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The Quantitative Estimation of Saponin in Pea (*Pisum sativum* L.) and Soya (*Glycine max*)

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ABSTRACT

A thin-layer chromatographic method is described for the analysis of saponins in pea and soya flours. The results are compared with those of methods using gas chromatography and high performance liquid chromatography. The levels of saponin found in the air-classified fractions of pea flour are reported. The observed levels of saponin in soya, as estimated by thin-layer and gas chromatography (0·35 % and 0·33 %), are much lower than that previously reported using the former approach and reasons for this discrepancy are discussed.

INTRODUCTION

Saponins are a diverse group of biologically active glycosides which are generally characterised as bitter or astringent (Rackis *et al.*, 1966). They are widely distributed in the plant kingdom being found in over 500 genera (Agarwal & Rastogi, 1974). Amongst plants grown for human foods, the presence of saponins in legumes such as soya and pea is particularly important because of the increasing use of such crops as sources of separated protein, starch and fibre (Sumner *et al.*, 1981). It is possible that the processing techniques which are necessary to achieve such separations will also cause the original plant saponins to be concentrated in certain fractions (Price *et al.*, 1985); this may in extreme cases lead to the occurrence of undesirable biological effects (Elkowicz & Sosulski, 1982). For this reason it is important that reliable methods are

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available for the determination of saponins in food plants and processed foods.

A number of different techniques have been applied to the analysis of saponins. Originally these included gravimetric methods (Van Atta *et al.*, 1961) or those using the foam-forming properties of saponins (O'Dell *et al.*, 1959). Subsequently bioassays were introduced, for example those relying on anti-fungal (Livingstone *et al.*, 1977) or haemolytic effects (Jones & Elliot, 1969). However, these are generally properties which relate to particular structural types of saponins, and cannot be used to determine these compounds *in toto*. Spectrophotometric methods have been described (Gestetner *et al.*, 1966) but suffer from the disadvantage that they are non-specific. Recently the content and nature of saponins in plant extracts have been determined using thin-layer chromatography (TLC) (Fenwick & Oakenfull, 1981), high performance liquid chromatography (HPLC) (Kitagawa *et al.*, 1984*b*) or gas chromatography (GC) (Kitagawa *et al.*, 1984*a*). Whilst the first two approaches allow the examination of the intact saponins, GC requires prior hydrolysis and derivatisation and thus is more accurately described as a technique for aglycone (sapogenin) estimation and identification.

The TLC method of Fenwick & Oakenfull (1981) has been widely used to determine saponin levels in plants and foodstuffs. However, in the course of a recent study we observed that the saponin content of pea and soya flours, as determined by this method, were inconsistent with sensory analyses (Price *et al.*, 1985), which suggested much lower concentrations. It was also observed that the saponin contents of other commodities determined using the published TLC method were not in agreement with more recent data obtained using GC or HPLC (Kitagawa *et al.*, 1984*a*,*b*; Ireland & Dziedzic, 1985). These findings prompted us to re-examine the TLC method, to introduce modifications and, using this improved method, to compare the saponin contents of soya and pea flours with those determined by GC.

EXPERIMENTAL

Materials

Pea flour (*cv.* Filby) and the four air-classified fractions of pea flour (i.e. protein; protein and starch; starch; testa) were kindly supplied by Dr

D. J. Wright of this Institute. Defatted soya was obtained by the hexane defatting of soyabean meal purchased from BDH Chemicals Ltd, Poole, Dorset, UK. All solvents were redistilled from BDH Analar grade. Reversed phase silica gel column packing was obtained from J. T. Baker Chemical Co., Phillipsburg, New Jersey, USA. Silica gel TLC plates (0·25 mm) were purchased from Merck. Soyasaponin I was isolated from defatted soya flour as described previously (Price & Fenwick, 1984). Bis(trimethylsilyl) trifluoroacetamide (BSTFA) was purchased from Pierce Chem. Co., Rockford, Illinois, USA and cholesteryl *n*-decylate from Sigma Chem. Co., Poole, Dorset. The GC column packed with 3% OV-1 on Diatomite C-NAW 60-80 mesh was bought complete from J.J.'s Chromatography Ltd, King's Lynn, Norfolk, UK.

METHODS

Extraction and clean-up procedure

This is outlined in Fig. 1. Defatted flour (1 g) was weighed into an extraction thimble and Soxhlet-extracted with methanol (100 ml) for approximately 30 h. The methanol extract was evaporated to dryness under reduced pressure and then dissolved up in a small volume (about 3 ml) of distilled water. This aqueous solution was applied to a flash chromatography column containing 3 g of reversed phase (RP) octadecylsilane (C_{18}) bonded to silica gel. The column was washed with water (20–30 ml) then eluted with methanol until no further colour was eluted off (20–30 ml). The methanol eluate was evaporated to dryness under reduced pressure.

Thin layer chromatography

The methanol eluate from the RP column was dissolved in 1·0 ml methanol. Ten microlitres of this solution was spotted onto a silica gel TLC plate (0·25 mm) alongside standard spots of soyasaponin I (1–30 μg). The TLC plate was developed in *n*-butanol:ethanol:0·880 ammonia (7:2:5) for a distance of 12 cm. The air-dried plate was sprayed with a mixture of anisaldehyde: glacial acetic acid: 97% sulphuric acid (1:100:2) and heated to 120 °C for 5–10 min to visualise the spots, purple–blue colours being characteristic of steroids and triterpenes.

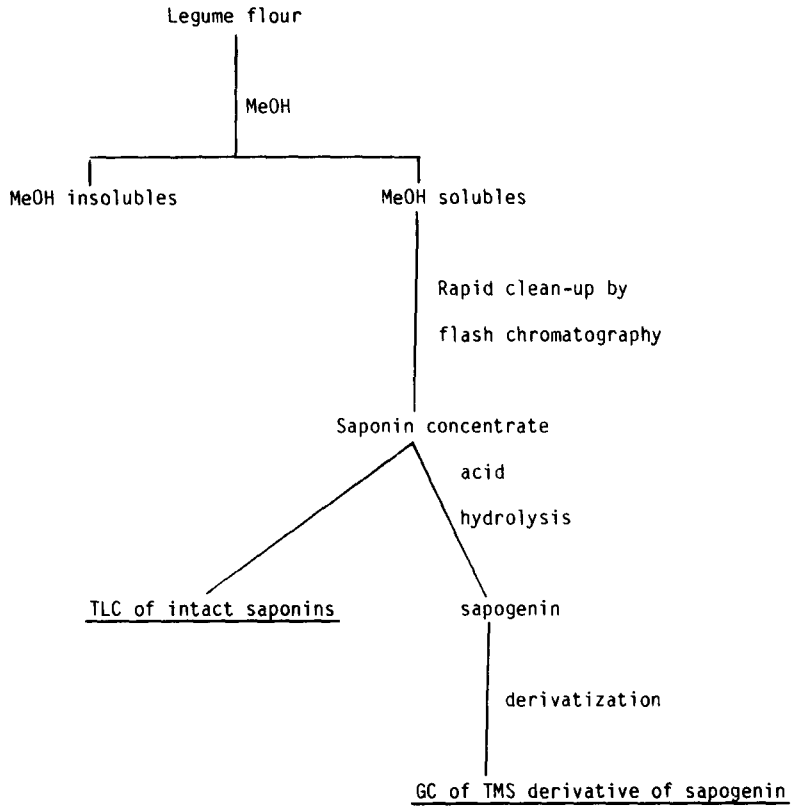

Fig. 1. Outline of saponin clean-up procedures.

Quantification

Photocopies of the sprayed and heated plates were used to quantify the saponin spots using a Vitatron Thin Layer Densitometer TLD100 linked to a Spectraphysics SP4270 integrator. Errors caused by changes in colour intensity of the plates due to the heating effects in the densitometer were eliminated by the use of the photocopies. A calibration curve was constructed from the standard soyasaponin I spots and the levels of saponin in the unknown calculated from the curve.

Hydrolysis of saponin extracts

The methanol eluate from the RP column was dissolved in 5 ml of 5% HCl in dry methanol and refluxed for 3 h. After cooling, the mixture was

neutralised with 5 M ammonium hydroxide solution then evaporated to dryness under reduced pressure. The product was dissolved in 5 ml of distilled water and extracted with 3 × 5 ml of ethyl acetate; combined ethyl acetate extracts were dried over anhydrous sodium sulphate, filtered and evaporated to dryness *in vacuo*.

Derivatisation

The hydrolysed saponin extract was treated with pyridine (0·2 ml) and BSTFA (0·2 ml) and heated in a sealed vial at 50 °C for 20 min.

Gas chromatography

Two microlitres of the derivatised solution was coinjected with 2 μl of a 2 mg/ml chloroform solution of cholesteryl *n*-decylate onto a Pye 104 GC, fitted with a 0·9 m × 3 mm i.d. glass column packed with 3% OV-I on Diatomite C-NAW 60-80 mesh and FID detector. The column temperature was 270 °C, the injector temperature 375 °C and the argon flow rate 35 ml/min. A standard sample of soyasaponin I was hydrolysed and derivatised as for the sample extracts and 1·25–15 μg samples were injected onto the GC to construct a calibration curve. Peak areas were integrated by a Trivector Trilab 2000 data station.

RESULTS AND DISCUSSION

Flour from the mature dried seed of *P. sativum* contains only one saponin, identified as soyasaponin I (Price & Fenwick, 1984). Some of this saponin is present in the acetylated form and may be deacetylated by treatment with alkali in methanol (K. R. Price, unpublished results); however, these acetylated components coelute in the TLC system described here. The absence of any other saponins on the chromatogram was confirmed by preparative TLC in the same solvent. Bands of silica corresponding to the saponin band and the areas above and below it were removed from the developed plate, extracted with methanol and the resulting extracts hydrolysed. TLC of these concentrated extracts only showed released sapogenin for the band corresponding to soyasaponin I. This was confirmed by mass spectrometry. The relationship between the weight of saponin (soyasaponin I) spotted onto the TLC plate and spot

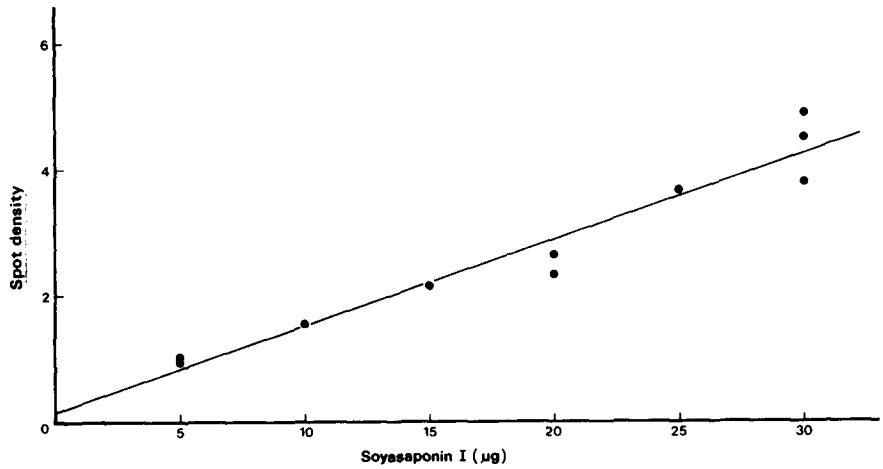

Fig. 2. Calibration curve for saponins using TLC method.

density is shown in Fig. 2. A linear fit on this calibration data gave a slope of 0·136 with a standard deviation of 0·0125 and a correlation coefficient of 0·964. The detection limit on the TLC plate was found to be approximately 1 μg of saponin which would correspond to a level of 0·01 % by weight of saponin in the flour analysed.

Gas chromatographic analysis of saponins involves the acid hydrolysis of the saponin mixture and derivatisation of the released sapogenins. The relationship between the weight of saponin (soyasaponin I), prior to the hydrolysis stage, and the peak area of the derivatised sapogenin (soyasapogenol B) by GC is shown in Fig. 3. A linear fit of this data gave a slope of 169·57 with a standard deviation of 9·93 and a correlation coefficient of 0·977. The detection limit was estimated to be 0·5 μg of saponin which would correspond to a level of 0·01 % by weight of saponin in the flour.

Table 1 compares the saponin content of four fractions derived from pea flour and the whole flour itself when measured by both of these methods. It will be noted that the fraction containing the highest concentration of saponin gave approximately twice the value by TLC as was obtained by GC. This difference is probably due to the presence of a co-eluting non-saponin component on the thin-layer chromatogram. The presence of this same component in the whole flour would also explain the slightly higher value obtained for saponin in that sample. The other three fractions show good agreement. Table 2 shows the saponin content

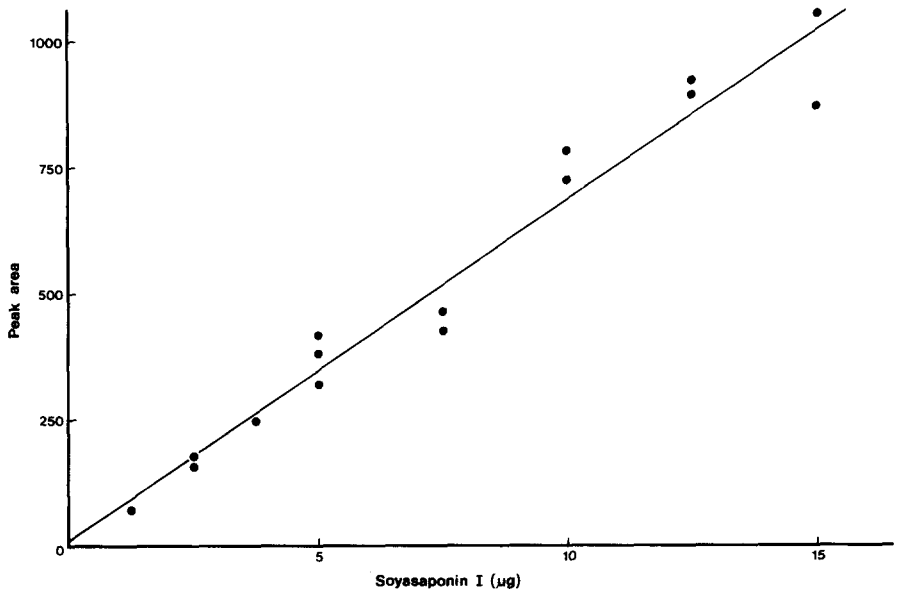

Fig. 3. Calibration curve for saponins using GC method.

based on the proportion that each fraction represents of the original whole flour (protein fraction 22·3 %, protein and starch mixture 10·5 %, starch fraction 56·3 % and testa 10·9 %). The sum of the figures by TLC and GC, 0·21 % and 0·15 % respectively, agree well with the figures obtained from the original whole flour of 0·18 % and 0·14 %, respectively.

The results reported here demonstrate that the levels of saponin present in pea flour can be estimated by a simple and relatively inexpensive TLC method but that errors may be introduced when processed flour is analysed.

TABLE 1
Saponin Content of Pea and Its Fractions

Flour fraction	*Saponin content (%)*	
	TLC	*GC*
Whole flour	0·18	0·14
Protein rich	0·54	0·25
Protein + starch	0·16	0·16
Starch rich	0·11	0·10
Testa	0·06	0·08

TABLE 2
Distribution of Saponin (Expressed as a Percentage of Whole Flour)

Flour fraction	*Saponin content (%)*	
	TLC	*GC*
Protein rich	0·12	0·06
Protein + starch	0·02	0·02
Starch rich	0·06	0·06
Testa	0·01	0·01
Total	0·21	0·15

A comparison of the saponin content found in soya by various methods reported in the literature is given in Table 3. The figures reported by Fenwick & Oakenfull (1981) are several times larger, a result which is most readily explained by the technique adopted by these authors, several spots on the chromatogram being considered to be saponins on the basis of their response to a relatively non-specific spray reagent. The agreement between the chromatographic methods described here and by Kitagawa *et al.* (1984*a*, *b*) and Ireland & Dziedzic (1985) is surprisingly good, bearing in mind the inherent variability of the biological materials examined. Our results show a total saponin content, as determined by GC, of 0·33% (comprising 0·24% combined soyasaponins I, II and III expressed as

TABLE 3
Comparison of Saponin Contents of Soya

Method	*Reference*	*Saponin (% defatted meal)*
TLC	Fenwick & Oakenfull (1981)	2·2
TLC	Present work	0·35
GC	Kitagawa *et al.* (1984*a*)	0·35[a]
GC	Present work	0·33
HPLC (intact saponins)	Kitagawa *et al.* (1984*b*)	0·31[a]
HPLC (sapogenins)	Ireland & Dziedzic	0·38
Spectrophotometry	Gestetner *et al.* (1966)	0·58[b]
Mould bioassay	Livingstone *et al.* (1979)	0·01[a]
Haemolysis	Elkowicz & Sosulski (1982)	0

[a] Corrected for assumed lipid content of 20% in the meal.
[b] Average of six varieties.

soyasaponin I and 0·09 % combined soyasaponin A_1 and A_2 expressed as soyasaponin A_1) in very close agreement with that determined by TLC, 0·35 %, where all the saponins are assumed to be present in a single spot, having an R_f of approximately 0·19. Kitagawa *et al.* (1984*a*, *b*) reports saponin levels determined by GC and HPLC (intact saponins) of 0·35 % and 0·31 % respectively, in close agreement both with each other and the levels reported here. Ireland & Dziedzic (1985) using a method involving the separation and quantification of the aglycones produced from soyasaponins have recently reported a figure of 0·38 %. Gestetner *et al.* (1966) found the mean saponin content of six varieties of soya to be 0·58 % (range 0·55–0·60 %) using a spectrophotometric approach. It is evident from the table that soyasaponins have little, if any, antifungal or erythrocyte-lysing activity; hence these methods of assay are clearly inappropriate for soya and pea products, although they continue to be used (Livingstone *et al.*, 1979). It is interesting to note that soya flour purchased in the UK has a significantly higher proportion of soyasaponin I (> 73 %) in the total saponin mixture when compared with figures reported by Kitagawa *et al.* (1984*a*) in soya from USA (44 %) and Canada (41 %) as determined by GC.

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