

DETERMINATION OF COUMESTROL IN SOYBEANS BY HIGH-PERFORMANCE LIQUID AND THIN-LAYER CHROMATOGRAPHY¹

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ABSTRACT

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Coumestrol, an estrogenic compound found in many forage plants, was determined quantitatively (0.05–30 ppm) in soybean (*Glycine max* L. Merrill) extracts by high-performance liquid chromatography (HPLC). The extraction

procedure required 3 hr and the HPLC analysis 12 min. A quantitative estimate of coumestrol in the extract was made by thin-layer chromatography in 25 min with a sensitivity limit of 10 ppm and in 2 min for a sensitivity of more than 50 ppm.

Coumestrol is an estrogenic compound found in many forage crops (1–5). In a test on 16 vegetables, 13 contained coumestrol, with the highest levels found in sprouted alfalfa and soybeans (5). Deleterious biologic effects, among which are increased gestation time, teat length, and uterine weight, have been reported for estrogenic substances present in forage crops (6–10). Beneficial effects of such substances also have been reported and include increased rate of growth and milk production (9–13).

Because the use of forage plants in both human and animal nutrition is increasing, a quick, reliable analytic determination is desirable. Paper chromatography (PC) methods, which involve detection by either ultraviolet (UV) fluorescence (3–5, 14,15) or color reactions (16), are time consuming and laborious; development of the chromatogram requires 24–36 hr, and densitometer readings must be made in quadruplicate.

This article presents two methods for quick analysis of coumestrol in extracts of soybean endosperms, germs, hulls, and whole beans. One of the methods is quantitative, involves high-performance liquid chromatography (HPLC), and requires about 12 min. The other method involves thin-layer chromatography (TLC) for the quantitative estimation of coumestrol and requires 2–25 min.

MATERIALS AND METHODS

Chemicals and Reagents

Water was distilled and deionized. All extraction solvents were analytic reagent grade. All other solvents were of high purity distilled-in-glass grade purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. Coumestrol, from Eastman Organic Chemicals, Rochester, NY, was used without further purification, since TLC showed one spot. Carl B. Overley, of Kansas State University, Manhattan, supplied soybean samples.

Separation of Soybean Endosperms From Hulls

Soybeans were passed three times through Tag-Heppenstall moisture rolls equipped with the large soybean shim. The broken soybeans were then passed

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through a McGill rice sheller set at 4-1/2 turns from open. The material, mostly hulls, aspirated off in the sheller was sifted on a Ro-Tap sifter equipped with a 10-mesh Tyler sieve. The overs (relatively pure hulls) of the 10-mesh sieve were aspirated on the South Dakota Seed Blower at a setting of 1-3/4. Germs still attached to hulls were separated by passing through the McGill rice sheller at a setting of five turns from open.²

Extraction and Cleanup

Soybean endosperms plus germs or whole beans (about 10 g) and soybean hulls (about 5 g), weighed to 10-mg accuracy, were ground and homogenized for 3 min in an Ultra-Turrax Model SDT with 50 ml of water at 10,000 rpm. The homogenized samples were allowed to hydrate for 1 hr, blended as before in the Ultra-Turrax with 100 ml of methanol, further solvated for 1/2 hr, and then centrifuged for 20 min at 1,000 × g. The supernatant was poured off and extracted by a modification of the procedures of Knuckles et al (3) and Livingston et al (14). The methanol-water supernatant was extracted with three 50-ml aliquots of pentane and the lipid-free, methanol-water layer concentrated on a rotary evaporator to 20 ml. The concentrate was extracted with three 25-ml aliquots of ethyl ether. The ether extracts were combined and concentrated on a rotary evaporator to 5 ml, quantitatively transferred into a 10-ml volumetric flask, and diluted to volume with 65:35 (v/v) ratio of methanol to water. That entire procedure required 3 hr. The solution was made ready for chromatography by passing through a 0.45- μ Gelman filter.

HPLC Analysis

All HPLC analyses were performed with a Tracor Model 6970 equipped with a 20- μ l sample loop on a Rheodyne Model 7120 injection valve and a Tracor Model 970 UV-VIS variable wavelength detector (wavelength 343 nm). A du Pont 5- μ m particle Zorbax-ODS column, 25 cm × 4.6 mm, was used in the reverse phase mode at room temperature. The solvent system was methanol and water (65:35, v/v) with a flow rate of 1.0 ml/min (1,900 psi), which gave a retention time for coumestrol of about 9 min. A dual pen recorder registered the UV and fluorescence traces. Fluorescence was detected with a Turner Designs Model 10 fluorometer equipped with special flow cell and filters. The fluorescence excitation and emission maxima in ethanol were 348 and 411 nm, respectively, and were determined on a Farrand Mark I spectrofluorometer.³ A Varian CDS-111C integrator quantitated the peak areas in microvolt seconds of the UV trace. The fluorescence trace was used to help identify coumestrol.

Standard coumestrol solutions containing 0.12, 0.24, 0.48, 0.96, 1.2, 2.4, 4.8, 9.6, 12.0, and 60.0 μ g of coumestrol/ml in absolute ethanol were prepared from a stock solution (120 μ g/ml). Concentrations were calculated by use of a standard curve prepared from the integrator responses (peak areas) for 20- μ l injections of standards and unknowns.

The following equation was used for the calculations:

$$C_s = \frac{(A_c - b)}{m} \cdot \frac{V_c}{W_s}$$

²L. C. Bolte and K. F. Finney. Private communication on machine fractionation of soybeans.

³G. L. Lookhart, R. A. MacQuarry, and P. E. Blatz. Private communication on fluorescence of coumestrol.

where m and b are the slope and intercept, respectively, of the standard curve; A_e is the peak area of coumestrol in the extract; V_e is the volume of final extract (10 ml); W_s is the weight of the original sample extracted; and C_s is the concentration of coumestrol in micrograms per gram (ppm) of soybean sample.

TLC Analysis

All TLC analyses were performed with Baker-flex 5×20 -cm precoated Silica Gel-G on plastic sheets. The coating was 0.25 mm thick and the sheets were cut in 5×5 -cm squares. The solvent was chloroform and acetone (88:12, v/v). The standards used in TLC analysis were the same as those prepared for HPLC analysis. For TLC analysis, 1.0-ml aliquots of extract were concentrated 20-fold by evaporating to dryness under nitrogen at 50°C . The dried material was dissolved in $50 \mu\text{l}$ of methanol, and 1.0- μl aliquots of that material and the TLC standards were spotted on the squares. The samples were allowed to air dry, developed in the TLC solvent for about 1 min, air dried, and viewed under long UV wavelength irradiation. The quantity of coumestrol in the sample was

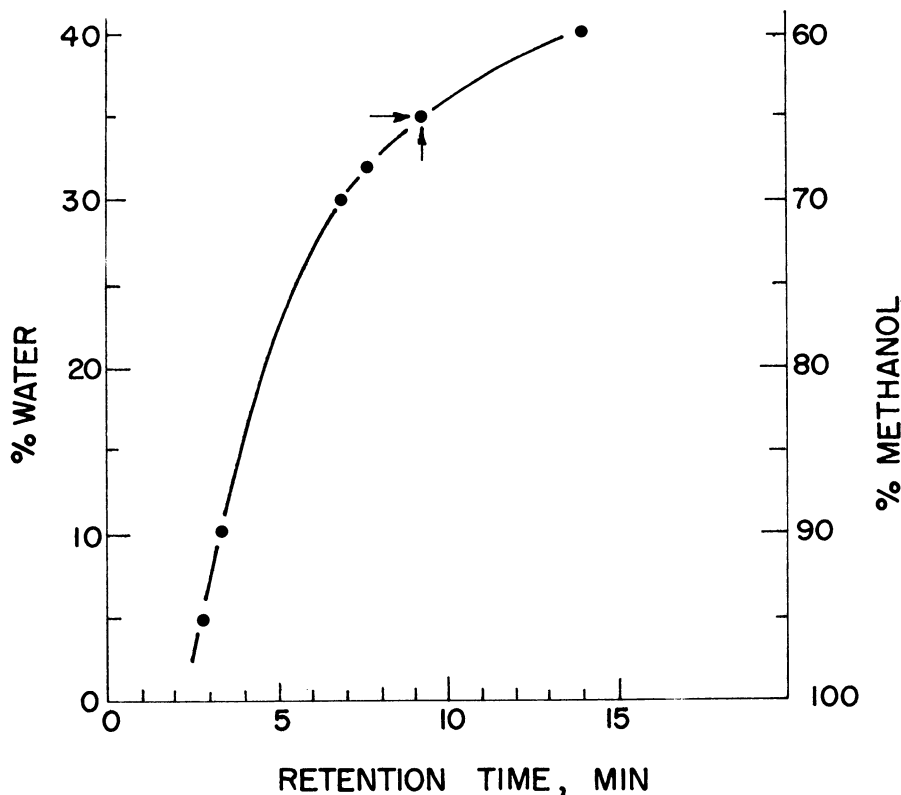


Fig. 1. Effect of methanol/water ratio on retention time. Arrows indicate retention time for coumestrol (9.2 min) and solvent composition (65:35, methanol/water) used in analytic method.

estimated by a visual comparison of the fluorescence intensities of the unknown and standard spots. The above procedure required 25 min. For concentrations above 50 ppm, the concentration step can be deleted and the time reduced to 2 min.

RESULTS AND DISCUSSION

Coumestrol in methyl alcohol (17) has UV maxima at 208, 243, and 343 nm, and was analyzed on the basis of its absorption at 343 nm. The absorption at 208 nm was not used, because most compounds absorb at that wavelength and background problems would have been great. Since the 243- and 343-nm data (6-nm bandpass in detector) gave nearly identical results and the 243-nm data was

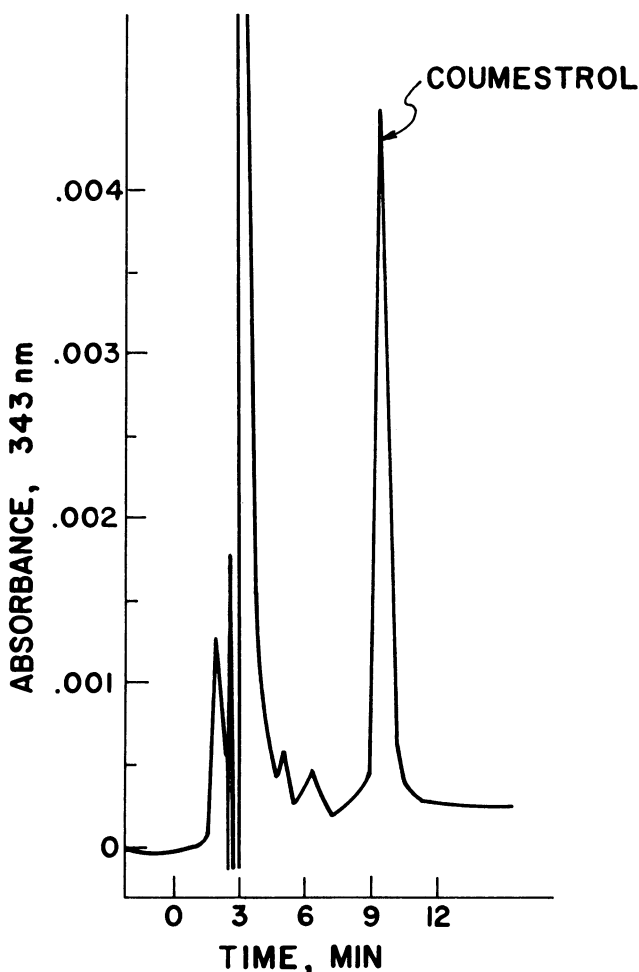


Fig. 2. HPLC chromatogram of soybean hull extract.

not sensitive below 1 ppm, only the 343-nm data are presented here.

Small changes in the methanol/water ratio composition of the HPLC solvent markedly affected the retention time as shown in Fig. 1. Hence, the retention time was checked daily, and the solvent adjusted to give retention times of about 9 min for coumestrol and a corresponding pressure drop of 1,900 psi. We optimized the solvent system by adjusting the percentage of water in methanol until the coumestrol peak in the soybean extract was separated from other material in the extract. The presence of coumestrol in other systems could be determined analogously.

Figure 2 is a typical chromatogram of the extract of soybean hulls. It shows that coumestrol could be separated quickly and quantitated in less than 12 min. The peaks in the chromatogram at times shorter than 6.5 min were due to solvent-solvent interactions and extracted impurities. The level of coumestrol in that sample was calculated to be 0.20 $\mu\text{g/g}$ of hulls. The standard curve was linear, with a slope of $0.193 \pm 0.007 \text{ ml } \mu\text{V-sec}/\mu\text{g}$ and an intercept of 0.

We analyzed the coumestrol content of whole soybeans, soybean endosperms (cotyledons), and soybean hulls in duplicate. Whole soybeans (Clark 63 variety) contained 0.05 $\mu\text{g/g}$, the cotyledons 0.05 $\mu\text{g/g}$, and hulls 0.20 $\mu\text{g/g}$. Duplicate samples were reproducible within 5–10%.

We were easily able to quantitate 1/400 the amount of coumestrol determined by other methods (3,14). Since the 10-ml extract did not require concentration for HPLC analysis, coumestrol could be quantitated in a relatively short time. The sensitivity of the method could be increased 1,000-fold if necessary by integration of the fluorometer output data.

The TLC sheets developed in 1 min were used for the quick estimation of coumestrol present in the 50–100 ppm range. Concentrations of coumestrol materially below 50 ppm required that the extract be concentrated 20- to 50-fold. That procedure took about 20 min. The fluorescence intensity of the sample spot at R_f , 0.50, was visually compared with that of the standard spots, and the quantity of coumestrol was estimated.

Some doubt about the reliability of the previously used PC method (3) is evidenced by TLC analysis, which showed spots close on either side of the coumestrol spot. Since the spots in PC are spread out over 100 times the area of the spots on the 5 × 5-cm squares used for TLC, the possibility of overlap and misreading of fluorescence intensity is appreciable. The TLC is good for estimating coumestrol levels, but does not compare with HPLC in accuracy of quantitation or sensitivity.

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