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Preparative chromatographic purification and surfactant properties of individual soyasaponins from soy hypocotyls

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Abstract

The amphipathic character of soyasaponins and their consequent biological and technological properties are well-recognised. However, mainly due to the absence of purified compounds, no data are available on the amphiphilic surfactant properties of individual soyasaponins. In this study we developed a preparative method for the purification of the main soyasaponins species from soy germ. Reversed-phase chromatography (Source 15 RPC) gave a good resolution of the various soyasaponins, and was used for the purification of non- and fully-acetylated soyasaponin Ab, DDMP-conjugated and unconjugated soyasaponins Ba and Bb. For these compounds, the critical micelle concentration (CMC), the minimal attainable surface tension (γ_{CMC}) and the surface density (Γ_{max}) were determined using the Wilhelmy plate method. The order of CMC values was as follows: soyasaponin Bb < Ba < βg < αg < Ab < non-acetylated Ab, and the CMC was found to range from 0.56 and to 3.2 g/L. It was concluded that the number of sugar side chains, the presence of acetyl groups and the presence of a DDMP-group are the main factors influencing the CMC of soyasaponins.

Keywords: Soyasaponin; CMC; Surface tension; Reversed-phase preparative chromatography

1. Introduction

Soyasaponins constitute a group of non-volatile, amphiphilic molecules found in a wide variety of legume seeds, such as soybeans, peas, lentils and lupins (Lasztity, Hidevegi, & Bata, 1998). Soybean (*Glycine max* L. Merill) and soy-based food products are major dietary sources of soyasaponins (Fenwick & Oakenfull, 1983; Oakenfull, 1981). Based on differences in substitution of the triterpenoid aglycone (or soyasapogenol), soyasaponins are generally categorised in mono- (group B) and bidesmosides (group A); i.e., carrying one and two sugar chains, respectively. In Fig. 1A and B the structures of the soyasaponins relevant to this study are presented; for a more detailed description of all currently known soyasaponins the reader is referred to our previously published paper (Decroos et al., 2005).

Group B soyasaponins can be conjugated to 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) at C-22 (Kudou et al., 1993). The latter is believed to be the genuine form of group B saponins in soybeans, as conditions applied during processing and analysis may result in loss of the DDMP-group, resulting in the formation of the unconjugated counterparts (Heng et al., in press; Kudou et al., 1994). The terminal glucosyl residue of the disaccharide C-22 side chain of soyasaponins Ab, may be acetylated at four positions (Fig. 1A). It is believed that, in soybean, group A soyasaponins mainly exist in their fully acetylated form (Decroos et al., 2005; Kitagawa et al., 1988a, Kitagawa, Wang, Taniyama, & Yoshikawa, 1988b).

Interest in soyasaponins has risen in recent years as more knowledge has become available on the biological activities of soyasaponins and their potential health-beneficial

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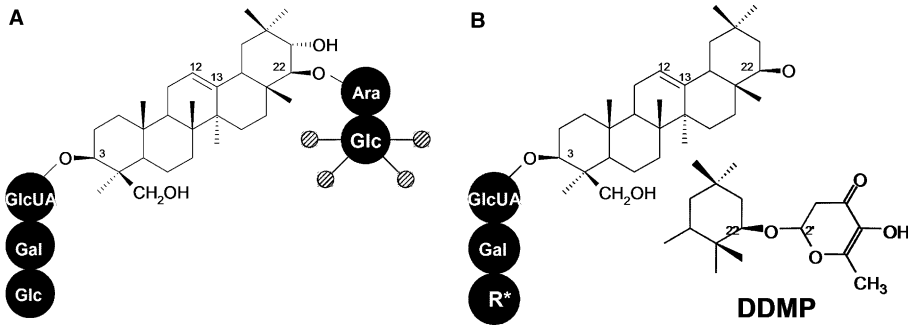

Fig. 1. Chemical structures of the components tested in this study. (A) Soysaponin Ab (gray circles represent acetyl groups; Glc, β-D-glucopyranosyl; Gal, β-D-galactopyranosyl; Ara, α-L-arabinopyranosyl; GlcUA, β-D-glucoronopyranosyl); (B) Group B soyasaponins $R^* = $ β-D-glucopyranosyl for soyasaponin Ba (unconjugated), and αg (DDMP-conjugated); $R^* = $ α-L-rhamnopyranosyl for soyasaponin Bb (unconjugated), and βg (DDMP-conjugated).

effects. Some of the biological activities attributed to saponins are related to their amphipathic character. The formation of mixed micelles with bile salts and cholesterol in the intestine is believed to cause a hypocholesterolaemic effect, as faecal excretion of these unabsorbable complexes causes a drain in the cholesterol pool in the body (Lee, Simons, Murphy, & Hendrich, 2005; Milgate & Roberts, 1995). Their amphiphilic nature enables saponins to interact with specific constituents of biological membranes, and to alter membrane permeability, hence their adjuvant properties (Oda et al., 2000, 2003; Sparg, Light, & van Staden, 2004). Other reported effects include immunostimulatory, anti-viral, hepatoprotective and antitumourigenic activities (Bae, Han, Choo, Park, & Kim, 2002; Berhow, Wagner, Vaughn, & Plewa, 2000; Gurfinkel & Rao, 2003; Lasztity et al., 1998; Milgate & Roberts, 1995; Philbrick, Bureau, Collins, & Holub, 2003; Rao & Sung, 1995; Rowlands, Berhow, & Badger, 2002).

An important limitation in soyasaponin research is the absence of purified components, which makes it difficult to unambiguously attribute the observed effects to individual molecules. Methods to purify specific soyasaponin species have been reported, but these methods mainly focus on the purification of a single soyasaponin species (Hu et al., 2002; Kinjo, Imagire, Udayama, Arao, & Nohara, 1998; Philbrick et al., 2003; Sasaki, Minowa, Kuzuhara, Nishiyama, & Omoto, 1998; Yoshiki, Kudou, & Okubo, 1998). However, procedures for the preparative-scale fractionation of mixtures of soyasaponins into single-component fractions are currently lacking.

Due to their amphiphilic structure, a hydrophobic aglycone (soyasapogenol) coupled to hydrophilic sugar moieties, soyasaponins are surface-active, i.e., they possess foaming and emulsifying properties, and can aggregate into unimolecular as well as in mixed micelles (Lasztity et al., 1998; Oakenfull, 1986). The micellar behaviour of a native mixture of saponins from *Quillaja saponaria*, which have a similar carbon skeleton as soyasaponins, has been investigated extensively by Mitra and Dungan (1997, 2000, 2001). They showed that the critical micelle concentration (CMC), being the minimal concentration of a surface-active compound at which it forms micelles in aqueous solution, varied between 0.1 and 0.8 g/L. For individual soyasaponins, however, no data on the micellar properties are available. As soyasaponins form a structurally diverse group, differences in values for the different parameters can be expected. The hydrophobic triterpenoid skeleton is the same for all the soyasaponins, but their decoration differs; particularly the hydrophilic saccharide side chains can differ in length, composition, number, and kind of attachment (ether- or ester-linked). In soybeans and soy-based foods, the soyasaponin composition may vary, depending on the soybean variety and the conditions applied during processing (Decroos et al., 2005; Gu, Tao, Gu, & Prior, 2002; Kudou et al., 1994; Shiraiwa, Harada, & Okubo, 1991, 1990). It is likely that this difference in composition affects the physico-chemical properties of the samples, and that different batches of soy (ingredients) behave differently in food applications. Therefore, it is important to know the properties of the individual soyasaponins, so that the behaviour of soyasaponin mixtures can be predicted from its composition.

In this study we developed a rapid and straight-forward protocol for the fractionation and purification of the different soyasaponins from soy hypocotyls, a rich source of the different soyasaponins, including group A, various group B, and various DDMP-conjugated group B saponins, using preparative liquid chromatography. Important quantitative parameters for defining the surface activity of a substance, being the CMC, the minimum attainable surface tension (γ_{CMC}) and the maximum surface density (Γ_{max}) were determined for the predominant purified soyasaponin species.

2. Materials and methods

2.1. Materials

Soy hypocotyls (variety HP204 USA, harvested in 2002 in Michigan, USA) were provided by Acatris Holding B.V. (Giessen, The Netherlands). Prior to the isolation of the hypocotyls, the soybeans were dried by a hot-air treatment for 20 min at 120 °C. The isolated hypocotyls were ground to a powder with an average particle size of 0.75 mm.

Equilenin, sodium cholate and Tween 80 were purchased at Sigma–Aldrich NV (Bornem, Belgium).

2.2. Extraction of soyasaponins from soygerms

The ground hypocotyls were defatted by extraction with hexane for 6 h under reflux conditions and air-dried afterwards. Subsequently, 10 g of defatted powder were extracted with 1 L of 70% (v/v) aqueous ethanol in an Erlenmeyer under constant agitation (150 rpm, 3 h, room temperature). After filtration over a paper filter (type 5971/2; Schleicher & Schuell, Gent, Belgium), the ethanol was removed by drying under vacuum at 30 °C. Next, the aqueous phase was applied onto a Supelpak™ XAD-2 (Supelco, Bellefonte, USA) column (26 × 210 mm), which had been conditioned with 200 mL 96% (v/v) aqueous ethanol, followed by 200 mL demineralised water, at a flow rate of 5 mL/min. After washing with 300 mL demineralised water, the column was eluted with 200 mL 96% (v/v) aqueous ethanol, and the eluate was evaporated to dryness under vacuum at 30 °C and denoted dried phytochemical extract. This extract consisted mainly of isoflavones and soyasaponins, as determined by HPLC.

2.3. Fractionation using Source 15 RPC

Four hundred milligrams of the dried phytochemical extract was dissolved in 5 mL 50% (v/v) aqueous methanol and applied onto a Source 15 RPC (Amersham Biosciences, Uppsala, Sweden) column (26 × 600 mm). The column was eluted with a solvent system consisting of water with 0.001% (v/v) acetic acid (A) and acetonitrile with 0.001% (v/v) acetic acid (B). The flow rate was 20 mL/min and the following gradient (Gradient 1) was applied: 0 → 10 min, 10% B (isocratic); 10 → 45 min, 10 → 25% B (linear gradient); 45 → 120 min, 25 → 35% B (linear gradient); 120 → 150 min, 35 → 100% B (linear gradient); 150 → 180 min, 100% B (isocratic). Eluting peaks were detected using a UV-detector at 205 and 295 nm, and fractions (2 mL) were pooled corresponding to the peaks in the resulting chromatogram. The organic phase was removed by vacuum drying at 30 °C, and the aqueous phase was removed by freeze-drying. As analysed by HPLC–MS, this yielded fractions each containing either no or a limited number of isoflavones or soyasaponins.

2.4. Purification of soyasaponins

One hundred milligrams of freeze-dried fractions obtained as described above, containing either soyasaponin Ab, αg or βg were re-solubilised in methanol, and resubjected to Source 15 RPC chromatography. Similar conditions as for the first run were used, except for the gradient employed (Gradient 2): 0 → 20 min, 10 → 25% B (linear gradient); 20 → 60 min, 25 → 35% B (linear gradient); 60 → 90 min, 35 → 100% B (linear gradient). Fractions (2 mL) were collected, and only appropriate fractions flanking the peak maximum were pooled, i.e., from two minutes before till two minutes after the retention time of the peak maximum (data not shown). The acetonitrile was evaporated under vacuum at 30 °C. The aqueous phase was subsequently dried by freeze-drying. In this way, soyasaponins Ab, αg and βg were obtained with a purity of >97% as determined by HPLC–MS.

Non-acetylated soyasaponin Ab and soyasaponins Ba and Bb were prepared by alkaline treatment. Fifty milligrams of soyasaponin Ab, αg and βg were dissolved in 50 mL of a 100 mM NaOH solution. After shaking for 30 min at 37 °C, an equivalent of acetic acid was added for neutralisation. The solutions were freeze-dried, and subjected to Source 15 RPC chromatography with Gradient 2. Fractions were collected and pooled as described above. The acetonitrile was evaporated under vacuum at 30 °C. The aqueous phase was dried by freeze-drying. In this way, non-acetylated soyasaponin Ab and soyasaponins Ba and Bb were obtained with a purity of >97% as determined by HPLC–MS.

2.5. HPLC analysis and mass spectrometry (HPLC–MS)

HPLC–MS analysis of soyasaponins samples was performed as described previously (Decroos et al., 2005).

2.6. Determination of the critical micelle concentration (CMC) using the Wilhelmy plate method

Surface tensions were measured with the Wilhelmy plate method using the Krüss 10 ST (Krüss, Charlotte, NC) equipped with a water bath maintained at 25 °C. Before measurement, the Wilhelmy plate was gently rinsed with chromosulfuric acid and deionised water and dried by heating. To verify the equipment's performance, the surface tension of water was determined between measurements; the deviation from the literature value was less than 0.5% at 25 °C. Dilution series of the test compounds were made in deionised water in flasks that had been thoroughly rinsed with chromosulfuric acid and deionised water, and were allowed to equilibrate for 2 h. The surface tension was then determined for each of the solutions. Experiments were performed in triplicate.

Experimentally, the CMC was determined from the inflection point of the plot of the surface tension versus the logarithm of surfactant concentration. Using linear regression, the equations describing the two linear parts in the plot were established. The CMC was then evaluated from the intersection of these two lines. The error in the CMC values obtained in this way was estimated from the standard deviation, using propagation of errors from the various parameters used in the linear regressions.

The minimal attainable surface tension (γ_{max}) was defined as the surface tension at the CMC. Density of the surfactants was determined from the slope of the decreasing line in the plot below the CMC, according to the Gibbs-equation: $\Gamma = -(1/RT)(-d\gamma/d\ln c)$ Γ_{max} is the surface

density (mol/m^2); $R = 8.314$ J/mol K; T is the absolute temperature (K); γ is the surface tension (mN/m); C is the surfactant concentration (mol/m^3).

2.7. Statistical analysis

All statistical analyses were performed using the SPSS 12.0 for Windows software.

3. Results

3.1. Soyasaponin and isoflavone composition of the phytochemical extract

The extraction of 50 g (dry weight basis) powdered soy hypocotyls yielded 2.79 g of dry matter after solid-phase extraction. This extract will be referred to as the phytochemical extract. The content of this extract was analysed by HPLC–MS (Fig. 2A). The eluting peaks were assigned based on the m/z ratio of the molecular ion $[M + H]^+$ or their sodium adducts $[M + Na]^+$ and their retention times (Decroos et al., 2005). The extract was shown to consist mainly of isoflavones and soyasaponins. Isoflavones eluted in the first part of the chromatogram (retention times between 15 and 35 min). The glycosyl conjugates (m/z of $[M + H]^+ = 417$, 447 and 433; RT (retention time) = 17.5, 17.8 and 19.5 min, respectively, daidzin, glycitin and genistin) eluted first, followed by the malonylated glycosides (m/z of $[M + H]^+ = 503$, 533 and 519; RT = 18.6, 19.1 and 20.5 min, respectively, 6-O''-malonyldaidzin, -glycitin and -genistin), acetylated glycosides (m/z of $[M + H]^+ = 459$, 489 and 475; RT = 24.3, 24.8 and 30.0 min, respectively, 6-O''-acetyldaidzein, -glycitin and -genistin), and finally the aglycones (m/z of $[M + H]^+ = 255$, 285 and 271; RT = 34.1, 34.7 and 40.5 min, respectively, daidzein, glycitein and genistein).

Soyasaponins eluted in the second part of the chromatogram (between 37 and 71 min). A detailed qualitative and quantitative analysis of soyasaponins using this HPLC method has already been described previously (Decroos et al., 2005). The total amount of soyasaponins measured in the extract was 0.63 ± 0.02 mg/mg extract, or 3.5 ± 0.1 mg/100 mg dry weight of the original sample. In Fig. 2B the procentual composition of the different soyasaponin groups (and the compounds of interest to this study) is represented. Group A soyasaponins were the most abundant soyasaponins present in this sample, with soyasaponin Ab as the main representative. Soyasaponin Aa and Ae were not detected. Group B soyasaponins appeared mainly in their DDMP-conjugated form, and from this group only soyasaponins Ba and Bb and their DDMP-conjugates were detected in a quantifiable amount.

3.2. Fractionation of soyasaponins using preparative-scale reversed-phase chromatography

The elution profile of 400 mg of the phytochemical extract on a Source 15 RPC column is presented in Fig. 3. Fractions were collected as is indicated, and analysed by

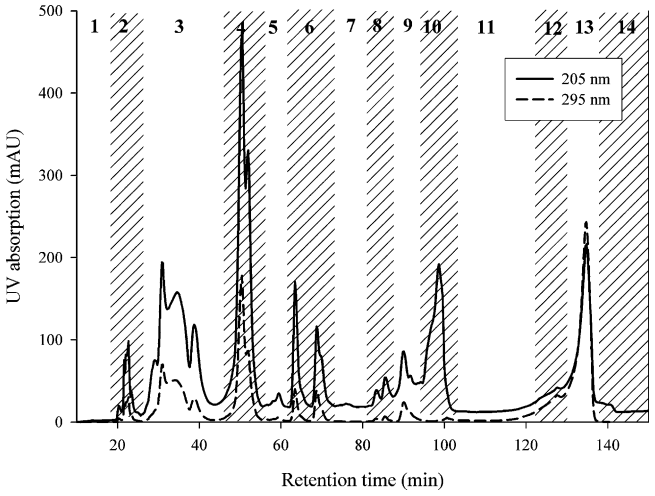

Fig. 3. Elution profile of the phytochemical extract on a Source 15 RPC column obtained with a UV-detector at 205 and 295 nm. The fractions that were collected are indicated by the hatched and open areas. Elution times and relative mass contributions of fractions were as follows (Fraction Number: Start Fraction (min) → End Fraction (min) (Relative Mass (%))): 1: 0 → 18 (0.0); 2: 18 → 26 (0.8); 3: 26 → 45 (8.4); 4: 45 → 56 (12.7); 5 :56 → 61.5 (1.2); 6: 61.5 → 72 (7.2); 7: 72 → 81.5 (0.2); 8: 81.5 → 88 (3.7); 9: 88 → 94 (9.5); 10: 94 → 102 (30.3); 11: 102 → 122 (0.0); 12: 122 → 130 (7.0); 13: 130 → 137.5 (19.0); 14: 137.5 → 150 (0.0).

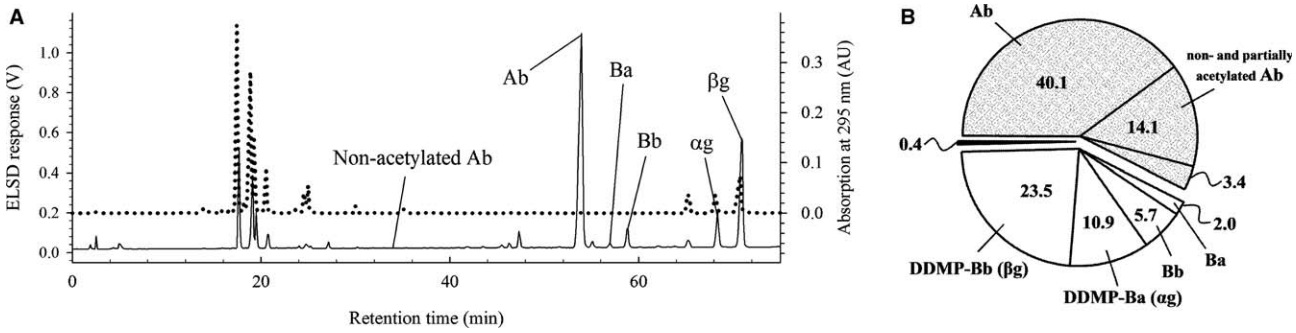

Fig. 2. Compositional analysis of the phytochemical extract. (A) Elution profile of the various soy germ components using RP-HPLC combined with ELS and UV detection. Solid line: ELSD signal; dotted line: UV signal at 295 nm. (B) Distribution of different soyasaponins in the soy germ sample. Hatched area: group A soyasaponins; white area: group B soyasaponins; black area: group E soyasaponins.

HPLC–MS. Peak identification was performed by comparing the observed m/z ratio of the molecular ion $[M + H]^+$ or their sodium adducts $[M + Na]^+$ with theoretical values in combination with the known retention times from an earlier study using the same HPLC method (Decroos et al., 2005). Neither soyasaponins nor isoflavones were found in fractions 1, 2, 11 and 14. All components detected in the phytochemical extract by HPLC–MS (supra), except for aglycone isoflavones and non-acetylated soyasaponin Ab, were also found back in the combined fractions. A similar elution order as for the analytical run was observed. Isoflavones eluted in the first (fractions 3–6) of the chromatogram. Dominating peaks in fraction 3 were malonlyldaidzin, -glycitin, and -genistin ($[M + H]^+ = 503.1$, 533.2 and 519.2, respectively), in fraction 4 daidzin and glycitin ($[M + H]^+ = 417.1$ and 447.2, respectively), in fraction 5 genistin ($[M + H]^+ = 433.1$) and in fraction 6 acetyldaidzin, -glycitin, and -genistin ($[M + H]^+ = 459.1$, 489.2 and 475.2, respectively). Soyasaponins eluted in the second part of the chromatogram. Fractions 7 and 8 contained, respectively, diacetyl soyasaponin Ab ($[M + H]^+ = 1353.2$) and triacetyl soyasaponin Ab ($[M + H]^+ = 1395.1$). Fraction 9 consisted mainly of a mixture of unconjugated group B soyasaponins (soyasaponins Ba, Bb, Bb′, Bc′, Bd and Be; $[M + H]^+ =$ 959.2, 943.1, 797.1, 957.2, 957.2 and 941.1, respectively). Remarkably, these soyasaponins eluted before the group A soyasaponins when using Source 15 RPC as a column material, whereas the reversed elution order was observed using an analytical Aquasil RP C18 column. Fraction 10 contained predominantly fully acetylated group A soyasaponins, mainly soyasaponin Ab ($[M + H]^+ = 1437.1$), but also small amounts soyasaponin Ac, Af, Ag and Ah ($[M + H]^+ = 1421.1$, 1275.1, 1173.2 and 1245.2, respectively) were detected. Fractions 12 and 13 contained mainly soyasaponin αg and βg ($[M + H]^+ = 1085.1$ and 1069.1, respectively), and traces of their respective non-DDMP-conjugated counterparts soyasaponin Ba and Bd ($[M + H]^+ = 959.1$ and 957.2, respectively) and Bb and Be ($[M + H]^+ = 943.2$ and 941.2, respectively), respectively. As calculated from the relative masses of the fractions (Fig. 3), isoflavones and soyasaponins accounted for 29.5% and 69.7%, respectively, of the total recovered material. Relatively pure fractions could be obtained for soyasaponin Ab (fraction 10) and soyasaponins αg and βg (fractions 12 and 13, respectively) with a purity between 88% and 94% as determined by RP-HPLC. The weight ratios between the soyasaponins were consistent with the data obtained from the analytical run (see Fig. 2).

3.3. Further purification of soyasaponins

The fractions 10, 12 and 13 that were collected from repetitive preparative runs were, respectively, combined. Each combined fraction was reapplied onto the Source 15 RPC column and eluted with Gradient 2. This yielded pure soyasaponins Ab, αg, and βg with a purity of >97%, as less than 3% impurities were detected using RP-HPLC and full scan MS (data not shown). Finally, 420, 95 and 252 mg of pure soyasaponins Ab, αg, and βg, respectively, could be derived from 50 g soygerms.

Non-acetylated soyasaponin Ab and soyasaponins Ba and Bb were prepared from soyasaponin Ab and soyasaponins αg and βg, respectively. A complete deacetylation of soyasaponin Ab was observed after 30 min of incubation in 0.1 M NaOH. This yielded 35.1 mg of non-acetylated soyasaponin Ab, starting from 50 mg of the fully acetylated counterpart. The same treatment on 50 mg of soyasaponins αg, and βg resulted in complete loss of the DDMP-group, and respectively, 38.5 and 39.3 mg of purified soyasaponins Ba and Bb, respectively, could be retrieved. The soyasaponins obtained through alkaline treatment had a purity of >97%, as determined by RP-HPLC and full scan MS (data not shown).

3.4. Surface tension, critical micelle concentration and surface density of purified soyasaponins

In the present study the surface tension method was used to determine the CMC and the surface density. In order to calibrate and validate the method used, the CMC of two well-known surfactants, the bile salt sodium cholate and the commercial anionic surfactant Tween 80, was determined as well. The CMC values we obtained for these two surfactants (Table 1) agree well with data reported earlier

Table 1
Critical micelle concentrations (CMC), surface densities (Γ_{max}) and minimal attainable surface tensions (γ_{CMC}), of the purified soyasaponins as determined by the Wilhelmy plate method

Component	CMC (g/L)[a]	95% confindence interval for CMC	Γ_{max}[a] (mg/m^2)	95% confindence interval for Γ_{max}	γ_{CMC} (mN/m)[a,b]
Soyasaponin Ab	0.56[a]	0.55–0.58	3.0[a]	2.9–3.1	48.6 ± 0.6[a]
Non-acetylated soyasaponin Ab	3.2[b]	3.0–3.4	1.7[b]	1.5–1.8	54.0 ± 0.4[b]
Soyasaponin Ba	0.10[c,e]	0.08–0.12	4.5[c]	4.1–4.9	50.1 ± 0.8[c]
Soyasaponin Bb	0.085[c]	0.07–0.10	4.6[c]	4.3–4.9	51.1 ± 0.6[c]
Soyasaponin αg	0.19[d]	0.16–0.21	2.1[a,d]	1.9–2.2	54.7 ± 0.5[b]
Soyasaponin βg	0.16[d,e]	0.11–0.22	2.6[a]	2.0–3.2	54.3 ± 0.9[b]
Sodium cholate	3.1	2.8–3.4	0.7[d]	0.7–0.8	44.0 ± 0.6[d]
Tween 80	0.022[g]	0.016–0.028	2.8[a]	2.6–3.0	48.3 ± 0.7[a]

Tween 80 and cholic acid were used as reference compounds.
[a] Values having a different superscript are significantly different ($p \leqslant 0.05$) within one parameter.
[b] Data are presented as mean ± SD ($n = 3$).

in the literature for the surface tension method, i.e., between 2.3 and 5.6 g/L for sodium cholate (Mitra & Dungan, 2000; Roda, Hofmann, & Mysels, 1983; Small, 1971) and between 0.015 and 0.036 g/L for Tween 80 at 25 °C (Coello, Meijide, Nunez, & Tato, 1995; Yeom, Ghosh, Cox, & Robinson, 1996; Zheng & Obbard, 2002), indicating the correctness of the method applied here. Also, the values for the surface tension at CMC (γ_{CMC}) were similar to values in the literature (Haque, Das, & Moulik, 1999; Small, 1971).

The surface tension of aqueous solutions of fully acetylated and non-acetylated soysaponin Ab and DDMP-conjugated and unconjugated soyasaponins Ba and Bb were measured at various concentrations. All solutions we tested were clear and RP-HPLC analysis of the test solutions after surface tension measurements showed that the tested soyasaponins were chemically stable, including the DDMP-conjugated soyasaponins αg and βg. When the surface tension was plotted against the logarithm of the concentration (Fig. 4), the decrease was linear ($R^2 > 0.96$ for all compounds, except for soyasaponin βg, where R^2 was 0.91). From the intersection between the two linear parts of the surface tension, below and above the estimated CMC, the CMC was calculated (Table 1). Significant differences were observed among the different soyasaponin species. The lowest CMC values were found for soyasaponins Ba and Bb, lower than their respective DDMP-counterparts, soyasaponins αg and βg. Between soyasaponin Ba and Bb and between αg and βg, no significant differences were found. The CMC value for soyasaponin Ab was clearly higher than the ones for group B soyasaponins. Deacetylation of soyasaponin Ab significantly increased the CMC.

The values for the minimal attainable surface tension, γ_{CMC}, are given in Table 1. The lowest minimal attainable surface tension was observed for soyasaponin Ab. There was no significant difference between the values for the non-DDMP-conjugated group B soyasaponins, but they were higher than the latter. The highest γ_{CMC} values were found for non-acetylated soyasaponin Ab and the DDMP-conjugated soyasaponins αg and βg.

From the slopes of the regression lines below the CMC the surfactant surface density was calculated (Table 1). Also for Γ_{max} significant differences were observed. Similar values were found for soyasaponins Ab, αg, βg, cholic acid and Tween 80. For non-acetylated soyasaponin Ab, the surface density appeared to be significantly lower, and for soyasaponins Ba and Bb significantly higher.

4. Discussion

The surfactant properties of soyasaponins are well-recognised. However, due to the absence of purified test compounds, limited data on the surfactant properties of individual soyasaponins are available. In this study, we developed a straight-forward method for separating the most abundant soyasaponins species from soy hypocotyls. Next, we investigated whether the differences in chemical structures among the individual soyasaponins had an impact on the interfacial tension, the surface density, and the CMC of the molecules.

4.1. Soyasaponin composition of soy hypocotyl

The starting material used in this study was raw soy hypocotyl, which is a rich source of both group A and group B soyasaponins (Decroos et al., 2005; Shiraiwa et al., 1991). Soyasaponin Ab, and its partially acetylated forms, comprised the largest part of the group A soyasaponins, whereas no soyasaponin Aa or Ae could be detected. This indicates that the hypocotyls originated from soybeans with the "Ab-genotype", i.e., containing soyasaponin Ab as the predominant group A soyasaponin (Shiraiwa et al., 1991). The group B soyasaponins occurred mainly in their DDMP-conjugated form. This was to be expected since

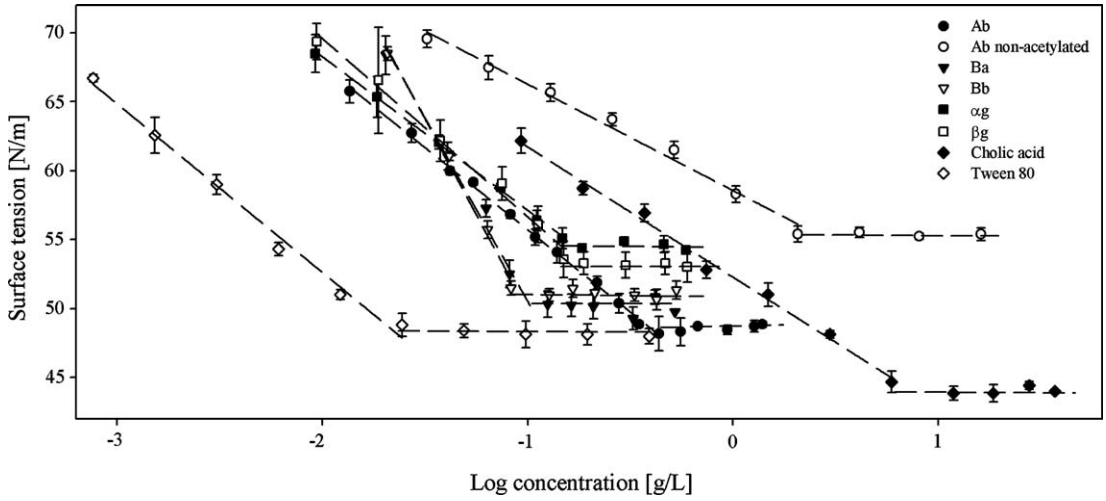

Fig. 4. Surface tension of aqueous solutions of the various purified soyasaponins as a function of their concentration. Tween 80 and cholic acid are included in the figure as reference compounds. Data are presented as mean ± SD (error bars) ($n = 3$).

raw hypocotyls were used as starting material and mild extraction conditions were applied. DDMP-conjugated soyasaponins are considered to be the natural form of group B soyasaponins in soybean (Kudou et al., 1993, 1994).

4.2. Preparative purification of soyasaponins

Application of the phytochemical extract on a Source 15 RPC column resulted in a satisfactory separation between soyasaponins and isoflavones on the one hand, and between the various soyasaponins on the other hand. Purified (>88%) soyasaponins Ab, αg and βg could thus be retrieved by collecting fractions in one chromatographic step. However, a second chromatographic step was necessary to obtain highly purified compounds (>97%). Non-acetylated soyasaponin Ab and soyasaponins Ba and Bb, respectively, were obtained from the soyasaponins mentioned before by alkaline treatment. The yield of these saponification step was 81.4%, 87.3% and 89.8%, respectively, of the theoretical maximal yield, which can be calculated taking into account the losses of four acetyl groups and the DDMP-group, respectively. It is known that aqueous saponification results in hydrolysis of the ester and ether bonds of group A and group B soyasaponins, resulting in respectively, deacetylation and deconjugation of the DDMP-group (Gu et al., 2002; Heng et al., in press; Hu et al., 2002; Yoshiki, Takagi, Watanabe, & Okubo, 2005). DDMP-conjugated soyasaponins are relatively instable molecules since the ether linkage of the DDMP-group is rather sensitive to heat and pH variations. However, soyasaponin αg and βg remained stable during the extraction and chromatographic procedures and further experiments. This can be explained by the presence of a substantial amount of organic solvent in the solute solvent during extraction and chromatography, and the fact that the temperature never exceeded 30 °C, neither during storage nor during further experiments (Heng et al., in press).

Reversed-phase chromatography with 15 RPC gave a good separation of all classes of soyasaponins present in the original soy germ sample. In contrast to our method, the procedures for isolation and purification of soyasaponins reported in the literature, often include a reversed-phase chromatography step devoted to the purification of one or a limited number of soyasaponins (Berhow et al., 2002; Ellington, Berhow, & Singletary, 2005; Hu et al., 2002; Philbrick et al., 2003; Yoshiki et al., 1998, 2005). The method described here can be used to separate group A soyasaponins (partially and fully acetylated) as well as DDMP-conjugated and unconjugated group B soyasaponins, as they occur in the original sample.

4.3. Critical micelle concentration of individual soyasaponins

The surface activity of a surfactant can be described by a number of quantitative parameters of which the CMC, the surface tension at the CMC and the surface density are the most important. In this study we determined the CMC of individual soyasaponins using the Wilhelmy plate method.

The order of CMC of the soyasaponins investigated in this study was as follows: soyasaponin Bb < Ba < βg < αϖ g < Ab < non-acetylated Ab. From our observations three major structural features having an important influence on the CMC can be derived: (i) number of sugar side chains, i.e. the monodesmosides have a lower CMC value than the bidesmosides; (ii) conjugation with a DDMP-group, i.e. the presence of a DDMP-group at the C-22 position increases the CMC value; and (iii) the presence of acetyl-groups, i.e., acetylated group A soyasaponins have a lower CMC value than their non-acetylated counterparts. The value of the CMC of a pure compound in aqueous solution depends on its molecular structure, temperature, pH, salt concentration (Mitra & Dungan, 1997). General rules for deriving the CMC value from the molecular structure are not established, but it has been suggested that the larger the contiguous hydrophobic area of the molecule, the lower the CMC value (Huibers, Lobanov, Katritzky, Shah, & Karelson, 1997; Roda et al., 1983).

The theory of contiguous hydrophobic area applies well for explaining the difference in the CMC of bidesmosides and monodesmosides. Because of the presence of the second sugar side chain at the C-22 position, the (relative) contiguous hydrophobic area becomes smaller, resulting in a higher CMC values for bidesmosides. The difference in CMC value between DDMP-conjugated and unconjugated soyasaponins, although smaller than between mono- and bisdesmosides, can be explained in the same manner. Because of the lower hydrophobicity of the DDMP-group compared to the triterpenoid skeleton, the (relative) contiguous hydrophobic area is reduced. Deacetylation of soyasaponin Ab resulted in a strong increase in CMC. The acetyl groups are more hydrophobic than the hydroxyl groups which are formed after deacetylation. They seem to play an important role in the formation of micelles. The same was observed for *Quillaja* saponins, where removal of the acyl group results in a marked increase in CMC value (Pillion, Amsden, Kensil, & Recchia, 1996). The CMC value of soyasaponin Ab determined in this study is similar to those found for *Quillaja* (0.51–0.72 g/L) (Mitra & Dungan, 1997) and *Gleditsia* (1.8 g/L) (Wang, Gu, & Li, 2005) saponins, under the same experimental conditions as applied here. The tested samples of both *Quillaja* and *Gleditsia* consisted of a mixture of bidesmosides, the structures of which were related to group A soyasaponins. However, these bidesmosides do not have their sugar side-chain attached to the C-22 position of the triterpenoid skeleton as in soyasaponin Ab (ether-linked), but ester-linked to the carboxyl group attached to the C-17 position of the skeleton (Marciani, Reynolds, Pathak, Finley-Woodman, & May, 2003; Zhang et al., 1999). Apparently, this does not seem to have a great influence on the CMC value.

4.4. Surface tension and surface density of individual soyasaponins

The lowest minimal attainable surface tension was observed for soyasaponin Ab. Deacetylation of the latter resulted in a significant increase in γ_{CMC}. Values for γ_{CMC} of soyasaponins were higher than values reported in literature for other saponins, i.e., between 34 and 36 mN/m for *Quillaja* and *Gleditsia* saponins (Mitra & Dungan, 2001; Wang et al., 2005).

The surface density Γ_{max} gives information on the arrangement of surfactants at the water–air interface. The highest Γ_{max} values were found for soyasaponins Ba and Bb, which had the lowest CMC values. Since there is little sterical hindrance, the triterpenoid skeletons are able to form a compact layer at the interface, resulting in a high surface density. Soyasaponin Ab and the DDMP-conjugated soyasaponins showed similar surface densities. The C-22 sugar chain and the DDMP-group at the same position hinder the triterpenoid skeletons from approaching each other, and consequently the interface can not be as densely packed as with soyasaponins Ba and Bb. The surface density of deacetylated soyasaponin Ab was found to be the lowest of all compounds tested. The removal of the acetyl groups makes the C-22 sugar chain more hydrophilic. At the interface, both sugar chains remain in the water phase and each individual saponin molecule occupies more space. Hence, a lower density is reached. Values for Γ_{max} determined in this study for soyasaponins were comparable with data reported earlier on other saponins (Mitra & Dungan, 2001; Wang et al., 2005).

4.5. Possible impact on biological and functional properties

Some biological effects of soyasaponins might be related to their amphipathic character. Mitra and Dungan (1997) showed that *Quillaja* saponin is capable of solubilising cholesterol in aqueous solutions, by incorporation in the apolar cavity of their micelles. They suggested that the lower the CMC value the lesser the amount of saponin required for cholesterol solubilisation. The hypocholesterolaemic effect of saponins has been attributed to the formation of mixed micelles with bile salts, in which cholesterol can be accommodated (Francis, Kerem, Makkar, & Becker, 2002; Oakenfull, 1981, 1986). Although in vivo comparative studies with purified compounds are necessary to establish a relationship, we hypothesise that soyasaponins with a low CMC value are more effective in bringing about a hypocholesterolaemic effect than those with higher CMC values. Thus, of the saponins investigated in this study, unconjugated soyasaponin B seems to be most promising. Other biological effects of saponins, such as adjuvant activity, do not seem to be related to their CMC (Oda et al., 2000, 2003).

It is widely known that surfactants reduce the interfacial tensions in foods, and thus promote the formation of emulsions and foams. Although many factors may influence the physicochemical properties in a complex food matrix, it is generally accepted that the lower the γ_{CMC} of a surfactant, the more efficiently it contributes to these processes (Mitra & Dungan, 1997). The difference found in CMC and γ_{CMC} between fully and unacetylated soysaponin Ab fits very well with the observations by Gohtani et al. (Gohtani, Shinomoto, Honda, Okubo, & Yamano, 1990), who reported that deacetylation of soyasaponin Ab resulted in partial loss of their foam formation capacity. The considerably lower γ_{CMC} value for soyasaponin Ab than for group B soyasaponins also indicates that the former has a greater potency to contribute to the formation of emulsions and foam.

It is important to note that processing conditions during preparation of soybean and soy-based food products have an impact on the soyasaponin composition. For instance, heat treatment and change of pH may lead to deconjugation of DDMP-conjugated group B soyasaponins or deacetylation of acetylated group A soyasaponins (Gu et al., 2002; Heng et al., in press). From the results of the present study it is clear that the soyasaponin's surfactant properties are affected by this. Furthermore, it was found that group A and group B soyasaponins have a different physicochemical behaviour. This indicates that relative proportion between group A and B soyasaponins, which is variable among soybean varieties (Decroos et al., 2005; Shiraiwa et al., 1991), may also influence the surfactant activity of the total soyasaponin fraction. Further studies with purified soyasaponins in specific food applications and biological systems are necessary to fully understand the extent of the functional and biological implications of the differences in physicochemical properties we found here among different soyasaponins.

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References

Bae, E. A., Han, M. J., Choo, M. K., Park, S. Y., & Kim, D. H. (2002). Metabolism of 20(*S*)- and 20(*R*)-ginsenoside R-g3 by human intestinal bacteria and its relation to in vitro biological activities. *Biological & Pharmaceutical Bulletin, 25*(1), 58–63.

Berhow, M. A., Cantrell, C. L., Duval, S. M., Dobbins, T. A., Maynes, J., & Vaughn, S. F. (2002). Analysis and quantitative determination of group B saponins in processed soybean products. *Phytochemical Analysis, 13*(6), 343–348.

Berhow, M. A., Wagner, E. D., Vaughn, S. F., & Plewa, M. J. (2000). Characterization and antimutagenic activity of soybean saponins. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis, 448*(1), 11–22.

Coello, A., Meijide, F., Nunez, E. R., & Tato, J. V. (1995). Aggregation behavior of bile salts in aqueous solution. *Journal of Pharmaceutical Sciences, 85*(1), 9–15.

Decroos, K., Vincken, J.-P., Heng, L., Bakker, R., Gruppen, H., & Verstraete, W. (2005). Simultaneous quantification of differently glycosylated, acetylated, and 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one-conjugated soyasaponins using reversed-phase high-per-

formance liquid chromatography with evaporative light scattering detection. *Journal of Chromatography A, 1072*(2), 185–193.

Ellington, A. A., Berhow, M., & Singletary, K. W. (2005). Induction of macroautophagy in human colon cancer cells by soybean B-group triterpenoid saponins. *Carcinogenesis, 26*(1), 159–167.

Fenwick, D. E., & Oakenfull, D. (1983). Saponin content of food plants and some prepared foods. *Journal of Science of Food and Agriculture, 34*, 186–191.

Francis, G., Kerem, Z., Makkar, H. P. S., & Becker, K. (2002). The biological action of saponins in animal systems: a review. *British Journal of Nutrition, 88*(6), 587–605.

Gohtani, S., Shinomoto, K., Honda, Y., Okubo, K., & Yamano, Y. (1990). Surface activity of soybean saponin Ab (acetylsoyasaponin A_1). *Nippon Nigei Kagaku Kaishi, 64*, 901–906.

Gu, L. W., Tao, G. J., Gu, W. Y., & Prior, R. L. (2002). Determination of soyasaponins in soy with LC-MS following structural unification by partial alkaline degradation. *Journal of Agricultural and Food Chemistry, 50*(24), 6951–6959.

Gurfinkel, D. M., & Rao, A. V. (2003). Soyasaponins: The relationship between chemical structure and colon anticarcinogenic activity. *Nutrition and Cancer – An International Journal, 47*(1), 24–33.

Haque, M. E., Das, A. R., & Moulik, S. P. (1999). Mixed micelles of sodium deoxycholate and polyoxyethylene sorbitan monooleate (Tween 80). *Journal of Colloid and Interface Science, 217*(1), 1–7.

Heng, L., Vincken, J.-P., Hoppe, K., van Koningsveld, G.A., Decroos, K., Gruppen, H., et al. Stability of pea DDMP saponin and the mechanism of its decomposition. Food Chemistry [in press], doi:10.1016/j.foodchem.2005.07.045.

Hu, J., Lee, S. O., Hendrich, S., & Murphy, P. A. (2002). Quantification of the group B soyasaponins by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry, 50*(9), 2587–2594.

Huibers, P. D. T., Lobanov, V. S., Katritzky, A. R., Shah, D. O., & Karelson, M. (1997). Prediction of critical micelle concentration using a quantitative structure–property relationship approach. *Journal of Colloid and Interface Science, 187*, 113–120.

Kinjo, J., Imagire, M., Udayama, M., Arao, T., & Nohara, T. (1998). Studies on the hepatoprotective drugs part 3 – studies on the constituents of the leguminous plants part 56 – structure–hepatoprotective relationships study of soyasaponins I–IV having soyasapogenol B as aglycone. *Planta Medica, 64*(3), 233–236.

Kitagawa, I., Taniyama, T., Nagahama, Y., Okubo, K., Yamauchi, F., & Yoshikawa, M. (1988a). Saponin and sapogenol .42. Structures of acetyl-soyasaponin-A1, acetyl-soyasaponin-A2, and acetyl-soyasaponin-A3, astringent partially acetylated bisdesmosides of soyasapogenol-a, from American soybean, the seeds of glycine-max merrill. *Chemical & Pharmaceutical Bulletin, 36*(8), 2819–2828.

Kitagawa, I., Wang, H. K., Taniyama, T., & Yoshikawa, M. (1988b). Saponin and sapogenol .41. Reinvestigation of the structures of soyasapogenol-a, soyasapogenol-B, and soyasapogenol-E, oleanene-sapogenols from soybean – structures of soyasap1onin-I, soyasaponin-Ii, and soyasaponin-Iii. *Chemical & Pharmaceutical Bulletin, 36*(1), 153–161.

Kudou, S., Tonomura, M., Tsukamoto, C., Uchida, T., Sakabe, T., Tamura, N., & Okubo, K. (1993). Isolation and structural elucidation of Ddmp-conjugated soyasaponins as genuine saponins from soybean seeds. *Bioscience Biotechnology and Biochemistry, 57*(4), 546–550.

Kudou, S., Tonomura, M., Tsukamoto, C., Uchida, T., Yoshikoshi, M., & Okubo, K. (1994). Structural elucidation and physiological properties of genuine soybean saponins. In M. T. Hang (Ed.). *Food phytochemicals for cancert prevention I* (vol. 546, pp. 340–348). Washington, DC: American Chemical Society.

Lasztity, R., Hidevegi, M., & Bata, A. (1998). Saponins in food. *Food Reviews International, 14*(4), 371–390.

Lee, S. O., Simons, A. L., Murphy, P. A., & Hendrich, S. (2005). Soyasaponins lowered plasma cholesterol and increased fecal bile acids in female golden Syrian hamsters. *Experimental Biology and Medicine, 230*(7), 472–478.

Marciani, D. J., Reynolds, R. C., Pathak, A. K., Finley-Woodman, K., & May, R. D. (2003). Fractionation structural studies and immunological characterization of the semi-synthetic Quillaja saponins derivative GPI-0100. *Vaccine, 21*(25–26), 3961–3971.

Milgate, J., & Roberts, D. C. K. (1995). The nutritional and biological significance of saponins. *Nutrition Research, 15*(8), 1223–1249.

Mitra, S., & Dungan, S. R. (1997). Micellar properties of quillaja saponin. 1. Effects of temperature salt and pH on solution properties. *Journal of Agricultural and Food Chemistry, 45*(5), 1587–1595.

Mitra, S., & Dungan, S. R. (2000). Micellar properties of quillaja saponin. 2. Effect of solubilized cholesterol on solution properties. *Colloids and Surfaces B – Biointerfaces, 17*(2), 117–133.

Mitra, S., & Dungan, S. R. (2001). Cholesterol solubilization in aqueous micellar solutions of quillaja saponin, bile salts, or nonionic surfactants. *Journal of Agricultural and Food Chemistry, 49*(1), 384–394.

Oakenfull, D. (1981). Saponins in food – a review. *Food Chemistry, 7*, 19–40.

Oakenfull, D. (1986). Aggregation of saponins and bile acids in aqueous solution. *Australian Journal of Chemistry, 39*, 1671–1683.

Oda, K., Matsuda, H., Murakami, T., Katayama, S., Ohgitani, T., & Yoshikawa, M. (2000). Adjuvant and haemolytic activities of 47 saponins derived from medicinal and food plants. *Biological Chemistry, 381*(1), 67–74.

Oda, K., Matsuda, H., Murakami, T., Katayama, S., Ohgitani, T., & Yoshikawa, M. (2003). Relationship between adjuvant activity and amphipathic structure of soyasaponins. *Vaccine, 21*(17–18), 2145–2151.

Philbrick, D. J., Bureau, D. P., Collins, F. W., & Holub, B. J. (2003). Evidence that soyasaponin B-b retards disease progression in a murine model of polycystic kidney disease. *Kidney International, 63*(4), 1230–1239.

Pillion, D. J., Amsden, J. A., Kensil, C. R., & Recchia, J. (1996). Structure–function relationship among *Quillaja* saponins serving as excipients for nasal and ocular delivery of insulin. *Journal of Pharmaceutical Sciences, 85*(5), 518–523.

Rao, A. V., & Sung, M. K. (1995). Saponins as anticarcinogens. *Journal of Nutrition, 125*, 717S–724S.

Roda, A., Hofmann, A. F., & Mysels, K. J. (1983). The Influence of bile-salt structure on self-association in aqueous-solutions. *Journal of Biological Chemistry, 258*(10), 6362–6370.

Rowlands, J. C., Berhow, M. A., & Badger, T. M. (2002). Estrogenic and antiproliferative properties of soy sapogenols in human breast cancer cells in vitro. *Food and Chemical Toxicology, 40*(12), 1767–1774.

Sasaki, K., Minowa, N., Kuzuhara, H., Nishiyama, S., & Omoto, S. (1998). Derivatization of soyasapogenol A and their hepatoprotective activities. *Bioorganic & Medicinal Chemistry Letters, 8*(6), 607–612.

Shiraiwa, M., Harada, K., & Okubo, K. (1991). Composition and content of saponins in soybean seed according to variety, cultivation year and maturity. *Agricultural and Biological Chemistry, 55*(2), 323–331.

Shiraiwa, M., Yamauchi, F., Harada, K., & Okubo, K. (1990). Inheritance of group-a saponin in soybean seed. *Agricultural and Biological Chemistry, 54*(6), 1347–1352.

Small, D. M. (1971). The physical chemistry of cholanic acids. In P. P. Nair & D. Kritchevsky (Eds.). *The bile acids* (Vol. 1, pp. 249). New York: Plenum Press.

Sparg, S. G., Light, M. E., & van Staden, J. (2004). Biological activities and distribution of plant saponins. *Journal of Ethnopharmacology, 94*(2–3), 219–243.

Wang, Z. W., Gu, M. Y., & Li, G. Z. (2005). Surface properties of gleditsia saponin and synergisms of its binary system. *Journal of Dispersion Science and Technology, 26*(3), 341–347.

Yeom, I. T., Ghosh, M. M., Cox, C. D., & Robinson, K. G. (1996). Micellar solubilization of polynuclear aromatic hydrocarbons in coal tar-contaminated soils (vol 29, pg 3015, 1995). *Environmental Science & Technology, 30*(6), 2104.

Yoshiki, Y., Kudou, S., & Okubo, K. (1998). Relationship between chemical structures and biological activities of triterpenoid saponins

from soybean. *Bioscience Biotechnology and Biochemistry, 62*(12), 2291–2299.

Yoshiki, Y., Takagi, S., Watanabe, M., & Okubo, K. (2005). Fractionation of soybean functional glycosides from soy-waste based on the chemical reaction of soyasaponin [beta] g. *Food Chemistry, 93*(4), 591–597.

Zhang, Z. Z., Koike, K., Jia, Z. H., Nikaido, T., Guo, D., & Zheng, J. H. (1999). Triterpenoidal saponins from *Gleditsia sinensis*. *Phytochemistry, 52*(4), 715–722.

Zheng, Z., & Obbard, J. P. (2002). Evaluation of an elevated non-ionic surfactant critical micelle concentration in a soil/aqueous system. *Water Research, 36*(10), 2667–2672.