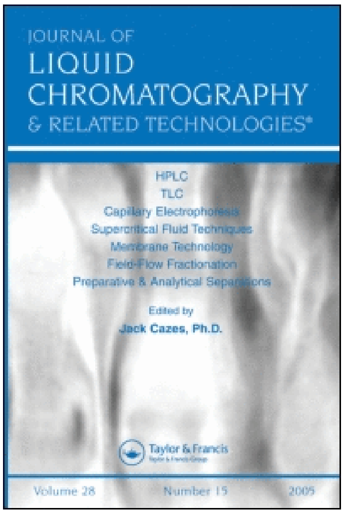

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DETERMINATION OF SAPONINS IN LUPIN SEED (LUPINUS ANGUSTIFOLIUS) USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: COMPARISON WITH A GAS CHROMATOGRAPHIC METHOD

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ABSTRACT

A quantitative method for the determination of intact saponins in *Lupinus angustifolius* seed by high-performance liquid chromatography (HPLC) is described. The seed was extracted under mild conditions with 70% aqueous ethanol containing 0.01% EDTA and quantified using an internal standard of α-hederin. Soyasaponin VI, also known as soyasaponin βg, a DDMP (2,3 -dihydro - 2,5 - dihydroxy - 6 - methyl - 4*H* - pyran - 4 - one)- conjugated form of soyasaponin I, was the only saponin detected in the lupin seed. The technique is compared with a gas chromatographic method which quantifies the sapogenol resulting from the acid hydrolysis of the saponins.

INTRODUCTION

Saponins are glycosides with terpenoid or steroid aglycones occurring primarily in plants, which are consumed by animals and man but usually processed industrially or domestically prior to consumption. A wide range of beneficial and deleterious properties has been ascribed to saponins, for example, hemolytic activity, cholesterol-binding ability, bitterness, and fungitoxic activity. However, these properties are better seen as characterizing particular types of saponin rather than being shared by all members of the chemically-complex group of saponins (1).

The quantitative determination of saponins has for a long time posed problems to the analytical chemist (2). A number of chromatographic methods have been used for saponin analysis and especial attention has focused on the use of gas chromatography (3) but it has the limitation that it can only be used for the separation and quantification of the aglycone portion of the saponin (after hydrolysis and suitable derivatization) which involves both the loss of structural information about the glycosidic portion of the molecule and potential loss of material during hydrolysis and derivatization.

Since relatively little is known about the effect of both industrial and domestic processing on the fate of the saponins in food and with recent work demonstrating a relationship between chemical structure and biological activity (4, 5), there is a requirement for analytical methods that can measure the individual saponins as they exist in the food matrix. However, the development of techniques for the analysis of these intact saponins, such as HPLC, has been limited due firstly to the difficulties with the detection of triterpene saponins which do not contain a UV-chromophore and secondly to the lack of appropriate standards. Derivatisation procedures have increased the sensitivity of HPLC methods but suffer from selectivity and stability problems (6). Kudou et al. (7), Yoshiki et al. (8) and Okubo et al. (9) have successfully separated, on a qualitative basis using HPLC, intact saponins from soybean (*Glycine max*), runner bean (*Phaseolus coccineus*) and American groundnut (*Apios americana*) respectively and the study reported here has further developed this work for the separation and quantification of the intact saponins present in lupin seed (*Lupinus angustifolius*) and compared the results obtained using the conventional GC method for the analysis of saponins.

MATERIALS

Lupin seed used was a commercial Australian variety of *L. angustifolius* called Warrah.

The internal standard used, α-hederin, was purchased from Apin Chemicals Ltd (Abingdon, Oxon, United Kingdom). Saponin βg (soyasaponin VI) was generously donated by Dr. K. Okubo and coworkers of the Faculty of Agriculture, Tohoku University. Soyasaponin I was isolated from soybean.

All solvents used during the extraction process were of analytical-reagent grade and water was purified using a Milli-Q system (Waters Chromatography Division, Millipore Corporation, Milford, USA).

METHODS

High-Performance Liquid Chromatographic Analysis

Extraction procedure

The lupin seed were milled with a coffee grinder (Janke & Kunkel, Ika-Werk). Four grams of the sample and 3 mg of powdered α-hederin were firstly soaked in 70%

aqueous ethanol containing 0.01% EDTA for 10 min and secondly stirred for 1 h at room temperature. The sample was then centrifuged at 8000 rpm for 15 min at 20°C. This extraction process was repeated three more times and the supernatants combined and filtered through a sinter funnel under reduced pressure. The extract was rotary-evaporated to dryness at less than 40°C and dissolved in a mixture of 2 ml of water and 1-butanol (1:1, v/v). After centrifugation, the 1-butanol layer was collected and analyzed by HPLC.

Apparatus

HPLC analyses were performed using a Philips PU 4100 liquid chromatograph coupled to a Philips PU UV 4025 detector and a Gilson 715 data collection system. Separations were performed on a column (25 cm x 4.6 mm ID) packed with Ultratechsphere 5μ C_{18} (HPLC Technology Ltd, Macclesfield, UK).

Chromatographic Conditions

Chromatographic runs were carried out with an acetonitrile-water gradient elution system. Solvents were acetonitrile-acetic acid (1000:0.3 v/v) (Solvent A) and water:acetic acid:EDTA (1000:0.3:0.15 v/v/w) (Solvent B). Solvents were degassed with helium. The gradient was run according to the following programme: 65% A isocratically for 18 min followed by a reduction to 58% A after a further 4 min, held at 58% A for 10 min then changed to 65% A over a further 4 min and finally held at 65% A for 8 min. The flow rate was 0.9 ml/min and detection was monitored by UV absorption at 205 nm.

Gas chromatography and Fast Atom Bombardment - Mass Spectrometry

These techniques are described by Price et al. (3).

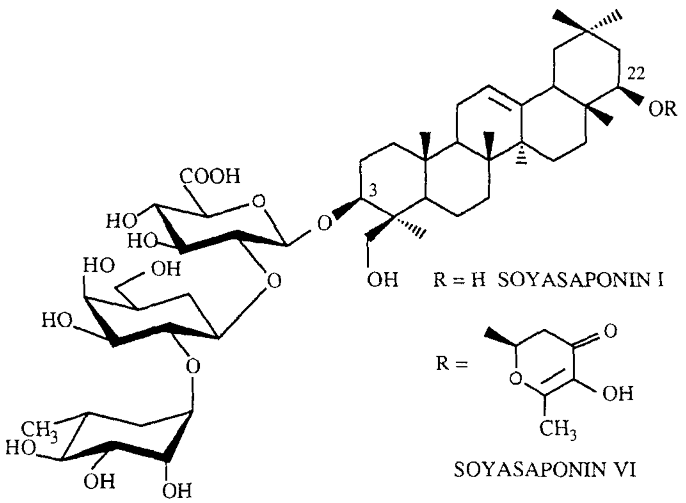

FIGURE 1. Chemical Structures of Soyasaponin I and Soyasaponin VI.

RESULTS AND DISCUSSION

HPLC analyses of soyasaponin I (SSI) and soyasaponin VI (SSVI), also known as soyasaponin βg (see figure 1), which are the saponins known to be present in *L. angustifolius* seed (10), together with the internal standard α-hederin revealed well resolved peaks whose retention times were 16.0, 28.3 and 32.3 min respectively (see figure 2).

Due to the relative instability of SSVI and in order to check its purity, data from the GC analysis of soyasapogenol B, which resulted from the hydrolysis of 1 mg of each of SSI and SSVI, showed SSVI to be 30% pure.

Peak-area ratio calibrations with the internal standard (HPLC) were then constructed for both SSI and SSVI with the purity of SSVI calculated from GC

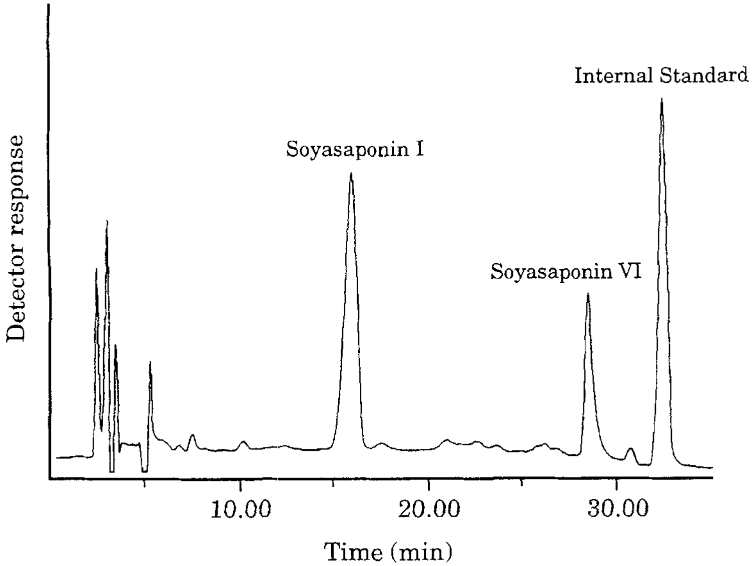

FIGURE 2. Chromatogram of a Mixture of Soyasaponin I, Soyasaponin VI, and α-Hederin.

measurements being included in these calculations. The equation constant, correlation coefficient, weight range and limit of detection for each saponin are illustrated in Table 1. The calibration plots showed good linearity for both SSI and SSVI (figure 3).

Fast atom bombardment-mass spectrometry (FAB-MS) of the seed extract containing the intact saponins after exhaustive hot solvent extraction as described in Price et al. (3), showed the presence of SSI, with a molecular weight of 942, and SSVI, with a molecular weight of 1068 (figure 1). The two saponins were calculated to be in roughly equimolar proportions. The total saponin content of the lupin seed was estimated to be 385 mg/kg by using a combination of the quantification of the acid released sapogenol by GC analysis and the ratio of sapogenol/saponin from the FAB mass spectral measurements.

TABLE 1

Chromatographic Constants for HPLC Analysis

Saponin	equation y = a + bx		r	Range (µg)	Internal Standard (µg)	Limit of Detection (µg)
SSI	-4.9228e-3	3.1573e-2	0.998	0 - 50	30	3.2
SSVI	-1.4656e-2	3.6615e-2	1.000	0 - 48.3	30	1.5

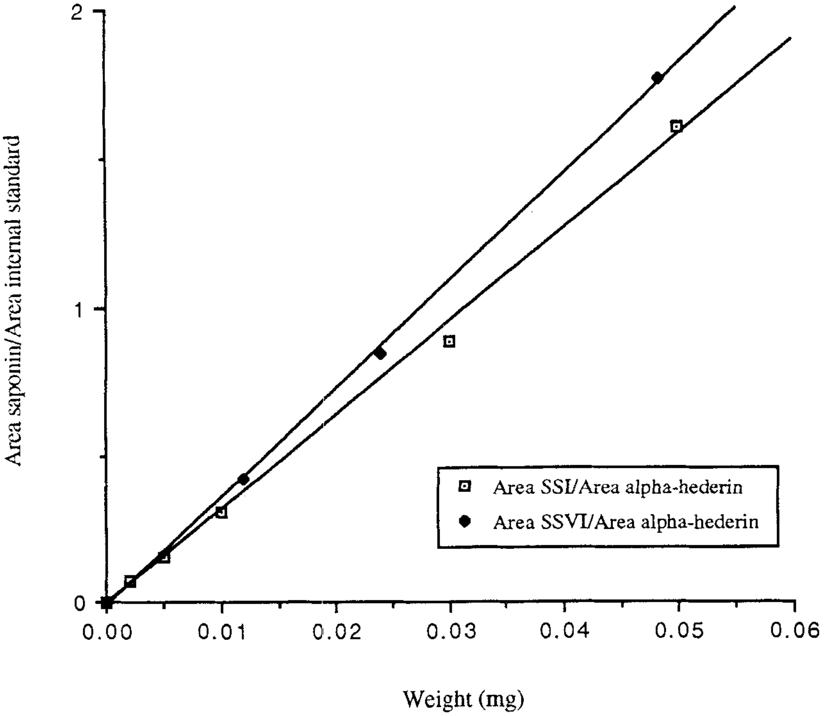

FIGURE 3. Calibration curves for Soyasaponin I and Soyasaponin VI.

In contrast the chromatogram resulting from the analysis of the lupin seed extract by HPLC following the mild extraction conditions described here is shown in figure 4. Retention time comparisons relative to the internal standard a-hederin and confirmation by co-chromatography of the reference saponins showed the presence of only SSVI. SSI was not detected. This finding is in agreement with Massiot et al. (11) who isolated SSVI from alfalfa and characterized it as the natural precursor of SSI and Kudou et al. (7) who postulated that these DDMP-conjugated saponins were, in fact, the genuine saponins in the case of native soybean seeds whilst soyasaponins I-V, the saponins normally associated with soya following conventional exhaustive hot solvent

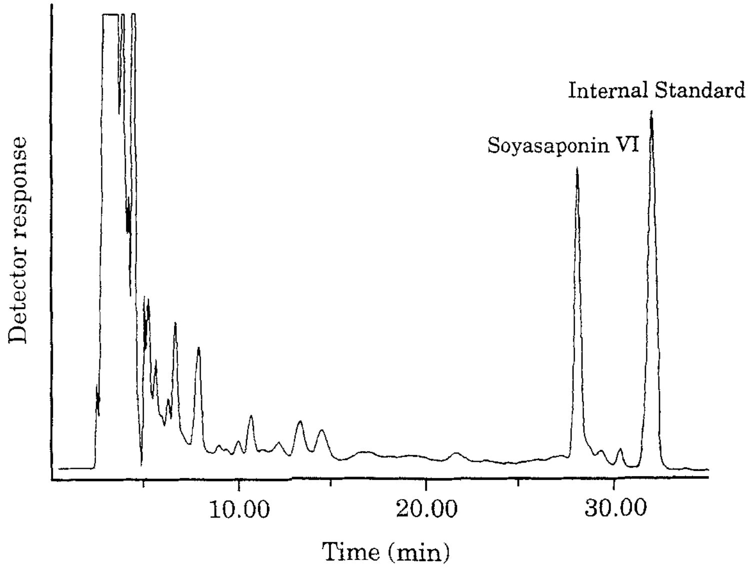

FIGURE 4. Chromatogram of *Lupinus Angustifolius* Seed Saponins (Soyasaponin VI) with Internal Standard (α-Hederin).

extraction, were in fact artifacts derived form degradation of the DDMP saponins during the extraction or concentration processes.

The saponin content of the lupin seed was estimated to be 450 mg/kg using the HPLC technique. This value was 14% higher than the one obtained from the GC technique, which suggests that saponin losses occurred during some of the stages involved in the latter method. Since the internal standard used for the GC analysis is not glycosidic in nature it cannot be added during the extraction, cleanup or hydrolysis stages and it is likely therefore that these would be the stages where saponin loss could occur. The mild extraction technique used here was confirmed to be quantitative by subsequent exhaustive hot solvent extraction and HPLC analysis of the extract in which neither SSI or SSVI were detected.

The HPLC method described here provides the quantification of intact, underivatised individual saponins in legumes with the use of a suitable internal standard and a proven mild quantitative extraction technique. It will therefore enable quantitative studies to be made for the first time on the effect of different kinds of processing not only on the total saponin content but also on the true saponin composition. These data will, in turn, allow the fate of the individual saponins, some of which have been shown to be relatively unstable (12) to be determined. Since some of the biological properties reported for saponins are now recognised to be dependent on their particular chemical structures (13), this type of information will allow further understanding of not only the relationship between chemical structure and bioactivity but also the impact of processing on that bioactivity in relation to both human and animal health.

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