Analysis by HPLC of Ryanodine and Dehydroryanodine Residues on Fruits and in Ryania Powdery Wood

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A simple and rapid HPLC method to evaluate residues of the major ryanoids (ryanodine and dehydroryanodine) on three fruits (olives, apples, and pears) has been developed. The pesticides were extracted from the fruits with hexane and acetone solution (1:1, v/v). Cleanup was carried out with aminopropyl-bonded silica cartridges. This method is characterized by recovery >75%, precision <11% RSD, and sensitivity of 0.020 mg/kg. The method can also be used to determine the level of active ingredients in ryania powdery wood.

Keywords: *HPLC; analysis; Ryania speciosa; residues; ryanodine; dehydroryanodine*

INTRODUCTION

A nonsystemic botanical insecticide is obtained from the ground stems of *Ryania speciosa* Vahl, a native plant of tropical America. Ten ryanoids are extracted from stem wood at 0.14% (w/w). Ryanodine and dehydroryanodine (Figure 1) are the main alkaloids and account for 34−43 and 47−54% (w/w), respectively, of the total ryanoids. The biological activity is attributable to the two major constituents, the potencies of which are similar (*1*−*5*). The mode of action of *Ryania* differs from that of acetylcholine esterase inhibitors (organophosphate and carbamate). *Ryania* alkaloids affect muscles by binding to the calcium channels in the sarcoplasmic reticulum. This causes calcium ions to flow into the cells, and death ensues very rapidly (*6*). *Ryania* extracts are used to control citrus thrips on citrus, codling moth (*Cydia pomonella*) on apples and pears, and European corn borer (*Ostrinia nubilalis*) on corn (*7*). *Ryania* extracts have been used commercially since the 1940s. They reached maximum use in the United States with 400000 pounds of active ingredients in the 1950s. In contrast, by 1990, the use of *Ryania* declined and only ∼200 pounds per year were used. The recent development in organic farming (∼1 million ha in Italy and ∼540000 ha in Great Britain are currently dedicated to organic farming) has created a new interest for natural pesticides. Although *Ryania* was registered in the United States from 1968 to 1997, to our knowledge no residue data in food has ever been reported in the literature. Perhaps this was due to a lack of analytical methods to determine ryanodine and dehydroryanodine residues in food. In this paper an HPLC method to determine ryanodine and dehydroryanodine residues in fruit is described. We also report on the determination of these active ingredients in stem wood.

MATERIALS AND METHODS

Chemicals and Materials. Methanol, acetone, and hexane were of HPLC grade (Merck, Milan, Italy); water was distilled and filtered through a Milli-Q apparatus (Millipore, Milan, Italy) before use. Ryanodine (95% purity) and a mixture of ryanodine/dehydroryanodine (45−51%) were purchased from Sigma Aldrich (Steinheim, Germany). Stock standard solutions of the pesticides (∼200 mg/kg each) were prepared in methanol. Dehydroryanodine concentration was calculated according to its percentage in the mixture. Working standard solutions of the pesticides were prepared by diluting with the mobile phase (methanol/water, 25:75, v/v). Aminopropyl SPE cartridges (1 mL/100 mg) were purchased from Varian (Harbor City, CA).

Apparatus. An Agilent Technologies (Waldbronn, Germany) model 1100 liquid chromatograph was used, fitted with a diode array detector (DAD), model UV6000LP (Termo Quest, San Jose, CA). A Spherisorb S5 ODS1 (250 × 4.6 mm, 5 μm) column was employed.

LC Analysis. The gradient profile for the separation of two active ingredients was as follows: initial 25:75 (v/v) methanol/water, reaching 75:25 (v/v) in 18 min. Before the next injection can be made, the LC system must be stabilized for 10 min with methanol/water (25:75, v/v). The sample injection volume was 100 μL, and the flow rate was 1 mL/min. The analysis was performed at the wavelength of 270 nm.

Extraction Procedure from Powdery Stem Wood. A 0.1 g sample of *R. speciosa* ground powdery wood was weighed in a 40-mL screw-capped tube, and 10 mL of chloroform was added. Tubes were placed in an ultrasonic bath for 15 min at a temperature of 60 °C and then centrifuged for 5 min at 4500 rpm. A 1 mL aliquot was removed, and organic solvent was dried under a nitrogen stream, taken up with 1 mL of mobile phase (water/methanol, 75:25, v/v), and filtered with a 0.45 μm PTFE membrane filter. The resulting solution was ready for HPLC analysis.

Extraction Procedure from Fruits. *(a)* Ten grams of well-mixed chopped apples and pears was weighed in a 40 mL screw-capped tube, and 20 mL of a hexane/acetone (1:1, v/v) solution and 10 g of NaCl were added. The tube was agitated for 10 min in a rotary shaker, and 2 mL of the mixture was dried under a nitrogen stream and dissolved in 1 mL of chloroform.

(b) Twenty-five grams of whole olives was weighed in a screw-capped flask, and 50 mL of a solution of hexane/acetone (1:1, v/v) was added. The mixture was agitated in a shaker (Stuart Scientific) for 10 min. Two milliliters of the mixture was dried under a nitrogen stream, and the residue was taken up with 1 mL of $CHCl_3$.

Cleanup. The extract, dissolved in 1 mL of $CHCl_3$, was applied to an aminopropyl SPE cartridge, which was preconditioned by sequential elution with 2 mL of methanol and 4 mL of $CHCl_3$. The eluate was discarded and the cartridge dried under a nitrogen stream. Analytes were eluted with 1 mL of

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10.1021/jf010224g CCC: $20.00
Published on Web 06/06/2001

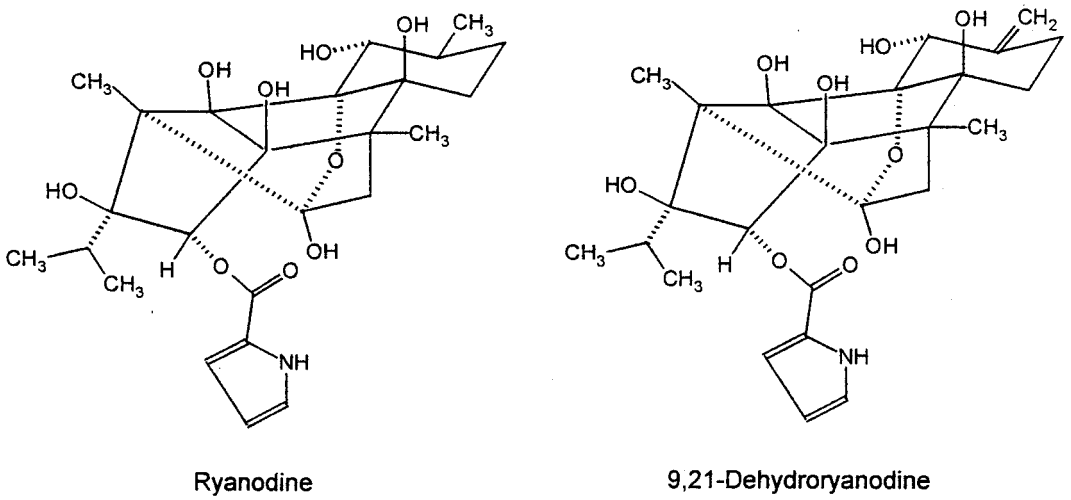

Figure 1. Structures of ryanodine and 9,21-dehydroryanodine.

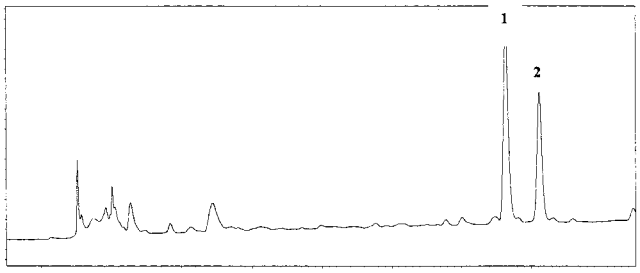

Figure 2. Chromatograms of ryanodine (peak 2) and dehydroryanodine (peak 1) in powdery stem wood under the operating conditions described in the text.

mobile phase (75% water/25% methanol). An aliquot of this eluate was used for HPLC analysis.

Recovery Assays. Samples of untreated olives, apples, and pears were fortified with the appropriate amount of the standard solutions to reach concentrations of 0.02, 0.05, 0.10, 0.50, and 1.20 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

Chromatography. Under isocratic conditions with different mobile phases of classic eluents (acetonitrile, methanol, and water), it was not possible to obtain a good separation of the two active ingredients. Gradient elution with methanol/water from a 25:75 initial to a 75:25 (v/v) final mobile phase in 18 min yielded a good resolution of ryanodine and dehydroryanodine (Figure 2). UV spectra were recorded during analysis with DAD. As expected, the spectra of the two compounds were very similar, because their structures differed only in the presence of a methylene, instead of a methyl, moiety and showed a maximum absorbance at 270 nm (Figure 3).

Linearity. Standard calibration curves of ryanodine and dehydroryanodine were constructed by plotting analyte concentrations against peak areas. Good linearity was achieved between 0.02 and 1.20 mg/kg with correlation coefficients of 0.9996 and 0.9998 for ryanodine and dehydroryanodine, respectively.

Cleanup. SPE cleanup was carried out using aminopropyl-bonded silica cartridges. Initial experiments showed that ryanodine and dehydroryanodine have a strong affinity for the aminopropyl-bonded silica phase.

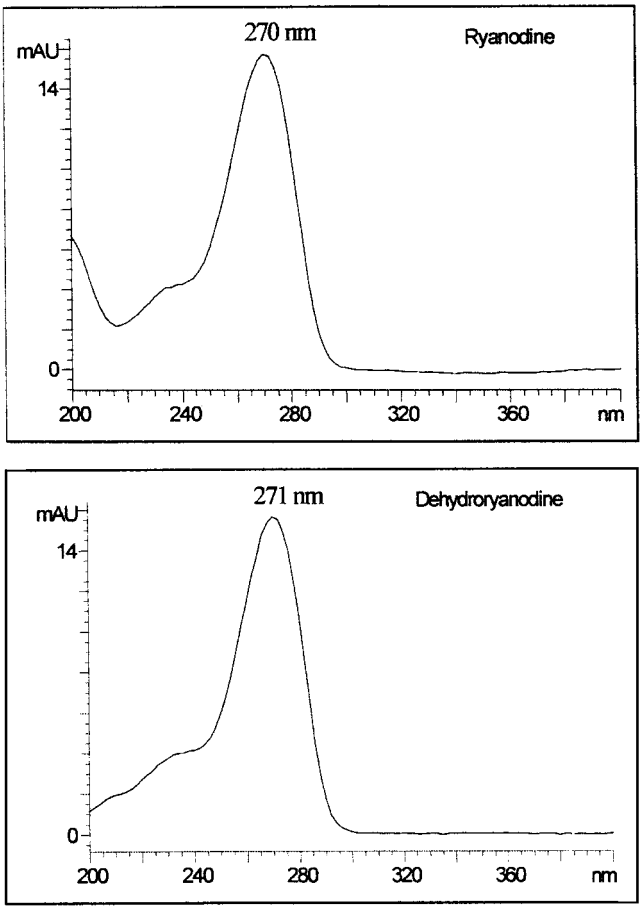

Figure 3. UV spectra of ryanodine and dehydroryanodine.

Aminopropyl-bonded silica retained the active ingredients contained in the $CHCl_3$ fruit extracts taken up while eliminating interfering compounds. The active ingredients were eluted using the initial mobile phase. Cleanup was efficient for all of the tested matrices (Figure 4).

Method Validation. Three matrices (apple, pear, and olive) were fortified with ryanodine and dehydroryanodine at 0.02, 0.05, 0.10, 0.50, and 1.20 mg/kg. Four replicate analyses were performed at each concentration. The recovery data are presented in Table 1. Recoveries ranged from 75 to 114%, with coefficients of variation between 1 and 11%. The limits of determina-

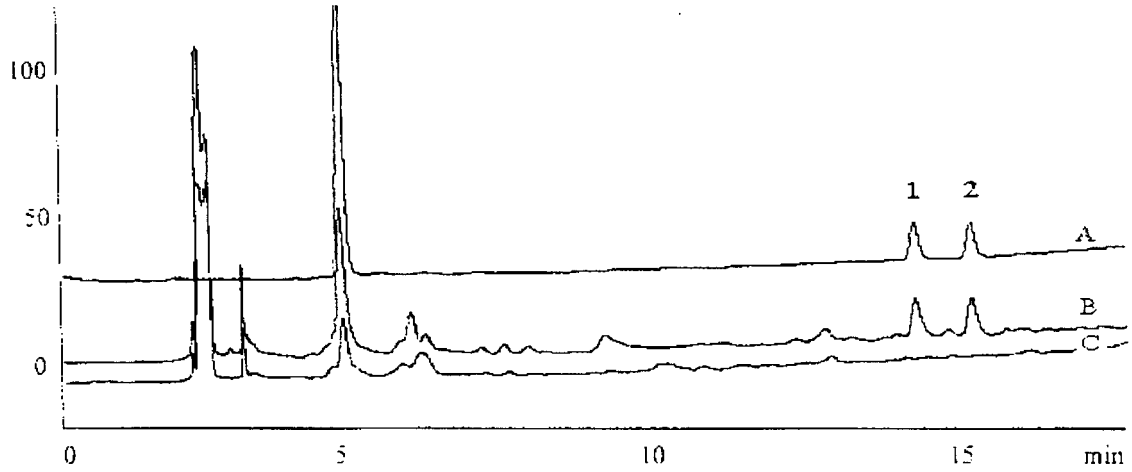

Figure 4. Chromatograms of extracts of pear under the operating conditions described in the text: (A) standard solution of ryanodine (peak 2) and dehydroryanodine (peak 1) at 0.1 mg/kg; (B) fortified sample at 0.1 mg/kg; (C) control.

Table 1. Recoveries (Percent ± RSD) of Ryanodine and Dehydroryanodine on Fruits

fruit	fortification level (mg/kg)	ryanodine	dehydroryanodine
pears	0.02	80 ± 6	81 ± 7
	0.10	114 ± 11	90 ± 10
	0.50	94 ± 2	81 ± 6
	1.20	97 ± 5	85 ± 4
apples	0.02	95 ± 11	103 ± 9
	0.10	83 ± 3	82 ± 3
	0.50	99 ± 1	80 ± 2
	1.20	100 ± 5	86 ± 3
olives	0.05	75 ± 10	84 ± 8
	0.10	79 ± 9	85 ± 10
	0.50	95 ± 5	102 ± 8
	1.20	103 ± 7	99 ± 6

tion for both active ingredients, according to Thier and Zeumer (*8*), were 0.02 mg/kg for pears and apples and 0.05 mg/kg for olives. 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. Due to the DAD used, it was possible to know the peak purity and confirm the active ingredients by overlapping the sample spectra with those of the standards.

Ryanodine and Dehydroryanodine in Powdery Stem Wood. This analytical method was used to determine the ryanodine and dehydroryanodine levels in powdery stem wood. Different extraction solvents were evaluated including chloroform, methanol, and ethanol. Chloroform was the solvent with the highest extraction power. Only one extraction with $CHCl_3$ was necessary to extract ryanodine and dehydroryanodine completely from powdery stem wood according to the above procedure because no ryanodine and dehydroryanodine residues were detectable in the second extract. No cleanup was necessary, because no interfering peaks were present at the retention times of the active ingredients (Figure 2). Concentrations of 690 and 487 mg/kg for ryanodine and 1315 and 725 mg/kg for dehydroryanodine were determined in two commercial samples. Dehydroryanodine was present in higher concentrations than ryanodine, accounting for 60−65% of the combined ingredients. These data were obtained from four replicates with an RSD <7%.

Conclusions. A residue method for the determination of ryanodine and dehydroryanodine was validated for three fruits: apple, pear, and olive. The procedures are simple, relatively rapid, and characterized by recovery >75%, precision <11% RSD, and sensitivity of 0.02 mg/kg. The method can also be used to determine the level of active ingredients in powdery wood.

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Received for review February 20, 2001. Revised manuscript received April 9, 2001. Accepted April 14, 2001.

JF010224G