Identification of *trans*-3,5-Dimethoxystilbene in Commercial Soy Protein Isolates

W.L. Boatright[a,*], J. St. Pyrek[b], J. Song[b], and A.D. Crum[a]

University of Kentucky, [a]Animal Science Department, and [b]Mass Spectrometry Facility, Lexington, Kentucky 40546-0286

ABSTRACT: A previously unidentified component of the lipid extracts from commercial soy protein isolates (SPI) was analyzed by gas chromatography–mass spectrometry (GC–MS), high resolution mass spectrometry (HRMS), ultraviolet-visible spectroscopy, and GC–Fourier transform infrared spectrometry (FTIR). All these data, together with mass spectra of derivatives obtained by hydrogenation, indicated the structure of an unsymmetrical dimethoxystilbene. Subsequently, standard *trans*-3,5-dimethoxystilbene, synthesized according to established procedures, was found to have identical retention times and spectra by GC–MS and GC–FTIR with the compound isolated from commercial SPI. Laboratory SPI prepared from Probst, Stressland, and Burlison variety soybeans contained no detectable amounts of either *trans*-3,5-dimethoxystilbene or dehydroabietinal.

JAOCS 75, 1651–1655 (1998).

KEY WORDS: Dehydroabietinal, *trans*-3,5-dimethoxystilbene, Fourier transform infrared spectrometry, mass spectrometry, nuclear magnetic resonance, protein, soy protein

In a previous investigation (1) the most abundant component of the volatile-nonpolar fraction of lipid extracts from commercial soy protein isolates (SPI) was an unidentified compound that constituted 0.616 and 1.36 ppm of the SPI (retention time of 44.65 min). In addition, dehydroabietinal and 22 other compounds were reported for the first time as components of SPI. This report describes further spectral analyses of this unidentified compound, together with its unambiguous identification employing the direct comparison with the standard *trans*-3,5-dimethoxystilbene. In addition to commercial SPI, extracts from laboratory-prepared SPI prepared from several different varieties of soybeans were analyzed to determine the presence of these compounds.

MATERIAL AND METHODS

SPI. SPI designated as Pro Fam 970 (samples A and B to designate different code dates) were obtained from Archer Daniels Midland Co. (Decatur, IL). Hexane-defatted soybean flakes were obtained from Archer Daniels Midland and Protein Technologies International (St. Louis, MO).

Burlison, Stressland, and Probst variety soybeans were obtained from the Purdue University U.S. Department of Agriculture, Agricultural Research Service soybean breeding and genetics program (West Lafayette, IN). Defatted soybean flour with a particle size of ≤0.825 mm was prepared by cracking the beans in a food blender and removing the hulls by aspiration. The dehulled bean pieces were then ground and passed through a 20-mesh screen. One part full-fat flour was mixed with 10 parts hexane, agitated for 10 min, and centrifuged at $1000 \times g$ for 10 min at 20°C. The hexane micella (supernatant) was decanted and discarded. The extraction was repeated two more times on the resulting pellet. Hexane was evaporated from defatted flour in a fume hood overnight.

The laboratory SPI were prepared by dispersing the laboratory-prepared hexane-defatted soybean flour in water (1 part flour to 10 parts water) followed by additions of 1 N NaOH, as needed, until pH 9 was achieved and maintained for 15 min. After centrifuging at $1500 \times g$ for 10 min, the supernatant was adjusted to pH 4.5 with 1 N HCl to precipitate proteins. Following centrifugation at $1500 \times g$ for 10 min, the precipitate was washed twice with water, and the protein isolate adjusted to pH 7 with 1 N NaOH and freeze-dried.

Lipid extraction. Lipid extractions from SPI were accomplished by a modification of the method of Bligh and Dyer (4) as previously described (1,5). Approximately 20 g of commercial SPI was extracted twice with 200 mL of chloroform/methanol/water. Laboratory SPI (1 g) was extracted twice with 20 mL of chloroform–methanol/water. The lipids obtained were brought to near-dryness with a rotary evaporator at 50°C with 0.7 kg/cm^2 vacuum followed by removal of the last few milliliters of solvent with a stream of dry nitrogen. The commercial extracts were dissolved in 10 mL hexane, and the laboratory SPI extracts were dissolved in 300 µL methylene chloride and stored in a freezer at −15°C. Throughout the extraction procedure these materials came into contact only with glass and Teflon rinsed with methanol/chloroform (2:1, vol/vol) and chloroform.

Prior to isolating compounds by high-performance liquid chromatography (HPLC), the commercial SPI extracts were separated on a 1.8×30 cm column of Florisil (60–100 mesh) preconditioned with 20% ethyl ether in hexane (vol/vol) fol-

*To whom correspondence should be addressed at University of Kentucky, Animal Science Department, 412 W.P. Garrigus Bldg., Lexington, KY 40546-0215.
E-mail: wlboat1@pop.uky.edu

lowed by 100% hexane. Extracts were applied in hexane, eluted first with hexane (200 mL), followed by 7% ethyl ether in hexane (200 mL). The residue from the 7% ethyl ether was dissolved in 1 mL of 0.1% vol/vol 2-propanol in hexane.

HPLC. HPLC separations were accomplished on a Rainin Gradient HPLC System (Ridgefield, NJ) with two model HPXL pumps, model 805 pressure module, Dynamax HPLC gradient controller, Dynamax model UV-D II variable ultraviolet detector set at 276 nm, and a 50 µL injection loop. Lipids were separated on a Dynamax Microsorb 5 µm silica column (4.6 × 250 mm) with a 5 µm silica guard column (4.6 × 15 mm). The flow rate was 1.0 mL per min with a solvent gradient of 0.1% (vol/vol) 2-propanol in hexane at the start to 4% 2-propanol by 20 min. *trans*-3,5-Dimethoxystilbene eluted at 13.8 min.

Gas–liquid chromatography–mass spectroscopy (GC–MS). GC–MS was done using either a Hewlett-Packard (Wilmington, DE) Model G1800A GCD System equipped with an HP-1 capillary column (30 m × 0.25 mm i.d.) or SE-54 capillary column (30 m × 0.25 mm i.d.) or a Finnigan INCOS-50 equipped with a DB-5 capillary column (15 m × 0.25 mm i.d.). The stationary phase of all capillary columns was 0.25 µm. The column temperature was held at 50°C for 2 min, then raised at 5°C/min to 300°C, at which point the temperature was held for 3 min. High-purity helium was the carrier gas at 1 mL/min.

Ultraviolet/visible (UV/Vis) spectra. These were obtained in the scan mode on a Gilford Response II UV/Vis spectrophotometer (Ciba-Corning Diagnostics Corp., Medfield, MA).

High-resolution mass spectra (HRMS). These were obtained with a Kratos, Concept IH (Manchester, England), electron impact mass spectrometer at the resolution of 10,000.

GC/Fourier transform infrared spectrometry (GC–FTIR). GC–FTIR was performed on a Hewlett-Packard Model 5890 Series 2 GC System equipped with an HP 5965B Fourier transform infrared detector and Chemstation controller. An HP-5 capillary column (30 m × 0.25 mm i.d.) was used at a column temperature of 50°C for 2 min, then raised to 300°C at 5°C/min, where the final temperature was held for 5 min. High-purity helium was the carrier gas at 1 mL/min.

^1H nuclear magnetic resonance (NMR). NMR spectra were recorded in $CDCl_3$ in reference to tetramethylsilane at 300 MHz using a Varian spectrometer (Palo Alto, CA).

A mixture of *cis* and *trans* isomers of 3,5-dimethoxystilbene was prepared by the Wittig reaction between 3,5-dimethoxy-benzaldehyde and the ylide obtained from benzyl triphenyl phosphonium bromide (2). The two isomers were separated by preparative thin-layer chromatography. The *trans*-isomer was more polar and was crystallized from methanol. It had the m.p. 53.5–54.0°C and the following NMR signals (compare to Fig. 1): δ 3.84 *s* (6H; 2 × OCH_3), 6.40 *t* (1H, *J* = 2.1 Hz, H-f), 6.67 *d* (2H, *J* = 2.1 Hz, protons H_2-e); 7.05 ABq (2H, *J* = 16 Hz, Δδ 0.04, H-d and d′), 7.26 *tt* (1H, *J* = 7.2 Hz and 1.2 Hz, H-a), 7.36 *tt* (2H, *J* = 7.5 Hz and 1.5 Hz, H_2-b), and 7.52 *dt* (2H, *J* = 6.9 and *J* = 1.5 Hz, H_2-c).

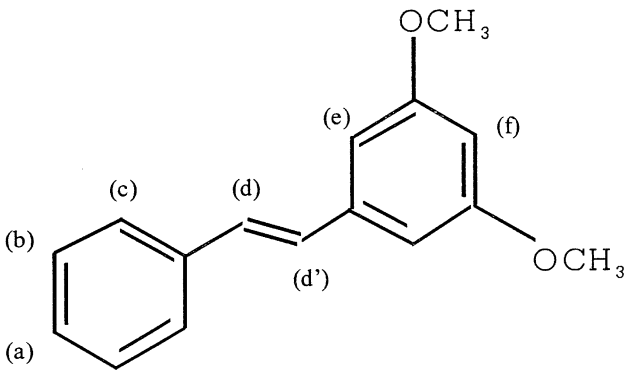

FIG. 1. Structure of 3,5-*trans*-dimethoxystilbene with proton designations.

These data were very close to those previously reported for *trans*-3,5-dimethoxystilbene (7). Dehydroabietinal was synthesized by the method of Carrau *et al.* (3).

RESULTS AND DISCUSSION

The unknown compound, separated from SPI by chloroform/methanol extraction followed by Florisil chromatography and HPLC purification, showed very strong UV absorption at 200 and 230 nm. Its mass spectrum (Fig. 2) indicated the presence of a prominent molecular ion at *m/z* 240 corresponding to the elemental composition of $C_{16}H_{16}O_2$ (measured mass 240.114, calculated mass 240.115). The highly unsaturated nature of this compound (equivalent of nine cycles/double bonds) was in agreement with the presence of the 1590 cm^{-1} band in its IR spectrum (Fig. 3) and the strong UV absorption mentioned above. This compound was unchanged upon attempted silylation and its IR spectrum indicated the lack of either a carbonyl or a hydroxyl group. The amount isolated from SPI was not sufficient to obtain NMR spectra and, consequently, the identification required procurement of suitable standards and their chromatographic and spectral comparison.

One of several possible structures in agreement with the elemental composition and the above spectral features was dimethoxystilbene. Considering *trans*-stilbene derivatives, there are six isomers with one methoxy group at each ring and six isomers with both methoxy groups placed at one ring. The first set of six dimethoxystilbenes was synthesized by the benzoin condensation of benzaldehyde, together with their respective *cis*-isomers (St. Pyrek, J., and J. Song, unpublished). All these compounds, when analyzed by GC–MS, produced similar mass spectra, which were very close to that of the unidentified SPI component. Their R_f values, however, were apparently different from the SPI component. This observation pointed to the *trans*-dimethoxy stilbenes with both methoxy groups on the same ring. This structure was further corroborated by the microhydrogenation reactions resulting in two products characterized by GC–MS. An intermediate product showed simple mass spectra with the following *m/z*

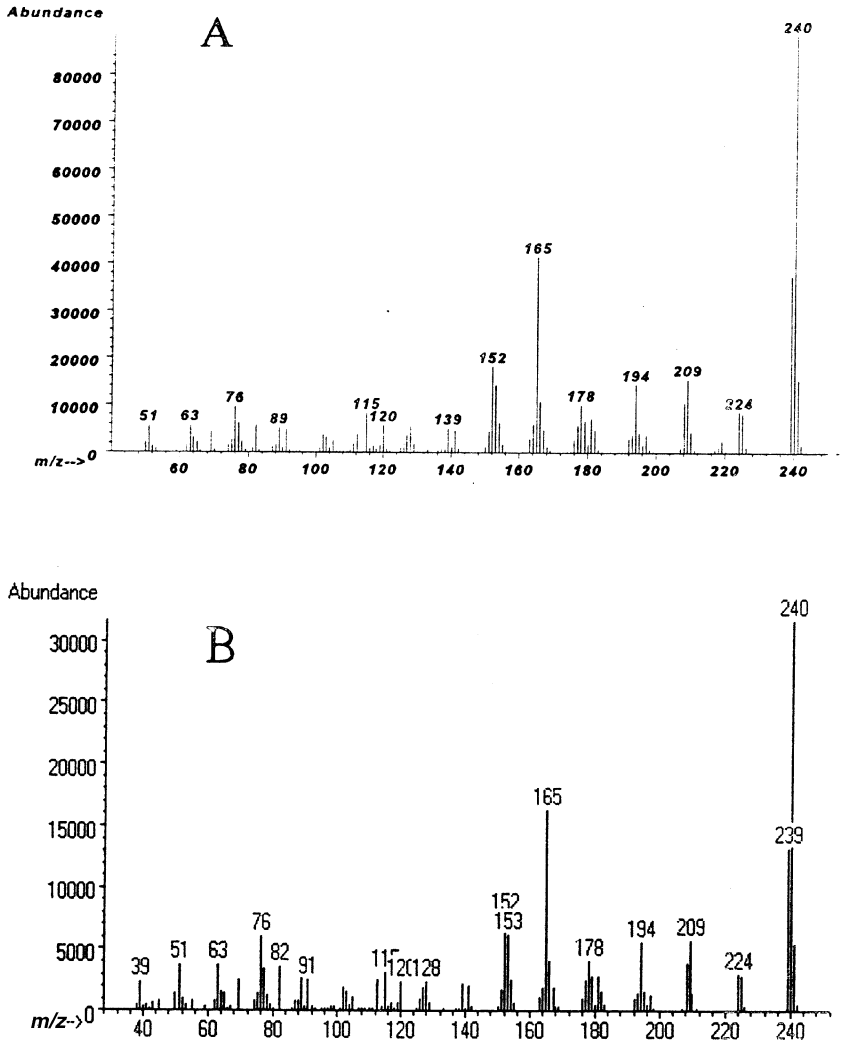

FIG. 2. Mass spectra of (A) a compound found in commercial soy protein isolate (SPI) that eluted at 44.65 min and (B) standard *trans*-3,5-dimethoxystilbene with same retention time.

ions: 242 (molecular ion), 227, 165, 151 (base peak), 138, 121, 91, 77, and 65. The final product showed ions at *m/z* 248 (molecular ion), 165, and 152 (base peak) (Fig. 4).

In an attempt to obtain standards of dimethoxystilbenes substituted on the same ring, 3,5-dimethoxystilbene isomers were selected as the first target. *Cis*- and *trans*-isomers were prepared both by the benzoin condensation of benzaldehyde with 3,5-dimethoxybenzaldehyde (data not shown) and by the Wittig reaction employed before (2). The *trans*-isomer, showing spectral data and m.p. identical to those reported before (7), was found to be entirely identical with the SPI-derived compound by the direct GC–MS and GC–FTIR comparison. Consequently, the syntheses of the other five isomers were not attempted.

This is the first reported occurrence of *trans*-3,5-dimethoxystilbene in soy products. The only previously reported natural occurrence of *trans*-3,5-dimethoxystilbene is from coniferous trees (6–8), primarily pine. This is the second class of compound reported in commercial soy products previously found associated primarily with conifers, the abietates being reported by Boatright and Crum (1).

Trans-3,5-dimethoxystilbene was found to be responsible for the red color that develops in tall oil fatty acids upon epoxidation (6). Owing to its relatively low levels in soy products compared to tall oil, it seems unlikely that *trans*-3,5-dimethoxystilbene would make a significant contribution to the discoloration of processed soy products.

SPI from different varieties of soybeans were prepared to determine if *trans*-3,5-dimethoxystilbene or dehydroabietinal is present in laboratory SPI. These SPI were analyzed by GC–MS along with the standards of *trans*-3,5-dimethoxystilbene and dehydroabietinal. Comparison of the retention times of the standards and their mass spectra revealed that neither of these compounds was detected in the laboratory SPI. This indicates that these may not be naturally occurring compounds from soybeans, but their presence may result from

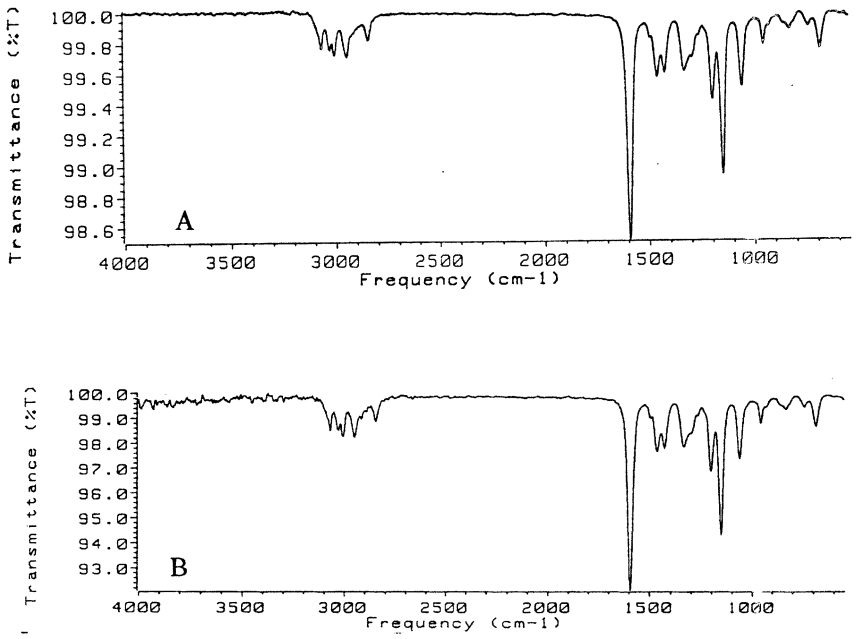

FIG. 3. Infrared spectra of (A) unknown compound found in commercial SPI and (B) standard *trans*-3,5-dimethoxystilbene. For abbreviation see Figure 2.

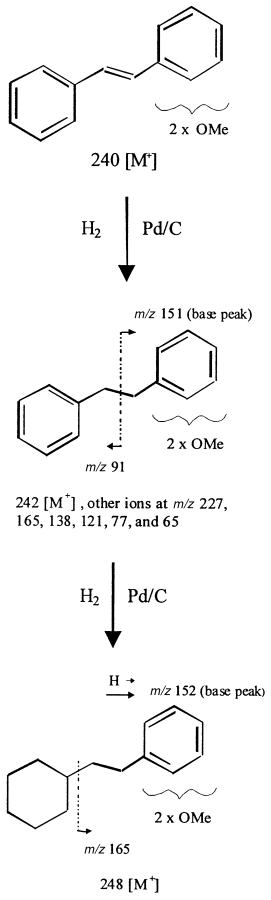

FIG. 4. Product from microhydrogenation of compound found in commercial SPI. For abbreviations see Figure 2.

contamination during commercial soybean processing. It is also possible that certain commercial processing practices that are unknown to the authors may bring about the synthesis of these compounds from naturally occurring soybean components. Whatever the source, it is likely the same for both compounds since they are both typically derived from the same source, conifers.

ACKNOWLEDGMENTS

The authors thank Dr. Paul Weston for valuable technical assistance with analysis by GC–FTIR and Dr. James Wilcox, Purdue University, for providing the soybeans used in this investigation. This project was funded in part by the Kentucky Soybean Promotion Board. Published with the approval of the Director of the Kentucky Agricultural Experiment Station as Journal Article # 98-07-45.

REFERENCES

1. Boatright, W.L., and A.D. Crum, Nonpolar-Volatile Lipids from Soy Protein Isolates and Hexane-Defatted Flakes, *J. Am. Oil Chem. Soc. 74*:461–467 (1997).
2. McKenna, E.G., and B.J. Walker, The Stereochemistry of Wittig Reactions of Ylide-Anions Derived from Semi-Stabilized Phosphonium Ylides, *Tetrahedron Lett. 29*:485–488 (1988).
3. Carrau, R., R. Freire, R. Hernandez, and E. Suarez, Dehydrogenation of Cyanamides. An Approach to Cyanimides and Carbonyl Compounds, SYNTBF, *Synthesis 12*:1055–1058 (1986).
4. Bligh, E.G. and W.J. Dyer, A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Phys. 37*:911–917 (1959).
5. Boatright, W.L., and H.E. Snyder, Soybean Protein Bodies: Phospholipids and Phospholipase D Activity, *J. Am. Oil Chem. Soc. 70*:623–628 (1993).

6. Min, D.B.S., and S.S. Chang, Isolation and Identification of *trans*-3,5-Dimethoxystilbene from High Quality Tall Oil Fatty Acids by Liquid Chromatography and Mass Spectrometry, *Ibid. 49*:675–677 (1972).
7. Suga, T., S. Ohta, K. Munesada, N. Ide, M. Kurokawa, M. Shimizu, and E. Ohta, Endogenous Pine Wood Nematicidal Substances in Pines, *Pinus massoniana*, *P. strobus* and *P. palustris, Phytochemistry 33*:1395–1401 (1992).
8. Rowe, J.W., C.L. Bower, and E.R. Wagner, Extractives of Jack Pine Bark: Occurrence of *cis*- and *trans*-Pinosylvin Dimethyl Ether and Ferulic Acid Esters, *Ibid. 8*:235–241 (1969).

[Received March 23, 1998; accepted July 19, 1998]