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Note

Spectrometric and thin-layer chromatographic quantification of sulfathiazole residues in honey

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Sulfathiazole (ST) is used for the prevention and treatment of the disease known as American foulbrood in honey bees. Although application of ST for this purpose is now regulated by the U.S. Food and Drug Administration, honey sold for human consumption may be contaminated if the product is imported into the U.S.A. or if bees were fed the drug illegally. A variety of methods for the detection and quantification of ST in honey have been reported, including colorimetry based on the Bratton-Marshall reaction[1], thin-layer chromatography (TLC)[2,3], and high-performance liquid chromatography (HPLC)[4,5]. In this paper, a previously described screening procedure[6] for residues of ST in honey, involving isolation of the drug on tandem alumina and anion-exchange columns and detection with Bratton-Marshall diazotization-coupling reagents, has been extended to provide quantification of ST by colorimetry at the 1-ppm level and by quantitative silica gel TLC in the concentration range 0.1–1 ppm.

EXPERIMENTAL

Stock solutions of ST and sulfaquinoxaline (SQO) (Sigma, St. Louis, MO, U.S.A.) (10.0 mg/ml) were prepared in 2 *M* hydrochloric acid, and dilutions of these solutions were made to prepare TLC standards containing 50, 100, 200, 400, 800 and 1200 ng ST/10 μl, each also containing 1000 ng SQO/10 μl. Spiking solutions of ST were prepared at 100–5000 ng/ml. A methanol solution containing 10 μg/ml of SQO internal standard was also prepared.

Bio-Rad (Richmond, CA, U.S.A.) AG MP-1 anion-exchange resin was prepared and converted to the bisulfate form as described earlier[6]. The upper column was composed of a glass chromatography tube, 13 cm × 1.5 cm, plugged loosely with glas wool, dry-packed with 7.5 g of neutral alumina (Alfa Products, Danvers, MA, U.S.A.), and topped with 1 cm of fine sand. The bottom column was prepared in a Fisher "large volume" pasteur pipet (No. 13-678-8). A 4-mm glass bead was dropped

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into the pipet, followed by a 5-mm layer of sand, 2 ml of well-stirred 0.2 g/ml resin slurry, and 5 mm of sand on top. A plastic funnel was attached via Tygon tubing to the top of the lower column to accept without loss the effluent from the upper column.

For colorimetry, honey (5.0 g) was weighed into a 100-ml beaker and dissolved in about 50 ml of distilled water. The solution was poured into the alumina column and the beaker rinsed several times with water. After the solution had percolated through both columns, the upper tube walls were washed down several times with water. After draining, the upper tube was removed, and the lower tube walls were washed down several times with water. After draining, the lower column was dried by drawing vacuum. A volume of 1 ml of 5% aqueous acetic acid was added to the lower column, and after draining, ST was eluted with 5 ml of 3.5 *M* hydrochloric acid collected in a 10-ml volumetric flask. One drop each of sodium nitrite, ammonium sulfamate, and N-1-(naphthyl)ethylenediamine dihydrochloride (NED) (Eastman-Kodak, Rochester, NY, U.S.A.) reagents, prepared as previously described[6], were added in order at 1-min intervals, with 10 s shaking after each addition. The solution was diluted to the line with distilled water. After standing in the dark for 15 min, absorbance was measured with a Varian DMS 90 spectrometer at 540 nm, the wavelength of maximum absorbance, and at 490 and 590 nm to correct for background absorbance.

For TLC, the same procedure was employed with the following changes: (i) 1 g of honey was weighed into a 50-ml beaker and dissolved in 25 ml of water; (ii) ST was eluted from the ion-exchange column with 5 ml of 4% trifluoroacetic acid in 95% ethanol collected in a tapered 5-ml silanized glass centrifuge tube. SQO internal standard solution (100 μl; 1 μg) was added, the tube was placed in a 60°C water bath, and the solution was evaporated just to dryness with a stream of nitrogen. The residue was reconstituted with about 75 μl of 2 *M* hydrochloric acid with 15 s of vortex mixing. The entire solution was spotted on the plate in small portions, with drying between applications.

TLC was performed on Whatman (Clifton, NJ, U.S.A.) LHPKD channeled, preadsorbent high-performance silica gel plates. Samples and standards were applied by streaking onto the preadsorbent area with a Drummond microdispenser, and the layer was developed with 2-propanol–conc. ammonium hydroxide (8:2, v/v) in a paper-lined glass tank. The dried chromatogram was sprayed with 1% sodium nitrite in 1 *M* hydrochloric acid, air dried, sprayed with 0.8% aqueous ammonium sulfamate, air dried again, and sprayed with 0.3% NED in 1 *M* hydrochloric acid. The sodium nitrite solution was removed from a refrigerator just before spraying. The first two sprays were applied uniformly until the layer just began to become dark from excess moisture; the NED was sprayed only until the red/purple zones no longer became darker. ST and SQO zones were scanned in the transmission mode with either a Kontes Model K-49500 or Model 800 fiber optics densitometer. The K-49500 was equipped with a baseline corrector and strip chart recorder, and the 5-mm light beam and "longwave UV" source were used. The Model 800 was interfaced with an HP 3390A integrator/recorder, and the 8-mm light beam and white phosphor source (440 nm peak emission) were used. Densitometry attenuation was set to give *ca.* 80% full scale deflection for the internal standard peak on each chromatogram. Calibration curves were plots of ST/SQO peak area ratios *vs.* nanograms of ST spotted. Standard curve slope and intercept data were used to calculate the level of ST in spiked and incurred samples from ST/SQO area ratios.

Recovery studies were done using a variety of honey samples that differed in color and viscosity. Samples were spiked at 1.00 ppm (5 μg ST/5 g honey) for colorimetry. For TLC, honey that was prescreened[6] and found to contain <25 μg/kg of ST was used, and appropriate volumes of the spiking solutions were added to honey to prepare samples fortified at 0.1, 0.5 and 1 ppm (0.1–1 μg ST/1 g honey). Recovery for TLC samples was calculated by comparing the theoretical ST amount to the amount calculated from the ST/SQO area ratio of the sample. The recovery by colorimetry for the 1-ppm fortified samples was calculated by dividing the difference between the corrected absorbances of the fortified and unfortified samples by the corrected absorbance of a standard containing the theoretical amount of ST. Corrected absorbance was calculated as the absorbance at 540 nm minus half the sum of the absorbance at 490 nm and 590 nm.

RESULTS AND DISCUSSION

Colorimetry

Table I shows recovery and precision data for the 1-ppm fortified samples analyzed by the colorimetric method. The table shows that the minimum recovery was about 76% for the different types of honeys tested. The average value and standard deviation for the five analyses of the palmetto honey was 0.875 ± 0.015 ppm. No more than three separate analyses were performed for any of the other samples. It was reported earlier[6] that spike levels as low as 0.5 ppm could be determined accurately by colorimetry using a smaller alumina and ion-exchange column and volumetric flask. Concentrations below 1 ppm were not analyzed in the present study.

TABLE I

RECOVERY OF SULFATHIAZOLE FROM HONEY SAMPLES FORTIFIED AT 1 ppm USING COLORIMETRIC DETERMINATION

Honey samples were provided by L. W. Doner, U.S. Department of Agriculture, Eastern Regional Research Center, Philadelphia, PA. U.S.A.

Honey	*Recovery (ppm)*	*Honey*	*Recovery (ppm)*
Light color		*Dark color*	
Alfalfa-clover	0.881	Gallberry	0.758
	0.884		0.914
Clover	0.890		0.854
	0.847	Canada	0.775
Medium color			0.831
Island blend	0.798	Avocado	0.843
	0.788		0.872
Alfalfa	0.881	Soybean	0.853
	0.814		0.850
Goldenrod-clover	0.984	Palmetto	0.888
Cotton sage	0.923		0.856
	0.923		0.875
	0.931		0.891
			0.864

TLC

Development with 2-propanol–conc. ammonium hydroxide (8:2, v/v) separated SQO and ST with respective R_F values of 0.70 and 0.60. Drugs were detected as flat red-purple bands spread across the layer channels. Old sodium nitrite and ammonium sulfamate spray solutions resulted in faint zones and non-white backgrounds, respectively. NED purchased from Eastman-Kodak gave the best detection when compared with reagent purchased from other companies. The minimum level for precise scanning was 20 ng of ST, although about 5–10 ng could be detected visually. The calibration curve was linear (correlation coefficient value > 0.990) for the 50–1200 ng range of ST used in this study.

Table II shows recovery and precision data for the analysis of fortified honey samples that were produced and purchased locally. Duplicate samples were analyzed in each case except for the 0.50-ppm alfalfa honey spike, for which five samples were run. The mean for the five replicates was 0.424 ppm sulfathiazole with a standard deviation of 0.026 ppm. Percent recoveries ranged from 96 to 76%, with an overall average recovery of 85.6%. A blank sample was chromatographed with each series of honey spikes, and no ST was detected (Fig. 1).

SQO was chosen as the internal standard because it can be separated on the TLC plate from ST, and it can be assumed that any loss in the blow down, reconstitution, and spotting steps will be similar for the two drugs. The sensitivity of detection

TABLE II

RECOVERY OF SULFATHIAZOLE FROM FORTIFIED HONEY SAMPLES USING QUANTITATIVE TLC DETERMINATION

Honey	*Amount added (ppm)*	*Amount found (ppm)*
Orange blossom (light color)	1.0	0.922
		0.901
	0.50	0.444
		0.454
	0.10	0.086
		0.080
Clover (medium color)	1.0	0.979
		0.901
	0.50	0.431
		0.462
		0.407
		0.428
		0.394
	0.10	0.080
		0.111
Buckwheat (dark, viscous)	1.0	0.933
		0.884
	0.50	0.409
		0.384
	0.10	0.083
		0.077

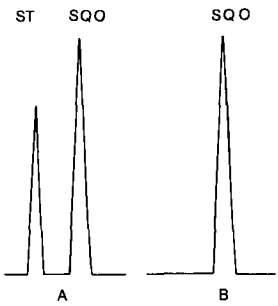

Fig. 1. Recorder tracings of TLC plate scans of (A) clover honey spiked with ST (R_F 0.60) at 0.5 ppm and (B) unfortified clover honey. SQO (R_F 0.70) internal standard was added to the column effluent before spotting. The peaks represent theoretical values of 500 ng for ST and 1000 ng for SQO. A Kontes K-49500 scanner with baseline corrector was used for measurement.

of SQO was about 30% lower than ST using the Bratton-Marshall reagent, based on relative scan areas of chromatograms containing equal weights of the two drugs.

An earlier quantitative TLC screening procedure for ST in honey was reported[3]. This method involved recovery of ST and added SQO by solvent extraction, followed by silica gel TLC, detection with fluorescamine, and scanning of induced fluorescence. A standard curve was prepared by spiking blank honey with ST and SQO and processing the standards the same way as samples. Spiked samples used for recovery studies were essentially identical to the spiked standards used to prepare the standard curve. The method described in this paper utilizes the Bratton-Marshall reagent, which is more selective for detection of ST and SQO than fluorescamine. In addition, the ion-exchange column was shown[6] to have specificity for only ST, SQO and sulfadimethoxine. Direct standards are employed rather than spiked standards, so that a blank honey is not required for each separate analysis, and a 1-g rather than 5-g honey sample is used. Internal standard was not added to the honey sample before being passed through the columns because exactly equal recoveries of ST and SQO could not be confirmed, and we did not want to have to process standards through the columns. The absolute recovery of ST was sufficiently adequate to preclude the need for addition of internal standard to the honey samples.

One dark and one light honey that tested positive during the earlier screening[6] of 110 samples were assayed for incurred residues by the TLC method. Duplicate analyses resulted in values of 0.515 ppm for the dark honey and 0.288 ppm for the light honey. These values were similar to the levels that could be visually estimated by the screening procedure with the use of permanent, artificial color standards[6].

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