Journal of Chromatography, 361 (1986) 410–416
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 683

Note

High-performance liquid chromatography of soyasaponins on silica phase with evaporative light-scattering detection

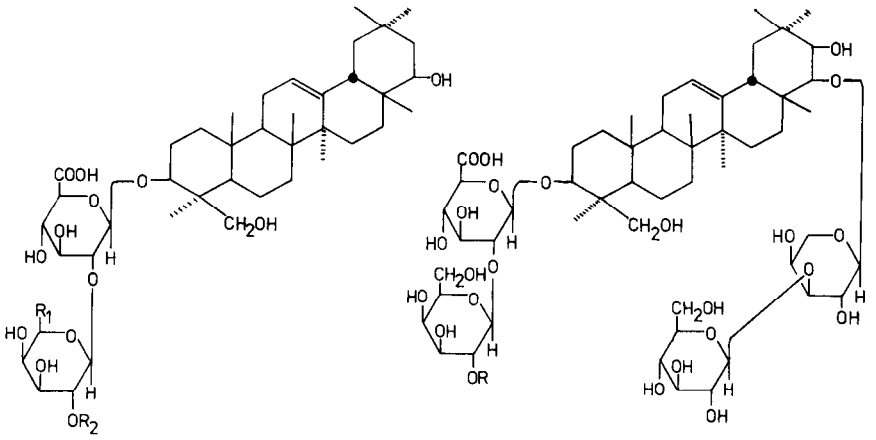
P. A. IRELAND* and S. Z. DZIEDZIC

Department of Food Science, University of Reading, Food Studies Building, Whiteknights, P.O. Box 226, Reading RG6 2AP (U.K.)

(First received February 24th, 1986; revised manuscript received March 26th, 1986)

Saponins are steroid or triterpenoid glycosides which exhibit a wide range of biological properties and are known to occur in a wide variety of food plants[1]. Five triterpenoid saponins: soyasaponins I, II and III[2], A_1[3] and A_2[4] have been isolated from soya (*Glycine max.* L. Merr) and their structures eluciated (Fig. 1). It has been noted that some of these saponins may exist in an acetylated form[5], and soyasaponin I has also been observed, both in free and acetylated forms, in pea (*Pisum sativum* L.)[6].

Recently four chromatographic methods for the determination of soya saponins have appeared[5–9]. A method based upon thin-layer chromatography (TLC) followed by densitometry[7], recently modified[6], yields information on total saponin con-



Soyasaponin I: R_1 = CH_2OH;
R_2 = α-L-rhamnopnraposyl
Soyasaponin II: R_1 = H;
R_2 = α-L-rhamnopyranosyl
Soyasaponin III: R_1 = CH_2OH;
R_2 = H

Soyasaponin A_1: R = β-D-glucopyranosyl
Soyasaponin A_2: R = H

Fig. 1. Structures of soyasaponins I, II, III, A_1 and A_2.

0021-9673/86/$03.50

tent. Two further methods, one using gas–liquid chromatography after derivatisation[8] and the other high-performance liquid chromatography (HPLC)[9], quantify the sapogenins (aglycones) released after hydrolysis of the saponins. These two methods[8,9] give the sapogenin profile and content, which can then be used to estimate saponin content by use of a sapogenin/carbohydrate ratio[10]. The fourth chromatographic method determines saponins by HPLC of the fluorescent coumarin derivatives[5]. Although the use of fluorescent coumarin derivatives overcomes difficulties in the detection of soya saponins, the isolation and derivatisation procedure used in this method is unable to provide information on the proportion of acetylated to free saponins and, also, unable to separate the coumarin derivatives of soyasaponins II and III, and quantify these two saponins individually. The fluorescent coumarin derivatives are formed by esterification with the carboxylic acid moiety of the glucuronic acid residue common to all five of the soyasaponins. It is not therefore possible to develop and extend this method to the analysis of neutral saponins.

Consequently, the aim of this study was to investigate the use of HPLC with the mass detector (an evaporative light-scattering detector) to separate and detect the soya saponins without derivatisation. The separation achieved was then used to investigate the saponin profile of some soya products whose saponin content had previously been determined by HPLC of the sapogenins after hydrolysis[11]. By observing the chromatographic behaviour of the saponin fractions before and after a deacetylation step, it was hoped to gain an insight into the presence and extent, if any, of acetylation of the saponins in the various soya samples.

EXPERIMENTAL

Materials

C_{18} Sep-Pak™ cartridges were obtained from Millepore-Waters (Harrow, U.K.). Samples of defatted soya flour (Arkasoy 50) and soya protein isolate (Ardex D.H.V.) were obtained from British Arkady Co. (Manchester, U.K.). Soya beans were obtained from British Soya Products (Ware, U.K.). Two separate samples of UHT soya milk were purchased locally. Chromatographic standards of soyasaponins I, II and III were kindly donated by Dr. G. R. Fenwick (AFRC, Norwich, U.K.) and standards of soyasaponins A_1 and A_2 were kindly donated by Professor I. Kitagawa (Osaka University, Osaka, Japan). Chromatographic standards of the isoflavone glycosides were isolated by preparative TLC and identified by mass spectrometry of the isoflavones after acid hydrolysis.

Sample preparation

Soya beans (U.S.D.A. Grade II) were milled (UDY cyclone sample mill, Tecator, Bristol, U.K.). The milled soya bean was defatted with light petroleum (b.p. 60–80°C) for 16 h in a Soxhlet apparatus.

The samples of soya milk were freeze dried.

Extraction and isolation of saponin fraction

A sample (5 g) of each of the two soya milks was defatted by refluxing with ethyl acetate (50 ml) for 15 min. The sample was filtered under suction and the defatting repeated by refluxing with a further portion of ethyl acetate (50 ml) for 15

min. The defatted sample was recovered by filtration under suction. The sample was then refluxed with methanol (50 ml) for 30 min, cooled, filtered under suction and the methanol extraction repeated. The combined methanol extract was divided into two equal portions from which the solvent was removed under reduced pressure. Each portion was then dispersed in distilled water (10 ml) and passed through a Sep-Pak C_{18} cartridge which had been preconditioned by washing with methanol (2 ml) followed by water (5 ml). The cartridge was washed with a further portion of water (10 ml) and the saponin fraction eluted from the cartridge with methanol (5 ml). The solvent was removed under reduced pressure and the saponin fractions dissolved in dry methanol (1 ml).

The above procedure, with the exception of the defatting stage, was repeated to give two identical saponin fractions from each of the following: milled, defatted soya beans, defatted soya flour and soya protein isolate.

Throughout the extraction and isolation procedure, to ensure no loss of compound, the location of the saponins was monitored by TLC on silica gel 60 G (Merck 5715) using chloroform–methanol–water (6:4:1) as eluent and visualising with 10% sulphuric acid in ethanol and charring.

Zemplén deacetylation of saponin fractions

A solution of sodium methoxide in dry methanol (1 *M*) was prepared by carefully dissolving sodium (2.3 g) in dry methanol (100 ml). Deacetylation was accomplished by the addition of ten drops of the sodium methoxide solution to one of the duplicates of each the saponin fractions isolated and stirring at room temperature for 20 min. The sample was neutralised by the addition of a small quantity of Dowex 50W-X8 resin, which was subsequently filtered off to yield the deacetylated saponin fractions.

Chromatography

The instrument used was a Gilson Model 704 gradient chromatograph with computerised integration and data handling (Anachem, Luton, U.K.). Detection was

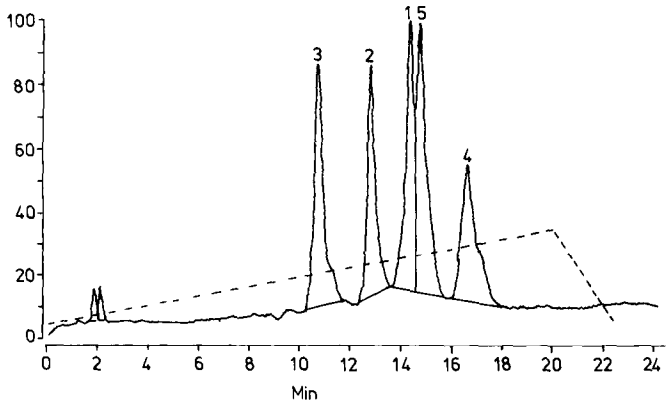


Fig. 2. Chromatogram of soyasaponin I (1), II (2), III (3), A_1 (4) and A_2 (5) standards. Column, 25 cm × 4.6 mm I.D. silica (5 μm); flow-rate, 1.5 ml min^{-1}; mass detector: attenuation, 1; photomultiplier sensitivity, 2; time constant, 5 s; nebuliser gas pressure, 22 p.s.i.; evaporator temperature setting, 50°C.

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). Columns (25 cm × 4.6 mm I.D.) of silica (Spherisorb 5 μm silica, Phase Separations, Queensferry, U.K.) packed in our laboratory were used. Solvent A was chloroform containing 1% (v/v) acetic acid and solvent B methanol–water–acetic acid (95:4:1). The linear gradient rose from 5% B to 35% B in 20 min. The flow-rate was 1.5 ml min^{-1}.

RESULTS AND DISCUSSION

The chromatogram of the standard soyasaponins is shown in Fig. 2. Although poorly resolved, soyasaponins I and A_2 are clearly evident.

Insufficient standard soyasaponins were available to calibrate the mass detector and consequently only qualitative observations, based upon spiking the samples with the standard soyasaponins, were available. An attempt to chromatograph the soyasaponins on a reversed-phase octadecylsilyl column proved unsuccessful as the high proportion of water required in the mobile phase was incompatible with the use of the mass detector because of incomplete vaporisation of the solvent eluate.

The chromatogram of the saponin extract isolated from the defatted soya flour (Fig. 3) indicates the presence of soyasaponins I and II together with a small amount of soyasaponin III. On deacetylation of the saponin fraction a component corresponding to soyasaponin A_1 is observed together with soyasaponins I and II (Fig. 4). The ratio of soyasaponin I to soyasaponin II increases upon deacetylation, as can be seen by comparing Figs. 3 and 4, thus it appears that in the original saponin extract soyasaponin A_1 is acetylated to an extent which causes it to be co-eluted with soyasaponin II. The other two main components in the chromatograms, before and after deacetylation, which are eluted before the saponins, are the isoflavone glycosides daidzin and genistin.

The soya protein isolate analysed displayed similar results to the soya flour: soyasaponins I and II together with a small amount of soyasaponin III in the original saponin fraction, and the appearence of soyasaponin A_1 with a corresponding drop

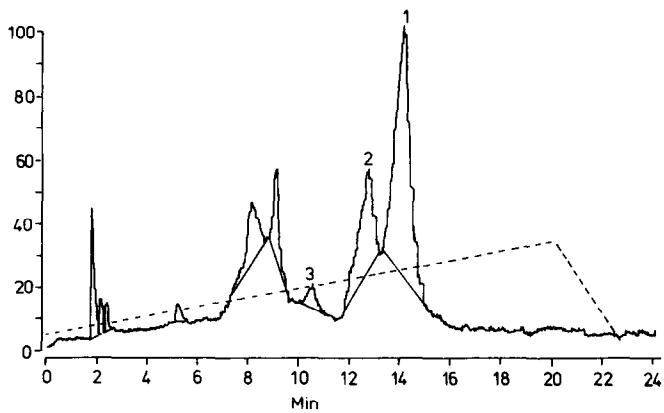


Fig. 3. Chromatogram of saponin extract of defatted soya flour. Conditions and peaks as in Fig. 2.

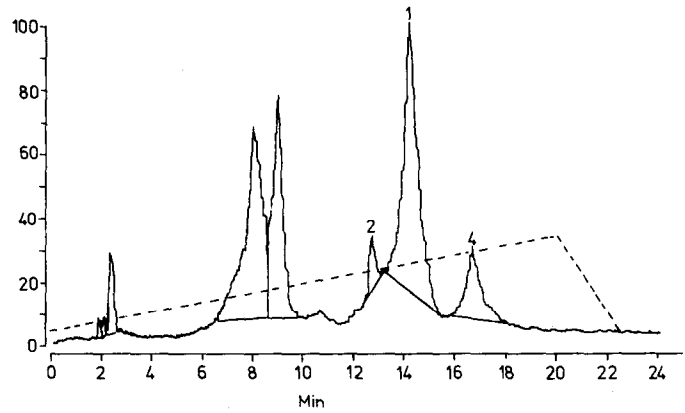


Fig. 4. Chromatogram of saponin extract of defatted soya flour after deacetylation. Conditions and peaks as in Fig. 2.

in the level of soyasaponin II upon deacetylation. One difference, however, between the soya protein isolate and the soya flour is the absence of the isoflavone glycosides in the soya protein isolate. The isoflavone glycosides appear to be removed during the repeated isoelectric precipitation steps used to prepare the protein isolate whilst the saponins are carried over with the protein, probably as a result of binding due to their surface activity.

The saponin fraction of the soya beans analysed gives a slightly different chromatogram (Fig. 5) to the soya flour. Soyasaponins I and II, the isoflavone glycosides and a small amount of soyasaponin III are present in the original saponin fraction as well as an additional component eluting between soyasaponins I and II, indicating the presence of an additional, unidentified saponin. However, after deacetylation (Fig. 6), this component is absent but a peak corresponding to soyasaponin A_1 appears, indicating that the unknown component is, in fact, soyasaponin A_1 in an acetylated form. The fact that, in the soya bean saponin fraction, the acetylated

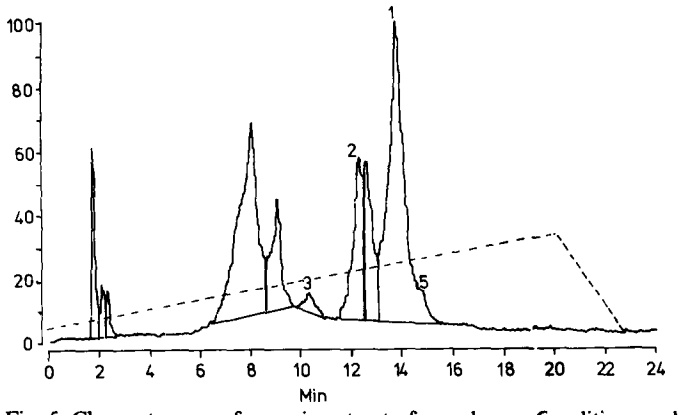


Fig. 5. Chromatogram of saponin extract of soya beans. Conditions and peaks as in Fig. 2.

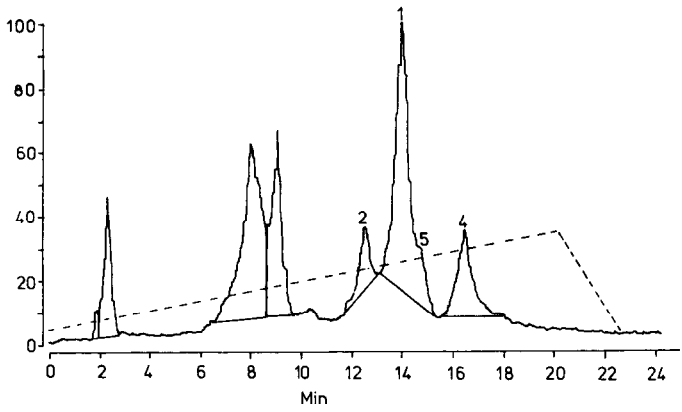


Fig. 6. Chromatogram of saponin extract of soya beans after deacetylation. Conditions and peaks as in Fig. 2.

soyasaponin A_1 component is not co-eluted with soyasaponin II indicates a different degree of acetylation between the forms of soyasaponin A_1 in the soya beans and the soya flour and protein isolate. The presence of soyasaponin A_2 is indicated in both the original and deacetylated saponin fractions of the soya beans (Figs. 5 and 6) but is poorly resolved from soyasaponin I.

Two commercially available UHT soya milks were analysed. Previous determination of the saponin content of the two soya milks by HPLC analysis of the aglycones[11] had revealed soyasapogenol B as the only aglycone present, indicating the absence of soyasaponins A_1 and A_2 which contain soyasapogenol A. One soya milk contained soyasaponin I (major) and soyasaponin II, deacetylation not altering the saponin profile of this sample. Thus soyasaponin III and the isoflavone glycosides were absent, as were soyasaponins A_1 and A_2 which had already been inferred from the analysis of the sapogenins of the sample[11]. The second soya milk also only contained soyasaponins I (major) and II as well as the isoflavone glycosides. As with the other soya milk, deacetylation did not alter the saponin profile and soyasaponins III, A_1 and A_2 were absent. Thus these results confirm the absence of soyasaponins A_1 and A_2 in these two soya milks, as previously inferred from the absence of soyasapogenol A after hydrolysis of the saponins[11], as well as indicating the absence of soyasaponin III.

The results show that it is possible to separate and detect soyasaponins I, II, III, A_1 and A_2 without recourse to derivatisation, although the resolution between soyasaponins I and A_2 is poor. It may be possible to improve the separation of these components by use of a polar bonded phase such as amino, nitrile or LiChrosorb Diol. Calibration of the mass detector would then lead to a rapid method for the quantitative determination of individual and total soya saponins that does not require derivatisation.

Additionally, quantification of individual saponins in the saponin fraction before and after deacetylation would result in information on the proportion of individual saponins in an acetylated form being available for the first time.

A lack of sufficient pure material has prevented quantitative analysis but puri-

fication of standard soyasaponins to enable the development of a quantitative method is in progress. It can be seen that HPLC with the mass detector can be applied to the analysis of saponins, both neutral and acidic, from other sources without the need for derivatisation.

ACKNOWLEDGEMENTS

We are grateful to Dr. G. R. Fenwick and Professor I. Kitagawa for donation of standard soyasaponins, and to British Arkady Co. Ltd. and British Soya Products Ltd. for donation of samples.

REFERENCES

1 D. Oakenfull, *Food Chem.*, 7 (1981) 19.
2 I. Kitagawa, M. Yoshikawa and I. Yosioka, *Chem. Pharm. Bull.*, 24 (1976) 121.
3 I. Kitagawa, M. Saito, T. Taniyama and M. Yoshikawa, *Chem. Pharm. Bull.*, 33 (1985) 1069.
4 I. Kitagawa, M. Saito, T. Taniyama and M. Yoshikawa, *Chem. Pharm. Bull.*, 33 (1985) 598.
5 I. Kitagawa, M. Yoshikawa, T. Hayashi and T. Taniyama, *Yakugaku Zasshi*, 104 (1984) 275.
6 C. L. Curl, K. R. Price and G. R. Fenwick, *Food Chem.*, 18 (1985) 241.
7 D. E. Fenwick and D. Oakenfull, *J. Sci Food Agric.*, 32 (1981) 273.
8 I. Kitagawa, M. Yoshikawa, T. Hayashi and T. Taniyama, *Yakugaku Zasshi*, 104 (1984) 162.
9 P. A. Ireland and S. Z. Dziedzic, *J. Chromatogr.*, 325 (1985) 275.
10 B. Gestetner, Y. Birk, A. Bondi and Y. Tencer, *Phytochemistry*, 5 (1966) 803.
11 P. A. Ireland, S. Z. Dziedzic and M. W. Kearsly, *J. Sci. Food Agric.*, in press.