Note on an Improved Method of Extracting and Quantitating Coumestrol from Soybeans[1]

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Coumestrol, an estrogenic compound, was found in many forage plants (Bickoff et al 1957; Guggolz et al 1961; Knuckles et al 1975, 1976; Wada and Yuhara 1964) and in 13 of 16 vegetables tested; levels were highest in sprouted alfalfa and soybean seeds (Knuckles et al 1976). Deleterious (Bickoff et al 1960, 1962; Bradbury and White 1954; Braden et al 1964; Magee 1963) and beneficial (Bickoff et al 1962, Bradbury and White 1954, Cheng et al 1953, Oldfield et al 1966, Story et al 1957) effects have been attributed to coumestrol. Because of increased use of soybean products to enhance protein and lysine contents of wheat products and meat substitutes, a reliable analytical method for coumestrol is desirable. Researchers in this laboratory modified published procedures (Knuckles et al 1975, 1976; Livingston et al 1961) to extract the coumestrol from soybeans. High performance liquid chromatography (HPLC) was used to detect coumestrol at the ppm level.

This article presents an improved method for extracting coumestrol from soybeans by considering the effect of the lipid fraction on extraction and the type of solvent on recovery. The coumestrol was then quantitated by HPLC as reported (Lookhart et al 1978).

MATERIALS AND METHODS

Chemicals and Reagents

Water was distilled and deionized; petroleum ether was analytical reagent grade; all other solvents were high purity HPLC grade. Coumestrol, from Eastman Organic Chemicals, was used without further purification because thin-layer chromatographic analysis yielded only one spot. Soybean samples were supplied by Carl B. Overley, associate professor, Agronomy Department, Kansas State University.

Extraction and Purification

Whole soybeans were ground in a Wiley Mill to pass a 20-mesh screen, and samples of about 3.0 g, weighed to 10-mg accuracy, were combined with 50 ml of petroleum ether. This mixture was homogenized for 2 min at 10,000 rpm on an Ultra-Turrax Model SDT, allowed to settle for 1 hr, and then centrifuged for 20 min at 1000 × *g*. The petroleum ether fraction, rich in lipids, was poured off and back-extracted with 50 ml of 75:25 (v/v) methanol/water. The centrifugate, now relatively free of lipids, was hydrated and extracted with 50 ml of 75:25 methanol/water by homogenizing for 2 min at 10,000 rpm on the Ultra-Turrax. The mixture was allowed to settle for 1 hr and centrifuged for 20 min at 1000 × *g*. The supernatant was decanted; the centrifugate was extracted a second time with 50 ml of 75:25 methanol/water by homogenizing for 2 min at 10,000 rpm, settling for 20 min, and centrifuging for 20 min at 1000 × *g*. The 1000 × *g* supernatants were combined and added to the methanol/water extract of the petroleum ether fraction. The combined methanol/water extracts were concentrated to about 20 ml on a rotary evaporator and extracted three times with 10-ml portions of ethyl ether. The ethyl ether fractions were combined and dried under vacuum. The residue was dissolved in two subsequent 1.0-ml aliquots of the liquid chromatography solvent; 65:35 methanol/water. The combined solution was filtered through a 0.45-μ Gelman disposable filter before analysis or refrigerated storage.

HPLC Analysis

All HPLC analyses were performed with a Tracor Model 6970 instrument with a Dupont 5-μm-particle Zorbax (ODS column, 25 cm × 0.46 cm). The samples were injected with an automatic sampler (Waters Associates WISP 710 system). The UV absorbance of the eluate was monitored at 343 nm. The eluting solvent was methanol/water (65:35, v/v) at a flow rate of 1 ml/min, which gave a retention time of about 9 min for coumestrol. The UV trace was registered on a recorder and peak areas were determined with a Varian CDS-111 C integrator.

A standard curve was constructed by plotting the peak area for coumestrol vs concentration of coumestrol by use of standards prepared from a 1,000-ppm stock solution as published (Lookhart et al 1978).

RESULTS AND DISCUSSION

In previous research (Lookhart et al 1978), coumestrol was extracted from soybeans with a method that was reported as 95–98% efficient for use on alfalfa (Knuckles et al 1975, 1976; Livingston et al 1961). When the extraction method for alfalfa was tested on soybeans, however, the percent recovery was very low and analyses of defatted and full-fatted soybeans showed large discrepancies. Apparently the lipids were responsible in part for the low extraction efficiency and should be the first material extracted from the soybeans. Recovery was checked on different extraction procedures to determine which gave the optimal yield. The normal defatting solvent, water-saturated-butanol, decreased the final extraction yield to less than 1%, so petroleum ether was used.

Ground soybean samples, 3.00 g each, were spiked with 0.50 ml of 100-ppm standard coumestrol in duplicate. Sample extraction methods listed in Fig. 1 were: (A) the procedure of Knuckles et al (1975, 1976) and Livingston et al (1961) with slight modification (Lookhart et al 1978); (B) one 50-ml petroleum ether extraction of the ground spiked soybeans, one 50-ml methanol/water extract of centrifugate; (C) one 50-ml petroleum ether extraction of the ground spiked soybeans, one 25-ml methanol water back extraction of petroleum ether fraction, one 50-ml methanol/water extraction of centrifugate; (D) two 50-ml petroleum ether extracts of the

[1]Specific instruments or trade names are mentioned for identification purposes only and do not imply any endorsement by the U.S. government.

ground spiked soybeans, one 25-ml methanol/water back extract, one 50-ml methanol/water extract of centrifugate; (E) one 50-ml petroleum ether extract of the ground spiked soybeans, one 25-ml methanol/water back extract of the petroleum ether fraction, two 50-ml methanol/water extractions of the centrifugate; and (F) same as E without soybeans (control). Following these extraction procedures, coumestrol was recovered from each methanol/water extract by extracting with three 10-ml ethyl ether portions, evaporation of the combined ether fractions to dryness, dissolving the residue in two subsequent 1.0-ml aliquots of methanol/water 65:35, and filtering that solution through a 0.45-μm filter.

More than 99% of the coumestrol standard added in the control was found in the final extract of method F. Phasic distribution is therefore not a problem. Efficiency of any extraction method can be checked by direct comparison with the control.

Previous researchers (Knuckles et al 1975, 1976; Livingston et al 1961) reported 95–98% extraction efficiency from alfalfa and used that recovery scheme for soybean analysis. That percent recovery was not a true extraction efficiency, because the standard was added to the filtered extract and not to the dried material directly. Therefore, the efficiencies reported were merely efficiencies for phasic distributions—not recoveries from the solid sample.

The lipid-coumestrol interactions in soybeans were partially responsible for the low recovery because the extraction efficiency was higher for defatted than for full-fatted soybeans; and using method A, only 8% of the added coumestrol spike was found in the final extract and 11% was found in the petroleum ether fraction.

The extraction efficiencies in the final extracts of methods B–E were: B, 48.0%; C, 58.0%, D, 58.0%; and E, 64.0%. The 10% difference between B and C was due to the back-extraction of the petroleum ether, lipid-rich fraction that must be included. Because there was no difference between methods C and D, the second petroleum ether extraction is not important or necessary. However, the increased yield of 6% from method C to E is due to the second

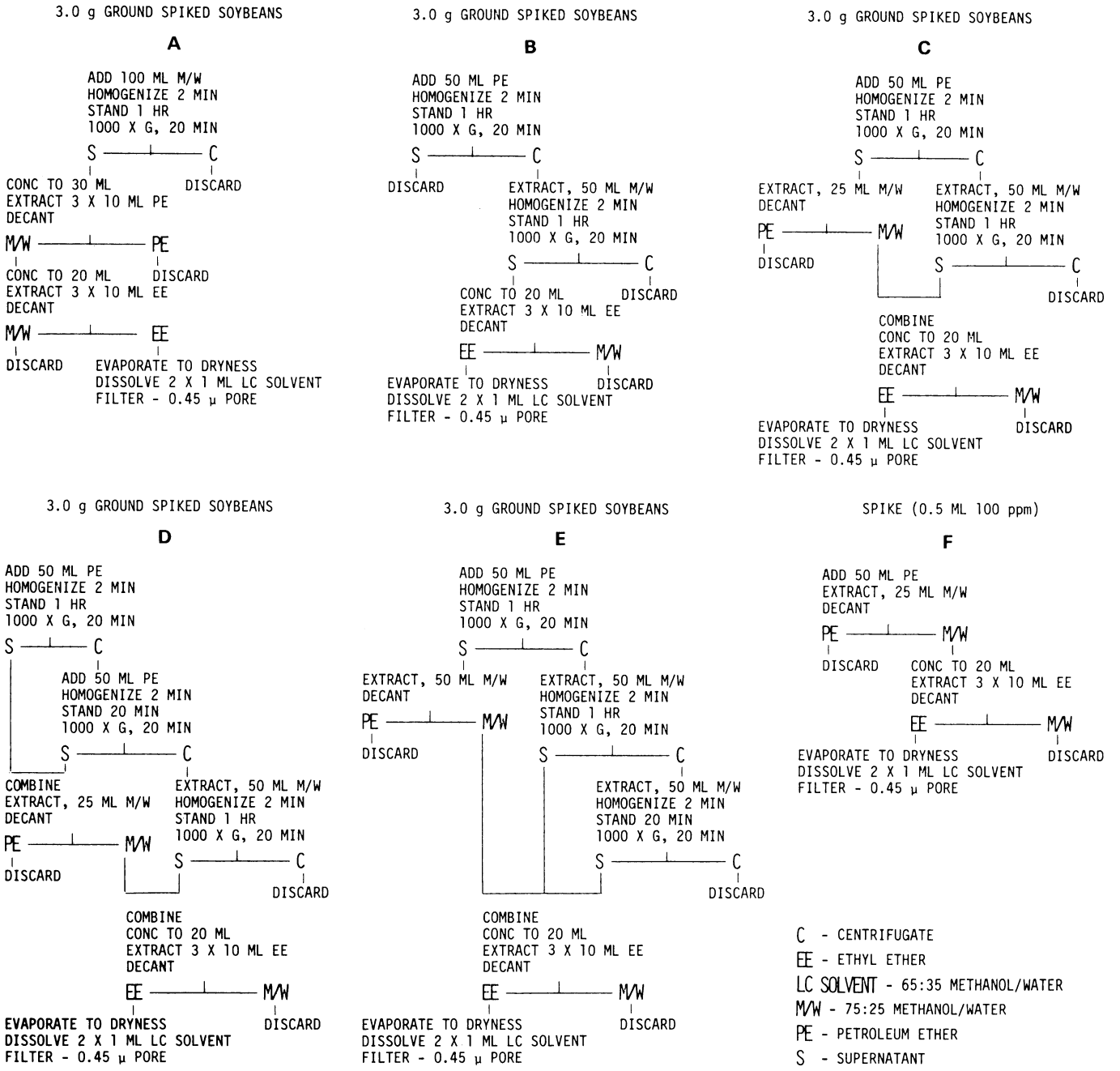

Fig. 1. Soybean extraction procedures for coumestrol. C = centrifugate, EE = ethyl ether, LC solvent = 65:35 methanol/water, M/W = 75:25 methanol/water, PE = petroleum ether, S = supernatant.

methanol/water extraction of the centrifugate and should be included in the extraction procedure. In a separate experiment methylene chloride was substituted for ethyl ether in the final extraction of the combined methanol/water fractions and found to be only half as efficient as ethyl ether.

In these tests, method E was the most efficient for extracting coumestrol from soybeans, and it should be considered for extraction of other estrogens from other lipid-rich materials.

The 36% nonextracted coumestrol apparently was tightly bound to the lipids and hence unextractable.

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