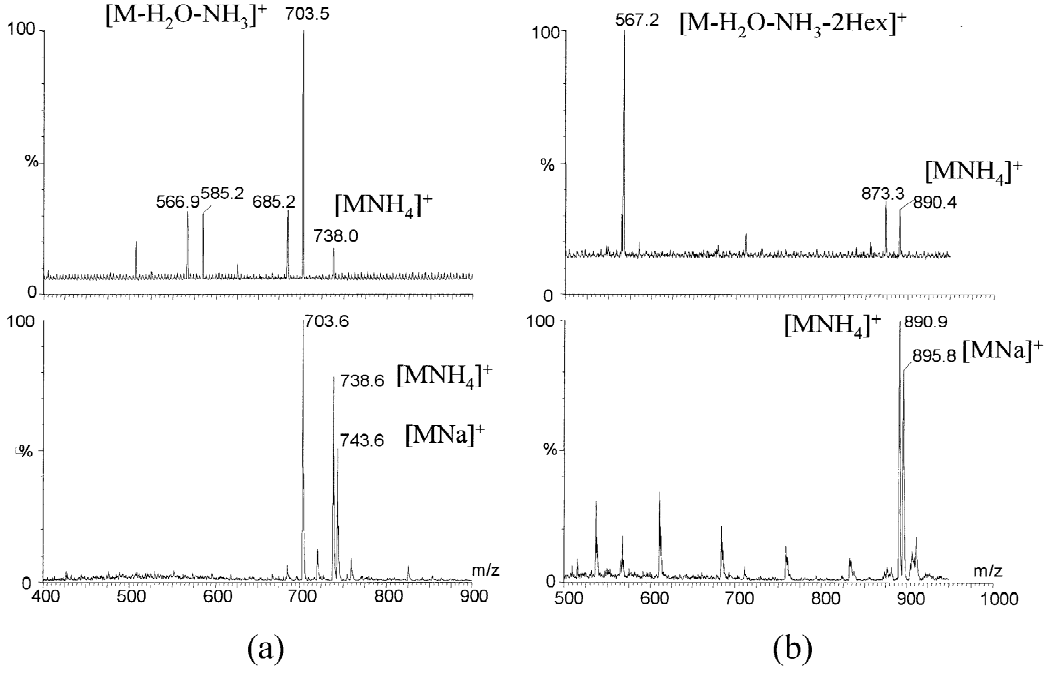

Fig. 2. The positive ion electrospray full scan mass spectra (bottom) and product ion spectra of ammonium adduct ion (top) of (a) azadirachtin and (b) abamectin acquired by infusion of 5 μg/ml standard solution (acetonitrile/water, 50:50) with 10 m*M* of ammonium acetate. Hex: monosaccharide group.

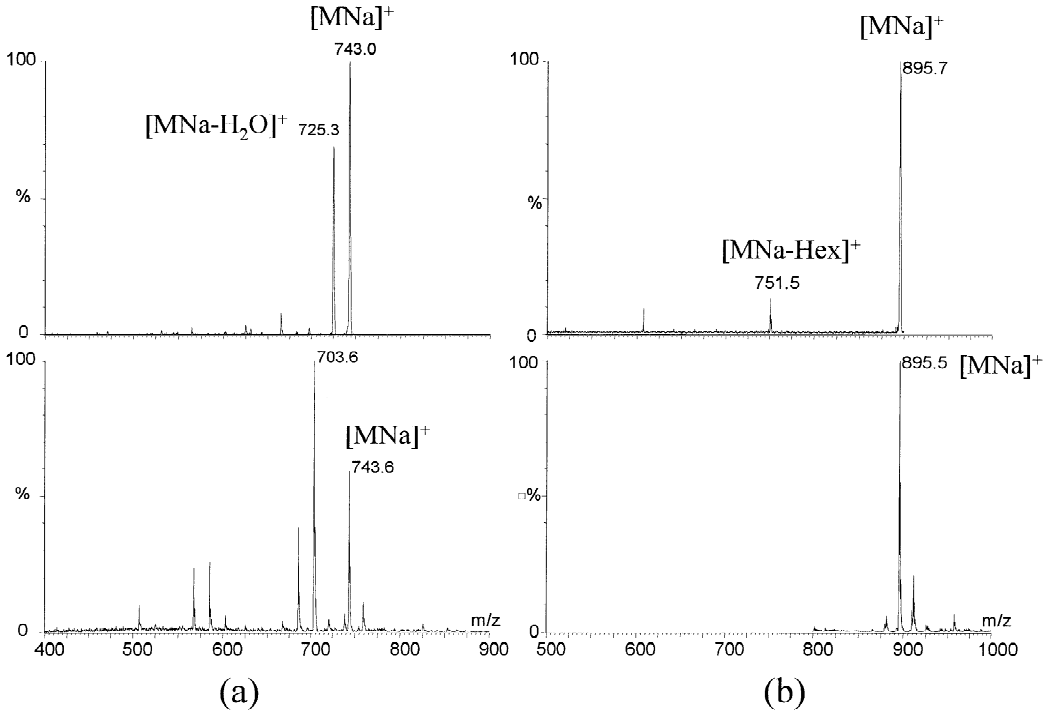

Fig. 3. The positive ion electrospray full scan mass spectra (bottom) and product ion spectra of sodium adduct ion (top) of (a) azadirachtin and (b) abamectin acquired by infusion of 5 μg/ml standard solution (acetonitrile/water, 50:50) with 60 m*M* of sodium acetate. Hex: monosaccharide group.

the orange extract. Increasing the buffer concentration in the mobile phase up to 50 m*M*, only led to a 10-fold signal reduction for the sodium adduct due to the effect of these high amounts of ammonium, but no signal for $[M+NH_4]^+$ was observed either for azadirachtin or abamectin. After these results, working with sodium adducts was selected.

Sodium is a non-volatile element than can precipitate in the sampling cone blocking it and, therefore, sodium is not considered an adequate cation to be introduced continuously in the interface with the mobile phase. In order to have constant amounts, the addition of an aliquot of sodium acetate to the sample extracts and to the standard solutions was checked. Thus, working with 30 m*M* sodium led to relative standard deviations (RSD) (three calibration curves, from 1 to 500 ng/ml, with each point injected in triplicate) between 11 and 24% for azadirachtin, and 9–34% for abamectin. When doubling the sodium amount (60 m*M*), the RSD dramatically decreased, achieving values lower than 9%, without affecting sensitivity.

In relation to the extraction step, two of the most typical organic eluents in LC were tested (methanol and acetonitrile) bearing in mind the direct injection of the orange extract without any pretreatment or solvent exchange. The recovery of abamectin was similar for both solvents (typically 70–80%). Azadirachtin was poorly recovered when the extraction was carried out with methanol (40%), while acetonitrile led to 80% recovery. In order to explain these differences, a methanol extract blank was spiked with a mixture of standards, obtaining the same results as above. Therefore, low recoveries with methanol should not be due to an inefficient extraction of the analyte, but to the matrix components soluble in methanol that interfered in the MS determination. As acetonitrile led to acceptable re-

coveries for both compounds, this solvent was finally selected for sample extraction.

3.3. LC optimization

Usually, the use of tandem mass spectrometry does not require optimal chromatographic separation, as is very rare to find molecules that share the same unique transition. However, when non-selective transitions are used (like the neutral loss of H_2O), some matrix components (or their isotopes or cone fragments) are able to present the same transition as the analyte, mainly if analysis without any sample pretreatment is carried out.

This is the case for azadirachtin in oranges samples. A direct injection chromatogram of blank extracts showed several peaks at retention times close to azadirachtin when 743.6→725.3 transition was monitored. Full scan chromatogram of orange extract reveals that these peaks corresponded to several major compounds with $m/z=742.6$, which exhibited a minor M-18 transition. Thus, the protonated molecule containing one ^{13}C isotope of these molecules shared the same transition with azadirachtin. Therefore, enough chromatographic resolution between these compounds and azadirachtin was required. To aid during the interference evaluation, an additional 742.6→724.3 transition was monitored.

In order to optimize chromatographic separation, mixtures of water/acetonitrile and water/methanol were assayed as mobile phases. The use of acetonitrile as organic modifier did not improve the selectivity between azadirachtin and co-extracted interferences, in spite of the fact that several gradients were tested. However, the use of methanol achieved the necessary resolution. However, abamectin determination was not affected by the organic modifier.

3.4. Validation study

Calibration curves ($n=9$) with standards in solvent showed good linearity between 1 and 500 μg/l, with correlation coefficients higher than 0.997 for both compounds. The method was precise (RSD< 7%) with instrumental limits of detection of 0.5 μg/l for azadirachtin and 2 μg/l for abamectin.

Although initially both compounds were analysed in one run, as they have similar applications in citric crops, better precision and accuracy was achieved by analysing the two compounds in two separate runs (Table 2).

The multi-residue method was found to be precise (RSD<4%) and accurate (around 70%) for azadirachtin determination. However, non satisfactory figures were obtained for abamectin determination, which was more affected by the signal suppression produced by co-eluting interferences.

The chromatographic separation was improved by optimizing fast individual gradients for each compound, minimizing in this way the signal suppression. Additionally, the injection volume for abamectin determination could be increased up to 25 μl, achieving the objective limit of quantitation (LOQ) of 0.01 mg/kg and obtaining good peak shape.

Under the experimental conditions shown in Table 1 for single determination, abamectin recoveries increased up to 80–100% and the detection limit decreased down to 0.007 mg/kg. Precision was also

Table 2
Validation study of the developed procedure for the determination of azadirachtin and abamectin in orange samples ($n=5$)

	Multi-residual determination		Individual determination	
	0.01 mg/kg	0.1 mg/kg	0.01 mg/kg	0.1 mg/kg
Azadirachtin	70[a] (4[b])	71 (2)	78 (4)	83 (2)
			103[c] (10)	92[c] (2)
Abamectin	53 (32)	66 (7)	96 (10)	80 (3)

[a] Recovery (%).
[b] Relative Standard Deviation (%).
[c] Twofold diluted extract.

improved as a consequence of lowering the abamectin retention time from 14 to around 7 min. This fact was possibly related to a more reproducible sodium adduct formation, as the amount of sodium in the interface was higher and more reproducible at short retention times, as sodium was introduced only during injection. As a consequence of the good sensitivity for azadirachtin determination (estimated LOD of 0.002 mg/kg), a twofold dilution of the extract was assayed in order to reduce matrix effects and increase recoveries up to 100% (Table 2). By optimizing chromatographic conditions, the total analysis time in two runs was only 4 min longer (24 instead of 20 min). Changing between the two methods can be done automatically. Typical chromatograms of standard solutions and orange samples (blank and 0.01 mg/kg spiked sample) are shown in Fig. 4.

4. Conclusion

This work has shown that LC–ESI-MS–MS is a rapid, sensitive and selective technique for the determination of azadirachtin and abamectin residues in orange samples. The analytical methodology developed allows to reach the quantification limits required for these pesticides in food samples from the regulatory point of view (0.01 mg/kg), without extensive sample pretreatment. Satisfactory results were obtained using acetonitrile for extraction of samples and methanol as organic modifier in the

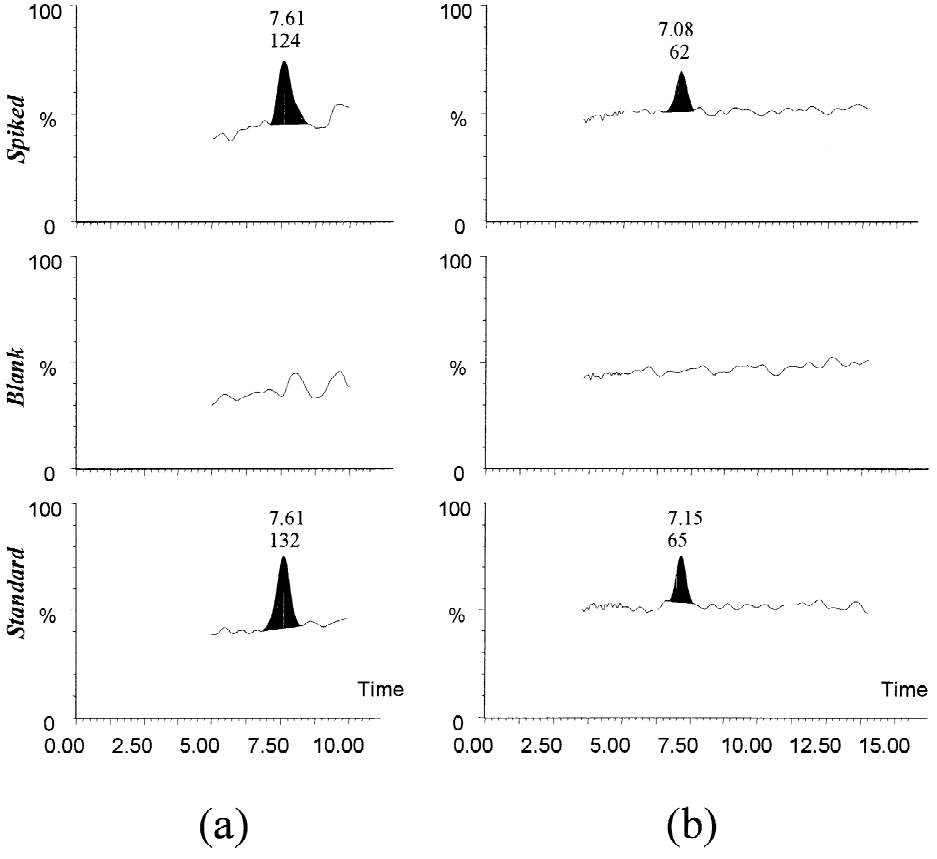

Fig. 4. LC–ESI-MS–MS chromatograms from individual analysis of standards (2.5 μg/l) and orange extracts (blank and spiked at 0.01 mg/kg): (a) azadirachtin (twofold dilution); (b) abamectin.

mobile phase. With a chromatographic run time of 20 min, acceptable results were achieved in the multi-residual analysis of both compounds. By optimizing chromatographic individual conditions, the total analysis time in two runs was only 4 min longer, with improved recoveries and precisions, mainly for abamectin at the 0.01 mg/kg concentration level. Addition of sodium acetate to the sample extract has proved to be an efficient way to obtain sodium adducts in LC–ESI-MS–MS, improving the precision and robustness of the analytical method.

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