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ANALYSIS OF SOYBEAN SAPOGENINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the analysis of soybean sapogenins is described. The method is based on the extraction of soybean saponins from a defatted sample. The triterpene glycosides are then hydrolysed with subsequent analysis of the liberated sapogenins by high-performance liquid chromatography using gradient elution and mass detection. By use of a sapogenin/carbohydrate ratio, an estimate of the total saponin content can be made.

INTRODUCTION

Saponins are glycosides which occur in a wide variety of plants. Hydrolysis yields the carbohydrate moiety and the sapogenin; either a steroid (usually a derivative of spirostan-3β-ol) or one of a number of triterpenoids.

Saponins have been shown to have diverse biological properties including fungistatic, haemolytic, insecticidal and miscellaneous pharmacological activities. The role of saponins as growth depressants in poultry and as a cause of ruminant bloat has been reported[1]. Recently, dietary saponins have been shown to lower plasma cholesterol levels in experimental animals[2] and may have a similar effect in the human diet[3].

Previous quantification of these compounds has been based upon (a) yield after a lengthy isolation procedure[4], (b) colorimetry after hydrolysis using a modified Liebermann-Burchard reagent[5] and (c) thin-layer chromatography followed by densitometry[6]. Previous estimates of the saponin content of soybean range from 0.5 (ref. 5) to 5% (ref. 6). Our work on the beneficial and/or detrimental properties of minor constituents in the human diet has dictated a need for a rapid, specific method for the analysis of these compounds in soybean and its products. The method developed analyses the soya sapogenins liberated upon acidic hydrolysis. Fig. 1 shows the recently revised[7] structures of soyasapogenols A, B, C and E. A fifth soyasapogenol, D, has been reported[8] but it has not been found, to date, in any of our investigations.

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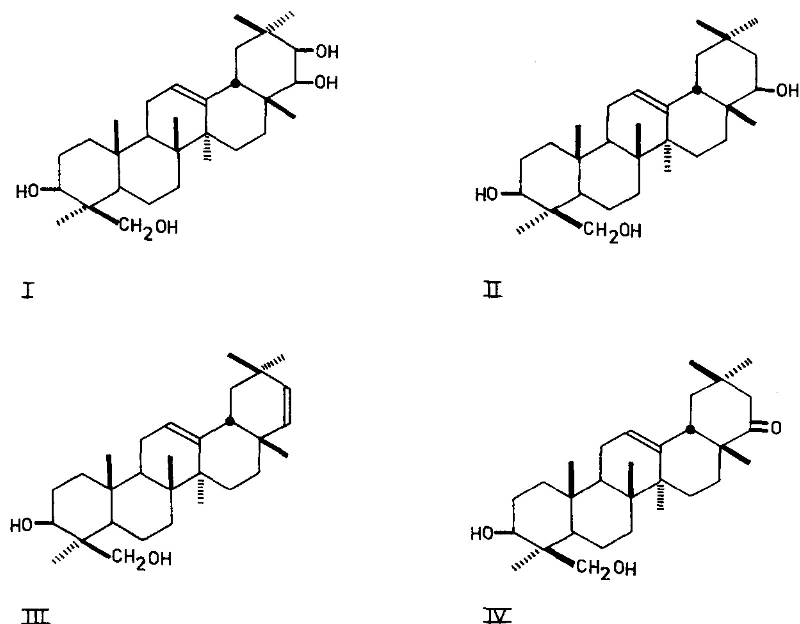

Fig. 1. Structures of soyasapogenol A (I), soyasapogenol B (II), soyasapogenol C (III), soyasapogenol E (IV).

EXPERIMENTAL

Materials

All solvents were of AR (analytical reagent) grade.

Isolation of soyasapogenols

Soyasapogenols A, B, C and E were isolated in our laboratory as detailed below.

Defatted soya flour was exhaustively extracted with methanol in a soxhlet apparatus. After removal of methanol under reduced pressure the extract was partitioned between *n*-butanol and 5% sodium chloride solution (w/v, pH adjusted to 4 by hydrochloric acid). The *n*-butanol layer was evaporated to dryness under reduced pressure and saponin present hydrolysed by refluxing with 3 *N* sulphuric acid in 1,4-dioxane–water (1:3, v/v) for 5 h. The sapogenins were extracted from the cooled hydrolysis mixture with ethyl acetate which was subsequently washed with 2% potassium hydroxide (w/v) and water before drying over magnesium sulphate.

The sapogenins were separated by repeated preparative thin-layer chromatography (TLC) on silica gel 60G (Merck, 7731) using light petroleum (b.p. 60–80°C)–ethyl acetate (4:3) as eluent and layers of 1 mm prepared in our laboratory. The compounds were visualised by spraying with 0.01% Rhodamine 6G in ethanol and viewing under UV. The bands corresponding to the soyasapogenols were scraped off, eluted with diethyl ether and rechromatographed by the same technique until pure. The separation was monitored by TLC using precoated silica gel 60 (Merck, 5715)

with the same eluent and visualisation with 10% sulphuric acid in ethanol and heating at 120°C for 5 min. The mass spectra of the isolated sapogenols using electron ionization displayed base peaks due to the expected retro Diels-Alder reaction characteristic of Δ^{12}-unsaturated pentacyclic triterpenes[9] (m/z 250, soyasapogenol A; m/z 234, soyasapogenol B; m/z 234, soyasapogenol B_1; m/z 232, soyasapogenol E and m/z 216, soyasapogenol C). Soyasapogenols A, B, and C had identical retention times under the high-performance liquid chromatography (HPLC) conditions used as authenic samples.

Extraction procedure

Soybeans (U.S.D.A. Grade II) were milled (UDY cyclone sample mill, Tecator, CO, U.S.A.). The milled soybean was defatted with light petroleum (b.p. 60–80°C) for 16 h in a soxhlet apparatus. A weighed quantity (*ca.* 18 g) of the soybean sample (either milled, defatted seed or defatted soya flour) was hydrolysed with 3 *N* sulphuric acid (133 ml) in 1,4-dioxane–water (1:3, v/v) for 5 h under reflux. The solution was cooled to room temperature, diluted with water (100 ml) and filtered under suction. The residue was washed with ethyl acetate (200 ml) which was subsequently used to extract the sapogenins from the hydrolysis solution. The residue and hydrolysis solution were extracted with two further portions of ethyl acetate (2 × 100 ml) and the combined extract was washed with 2% potassium hydroxide (w/v, 2 × 100 ml), water (2 × 100 ml) and dried over magnesium sulphate. The sapogenin extract was evaporated under reduced pressure and dissolved in chloroform to constant volume (5 ml).

Chromatography

The instrument used was a Gilson Model 704 gradient chromatograph with computerised integration and data handling (Anachem, Luton, U.K.). Detection was with a mass detector, Model 750/14 (Applied Chromatography Systems, Luton, U.K.). Injection was carried out via a Rheodyne injection valve, Model 7125 (20 µl loop). Commercially packed (Hichrom, Reading, U.K.) columns (25 cm × 4.6 mm) of silica (LiChrosorb Si 60, 5 µm) were used. The mobile phase consisted of a light petroleum (b.p. 60–80°C)–ethanol gradient as shown in Table I using a flow-rate of 1.5 ml min^{-1}.

TABLE I

HPLC GRADIENT EMPLOYED

Solvent A, light petroleum (b.p. 60–80°C); solvent B, ethanol.

Time (min)	*%B*
0	0
7.5	7.5
15	7.5
20	20

RESULTS AND DISCUSSION

The detection system used was the mass detector, a device which relies upon light scattering of the eluate after nebulisation and solvent evaporation. The calibration curves for soyasapogenols A, B, C and E under the conditions used for their HPLC separation are shown in Fig. 2.

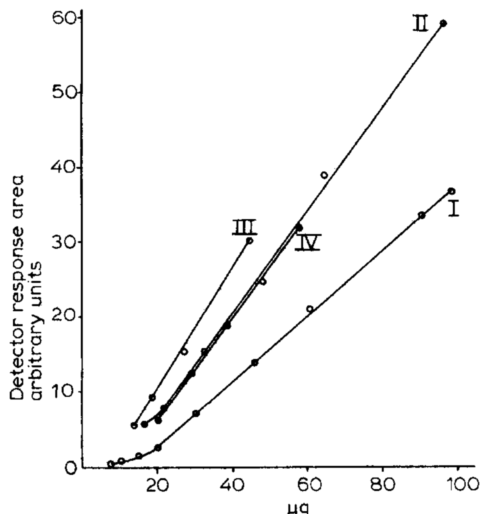

Fig. 2. Calibration of mass detector for Soyasapogenols.

The variation in the slopes of the calibration curves is probably due to the individual sapogenols being eluted in an eluate of differing solvent composition resulting from the gradient employed and the differing volatilities of the two solvents under the evaporation conditions. This effect can also be used to explain the similarity of the calibration curves for soyasapogenols B and E if they are both eluted whilst solvent from the linear portion of the gradient is emerging from the column.

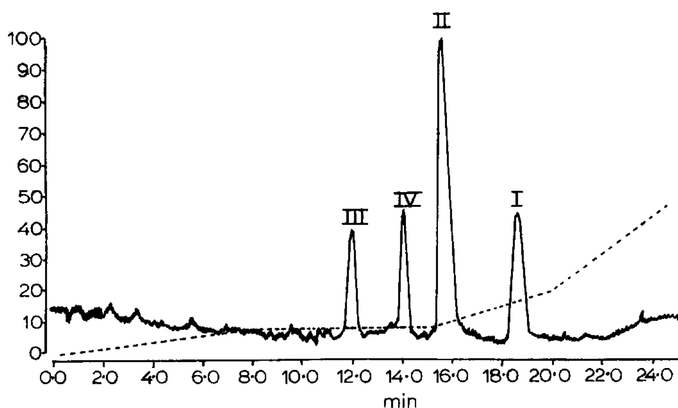

Fig. 3. Chromatogram of soyasapogenol standards. Column: 25 cm silica (5 µm); flow-rate: 1.5 ml min^{-1}; gradient: see Table I; mass detector: attenuation 1; photomultiplier sensitivity, 2; time constant, 5 sec; nebuliser gas pressure, 22 p.s.i.; evaporator temperature setting, 50°C.

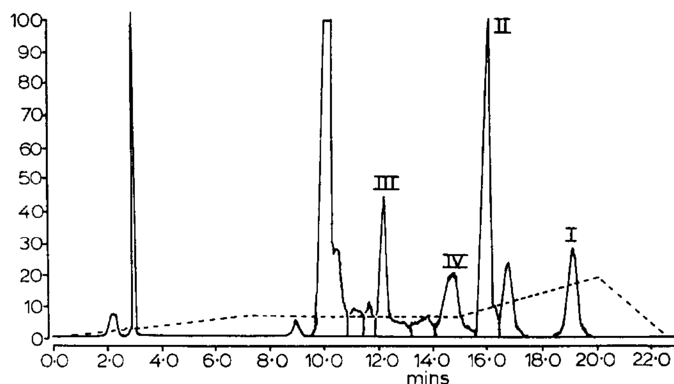

Fig. 4. Chromatogram of sapogenin extract of defatted soya flour. Conditions as Fig. 3.

The chromatogram of the soyasapogenin extract (Fig. 4) contains an additional peak, eluting between soyasapogenols A and B, when compared to a standard mixture of soyasapogenols A, B, C and E (Fig. 3). A small amount (*ca.* 1 mg) of this compound, referred to as B_1, was isolated and the mass spectrum obtained closely resembled soyasapogenol B. Insufficient soyasapogenol B_1, was available for calibration of the mass detector, however it was assumed to give the same response as soyasapogenols B and E.

Table II gives the sapogenin profile of the soybeans and defatted soya flour investigated. This is the first time that the sapogenin profile has been available; previously only the profile of radioactive soyasapogenols[10] obtained upon incubating germinating soybeans with radioactively labelled precursors (either [2-^{14}C]mevalonic acid or [^{14}C]squalene) was available. The possibility of artefact formation upon acid hydrolysis of soya saponins has been noted[11] and this is currently being investigated in our laboratory using the HPLC method developed to monitor the sapogenin profile.

Table III compares the saponin content of soybean and defatted soya flour obtained with those published previously. The saponin content has been calculated by determining the total sapogenin content and applying a sapogenin to saponin conversion factor of 2 dèrived from a carbohydrate/sapogenin ration of 1:1 (w/w)

TABLE II

SAPOGENIN PROFILE OF SOYBEANS

N.D. = not detected.

Soyasapogenol	*Whole seed (%)**	*Defatted flour (%)**
A	22.4	22.3
B	24.6	28.7
B_1	18.3	14.7
C	18.1	13.5
D	N.D.	N.D.
E	16.6	20.8

* Mean of triplicates.

TABLE III

SAPOGENIN CONTENT OF SOYBEAN

	*Sapogenin content (%)**
Whole soybean	0.073**,***
Literature whole soybean[6]	5.0§
Literature whole soybean[5]	0.46–0.50**
Defatted soya flour	0.38**,***
Literature defatted soya flour[6]	2.0§
Literature defatted soya flour[5]	0.55–0.60**

* Wet weight basis.
** Determined by analysis of sapogenins and applying a carbohydrate/sapogenin ratio of 1:1 (w/w).
*** Mean of triplicates.
§ Recalculated from original source[6] to wet weight basis.

obtained for soya saponins by Gestetner *et al.*[5]. The value for whole soybean is low and could result from the variety of seed analysed having a low saponin content, but this is unlikely. The saponin content of the defatted soya flour examined, although slightly lower than that found by Gestetner *et al.*[5], indicates that the saponin content of defatted soya flour is at the lower end of the range of 0.5 to 5% and not as high as that found by Fenwick and Oakenfull[6].

The low values obtained could be a consequence of incomplete hydrolysis of the saponins or losses of saponins during the extraction procedure. The effect of time of hydrolysis on the total sapogenin yield has been studied[12] and the optimum time for maximum yield of sapogenins from a crude soya saponin extract was found to be 5 h. All waste aqueous layers produced during the extraction procedure were re-extracted with diethyl ether and monitored by thin-layer chromatography to ensure complete extraction of sapogenins; no evidence of incomplete extraction or losses was found. Although further work needs to be carried out to improve the quantitative aspect of this method, the results presented indicate that the levels of saponins in soya may not be as high as has recently been suggested[6].

ACKNOWLEDGEMENTS

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