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Fast and Versatile Multiresidue Method for the Analysis of Botanical Insecticides on Fruits and Vegetables by HPLC/DAD/MS

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A simple multiresidue method for screening analysis of 12 botanical insecticides used by organic farmers has been developed. The method involves a rapid and small-scale extraction procedure with acetonitrile. For all fruit and vegetable samples, there was no need for clean up. Rotenone, azadirachtin, ryanodines, and pyrethrins can be separated by high-performance liquid chromatography, quantified, and confirmed with a diode array detector (DAD) and atmospheric pressure chemical ionization mass spectrometry (APCI−MS) in the select ion-monitoring mode (SIM). The majority of pesticide recoveries for various fruits and vegetables were >70% in the concentration range from 0.01 to 5 mg/kg. The limit of quantitation for most of the pesticides was 0.01 mg/kg, with the majority of relative standard deviations (RSD) mostly below 10%.

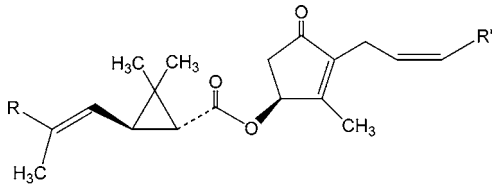
KEYWORDS: HPLC/DAD; LC−APCI−MS; rotenone; deguelin; pyrethrins I; pyrethrins II; cinerin I; cinerin II; jasmolin I; jasmolin II; azadirachtin; ryanodine; dehydroryanodine; piperonyl butoxide; 12aβ-hydroxy-rotenone

INTRODUCTION

In recent years, an increased consumer awareness of food safety issues and environmental concerns has contributed to the growth of organic farming (*1*). Recent studies have shown a possible relationship between certain pesticides and Parkinson's disease. Among them, a new study showed that rats chronically treated with the mitochondrial inhibitors rotenone and deguelin, nonsystemic botanical insecticides, develop neuropathological and behavioral symptoms of Parkinsonism (*2*).

Since the beginning of the 1990s, organic farming has rapidly developed in almost all European countries, with more than 5.6 million hectares managed organically by around 143 000 farms in the 25 countries of the European Union (EU) (*3*). This constitutes 3.4% of the agricultural area in the EU. One-fifth of the EU's organic land and almost a quarter of its organic farms are located in Italy.

In 2003, according to the research institute of organic agriculture, the market value of organic products worldwide reached 25 billion U.S. dollars (*4*). The use of biopesticides in the EU is controlled by the regulation EEC 2092/91, which allows farmers to use azadirachtin, rotenone, and pyrethrins on different crops. Chemical structures of pyrethrins I and II are designed in **Figure 1**, and structures of rotenone, ryanodine, azadirachtin, and their analogues deguelin, 12aβ-hydroxy-rotenone, 9,21-dehydroryanodine, and piperonyl butoxide (PB) are reported in **Figure 2**.



Pyrethrins	R	R'
pyrethrins I – esters of chrysanthemic acid		
pyrethrin I	CH_3	$CH{=}CH_2$
cinerin I	CH_3	CH_3
jasmolin I	CH_3	CH_2CH_3
pyrethrins II – esters of pyrethric acid		
pyrethrin II	$CH_3OC(O)$	$CH{=}CH_2$
cinerin II	$CH_3OC(O)$	CH_3
jasmolin II	$CH_3OC(O)$	CH_2CH_3

Figure 1. Chemical structures of pyrethrins I and II.

Azadirachtin is a limonoid of the tetranortriterpenoid type extracted from the oil obtained from the seed of the neem tree (*Azadirachta indica*). Neem extracts show an insecticidal activity and antifeedant and repellent properties. Neem extracts and pure azadirachtin are used to control *Lepidoptera* and *Diptera* both by contact and ingestion. In Italy, these insecticides are registred for many crops with a maximum residue level (MRL) of 0.5 mg/kg and a preharvest interval (PHI) of 3 days for all crops (*5*).

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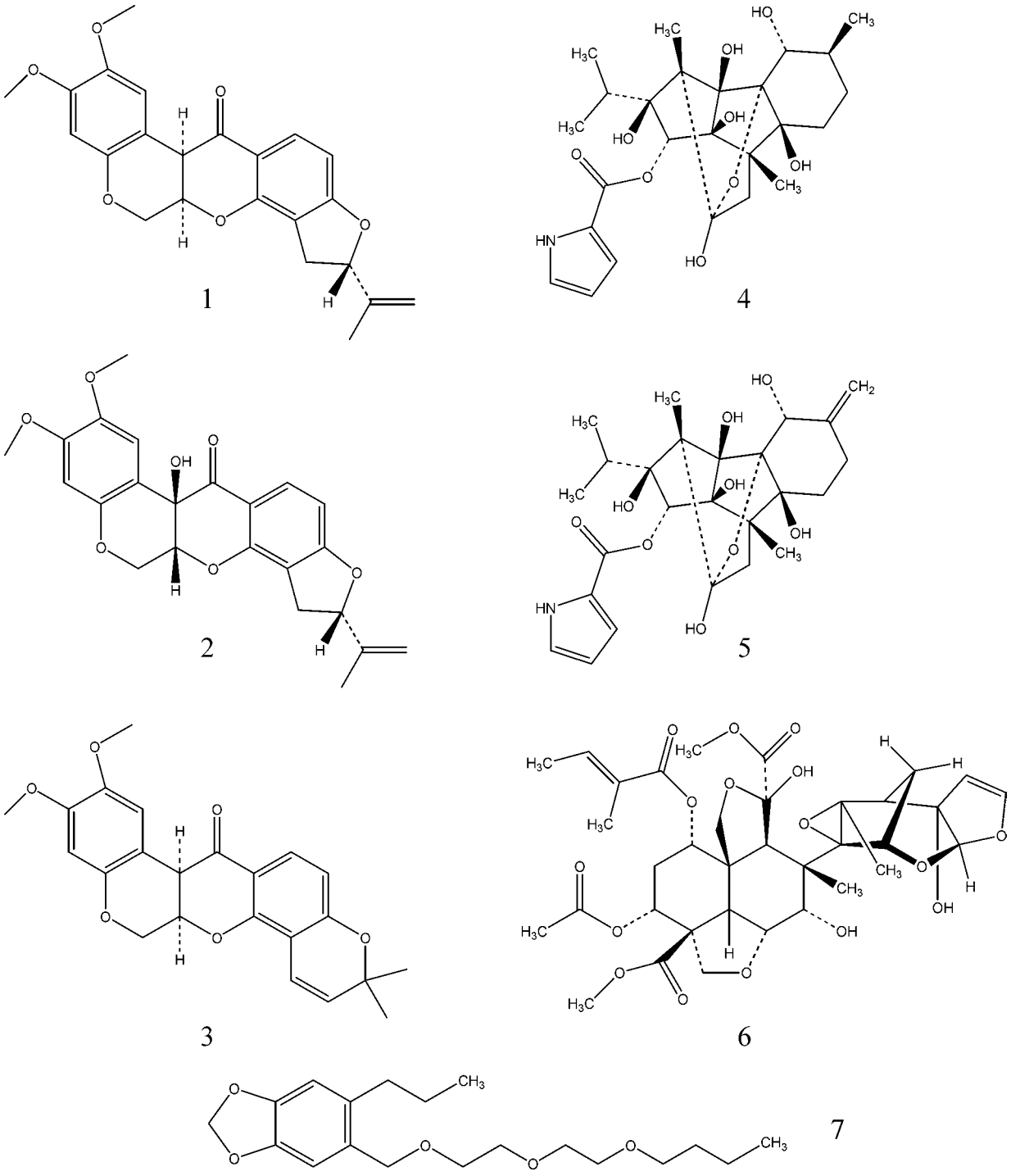

Figure 2. Chemical structures of 12aβ-hydroxyrotenone (2), deguelin (3), ryanodine (4), 9,21-dehydroryanodine (5), azadirachtin (6), and PB (7).

Pyrethrum is the extracts from the dried, powdered flowers of *Tanacetum cinerariaefolium* and has been used as an insecticide from ancient times (*6*). The active ingredient consists of six esters called pyrethrins and identified as pyrethrin I and II, cinerin I and II, and jasmolin I and II, which are obtained from the combination of chrisanthemic acid and pyrethric acid with three alcohols: cinerolone, pyrethrolone, and jasmolone (*7*). The rapid knockdown action and lethal effects of pyrethrins on insects depends upon the ability of these compounds to disrupt the normal functioning of the insect nervous system (*8*). Pyrethrum extracts are used to control a wide range of insects, and its use is recommended on fruits and vegetables as field crops. The MRL for pyrethrins (expressed as the sum of pyrethrin I and II) is 1 mg/kg with a safety interval of 2 days. The insecticidal activity of the six individual pirethrins in their mixture in pyrethrum extracts is enhanced by PB, which retards the rate of the P-450 oxidative detoxification (*9*).

Rotenone is a nonsystemic botanical insecticide obtained from leguminous plants such as *Derris elliptica*, *Lonchocarpus nicou*, and *Tephrosia vogelii*. This insecticide is used to control a wide range of arthropod pests including aphids, suckers, and other insects on fruits and vegetables with contact and stomach action (*10*). Rotenone and related rotenoids such as deguelin and 12aβ-hydroxyrotenone are potent complex I (NADH:ubiquinone oxidoreductase) inhibitors. The MRL for rotenone is 0.05 mg/kg with a safety interval of 10 days for fruits and vegetables, except for melons and strawberries, where MRL is 0.10 mg/kg with a PHI of 3 days.

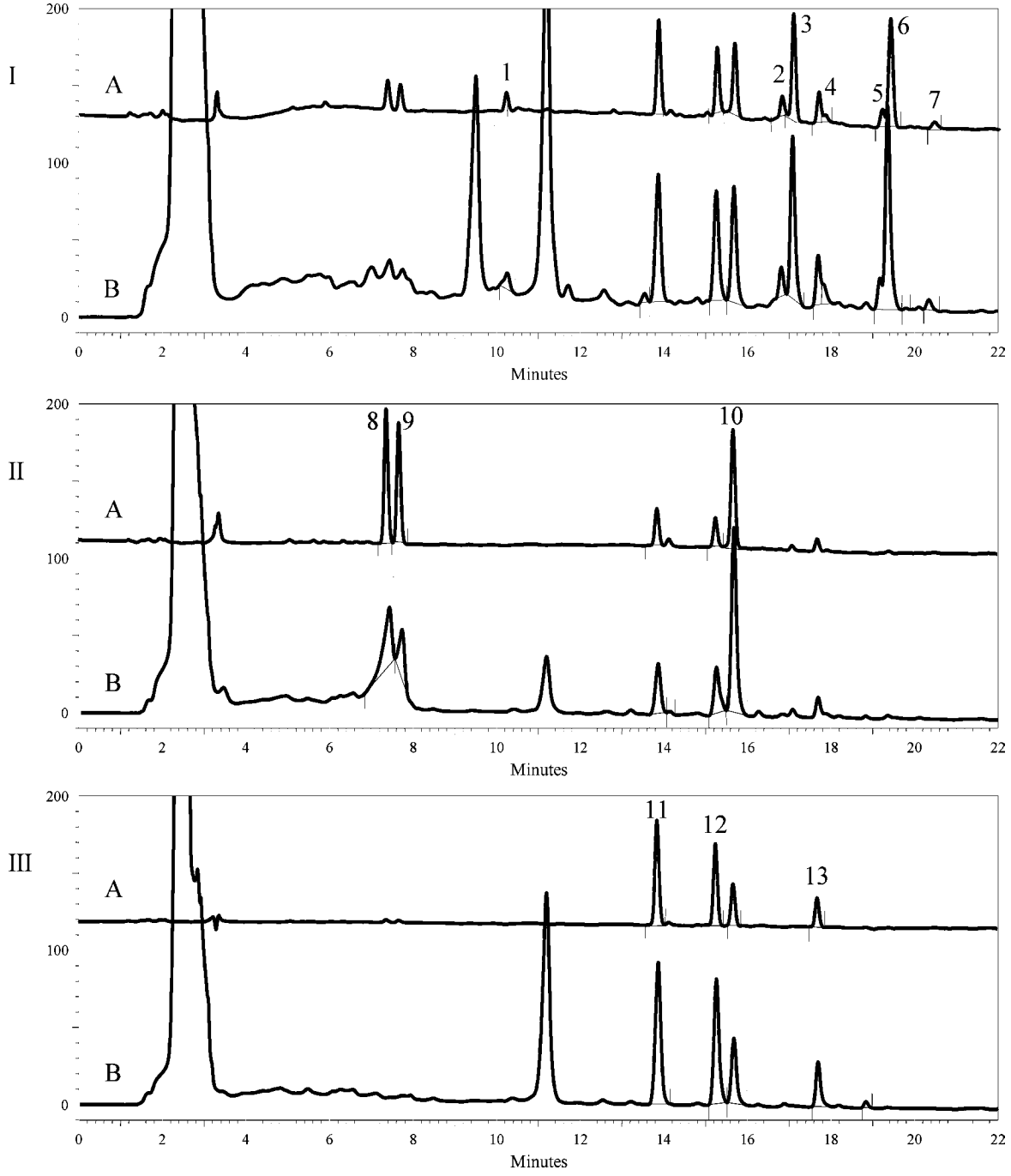

Figure 3. Chromatograms at 230 (I), 270 (II), and 295 (III) nm for the analysis of azadirachtin (1), pyrethrins (cinerin II, pyrethrin II, jasmolin II, cinerin I, pyrethrin I, and jasmolin I) (2–7), 9,21-dehydroryanodine (8), ryanodine (9), deguelin (10), 12aβ-hydroxyrotenone (11), rotenone (12), and PB (13). (A) Standards containing all compounds. (B) Blank fortified at 0.5 mg/kg.

Ryania extracts are nonsystemic botanical insecticides, obtained from the ground stems of *Ryania speciosa*, a native plant of tropical America (*11*). Ryanodine and 9,21-dehydroryanodine are the main alkaloids and affect muscles by binding to the calcium channels in the sarcoplasmic reticulum of insects causing calcium ions to flow into the cells (*12*). Currently, *Ryania* extracts are not registered in Italy as insecticides. Although they are currently not registered in the EU, *Ryania* extracts are regarded as promising biopesticides.

Several publications report analytical methods for determining organic insecticides in foods, fruits, and vegetables, as well as in botanical extracts (*13*−*15*). To date, very little is known about residue levels of organic insecticides in foods because of the lack of easy, rapid, inexpensive, and rugged analytical methods. Modern residue monitoring programs, however, are expected to be responsive to the latest development in agriculture and new legislation.

For this reason, we have developed a fast HPLC/DAD/MS analytical procedure for the identification and quantitation of a wide number of biopesticides, namely, azadirachtin, rotenone, pyrethrins, and ryanodine, on fruits and vegetables.

MATERIAL AND METHODS

Chemicals. Methanol and acetonitrile were HPLC-grade (Merck, Milan, Italy); sodium chloride, anhydrous magnesium sulfate, trifluoroacetic acid 99% (Sigma−Aldrich, Steinheim, Germany), and water was distilled and filtered through a Milli-Q apparatus (Millipore, Milan, Italy) before use. Standards of azadirachtin (95% purity), ryanodine,

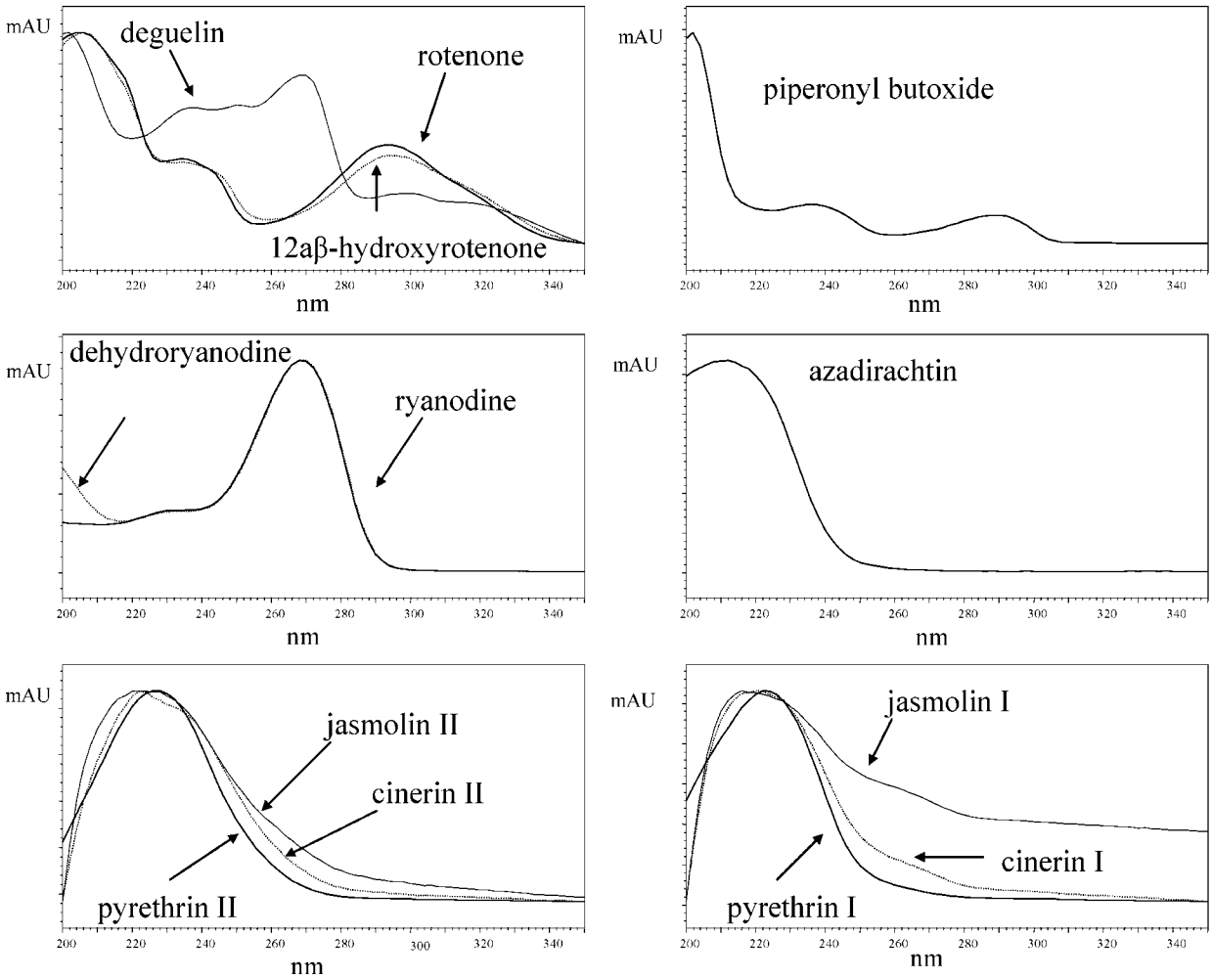

Figure 4. UV spectra of biopesticides, their analogues, and PB.

9,21-dehydroryanodine (99% purity), pyrethrins (technical grade 21.58%: cinerin II, 1.25%; pyrethrin II, 7.0%; jasmolin II, 0.50%; cinerin I, 1.51%; pirethrin I, 10.62%; jasmolin I, 0.70%), and PB (90% purity) were purchased from Sigma−Aldrich (Steinheim, Germany). Rotenone (98% purity) was purchased from Ehrenstorfer (Augsburg, Germany), and deguelin (>98% purity) and 12aβ-hydroxyrotenone (>98% purity) were kindly provided by John Casida (University of California at Berkeley).

Stock Standard Solutions. A stock standard solution of each insecticide (1000 mg/L for all compounds, except 500 mg/L for azadirachtin) were prepared in acetonitrile or methanol by weighing approximately 0.01 g of the analyte into a 10 mL volumetric flask and diluting to volume. An intermediary mixed standard solution was prepared daily by diluting with the mobile phase (25:75 acetonitrile/water. v/v), except for the HPLC/MS analysis (25:75 acetonitrile/aqueous 0.1% trifluoroacetic acid, v/v). All standard solutions were stored in the dark at −20 °C until usage.

Instrumentation and Sample Analysis. *HPLC/DAD Analysis*. An Agilent Technologies (Waldbronn, Germany) model 1100 high-performance liquid chromatograph was used, fitted with a diode array detector (DAD) mode. UV6000LP linked to a PC computer running the ChromQuest version 2.51 software program (TermoQuest, San Jose, CA). An analytical column Waters XTerra RP18 (250 × 4.6 mm, 5 μm particle size) (Milford, MA) was employed.

For HPLC analysis, an aliquot (100 μL) was injected into the column and eluted at room temperature. For the analytical separation, the gradient profile of the mobile phase, at the constant flow of 1 mL/min, was as follows: initial (25:75, v/v) acetonitrile/water reaching in 15 min (80:20, v/v) and hold to 25 min. Before the next injection, the HPLC system must be stabilized for 10 min with acetonitrile/water (25:75, v/v). Detection was carried out at wavelengths between 200 and 400 nm, and quantification analyses involved peak area comparisons with synthetic standards and absorbance measurements at 230 nm for pyrethrins and azadirachtin, at 270 nm for deguelin, ryanodine, and 9,21-dehydroryanodine, and at 295 nm for rotenone, 12aβ-hydroxyrotenone, and PB.

HPLC−MS Analysis. An HPLC system (Shimadzu, Milan, Italy) equipped with an SPD11 Avp DAD detector, an SIL 11 AD vp auto injector, and a LC 10 AD binary pump coupled on-line with an MS2010 mass spectrometer (Shimadzu, Milan, Italy) was used. UV and MS data were acquired and processed using Shimadzu "LCMS solution" software. Gradient elution was with acetonitrile/aqueous 0.1% trifluoroacetic acid 99% (30:70, v/v) for 40 min. The used column was a 150 × 2.1 i.d., 3.5 μm particle size Waters Symmetry C18. The injection volume was 20 μL, and the flow rate was 0.4 mL/min. The APCI−MS interface was operated in the positive mode: APCI source probe, 245 °C; CDL, 245 °C; block, 230 °C; flow gas (N_2), 4.5 mL/min; probe voltage, 4.5 kV; scan, 150−850 amu.

Extraction Procedure from Fruits and Vegetables. Fresh and uncontaminated samples of fruits and vegetables were purchased at local markets in Cagliari, Italy. Samples were analyzed, unwashed, and in a raw state. Samples of fruits or vegetables were placed in a blender/cutter (Malavasi, Bologna, Italy) and chopped for 30 s.

A portion (5 g) of well-homogenized chopped grapes, pears, eggplants, peppers, and tomatoes were weighed in a 40-mL screw-capped glass tube containing 4 g of sodium chloride and 1 g of anhydrous

Table 1. Mean Recoveries (% ±RSD) of Biopesticides on Tomatoes[a]

	fortification level (mg/kg)						
compound	0.01	0.02	0.05	0.1	0.5	1	5
9,21-dehydroryanodine	89 ± 2	93 ± 11	83 ± 14	96 ± 3	87 ± 5	84 ± 5	87 ± 2
ryanodine	87 ± 3	95 ± 6	99 ± 12	93 ± 11	92 ± 4	95 ± 7	76 ± 7
azadirachtin	nd	nd	80 ± 1	90 ± 1	110 ± 4	96 ± 1	93 ± 9
12aβ-hydroxyrotenone	106 ± 9	93 ± 0	100 ± 4	99 ± 10	88 ± 9	97 ± 2	86 ± 6
rotenone	88 ± 11	107 ± 4	115 ± 4	101 ± 5	93 ± 8	110 ± 8	104 ± 3
deguelin	109 ± 4	98 ± 4	109 ± 8	111 ± 3	87 ± 10	85 ± 3	114 ± 8
piperonyl butoxide	91 ± 7	106 ± 11	93 ± 4	106 ± 1	92 ± 8	101 ± 13	97 ± 4
cinerin II[b]	nd	nd	83 ± 6	104 ± 8	91 ± 6	103 ± 4	108 ± 9
pyrethrin II[b]	96 ± 8	96 ± 3	97 ± 2	102 ± 5	95 ± 4	107 ± 1	110 ± 4
jasmolin II[b]	nd	nd	nd	84 ± 6	98 ± 1	85 ± 5	98 ± 11
cinerin I[b]	nd	nd	73 ± 7	101 ± 5	95 ± 5	96 ± 17	102 ± 4
pyrethrin I[b]	84 ± 2	101 ± 2	99 ± 3	105 ± 2	91 ± 10	97 ± 5	108 ± 5
jasmolin I[b]	nd	nd	nd	89 ± 5	90 ± 6	90 ± 2	80 ± 2

[a] nd = not detected. [b] Referred to as the total content of pyrethrins.

Table 2. LC/MS (APCI+) Characteristics of Biopesticides, Their Analogues, and PB

compound	formula	log p[a] HPLC	t_R (min)	molecular weight	LC/MS (APCI) m/z (amu) (% relative abundance)
9,21-dehydroryanodine	$C_{25}H_{33}NO_9$	–0.40	7.31	491	379 $[M - C_5H_6NO_2]^+$ 100, 490 $[M - H]^+$ 80, 361 $[M - C_5H_8NO_3]^+$ 35
ryanodine	$C_{25}H_{35}NO_9$	–0.05	7.61	493	381 $[M - C_5H_6NO_2]^+$ 100, 492 $[M - H]^+$ 45, 363 $[M - C_5H_8NO_3]^+$ 15
azadirachtin	$C_{35}H_{44}O_{16}$	–0.52	10.15	721	685 $[M - 2H_2O]^+$ 100, 703 $[M - H_2O]^+$ 76, 722 $[M+H]^+$ 35
12aβ-hydroxyrotenone	$C_{23}H_{22}O_7$	2.27	13.81	410	393 $[M - H_2O]^+$ 100, 411 $[M + H]^+$ 15, 434 $[M + H + Na]^+$ 10
rotenone	$C_{23}H_{22}O_6$	2.90	15.21	394	395 $[M + H]^+$ 100, 436 $[M + H + CH_3CN]^+$ 18
deguelin	$C_{23}H_{22}O_6$	2.82	15.71	394	395 $[M + H]^+$ 100, 393 unidentified 62, 436 $[M + H + CH_3CN]^+$ 19
cinerin I	$C_{20}H_{28}O_3$	3.61	16.81	316	317 $[M + H]^+$ 100, 358 $[M + H + CH_3CN]^+$ 39
pyrethrin I	$C_{21}H_{28}O_3$	3.76	17.11	328	329 $[M + H]^+$ 100, 370 $[M + H + CH_3CN]^+$ 12
jasmolin I	$C_{21}H_{30}O_3$	4.03	17.91	330	331 $[M + H]^+$ 100, 346 unidentified 71, 372 $[M + H + CH_3CN]^+$ 24
cinerin II	$C_{21}H_{28}O_5$	2.94	19.21	360	361 $[M + H]^+$ 100, 402 $[M + H + CH_3CN]^+$ 44, 378 $[M + H_2O]^+$ 31
pyrethrin II	$C_{22}H_{28}O_5$	3.09	19.41	372	373 $[M + H]^+$ 100, 390 $[M + H_2O]^+$ 20, 414 $[M + H + CH_3CN]^+$ 12
jasmolin II	$C_{22}H_{30}O_5$	3.36	20.51	374	375 $[M + H]^+$ 100, 416 $[M + H + CH_3CN]^+$ 27, 392 $[M + H_2O]^+$ 18
piperonyl butoxide	$C_{19}H_{30}O_5$	3.85	17.61	338	356 $[M + H_2O]^+$ 100, 377 $[M + K]^+$ 19

[a] log p values were calculated with CS ChemDraw Pro Cambridge Software Corporation, Cambridge, MA.

magnesium sulfate, and finally, 10 mL of acetonitrile were added. The tube was agitated for 5 min in a rotary shaker at 9 rpm (FALC Instrumentals, Bergamo, Italy) at room temperature, and 1 mL of the mixture was submitted for HPLC analysis.

Recovery Assays. A 50 μL aliquot of the pesticide solution at the desired concentration was added to each 5 g sample of untreated grapes, pears, eggplants, peppers, and tomatoes. The fortification levels used were 0.01, 0.02, 0.05, 0.10, 0.50, 1.0, and 5.0 mg/kg for fruits and vegetables, except for grapes, where fortification levels were 0.01, 0.04, 0.10, 0.20, 1.0, and 4.0 mg/kg. The samples were allowed to settle for 30 min prior to extraction. They were later processed according to the above extraction procedure. Four replicates of each matrix were analyzed.

RESULTS AND DISCUSSION

A good separation of pyrethrins, rotenoids, ryanoids, and PB was obtained by gradient analysis using acetonitrile/water (25:75, v/v) reaching in 15 min (80:20, v/v) and held in this condition for 25 min (**Figure 3**). Retention times for 9,21-dehydroryanodine, ryanodine, azadirachtin, 12aβ-hydroxyrotenone, rotenone, deguelin, cinerin II, pyrethrin II, PB, jasmolin II, cinerin I, pyrethrin I, and jasmolin I were 7.31, 7.61, 10.15, 13.81, 15.21, 15.71, 16.81, 17.11, 17.61, 17.91, 19.21, 19.41, and 20.51 min, respectively.

According to the UV spectra, biopesticides were detected at 270 nm for dehydroryanodine, ryanodine, and deguelin; 230 nm for azadirachtin, cinerin II, pyrethrin II, jasmolin II, cinerin I, pyrethrin I, and jasmolin I; and 295 nm for 12aβ-hydroxyrotenone, rotenone, and PB (**Figure 4**).

Calibration solutions were prepared by adding the appropriate amounts of standards to the extract obtained from untreated fruits and vegetables. Standard calibration curves were obtained by plotting analyte concentrations against peak areas. Good linearity was achieved between 0.01 and 5 mg/kg, with correlation coefficients between 0.9996 and 0.9999.

The pesticide extraction was performed with acetonitrile. It is noteworthy that no clean up was necessary because no interfering peaks were present in the analytical region of interest. The recovery data for tomatoes are summarized in **Table 1**. Mean recoveries ranged from 73 to 115%, with coefficients of variation between 1 and 14%.

Both the precision under conditions of repeatability and intermediate precisions were determined, by performing either

six injections of 0.05, 0.1, and 0.5 mg/L standards in the same day or six injections of the same standards in different days, respectively. The highest and the lowest coefficients of variation were 12.2 and 0.1% for repeatability and 12.9 and 1.1% for intermediate precision, respectively.

According to Their and Zimmer (*16*), the limit of quantitation (LOQ) was 0.01 mg/kg for ryanodine, 9,21-dehydroryanodine, rotenone, 12aβ-hydroxyrotenone, deguelin, and PB and 0.02 mg/kg for azadirachtin. LOQ for pyrethrins I and II was 0.05 mg/kg, and for the cinerin and jasmolin I and II series, LOQ was 0.1 mg/kg, with both LOQs referred to as total pyrethrins. These low detection limits were obtained as the result of the high sensitivity of the detector, which was due to a cell path length of 50 mm. Utilizing the DAD made it possible to know the sample spectra and the peak purity and confirm the active ingredients by overlapping the sample spectra with those of the standards. HPLC and LC/MS were the critical methods for compound identification, matching t_R values and APCI fragmentation patterns with authentic standards. The analysis performed in the positive APCI mode gave the fragments and abundances reported in **Table 2**. It is noteworthy that compounds belonging to the class II pirethrins gave $[M + H]^+$, $[M + H_2O]^+$, and $[M + H + CH_3CN]^+$ adducts; on the other hand, those belonging to the class I gave $[M + H]^+$ and $[M + H + CH_3CN]^+$ adducts.

Ryanodine and 9,21-dehydroryanodine gave the $[M - H]^+$ fragments followed by the loss of the pyrrole-2-carboxylic acid moiety and water: *m*/*z* 381 (100) $[M - C_5H_6NO_2]^+$, *m*/*z* 492 (45) $[M - H]^+$, and *m*/*z* 363 (15) $[M - C_5H_8NO_3]^+$ and *m*/*z* 379 (100) $[M - C_5H_6NO_2]^+$, *m*/*z* 490 (80) $[M - H]^+$, and *m*/*z* 361 (35) $[M - C_5H_8NO_3]^+$, respectively.

In conclusion, the multiresidue method proposed here is suitable to determine 11 biopesticides in fruit and vegetable matrixes. The use of acetonitrile as an extraction solvent allows quantitative determination without significant interferences. The method showed a good linearity, precision, and accuracy and is highly sensitive. The synergist PB and 12aβ-hydroxyrotenone, the main rotenone metabolite, can also be detected and quantified with this procedure.

ABBREVIATIONS USED

DAD, diode array detector; HPLC/MS, liquid chromatography/mass spectrometry; APCI, atmospheric pressure chemical ionization.

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