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HPLC Determination of Thiamin and Riboflavin in Soybeans and Tofu

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A high-performance liquid chromatographic (HPLC) methodology has been developed to determine thiamin and riboflavin in soy products. An aqueous reversed-phase C_{18} system with fluorescence detection was employed. Sample preparation included acid hydrolysis and an oxidation step to produce thiochrome from thiamin. Fluorescent chromatograms indicated the presence of many other fluorescing compounds in addition to the vitamins. Thus, vitamin contents of soy products are less than reported in the literature with use of the AOAC methods. Consistent recoveries of 84% for thiamin and 95% for riboflavin were routinely obtained in whole soy flour samples. The contents ranged from 6.26 to 6.85 and 0.92 to 1.10 $\mu g/g$ for thiamin and riboflavin, respectively, in three soybean varieties. Examination of the vitamin distribution on the processing of tofu revealed that the ranges of retention of thiamin and riboflavin were 7.6-15.7% and 11.7-21.1%, respectively. Retention of these water-soluble vitamins in tofu stored in water indicated remarkable losses due to leaching.

The growth of the soy foods industry and the advent of labeling requirements have necessitated the quantitation of the vitamins thiamin and riboflavin in soy products. Attempts to use the standard Association of Official Analytical Chemists (AOAC) method (AOAC, 1984) for the quantitation of riboflavin were not successful, especially because of the interference caused by nonvitamin compounds. Not only did the sample analyzed fluorometrically have a high background fluorescence but the fluorescence also kept changing and did not reach an equilibrium in a reasonable time. The standard AOAC method for the analysis of thiamin is lengthy and involves the use of an enzyme hydrolysis step. Extraction into organic phase and cation-exchange column purification lengthens the procedure and may impart inaccuracy (Pippen and Potter, 1975). Enzymes such as clarase and takadiastase impart fluorescing interferences to the analysis of riboflavin in the same sample extract. Compounds coeluting and cofluorescing with riboflavin are reported to be present in these enzymes (Egberg and Potter, 1975; Edijala, 1979).

Vitamins thiamin and riboflavin are quantitated in food samples by ultraviolet (UV) absorption of thiamin and riboflavin and the fluorescence of thiochrome and riboflavin. The high content of proteins and phospholipids (Smith and Circle, 1978) in soy systems ruled out the use of UV detection because of its low selectivity and sensitivity. Using UV detection techniques, Ayi et al. (1985) were not able to detect significant levels of thiamin in unfortified soy products. The use of manual and automated nonchromatographic methods for thiamin and riboflavin was not promising because of their inability to isolate the vitamin from other interfering components before fluorometric observation. Thus, development of a rapid, accurate, and adequately sensitive method for the analysis of these vitamins, thiamin and riboflavin, in soy products was necessitated.

The method presented in this paper was used to examine the vitamin distribution in the processing of soybeans to tofu. The stability of these vitamins on refrigerated storage of tofu in water was examined. Attempts were made to account for the observed loss of thiamin and riboflavin from tofu on storage. USDA Agriculture Handbook 456 (Adams, 1975) reports the thiamin and riboflvain content of whole soyflour, soy milk, and tofu. The handbook data reported were the average vitamin contents of two samples of unknown soybean varieties. The possible varietal differences in the content of these two vitamins were examined in our study.

MATERIALS AND METHODS

Soybeans. Three soybean varieties (Prize, Vinton, Weber) grown in 1985 in Hudson, IA, were donated by Strayer Seed Co., Hudson, IA. Soybeans were ground in a coffee and spice mill to pass a No. 60 sieve and used in the vitamin analysis.

Tofu Manufacture. The traditional method of tofu manufacture was adapted from Johnson (1984). Soybeans (900 g) were soaked and ground with 6 L of tap water in a Cherry-Burrell vibroreactor. The slurry was cooked at 95 °C for 7 min together with 1 L of water in a steam-jacketed kettle. The cooked slurry was filtered through two cloth sacks. The residue (okara) was washed with 1 L of water. A 20-mL aliquot of filtrate was removed to measure the solids content by using the light-scattering technique of Johnson and Snyder (1978). Upon adjustment of the solids content to 5%, the combined filtrates were reheated to 85 °C and calcium sulfate was added at a concentration of 0.018 N for coagulation. After a 5-min coagulation, the coagulum was cut and poured into a cheesecloth-lined, stainless steel box with perforations on all sides. The coagulum was pressed for 15 min. The resulting tofu was stored at 4 °C in a

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Table I. Recovery Data for Thiamin and Riboflavin

fraction	quantity used[a]	% recovery thiamin[b]	% recovery riboflavin[c]
okara	5.000 (g)	63 ± 5	71 ± 4
whey	5.0 (mL)	98 ± 8	82 ± 11
tofu	10.00 (g)	43 ± 1	48 ± 1
whole bean	0.500 (g)	84 ± 3	95 ± 7

[a] Preceding chromatographic analysis the solution was diluted to 25 mL. Three samples per level of vitamin spiking were analyzed. [b] Amounts of recovery material used were 1.50, 2.00, and 2.50 μg of thiamin/sample quantity indicated. [c] Amounts of recovery material used were 0.20, 0.25, and 0.30 μg of riboflavin/sample quantity indicated.

water-filled plastic container. The weight of okara, whey, and tofu was obtained, and samples of okara and whey were removed for analysis. Moisture analysis was performed according to AOAC Method 14.081 (AOAC, 1984).

High-Performance Liquid Chromatography. The chromatographic system consisted of a reversed-phase 4.6 × 150 mm, 5-μm Ultrasphere (Beckman) C_{18} column with a 3-cm RP-18 guard column (Brownlee Laboratories). The mobile phase was acetonitrile–0.01 M acetate buffer (13:87), pH 5.5, metered at a flow rate of 1.2 mL/min. A 250-μL sample loop was used. A 5-min washing step at 2.3 mL/min was employed with acetonitrile–water (90:10) to regenerate the column after 6 or 10 min of mobile phase for thiamin and riboflavin, respectively. The column was reequilibriated in the mobile phase for 7 min before the next run. A Beckman/Altex Model 420 microprocessor/controller, a Beckman Model 110A pump connected to two solvent ports via a three-way stream switching valve, and a solenoid interface (Rainin Instruments, Inc.) were utilized to maintain the solvent program. Thiamin hydrochloride, thiamin monophosphate, and riboflavin were purchased from Sigma Chemical Co. Standard solutions were prepared as in the AOAC method (AOAC, 1984). Riboflavin was monitored fluorometrically in a Turner Model 112 fluorometer equipped with a 7.5-μL continuous-flow cell, using a No. 47B (436-nm, narrow-pass) excitation filter and a No. 16 (535-nm, sharp-cut) emission filter. Thiamin was oxidized to thiochrome in a precolumn process. A 2.5-mL sample aliquot was treated with 2.5 mL of 1% $K_3Fe(CN)_6$ in 15% NaOH and neutralized with 0.375 mL of concentrated H_3PO_4 after 45 s. Thiochrome was monitored fluorometrically by using a No. 7-60 (364-nm, narrow-pass) excitation filter and a No. 47B emission filter.

Sample Preparation. Suitable aliquots (Table I) of dry samples were hydrated in H_2O at 4 °C for 10 h. The hydrated soy flour and okara were heated at 90 °C for 30 min. The medium was adjusted to pH 2 with 5 N HCl and autoclaved at 20 psi for 15 min. The pH of the cooled samples was increased to 4.5 by 1 N NaOH, centrifuged at 12 000 rpm for 5 min at 20–25 °C, and filtered through Whatman No. 41 filter paper. The volume was brought to 25 mL with distilled water, refiltered through 0.45-μm membrane filter (Fisher Scientific), and used in the quantitation of riboflavin via HPLC or subjected to oxidation for thiamin analysis followed by HPLC analysis. The peak heights (Snyder and Kirkland, 1979) were used in the quantitation.

Identities of the peaks of interest were determined by comparison with retention times of standards and by spiking samples with standards. The purity of the peak was established by destroying the riboflavin ($Na_2S_2O_4$ reduction) or by preventing the oxidation of thiamin to thiochrome (benzenesulfonyl chloride) (Soliman, 1981). Recovery efficiency was analyzed by spiking samples before hydration at three different levels for each vitamin. Data obtained were compared with those obtained by using the AOAC method for riboflavin and thiamin (AOAC, 1984).

Statistical analysis of the data was performed with the SAS (1985) package. The means of vitamin concentrations were compared by using Fisher's least significant difference test. First-order rate constants were derived from linear regression equations.

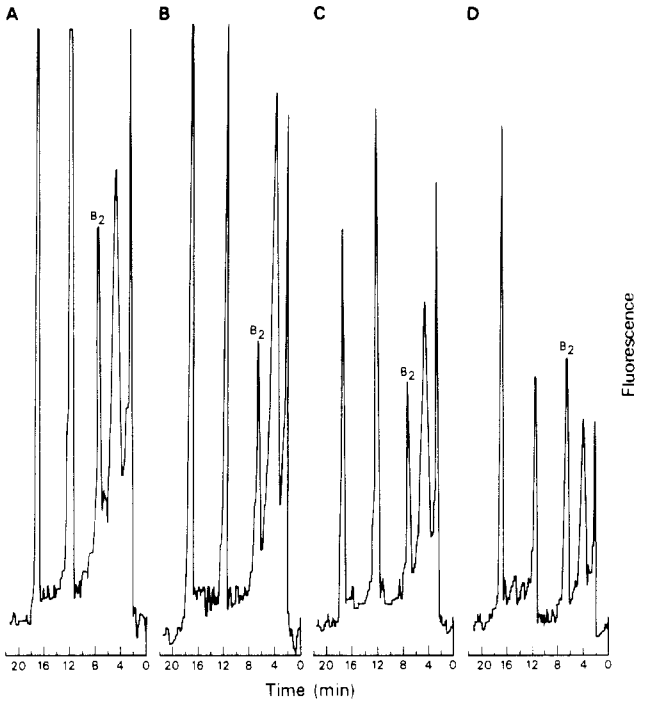

Figure 1. Reversed-phase (C_{18}) HPLC separation of riboflavin in whole soy flour (A), okara (B), whey (C), and tofu (D) sample extracts of Vinton soybeans. Retention time for riboflavin was 7 min. The column-washing step was initiated at 10 min.

Vitamin Distribution and Storage Studies. Okara, whey, and tofu samples were analyzed for the thiamin and riboflavin content as already described. The fractionation of these vitamins was evaluated by using the weight and percent recovery of each sample. Tofus of all three varieties were stored at 4 °C, sampled, and analyzed daily over a 2-week duration. The apparent first-order rate of loss was calculated, and the shelf-life was estimated as based on 20% loss of thiamin and riboflavin from freshly prepared tofu. A block of Vinton tofu was cut into 16 equivalent cubes and stored separately in 100 mL of water each at 4 °C. These tofu cubes and the leach water were analyzed daily over a 10-day duration for both vitamins.

RESULTS AND DISCUSSION

Most literature values for thiamin content in soy products were obtained by the AOAC thiochrome method. The literature values for thiamin were in the same range as those obtained in our study (Ayi et al., 1985; Sebecic and Vedrina-Dragojevic, 1986; Miller et al., 1952; Heneberg et al., 1983; Abdullah and Baldwin, 1984; Campbell et al., 1985). Previously reported riboflavin analyses in soy products were based on either the standard AOAC method or the lumiflavin method (Egberg and Potter, 1975; Miller et al., 1952; Watson, 1976; Heneberg et al., 1983; Abdullah and Baldwin, 1984; Campbell et al., 1985; Vedrina-Dragojevic and Sebecic, 1986). The standard AOAC method of analysis had considerable interference in soy products because of the high background fluorescence (Figure 1) from proteins and phospholipids (Smith and Circle, 1978). The lumiflavin method involves a high-pH treatment that has the potential of causing further degradation of lumiflavin (Wagner-Jauregg, 1972). Literature values for riboflavin content are much higher than observed in our study.

The method reported here is simple, rapid, and adequately sensitive for a soy system. The low concentration of these vitamins in soy products necessitated the operation of the fluorometer at greatest sensitivity (×30). The minimum detection limit of thiamin and riboflavin

Table II. Detectable Thiamin and Riboflavin Contents in Whole Soy Flour

variety	μg B_1/g sample[a] (wet wt)	μg B_2/g sample (wet wt)	% moisture
Prize	6.68 ± 0.11[p]	0.92 ± 0.03[x]	9.73
Vinton	6.85 ± 0.01[p]	0.96 ± 0.01[x]	10.99
Weber	6.26 ± 0.05[q]	1.10 ± 0.05[y]	10.79

[a] Value indicated as micrograms of thiamin/gram of sample (wet weight). (p, q, x, y) Values followed by the same superscript letter are not significantly different with a 5% level of significance.

was 5 and 2 ng/mL of sample filtrate when a 250-μL loop was used. Hydration overnight for all samples and additional heat treatment for soy flour and okara ensured better release of these protein-bound vitamins. A 0.1 N HCl medium and 15-min autoclaving duration efficiently hydrolyzed the vitamins with minimum levels of browning. Increasing the hydrolysate to pH 4.5 was adequate to isoelectrically precipitate most of the proteins. Prior adjustment to pH 6.0 was not necessary (Bechtel, 1962). Centrifugation before filtration enabled efficient filtration.

Acetate was preferred over phosphate as the buffer in the mobile phase. Acetate buffered better at lower pH (Snyder and Kirkland, 1979) and eluted riboflavin as a sharper peak as compared with a broader peak observed with phosphate buffers. Incorporation of 13% acetonitrile and precise adjustment of the mobile phase to pH 5.5 optimized the resolution of riboflavin. Upon elution of the vitamin peak, a solvent step program was necessary to elute nonpolar compounds from the column. The column was equilibriated in the initial eluting solvent before injection of the next sample. A reversed-phase C_{18} column yielded adequate resolution of these vitamins (Abdullah and Baldwin, 1984; Skurray, 1981; Fellman et al., 1982; Finglas and Faulks, 1984; Mauro and Wetzel, 1984; Wehling and Wetzel, 1984; Wills et al., 1985). Neutralization of the oxidized thiamin by concentrated H_3PO_4 ensured a pH level acceptable to the C_{18} column and eliminated possible pH-dependent alkaline degradation of thiochrome to its disulfide (Fellman et al., 1982). Three different concentrations of each vitamin were analyzed daily along with the samples. Linearity was observed in the chromatographic response to the concentration of the vitamin in the sample analyzed. The linear regression equation of the calibration curve obtained for thiamin hydrochloride was $y = 110.27x + 0.05$ ($r = 0.997$), and that for riboflavin was $y = 627.46x + 0.33$ ($r = 0.990$) (y = peak height, x = concentration of the vitamin in micrograms per gram). Duplicate analyses were performed on three whole soy flour samples of each soybean variety. Good reproducibility of data was indicated by the low standard deviations observed. The thiamin and riboflavin contents in whole soy flour were as shown in Table II. Total chromatographic analysis times of 18 and 22 min for thiochrome and riboflavin were observed, as illustrated in Figures 2 and 1.

Simultaneous determination of these vitamins was not attempted because of the lack of another fluorometer. Because all the chromatographic conditions (except the fluorometric filters and the oxidation of thiamin) used were the same for both vitamins, we suggest the possibility of simultaneous detection by using a postcolumn oxidation step and a second fluorometer (Fellman et al., 1982; Finglas and Faulk, 1984; Mauro and Wetzel, 1984; Wehling and Wetzel, 1984; Wills et al., 1985; Wimalasiri and Wills, 1985). Chromatographic separation prior to detection enabled the omission of $KMnO_4$–H_2O_2 treatment in the analysis of riboflavin and enzyme treatment, Decalso column purifi-cation, and isobutyl alcohol extraction in the analysis of thiochrome. $KMnO_4$–H_2O_2 is known to quench the fluorescence of riboflavin (Woodrow et al., 1969; Rashid and Potts, 1980; Roy et al., 1976). Elution of thiochrome from a Decalso column has been reported to be incomplete (Pippen and Potter, 1975). An attempted hydrolysis step with enzyme takadiastase failed to increase the yield of thiochrome and caused fluorescent interference of riboflavin in the sample extract. Thiochrome monophosphate coeluted with thiochrome. Thus, the peak representing thiochrome was considered as the sum of the vitamin B_1 present in the sample.

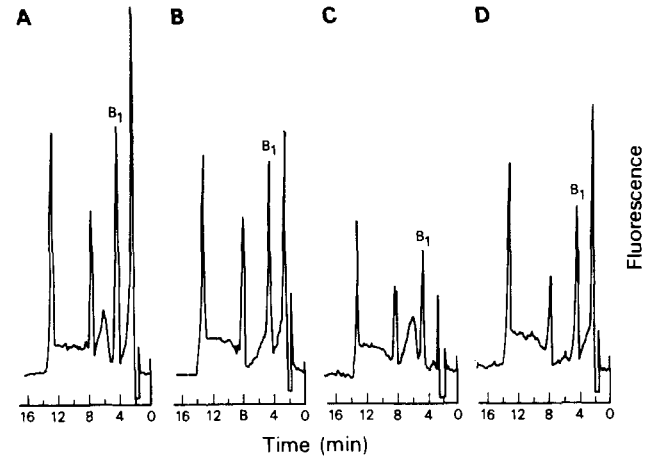

Figure 2. Reversed-phase (C_{18}) HPLC separation of thiamin (as thiochrome) in whole soy flour (A), okara (B), whey (C), and tofu (D) sample extracts of Vinton soybeans. Retention time for thiamin was 5 min. The column-washing step was initiated at 6 min.

Table III. Thiamin Mass Balance in the Tofu Manufacture (Adjusted for Recovery)

fraction	thiamin content, μg/g wet wt			% retained		
	Prize	Vinton	Weber	Prize	Vinton	Weber
okara	0.643	0.826	0.630	16	19	18
whey	0.378	0.386	0.482	37	41	53
tofu	0.621	0.643	0.357	15	16	8
total				68	76	79
whole bean	7.956	8.167	7.467	100	100	100

Weber soybeans were significantly lower in thiamin and higher in riboflavin than the other two varieties (Table II). Varietal differences in the thiamin and riboflavin content could not be established at a 5% level of significance for Prize and Vinton. Varietal differences in protein content (Cartter and Hopper, 1942) and tocopherol content (Guzman and Murphy, 1986) have been established for similar varieties.

Processing of soybeans into tofu resulted in a significant loss of these water-soluble vitamins as shown in Tables III and IV. The overall variation in the distribution of these vitamins among the fractions was approximately 5%. Inaccurate estimation of the amounts of okara and whey plus thermal degradation, especially of thiamin (Stecher, 1960; Labuza and Kamman, 1982), may account for the deficit in the mass balance of thiamin observed. These data are in contrast to the significant retention of the fat-soluble vitamin E reported for tofus of these same soybean varieties (Guzman and Murphy, 1986).

Efficiency of recovery observed (Table I) was adequate for this study. High protein content of these soy products and occlusion of vitamins in the residue may

Table IV. Riboflavin Mass Balance in the Tofu Manufacture (Adjusted for Recovery)

fraction	riboflavin content, μg/g wet wt			% retained		
	Prize	Vinton	Weber	Prize	Vinton	Weber
okara	0.084	0.087	0.090	17	16	16
whey	0.075	0.070	0.065	60	60	48
tofu	0.101	0.106	0.084	20	21	12
total				97	97	76
whole bean	0.967	1.011	1.156	100	100	100

Table V. Apparent First-Order Rate of Loss of Thiamin and Riboflavin from Tofu Stored at 4 °C

variety	vitamin B_1 loss		vitamin B_2 loss		shelf life,[b] days
	rate, day^{-1}	p_1[a]	rate, day^{-1}	p_1[a]	
Prize	0.0318	0.0021	0.0297	0.0001	7
Vinton	0.0419	0.0002	0.0373	0.0001	5
Weber	0.0720	0.0001	0.0338	0.0001	3

[a] Probability of goodness of fit for a first-order kinetic model. [b] Calculated on 20% loss of vitamin from tofu.

Table VI. Comparison of HPLC and AOAC[a] Data for Riboflavin Content in Whole Soy Flour

variety	μg B_2/g flour	
	HPLC	AOAC
Vinton	0.96 ± 0.01	2.24 ± 0.23
Prize	0.92 ± 0.03	2.78 ± 0.53
Weber	1.10 ± 0.05	3.17 ± 0.25

[a] Samples analyzed according to the AOAC method (1984).

account for the incomplete recovery. The low content of these vitamins in soy products required the use of large sample aliquots.

Tofu lost vitamins B_1 and B_2 rapidly on storage in water at 4 °C (Table V). Approximately 91% of the thiamin and 78% of the riboflavin could be accounted for in the sum total of the tofu and its leach water (data not shown). Leaching was concluded to be the main factor responsible for the loss of these vitamins because most of the thiamin and riboflavin lost by the tofu appeared in the leach water. The percent U.S. RDA values per 300-g serving of fresh tofu calculated according to the FDA nutrition labeling requirements (basis: 1.5 mg of thiamin and 1.7 mg of riboflavin U.S. RDA per adult) were 4 and 0, respectively, for thiamin and riboflavin in all three varieties. In contrast, a commercial product (Tofu, Morinaga Brand Minato-ku, Japan)reported having 2% of the U.S. RDA of thiamin and 10% of the U.S. RDA of riboflavin. These data suggest that current nutrition label information for tofu may be overestimating riboflavin and thiamin contents. Riboflavin and thiamin contents may be even lower because the tofu soak water is usually discarded by the consumer.

Comparison of data obtained by our method with those obtained by the AOAC method (1984) revealed similar results for thiamin but remarkably lower values for riboflavin (Table VI). Analysis of riboflavin in samples prepared for HPLC analysis by using AOAC (1984) Method 43.040-43.042 gave data similar to those previously reported (Watson, 1976; Abdullah and Baldwin, 1984; Campbell et al., 1985; Vedrina-Dragojevic and Sebecic, 1986). Treatment of the sample prepared for HPLC analysis with HOAc–$KMnO_4$–H_2O_2 before HPLC analysis failed to eliminate all the nonvitamin peaks from the fluorescent chromatograms. Treatment of the sample solution with HOAc–$KMnO_4$–H_2O_2–$Na_2S_2O_4$ preceding HPLC analysis destroyed the peak representing riboflavin. When milk was analyzed according to the same procedures, only a single peak coeluting with riboflavin was observed (chromatogram not shown). This supports the purity of the peak attributed to riboflavin. It indicates that the fluorescence of other compounds was being attributed to riboflavin fluorescence when the AOAC method was used for soy products (Figure 1). Thus, we recommend the use of the method reported here in preference to the AOAC method for the analysis of thiamin and riboflavin in soybeans and soy products.

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Registry No. Thiamin, 59-43-8; riboflavin, 83-88-5.

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Determination of Benomyl by High-Performance Liquid Chromatography/Mass Spectrometry/Selected Ion Monitoring

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Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl) was analyzed by reversed-phase high-performance liquid chromatography/mass spectrometry/selected ion monitoring (HPLC/MS/SIM) using 2-aminobenzimidazole (2-AB) as internal standard. Benomyl was quantitatively converted to methyl 2-benzimidazolecarbamate (carbendazim) by acid hydrolysis, and the latter was recovered by partitioning into ethyl acetate. The minimum detectable level in apples, peaches, and tomatoes was 0.025 ppm. Recoveries of benomyl fortified at 0.1 ppm were in the range 85–110% in all three commodities. Twenty-five samples each of tomatoes, peaches, and apples were analyzed. Four apple samples contained levels of 0.15–0.59 ppm benomyl, while eighteen of the peach samples analyzed had levels of 0.25–3.48 ppm. Trace levels of benomyl were also found in three tomato samples and one apple sample.

Benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] is a fungicide that is widely used for the control of a variety of plant diseases. Benomyl has proven to be a difficult analytical target because it readily decomposes in many common organic solvents, as well as in water (Calmon and Sayag, 1976). Because of this instability, residues of benomyl in crops are determined by acid hydrolysis of benomyl to the stable compound, carbendazim, which is then analyzed by HPLC on strong cation-exchange or reversed-phase (Bardalaye and Wheeler, 1985) columns.

We have adapted these methods to the determination of benomyl in peaches, apples, and tomatoes by reversed-phase HPLC/MS.

MATERIALS AND METHODS

Chemicals. Benomyl and carbendazim reference standards were donated by the EPA (Research Triangle Park, NC). 2-Aminobenzimidazole (2-AB) was purchased from Alfa Products, (Danvers, MA). Acetonitrile, ammonium acetate, ethyl acetate, and hexane were high-purity HPLC grade (Fisher Scientific, Springfield, NJ). ACS-grade anhydrous sodium sul-

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