Journal of Chromatography, 386 (1987) 315–323
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 956

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PHYTOESTROGENS IN SOY PROTEIN PREPARATIONS WITH ULTRAVIOLET, ELECTROCHEMICAL AND THERMOSPRAY MASS SPECTROMETRIC DETECTION

K. D. R. SETCHELL and MARY BETH WELSH
Department of Pediatric Gastroenterology, Children's Hospital Medical Center, Cincinnati, OH 45229 (U.S.A.)
and
C. K. LIM
Division of Clinical Cell Biology, Clinical Research Center, Watford Road, Harrow, Middlesex HA1 3UJ (U.K.)

SUMMARY

The phytoestrogens daidzein, genistein, coumestrol, formononetin, and Biochanin A are separated on a C_{18} reversed-phase column (Hypersil ODS) with methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (60:40, v/v) as eluent. The retention and resolution are affected by buffer concentrations, pH type, and proportion of organic solvent in the mobile phase. Detection in the (low pg range) is achieved with an electrochemical detector, and the compounds are positively identified by high-performance liquid chromatography–thermospray mass spectrometry. Daidzein and genistein were found in high concentrations in all soy protein preparations analyzed.

INTRODUCTION

The phytoestrogens are a group of naturally occurring plant products[1,2] possessing weak estrogenic activity[3–6]. Their existence in soybeans has been known for some time[7], and recently several phytoestrogens and their metabolites were identified in biological fluids of man[8–11]. In particular, the ingestion of soy protein has been shown to be associated with a vast increase in the urinary excretion of these compounds, and levels in vegetarians generally are higher than those for the general population[8,9,12]. Given the strong association between diet and disease[13], the potential implications, whether beneficial or deleterious, of ingesting biologically active compounds, such as phytoestrogens requires examination. This is particularly the case with the increasing use of soy-based products for human consumption[9], and important to such studies is the requirement of suitable techniques for the detection of these compounds in diets.

Methods for the separation of phytoestrogens in plant extracts by high-performance liquid chromatography (HPLC) have been described[14–22], however, these

0021-9673/87/$03.50

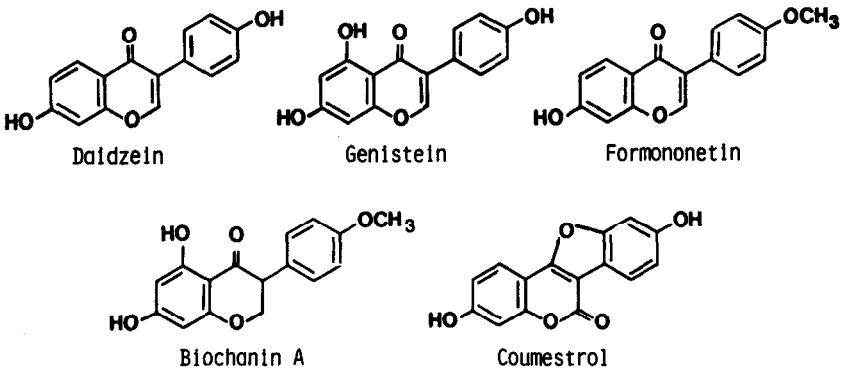

Fig. 1. Chemical structures of the principal plant phytoestrogens.

have generally used gradient elution systems. This paper describes a simple isocratic reversed-phase system, with methanol–0.1 *M* ammonium acetate, pH 4.6 (60:40, v/v) as mobile phase on an Hypersil ODS column, for the rapid and effective separation of the phytoestrogens daidzein, genistein, coumestrol, formononetin, and Biochanin A (Fig. 1). The effects that buffer concentrations, pH, and the type and concentration of organic solvent in the mobile phase have on the retention and resolution have been studied. The sensitivity of ultraviolet (UV) and electrochemical detection (ED) systems has been compared and conditions have been established for HPLC–thermospray mass spectrometry (MS) to allow the identity of individual phytoestrogens in the HPLC effluent to be confirmed. The method has been successfully applied to the analysis of phytoestrogens in a range of soybean products, including soy-based milk formulae and animal diets.

EXPERIMENTAL

Materials and reagents

Biochanin A, genistein, daidzein, and formononetin were from K & K Rare and Fine Chemicals (Plainview, NY, U.S.A.) and coumestrol from Kodak (Rochester, NY, U.S.A.). Textured soy and soy flakes were from Arrowhead Mills, Inc. (Heuford, TX, U.S.A.). Isomil was from Ross Laboratories (Columbus, OH, U.S.A.) and ProSobee was from Mead Johnson (Evansville, IN, U.S.A.). Ammonium acetate, glacial acetic acid, and EDTA were AnalaR-grade from BDH (Poole, U.K.). Acetonitrile and methanol were HPLC grade from Rathburn (Walkerburn, U.K.). The enzyme preparations, β-glucosidase and Helix pomatia (β-glucuronidase and sulfatase) were obtained from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

Samples of Isomil (50 ml), ProSobee (50 ml), or individually homogenized samples of textured soy (5 g) and soy flakes (5 g) were refluxed in 80% aq. ethanol (total volume 250 ml) for 2 h to extract all isoflavones, polar isoflavone conjugates, and related compounds. The organic extracts were cooled, centrifuged, and the supernatant was removed. The ethanol was evaporated in a rotary evaporator, and the lipids were extracted from the remaining aqueous extract by partitioning twice into

four volumes of hexane for textured soy and soy flakes, and partitioning four times for Isomil and ProSobee. The aqueous extract was taken to dryness. Hydrolysis of isoflavone conjugates was carried out using several enzyme preparations. The samples were first subjected to hydrolysis with a β-glucosidase preparation in 0.1 *M* acetate buffer (pH 5.0) overnight at 37°C. The hydrolysate was passed through a cartridge of reversed-phase octadecylsilane-bonded silica (Bond-Elut C_{18}; Analytichem, Harbor City, CA, U.S.A.) to extract all isoflavones, and after washing the cartridge with water, the isoflavones were recovered by elution with 5 ml methanol. After evaporation of the methanol to dryness a second hydrolysis was performed using 0.2 ml of a combined β-glucuronidase and sulfatase preparation (Helix pomatia) in 20 ml 0.5 *M* acetate buffer (pH 4.5) for 24 h at 37°C. The hydrolysate was again passed through a Bond-Elut cartridge to extract the isoflavones, which were recovered by elution with 5 ml methanol and taken to dryness under nitrogen on a 65°C heating block, and the residue was reconstituted prior to assay.

HPLC

A Varian (Walnut Creek, CA, U.S.A.) Model 5000 liquid chromatograph and a Varian UV-100 variable-wavelength detector or a LCA-15 electrochemical detector (EDT Research, London, UK) were used. The electrochemical detector was of the wall-jet type with a glassy-carbon working electrode and a Ag/AgCl reference electrode. Samples were injected via a Rheodyne 7125 injector (Cotati, CA, U.S.A.), fitted with a 100-μl loop.

The column (25 cm × 4.6 mm I.D.) was Hypersil ODS, 5 μm spherical silica, chemically bonded with a monolayer of octadecylsilyl groups from Shandon Southern Products (Runcorn, UK). The mobile phase was methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (60:40, v/v), containing 0.25 mmol/l EDTA. The mobile phase was continuously degassed with a stream of helium during ED. This is unnecessary for UV detection, and EDTA can also be omitted from the mobile phase. The flow-rate was 1 ml/min at ambient temperature and UV detection was at 260 nm. ED of phytoestrogens was achieved at different operating potentials in the range +0.4 to +1.2 V.

Mobile phases of different buffer concentrtions, pH, and with acetonitrile and acetonitrile–methanol mixtures as organic modifiers were used to study their influence on retention and resolution of the individual phytoestrogens.

HPLC–MS

The Varian Model 5000 high-performance liquid chromatograph was coupled to a Finnigan 4635 quadrupole mass spectrometer via a thermospray interface (Finnigan). The mass spectrometer was operated in continuous scanning mode over a mass range of 110–300 daltons. The optimum interface temperatures at the flow-rate used for HPLC separation of the phytoestrogens were determined by multiple injection of standards and varying heater temperatures and repeller voltages. Optimum conditions for the ionization of all the phytoestrogens studied were obtained with a vaporizer temperature of 135°C and a jet-block temperature of 215°C. Solvent flow-rate was 1.0 ml/min, and the HPLC conditions were as described above.

RESULTS AND DISCUSSION

HPLC

The separation of a standard mixture of phytoestrogens on Hypersil ODS with methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (60:40) as eluent is shown in Fig. 2. The elution of these compounds was in the order: daidzein, genistein, coumestrol, formononetin, and Biochanin A. Genistein with three phenolic groups (Fig. 1) is expected to be less hydrophobic than, and therefore eluted before, daidzein, having two phenolic groups. Similarly, Biochanin A with two phenolic groups should, under normal circumstances, be eluted before formononetin with only one phenolic group. The observed reversal in elution order is probably due to the ability of genistein and Biochanin A to form intramolecular hydrogen bonds between one of the phenolic group and the keto group, as shown in Fig. 3. Intramolecular hydrogen bonding will decrease the polarity (increase hydrophobicity) of the molecules, leading to longer retention[21].

Methanol is a better modifier than acetonitrile and is essential for the separation of genistein and coumestrol. The resolution of these two compounds is lost when methanol is replaced with acetonitrile as the modifier (Fig. 2). However, ternary systems which include methanol as one of the organic components will still resolve genistein and coumestrol. For example, a ternary system of acetonitrile–methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (10:50:40) gave a resolution similar to that with the methanol–buffer system.

The pH and buffer concentration of the mobile phase affects the retention but not the resolution. Increasing the pH and/or the buffer concentration decreases the retention of all compounds while maintaining the resolution. A 0.1 *M* buffer at pH

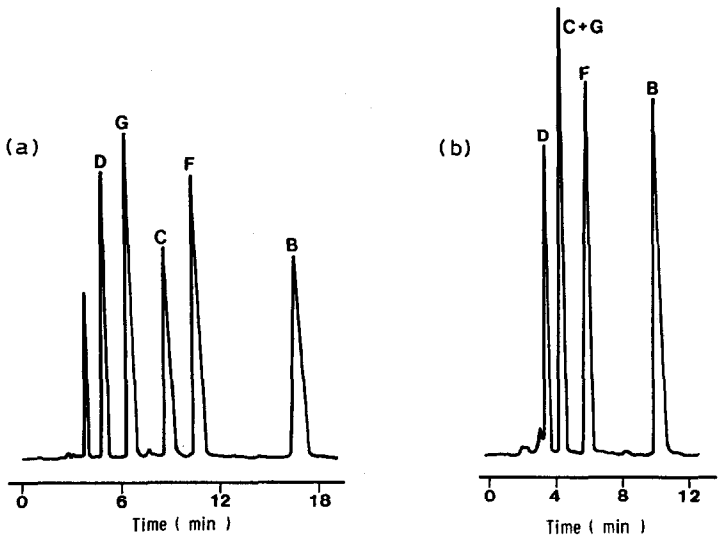

Fig. 2. HPLC separation of phytoestrogen standards, illustrating the effect of varying the mobile phase. (a) Mobile phase: methanol–0.1 *M* ammonium acetate, pH 4.6 (60:40); (b) acetonitrile–0.1 *M* ammonium acetate, pH 4.6 (47:53). Flow-rate 1 ml/min. 260 nm. The following compounds are indicated: D = daidzein; G = genistein; C = coumestrol; F = formononetin; B = Biochanin A.

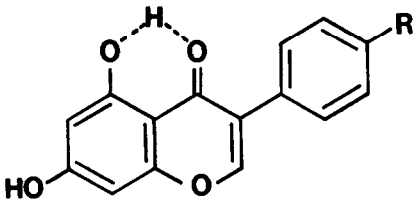

Fig. 3. Intramolecular hydrogen bonding between the phenol and keto groups in the structures of the phytoestrogens genistein (R = OH) and Biochanin A (R = OCH_3).

4.6 was chosen to provide rapid and yet adequate separation of the phytoestrogens, particularly of the early eluted peaks, from possible interferences in sample extracts.

Choice and sensitivity of detector

The phytoestrogens can be detected by UV absorption at 260–280 nm with a detection limit of about 5 ng injected (signal-to-noise ratio of 3 at 0.002 a.u.f.s.). The phytoestrogens are also electroactive, due to the presence of phenolic groups, and can therefore be detected with ED. Coumestrol is the most electroactive compound, followed by genistein and daidzein. The voltammagram for these three compounds is shown in Fig. 4. The optimum potential for the simultaneous sensitive detection of all three compounds is +0.75 V. At a detector sensitivity of 3 nA, the detection limits of coumestrol, genistein and daidzein are 5, 10, and 15 pg injected, respectively. Thus, ED is much more sensitive than the UV detector. However, the satisfactory ED of formononetin and Biochanin A required an operating potential above +1.2 V. At this detector potential, baseline stability becomes a problem. These two compounds are therefore better detected with an UV detector. An UV detector may also be coupled in series with an electrochemical detector for the detection of a wide range of phytoestrogens. However, in preparations containing only daidzein and genistein, ED is the obvious detection system of choice. It allows a much smaller sample size to be used, and therefore a simpler and a cleaner matrix is obtained. For the specific detection of coumestrol a lower operting potential (+0.45 to +0.5 V) may be used, since few compounds are electroactive at these low potentials.

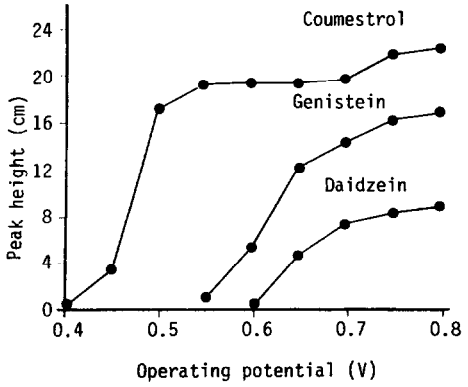

Fig. 4. Voltammagrams for the phytoestrogens coumestrol, genistein and daidzein.

HPLC–thermospray MS

With the development and introduction of the thermospray interface[23–26], many classes of compounds which previously were difficult to analyse by MS can now successfully be analyzed by direct HPLC–MS. Such compounds include those which are highly polar, non-volatile, or thermally labile, such as the phytoestrogens. MS analysis of phytoestrogens in biological fluids has previously necessitated extraction, hydrolysis, purification, and the preparation of volatile derivatives, suitable for introduction into the mass spectrometer via the gas chromatographic outlet[8,10,27,28], techniques which are time consuming.

HPLC–thermospray MS was investigated for its potential to identify individual phytoestrogens in the HPLC effluent under the conditions used here. For all compounds tested the best ionization was achieved at or about a vaporiser temperature of 135°C and a jet-block temperature of 215°C, when the flow-rate was 1 ml/min. Fig. 5 illustrates the total ion current chromatogram, obtained following continuous scanning over the mass range 110–300 *m/z* for a mixture of the five phytoestrogen standards. With the exception of coumestrol, the sensitivity of this technique was comparable to HPLC with UV detection, and no significant loss in chromatographic resolution was observed as a result of interfacing the column with the mass spectrometer. The mass spectra generated in the thermospray ionization process (Fig. 6) were characterized by intense protonated molecular ions, $[MH^+]$, for all of these compounds, and this soft ionization method yielded no significant fragmentation of the molecule.

Since most of the ionization resides in a single ion, selected ion monitoring of the $[MH^+]$ for each phytoestrogen affords a more specific method of detecting these

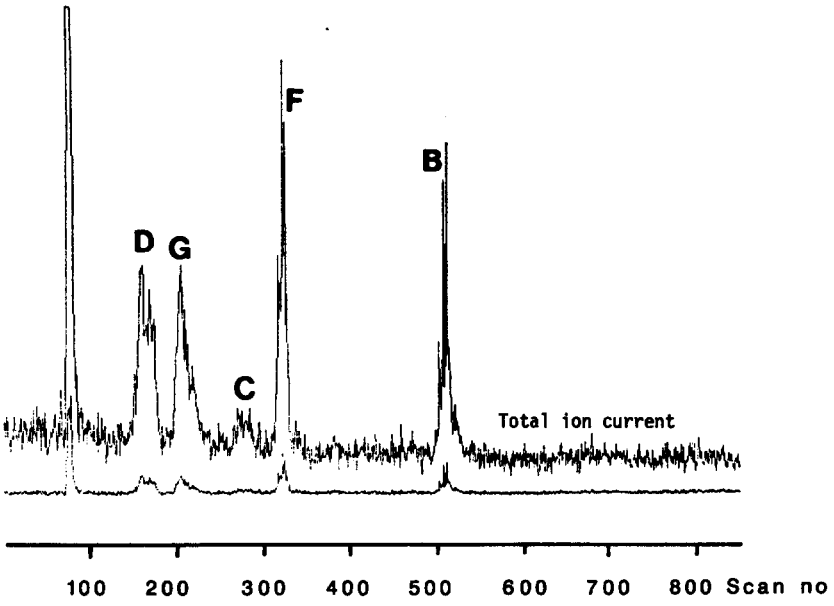

Fig. 5. Total-ion current chromatograms obtained for the HPLC–thermospray MS analysis of a mixture of the phytoestrogen standards listed in Fig. 2.

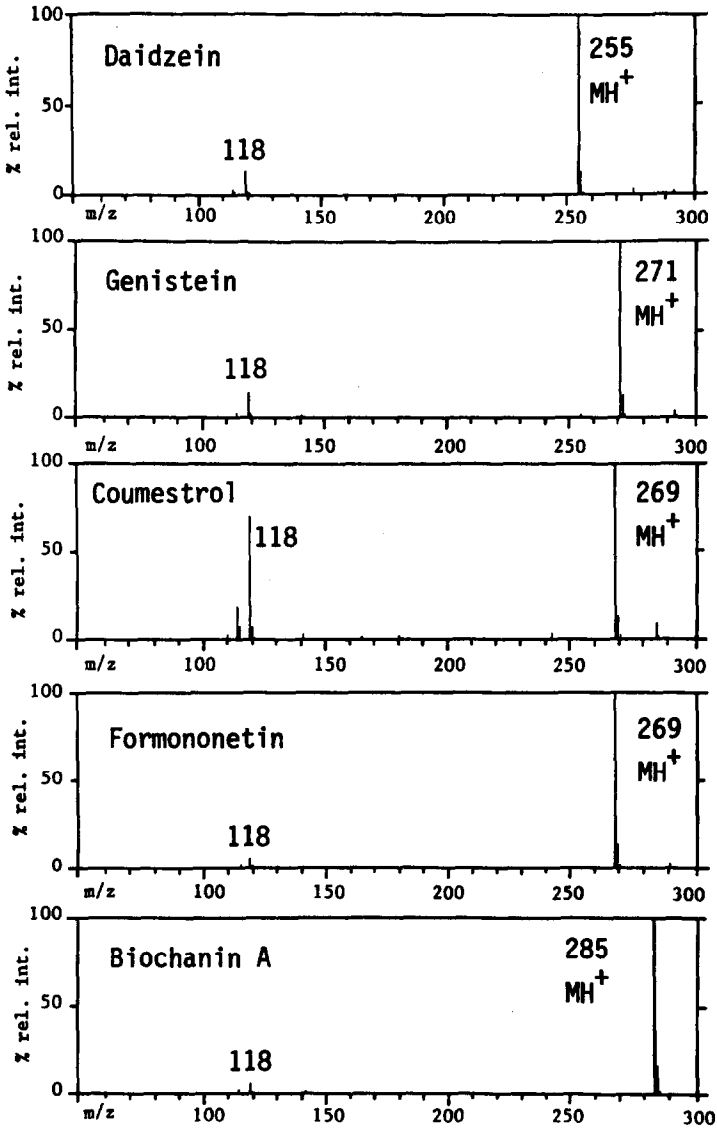

Fig. 6. Mass spectra obtained by thermospray ionization, during HPLC–MS analysis of authentic standards of phytoestrogens.

compounds with a 100-fold improvement in sensitivity over the scanning mode or UV detection alone. Furthermore, we suggest that these compounds would be ideally suited to HPLC–MS–MS detection, where, after focusing the [MH^+] ion, collision-induced dissociation would yield fragmentation specific for each compound, thereby assisting structural elucidation of these and unknown phytoestrogens or metabolites, separated by HPLC. This approach is under evaluation.

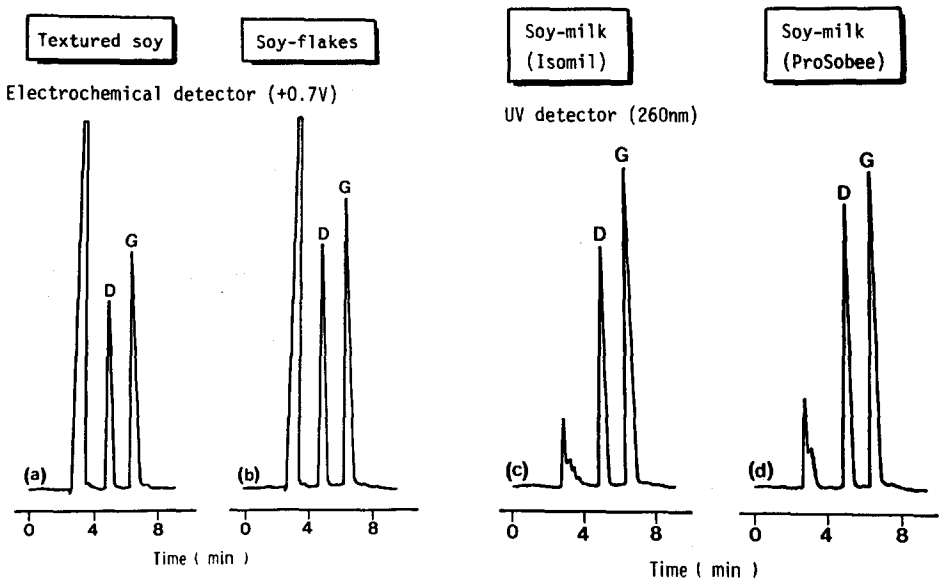

Fig. 7. HPLC profiles of phytoestrogens isolated from samples of soybean products. Both ED and UV detection are illustrated to demonstrate their applicability.

Analysis of soy protein products

Since the phytoestrogens exist in plants mainly as glycoside conjugates[1,2,17,29] or in biological fluids from man and animals as glucuronide or sulphate conjugates[8,10,27], hydrolysis of the conjugate moiety is required prior to HPLC analysis. A general scheme for the analysis of diets or biological fluids was therefore developed to include hydrolysis with glucosidase and/or glucuronidase–sulfatase. Where pure soy protein preparations are to be analyzed, the latter step is unnecessary, but with animal tissues or fluids this step should be considered essential[30]. Ideally, it would be better to develop a system for the direct analysis of the intact conjugates, but at this time the lack of readily available conjugated phytoestrogen standards makes this difficult.

Fig. 7 shows the HPLC analysis for textured soy and soy-flakes by ED and for the soy-milk formulae, Isomil and ProSobee, by UV detection. In all of these soybean products, daidzein and genistein were the only phytoestrogens detected, and

TABLE I

CONCENTRATIONS OF PHYTOESTROGENS IN SOY-BASED PRODUCTS DETERMINED BY HPLC

Soy product	*Daidzein (μg/g)*	*Genistein (μg/g)*
Textured soy	568	568
Soy flake	221	280
Soy-milk formula (ProSobee)	17.1	21.8
Soy-milk formula (Isomil)	19.1	22.6

their concentrations are indicated in Table I. Confirmation of the peaks in each product was made from the mass spectra, obtained by HPLC–thermospray MS, which were identical to those of the authentic compounds.

In earlier reports of the phytoestrogen content of soybean products, daidzein and genistein were the most abundant compounds identified, the latter in slightly higher concentrations[14,15,17–19], but it is evident that there is considerable variability between the different species of soybean and processed products[15].

REFERENCES

1 R. B. Bradbury and D. E. White, *Hormones*, 12 (1954) 207.
2 N. R. Farnsworth, A. S. Bingel, G. A. Cordell, F. A. Crane and H. H. Fong, *J. Pharm. Sci.*, 64 (1975) 717.
3 D. A. Shutt and R. I. Cox, *J. Endocrinol.*, 52 (1972) 299.
4 M. Shemesh, H. R. Lindner and N. Ayalon, *J. Reprod. Fertil.*, 29 (1972) 1.
5 K. Verdeal, R. R. Brown, J. Richardson and D. S. Ryan, *NCI, J. Natl. Cancer Inst.*, 64 (1980) 285.
6 B. Y. Tang and N. R. Adams, *J. Endocrinol.*, 85 (1980) 291.
7 E. Walz, *Justus Liebigs Ann. Chem.*, 489 (1931) 118.
8 M. Axelson, J. Sjovall, B. E. Gustafsson and K. D. R. Setchell, *J. Endocrinol.*, 102 (1984) 49.
9 K. D. R. Setchell, S. P. Borriello, P. Hulme, D. N. Kirk and M. Axelson, *Am. J. Clin. Nutr.*, 40 (1984) 569.
10 C. Bannwart, T. Fotsis, R. Heikkinen and H. Adlercreutz, *Clin. Chim. Acta*, 136 (1984) 165.
11 C. Bannwart, H. Adlercreutz, T. Fotsis, K. Wahala, T. Hase and G. Brunow, *Finn. Chem. Lett.*, (1984) 120.
12 H. Adlercreutz, T. Fotsis, C. Bannwart, T. Makela, K. Wahala, G. Brunow and T. Hase, Abstract presented at *Estrogens in the Environment: Influences on Development*, Research Triangle Park, NC, 1985.
13 *Diet Nutrition and Cancer*, Committee on Diet Nutrition and Cancer, Assembly of Life Sciences, National Research Canine, National Academy Press, Washington, DC, 1982.
14 A. C. Eldridge, *J. Chromatogr.*, 234 (1982) 494.
15 P. A. Murphy, *Food Technol.*, 34 (1982) 60.
16 S. Z. Dziedzic and J. Dick, *J. Chromatogr.*, 234 (1982) 497.
17 A. C. Eldridge, *J. Agric. Food Chem.*, 30 (1982) 353.
18 H. Pettersson and K-H Kiessling, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 503.
19 A. Seo and C. V. Morr, *J. Agric. Food Chem.*, 32 (1984) 530.
20 G. F. Nicollier and A. C. Thompson, *J. Chromatogr.*, 249 (1982) 399.
21 J. Sachse, *J. Chromatogr.*, 298 (1984) 175.
22 E. Farmakalidis and P. A. Murphy, *J. Chromatogr.*, 295 (1984) 510.
23 C. R. Blakley, M. J. McAdams and M. L. Vestal, *J. Chromatogr.*, 158 (1978) 261.
24 C. R. Blakley, J. J. Carmody and M. L. Vestal, *J. Am. Chem. Soc.*, 102 (1980) 5931.
25 M. L. Vestal, *Int. J. Mass Spectrom. Ion Phys.*, 46 (1983) 193.
26 C. R. Blakley and M. L. Vestal, *Anal Chem.*, 55 (1983) 750.
27 M. Axelson and K. D. R. Setchell, *FEBS Lett.*, 123 (1981) 337.
28 K. D. R. Setchell, in J. McLachlan (Editor), *Estrogens in the Environment: Influence on Development*, Elsevier, New York, 1985, p. 69.
29 M. Naim, B. Gestetner, S. Zilkah, Y. Birk and A. Bondi, *J. Agric. Food Chem.*, 22 (1974) 866.
30 K. D. R. Setchell, S. J. Gosselin, M. B. Welsh, J. O. Johnston, W. F. Balistreri, L. W. Kramer, B. L. Dresser and M. J. Tarr, *Gastroenterol.*, (1986) in press.