Table III. Comparative HPLC and Microspectrofluorometry Techniques for Ferulic Acid Determination

sample[a]	HPLC ferulic acid, μg/g	rel fluorescence intens mean	std dev	std error
		Wheat 6		
1BK	15.5	33.84	2.01	.39
2BK	15.6	32.16	2.59	.52
grader	21.4	35.64	3.01	.60
3BK	47.6	44.64	4.10	.82
1M	11.7	30.80	2.39	.48
2M	16.1	34.20	3.28	.66
3M	29.8	39.40	4.31	.86
1MR	12.4	31.61	2.52	.50
4M	85.4	53.08	8.93	1.79
5M	348.5	77.96	11.39	2.28
		Wheat 7		
2BK	12.6	32.01	1.47	.29
4M	73.4	47.52	5.65	1.13
5M	396.9	76.08	9.98	1.99
		Wheat 8		
2BK	14.6	32.52	2.26	.45
5M	484.2	77.08	6.63	1.33
		Wheat 9		
5M	574.9	85.16	12.58	2.52

the high correlation between the two methods establishes the potential for development of rapid, sensitive assays of ferulic acid (and hence bran) content of flour streams using strictly optical systems. Furthermore, the extremely low ferulic acid content of the endosperm makes it possible to establish an absolute value for judging the purity of flours, while the sensitivity and specificity of the HPLC procedure provide an authentic basis for measuring bran carryover and for standardizing future rapid quality control methods for ferulic acid.

Registry No. Ferulic acid, 1135-24-6.

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Received for review November 12, 1986. Revised manuscript received May 27, 1987. Accepted January 11, 1988. Contribution No. 86-511-J, Kansas Agricultural Experiment Station, Manhattan. Contribution No. 935, Plant Research Centre, Agriculture Canada.

An Analytical Method for Residues of Imazalil in Tomatoes and Bell Peppers after Postharvest Application and Storage

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A method using gas–liquid chromatography with electron capture detection was developed for residues of the fungicide imazalil in tomatoes and bell peppers. Recoveries from fortified controls ranged from 90.1 to 95.7% for tomato samples and from 73.6 to 79.2% for pepper samples. Both fruits were dipped in aqueous solutions containing 50, 100, or 250 mg/L imazalil, and the residues were determined after various storage times. The fruits were stored at 10 °C for 3 days and 21 °C thereafter to simulate commercial shipping and storage. Residues of imazalil in tomatoes for the three concentrations of dip were 0.24, 0.45, and 0.84 mg/kg 2 h after dipping and 0.13, 0.25, and 0.53 mg/kg after 15 days of storage. Similarly, residue levels in bell peppers were 0.51, 0.55, and 1.51 mg/kg 2 h after dipping and 0.05, 0.11, and 0.28 mg/kg after 12 days of storage.

The use of imidazole-based compounds to control many fungal and some bacterial disorders in both agricultural and medical fields has steadily increased in recent years. Medical uses of imidazole containing antifungal drugs have recently been reviewed by Beggs et al. (1981). The present work concerns the agricultural uses of one imidazole-based compound, imazalil, 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole, which has been shown to have remarkable activity on penicillium citrus fruit rots and significant activity on many other fruit rots such as alternaria rot, stem end rot, and sour rot (Laville et al., 1977). Subsequently imazalil has been found to be effective in controlling postharvest alternaria rot of tomatoes and peppers (Spalding, 1980). The analysis of imazalil by gas–liquid chromatography (GLC) and high-pressure liquid chromatography (HPLC) has been reported by several

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authors. The analysis of imazalil in citrus using GLC with electron capture (EC) and flame ionization detectors (FID) has been reported by Wynants (1977), who used a hexane–isoamyl alcohol (5:95) mixture for extraction and an acid–base cleanup procedure, and by Greenberg and Resnik (1977), who extracted with acetonitrile and used a Florisil column cleanup. Subsequent to this work, Stein et al. (1981) assayed imazalil in grapefruit using a modification of Wynants method. A GLC method was reported for the simultaneous determination of imazalil and thiabendazole (Camps Alemany et al., 1980). Reversed-phase HPLC analyses of imazalil using acetonitrile admixed with aqueous phosphate buffers have been described (Norman and Fouse, 1978; Wynants, 1979).

Tolerances for imazalil residues have been established for citrus fruit, citrus products, meat products, and bananas by the U.S. Environmental Protection Agency (1983). Imazalil residue data are needed for peppers and tomatoes treated to control fungal decay to aid in the establishment of tolerance levels for residues in these fruit. We report a simplified and reproducible GC–ECD method for the assay of imazalil in these substrates based on modification of the Wynants (1977) method.

EXPERIMENTAL SECTION

Reagents. Imazalil (No. ZR23979) used for the analytical standards was provided by Janssen Pharmaceutica, 501 George St., New Brunswick, NJ 08903. The standard was colorless and exhibited no extraneous peak upon EC-GC analysis. Technical-grade material is specified to be 98–99% pure with a light brown color. A sample of a commercial formulation, also provided by Janssen Pharmaceutica, containing 50% imazalil and emulsifiers was used to prepare solutions used to dip fruit. Sodium chloride (AR), anhydrous sodium sulfate (AR), sulfuric acid (AR), sodium hydroxide pellets (AR), and nanograde benzene were products of Mallinckrodt, Inc. Because of the hazards of working with benzene, we recommend, and currently use, toluene as an extraction solvent for imazalil residues. Solutions of sodium hydroxide (50 g/L) and sulfuric acid (5 g/L) were prepared with distilled water. None of the reagents exhibited interferences for the gas chromatographic conditions and residue concentration levels encountered.

Equipment. A Hewlett-Packard Model 5700 gas chromatograph equipped with a linear ^{63}Ni electron capture detector and a $^{1}/_{16}$-in. stainless steel transfer line from the column to the detector was used for the imazalil assays. A 4 mm (i.d.) × 44 cm glass column packed with 20% OV-17 on 80–100 mesh Gas-chrom Q was used with the following conditions: carrier gas, 5% methane in argon at 80 mL/min flow,; injection port, 250 °C; oven temperature, 260 °C; detector temperature, 300 °C. Injections of 2 μL were made with use of a 10-μL Hamilton syringe equipped with a Chaney adapter.

Treatment. Mature green tomatoes (Flora-Dade cultivar) were dipped for 10 s and mature green peppers for 30 s in aqueous solutions of 0, 50, 100, or 250 mg/L of imazalil. Fruits were drained of excess liquid, placed in plastic trays, and loosely covered with polyethylene bags. Fruits were then held for approximately 2 weeks—the first 3 days at 10 °C and the remaining time at 21 °C—to simulate shipping and marketing conditions (Spalding and King, 1980).

Analytical Procedures. The whole pepper or tomato was weighed, quartered, and placed in a 500-mL Eberbach blending container. The container was sealed with a Teflon-lined lid and the sample blended on low speed of a two-speed Waring blender. A 20-g aliquot of blended sample was weighed into a 25 mm × 150 mm Kimble culture tube. The sample was then adjusted to ca. pH 10.5 by the addition of 1 or 0.5 mL of NaOH solution (50 g/L) for tomato and pepper samples, respectively. The tube was then sealed with a Teflon-lined cap, shaken vigorously for about 30 s, and then allowed to settle for 30 min. A 20-mL portion of benzene was then added and the culture tube shaken by hand to evenly distribute the benzene but not vigorously enough to create a complete emulsion. Tomato samples were then mechanically shaken at about 80 excursions/min for 60 min in a Dubnoff shaking incubator at ambient temperature and then centrifuged at 1000 rpm for 10 min. This procedure was found to result in low recoveries of imazalil in pepper samples so the following procedure was used: 2 g of NaCl was added to pepper samples that were then gently shaken by hand, mechanically shaken for 30 min, and centrifuged for 10 min. If emulsions remained after the samples were centrifuged, 1–2 g of NaCl was added to each tube, gentle hand shaking was then used to break the emulsion, and the samples were recentrifuged. For cleanup, a 3-mL protion of the benzene layer was transferred, via a volumetric pipet, to a 16 mm × 100 mm culture tube containing 5 mL of a 0.5% aqueous solution of sulfuric acid of pH ca. 1.5. The tube was then manually shaken for 30 s and centrifuged at 1000 rpm for 5 min. The benzene layer was removed by pipet and discarded. The tube was then placed in a Thermoline Dri Bath at 35 °C and a stream of dry nitrogen used to evaporate the remaining benzene. A 3-mL portion of benzene and 0.5 mL of 5% NaOH were added, with caution because of possible spattering, to the cooled culture tube, and the mixture was vigorously shaken for 30 s. The layers were allowed to separate; the benzene layer was removed, dried over anhydrous sodium sulfate, and assayed by GC-ECD.

Table I. Recovery of Imazalil from Fortified Tomato and Bell Pepper Samples[a]

imazalil added, mg/kg	imazalil recd, mg/kg	% recovery
	Tomatoes	
1.250	1.169 ± 0.014	93.5 ± 1.1
0.700	0.667 ± 0.009	95.7 ± 1.4
0.125	0.112 ± 0.00	90.1 ± 3.0
0.070	0.067 ± 0.00	93.0 ± 1.4
	Peppers	
2.000	1.585 ± 0.400	79.2 ± 2.0
0.900	0.705 ± 0.031	78.3 ± 3.4
0.070[b]	0.052 ± 0.001	73.6 ± 0.7

[a] Three replicates at each level were assayed to obtain the means and standard deviations shown. [b] Acid–base cleanup was used for these samples.

RESULTS AND DISCUSSION

Efficiencies of extraction, shown in Table I, were determined by blending a whole fruit and taking four 20-g portions for three spiked replicates and one control. Standards were prepared in acetone so that 5 μL of solution would give the desired amount of imazalil to fortify the spiked samples. After 5 μL of the appropriate imazalil solution, or pure acetone in the case of controls, was added, the samples were shaken, allowed to equilibrate for 60 min, and then extracted and assayed as previously described. Recoveries were determined by comparison of samples to 20-mL portions of benzene fortified with the same amount of acetone standard. Good recovery and reproducibility were obtained for imazalil in both tomatoes and peppers. Tomato samples, for example, spiked at levels of 0.07–1.25 mg/kg gave relative standard deviations (RSD) ranging

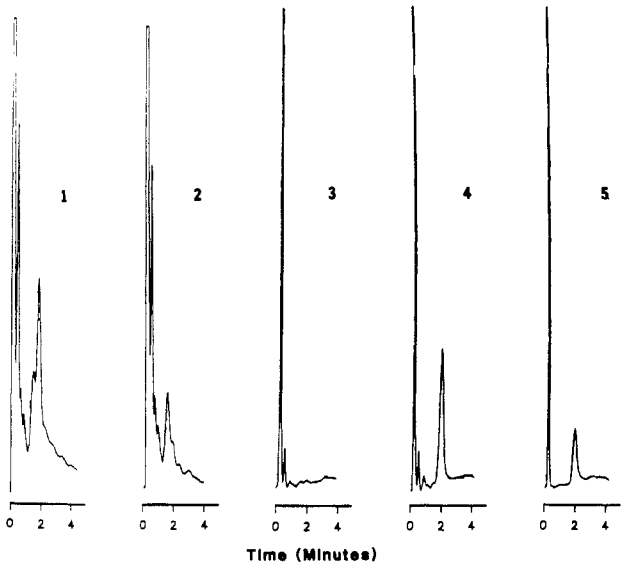

Figure 1. Typical chromatograms of imazalil residues in bell pepper samples: samples 1 and 4, spiked with 1.4 mg/kg imazalil; samples 2 and 3, unspiked controls. An acid–base cleanup was used for samples 3 and 4 but omitted for samples 1 and 2. Sample 5 was a 0.025 mg/L imazalil standard in benzene, which is equivalent to 0.025 mg/kg in a sample.

Table II. Imazalil Residues (mg/kg) in Tomatoes after Dip and Storage[a]

time, days	imazalil dip concentration		
	50 mg/L	100 mg/L	250 mg/L
0[b]	0.24 ± 0.00	0.45 ± 0.07	0.84 ± 0.23
3	0.18 ± 0.01	0.47 ± 0.08	0.84 ± 0.14
9	0.16 ± 0.03	0.34 ± 0.04	0.64 ± 0.14
15	0.13 ± 0.03	0.25 ± 0.07	0.53 ± 0.20

[a] Three individual fruits were assayed to obtain the means and standard deviations listed. The fruit was stored at 10 °C for 3 days and 21 °C for the remainder of the test. [b] Two hours after dip.

from 1.11 to 2.96% and average recoveries of 90.1–95.7% for three replicates at each level. Similarly, recoveries from spiked peppers ranged from 73.6 to 79.2% with the RSD range of 0.67–1.98%. Reduced recoveries from peppers may be due to an interaction between unsaturated fatty acids and substituted imidazoles as suggested by Beggs et al. (1981) in a review of the antifungal properties of imidazoles.

Five typical chromatograms from pepper samples, as described above, are shown in Figure 1. Chromatograms 1 and 4 are from samples spiked with 1.4 mg/kg imazalil and chromatograms 2 and 3 are from control samples. Chromatograms 3 and 4 are from samples carried through the entire cleanup procedure whereas 1 and 2 are from samples with the acid–base cleanup step omitted. Chromatogram 5 is from a 0.025 mg/L imazalil standard in benzene, which is equivalent to 0.025 mg/kg in a sample. Comparison of chromatogram 3, from a control sample with acid–base cleanup, with the peak of the standard in chromatogram 5 indicates that the interferring peak is equivalent to about 0.002 mg/kg of imazalil residue, and therefore the lowest detectable level, based on twice the background interference, is 0.004 mg/kg.

Results obtained from the treatment and storage of fruit are given in Tables II and III. Residues in pepper sampled 2 h after dipping were higher than in the corresponding tomato samples. This is likely due to the high wax content of the surface of the pepper peels and the higher surface area to mass ratio of peppers compared to tomatoes. After

Table III. Imazalil Residues in Bell Peppers after Dip and Storage[a]

time, days	imazalil dip concentration[b]		
	50 mg/mL	100 mg/mL	250 mg/mL
0[c]	0.516 ± 0.073	0.553 ± 0.223	1.512 ± 0.123
3	0.398 ± 0.077	0.608 ± 0.097	1.348 ± 0.271
6	0.254 ± 0.052	0.247 ± 0.015	0.656 ± 0.068
2	0.046 ± 0.021	0.113[d]	0.277[d]

[a] After dipping, the fruit was stored at 10 °C for 3 days and then at 21 °C to simulate commercial shipping and storage. [b] Two individual fruit were assayed to determine means and standard deviations. The residue concentrations are expressed in milligrams/kilogram. [c] Samples were taken 2 h after dip to allow the fruit to dry. [d] Only one fruit run due to poor condition of second.

6 days for peppers and 15 days for tomatoes the residues had decreased to about half that present at 2 h. After 12 days of storage, imazalil residues in bell peppers decreased to about 9–20% of the original amount to levels ranging from 0.05 to 0.28 mg/kg. During similar storage of tomatoes for 15 days imazalil residues decreased only to about 53% of the initial value to levels ranging from 0.13 to 0.53 mg/kg. The greater loss of residue in bell peppers, compared to tomatoes, may be real or could be due to the formation of bound residues that were not extracted and therefore not determined by the method used for these assays. In any case, these results indicate that when samples reach consumers, more than half the initial amount of residue will likely remain. Since we had neither labeled imazalil nor equipment for scintillation counting, no investigation of bound residues, metabolites, or degradation products of imazalil was conducted.

Because of the problems encountered in the analysis of imazalil residues, some discussion of the method is warranted. The use of several column packings (i.e. 3% OV-17, 5% OV-210, and 3% OV-25 on Gas-Chrom Q or Chromosorb WHP) resulted in tailing and lack of reproducibility of peak heights or areas. Because this problem is likely due to degradation caused by active sites, a heavily loaded support, 20% OV-17 on Gas-Chrom Q, was selected. Under the GLC conditions previously described, symmetrical peaks were obtained with a retention time of about 1.8 min. Standards were injected before each sample until a constant response was obtained and after each sample to ensure reproducibility. Syringes were cleaned between samples with alcoholic KOH and rinsed with benzene to eliminate previously reported (LeBel and Williams, 1979) contamination problems. This was especially important when switching from high- to low-concentration solutions.

ACKNOWLEDGMENT

We are grateful to William F. Reeder for technical assistance.

Registry No. Imazalil, 35554-44-0.

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Received for review June 1, 1987. Accepted January 14, 1988. Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

Determination of Both Tannin and Protein in a Tannin–Protein Complex

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A method for determination of both protein precipitation capacity of tannins and protein-precipitable phenolics and the ratio of protein to tannin in a tannin–protein complex is presented. The relationships of both bovine serum albumin (BSA) precipitated and the protein-precipitable phenolics with tannic acid and the ratio of BSA and tannins in the protein–tannic acid complex agreed well with those obtained from the methods already available. The above parameters were also determined for *Quercus incana* leaves at different stages of maturation. The protein precipitation capacity and protein-precipitable tannins decreased substantially with maturation, whereas the ratio decreased slightly. As the present method allows determination of these parameters from a tannin–protein complex, it is convenient, takes less time, can handle a large number of samples at a time, and requires less tannin-containing extracts and chemicals compared to other available methods.

Protein-precipitation methods for quantitation of tannins involve the formation of a tannin–protein complex. The method of Hagerman and Butler (1978) measures tannins, and those of Makkar et al. (1987) and Marks et al. (1987) measure proteins in the complex. Not much attention has been given to another parameter, i.e., the ratio of protein to tannin in a tannin–protein complex, probably because of nonavailability of simple methods to quantitate protein in a tannin–protein complex. This ratio represents the amount of protein bound by an unit of phenolics/tannins in the complex. This could provide valuable information regarding changes in the nature of tannins, vis-à-vis their protein-binding capacity associated with, for example, development, maturity (Makkar et al., 1988), and postharvest storage of foods and feeds. In addition, insight can be had into the differences in the nature of tannins that bind proteins in bird- and mold-resistant and susceptible varieties. One way of obtaining the protein to tannin ratio in the complex is to measure protein in a complex (Makkar et al., 1987) and tannins (Hagerman and Butler, 1978) in another complex formed under the identical conditions. This takes more time and labor and requires more chemicals and extracts of the sample containing tannins. The present paper reports simple modifications of the methods of Hagerman and Butler (1978) and Makkar et al. (1987), which enable measurements of both tannin and protein in a complex. Besides alleviating the above-mentioned problems associated with separate measurements of tannins and proteins, a large number of samples can be handled and the variation in the protein to tannin ratio is expected to be smaller by the present method. The utilization of *Quercus incana* leaves as feed for ruminants is a common practice in hilly regions of India and various other countries (Makkar et al., 1988). The protein precipitation capacity, protein-precipitable phenolics, and ratio of protein to tannin in protein–tannin complex were measured for *Q. incana* leaves at different stages of maturation by the method reported here.

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MATERIALS AND METHODS

Materials. Chemicals and equipment were the same as described earlier (Makkar et al., 1987).

The leaves of oak (*Q. incana*) were collected from a tree in the vicinity of the Research Station. On leaf sprouting, the first collection of leaves (∠4 days old) was made in April 1987. The collected leaves were divided into two groups of sizes 3–5 and 6–9 cm. Next collections were made 3 and 10 days after the first collection. Two more collections were made at an interval of 14 days. The old leaves (aged leaves), which were about to fall after the sprouting of new leaves, were also collected. The leaves were dried at 50 °C.

Methods. The extracts were prepared as described by Martin and Martin (1982).

Preparation of the Complex and Solubilization. The protein–tannin complexes were formed as described earlier (Makkar et al., 1987). The complex was dissolved in 1.5 mL of sodium dodecyl sulfate (SDS) (1% (w/v) in distilled water).

Assay of Tannins in the Complex. The method of Hagerman and Butler (1978) was used with slight modi-

0021-8561/88/1436-0523$01.50/0