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Journal of Chromatography A, 1072 (2005) 185–193
JOURNAL OF CHROMATOGRAPHY A
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Simultaneous quantification of differently glycosylated, acetylated, and 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one-conjugated soyasaponins using reversed-phase high-performance liquid chromatography with evaporative light scattering detection

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Received 24 May 2004; received in revised form 28 January 2005; accepted 3 March 2005
Available online 25 March 2005

Abstract

A novel method utilizing high-performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD) and electrospray ionisation mass spectrometry (ESI-MS) was developed for the analysis of soyasaponins, a divers group of triterpenic compounds with one or two sugar side chains, occurring in soy. Group A soyasaponins in different degrees of acetylation, as well as group B soyasaponins in both their 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP)-conjugated and non-conjugated forms could be separated and quantified using authentic soyasaponin standards, in one single run. The method was tested by the determination of the soyasaponin content and composition of eight soygerm samples of different origin. Differences in the composition and the degree of acetylation of the group A soyasaponins were observed among these samples. The group B soyasaponins showed much less variability and they were mainly present in their DDMP-conjugated form.

Keywords: Soy; Soygerm; Soyasaponin; Reversed phase HPLC; Evaporative light scattering detection; DDHP

1. Introduction

Saponins form a diverse group of amphiphilic molecules and are found widespread over the plant kingdom. Soybean (*Glycine max* L. Merill) and soy-based food products are major dietary sources of saponins [1,2]. Soyasaponins, which are triterpenoid glycosides, are divided into three major groups, based on differences in substitution of the C-22 and C-23 position of the aglycone (or soyasapogenol): group A, B and E soyasaponins (Table 1). Group A soyasaponins have a glycosyl chain attached to the C-3 and the C-22 position of the aglycone. Group B soyasaponins carry only one glycosyl chain (connected to the C-3 position), and they can be conjugated to 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) at C-22 [3]. According to Kudou et al.'s findings [4], the latter is the genuine form of group B saponins in soybeans, but conditions applied during processing can easily result in loss of DDMP, and formation of the non-DDMP counterparts. Group E soyasaponins are the least abundant of the three, and they are considered to be photo-oxidation products of group B soyasaponins [5]. The C-3 sugar chain is similar for group A, B, and E soyasaponins; it starts with a glucuronyl residue, followed by a galactosyl or arabinosyl residue, and in most cases followed by a glucosyl or rhamnosyl residue [6,7]. The C-22 side chain of group A soyasaponins consists of two sugar residues, starting with an arabinosyl. The terminal sugar moiety is a xylosyl or glucosyl residue, which can be acetylated at three or four positions, respectively. It is believed that in soybean group A soyasaponins are fully acetylated [5,8].

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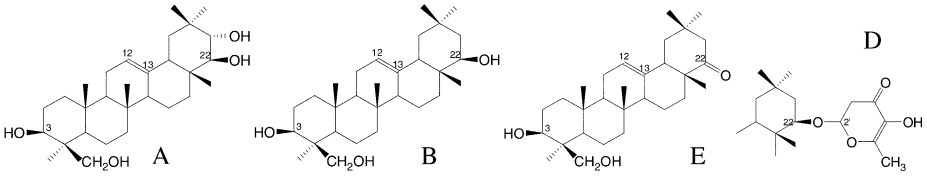
doi:10.1016/j.chroma.2005.03.021

Table 1
Structures of known soyasaponins and their calculated molecular weight; the retention times of the various soyasaponins in the chromatograms obtained with the method applied in this study are indicated as well



A: soyasapogenol A; **B**: soyasapogenol B; **E**: soyasapogenol E; D: DDMP conjugated to soyasapogenol B

Name	Structure[a]	MW[b]	RT[c]
Aa	glc(1→2)gal(1→2)glcUA(1→3)**A**(22←1)ara(3←1)xyl(2,3,4-tri-*O*-acetyl)	1364	52.0
Ab	glc(1→2)gal(1→2)glcUA(1→3)**A**(22←1)ara(3←1)glc(2,3,4,6-tetra-*O*-acetyl)	1436	54.0
Ac	rha(1→2)gal(1→2)glcUA(1→3)**A**(22←1)ara(3←1)glc(2,3,4,6-tetra-*O*-acetyl)	1420	55.3
Ad	glc(1→2)ara(1→2)glcUA(1→3)**A**(22←1)ara(3←1)glc(2,3,4,6-tetra-*O*-acetyl)	1390	56.1
Ae	gal(1→2)glcUA(1→3)**A**(22←1)ara(3←1)xyl(2,3,4-tri-*O*-acetyl)	1202	56.7
Af	gal(1→2)glcUA(1→3)**A**(22←1)ara(3←1)glc(2,3,4,6-tetra-*O*-acetyl)	1274	59.5
Ag	ara(1→2)glcUA(1→3)**A**(22←1)ara(3←1)xyl(2,3,4-tri-*O*-acetyl)	1172	64.5
Ah	ara(1→2)glcUA(1→3)**A**(22←1)ara(3←1)glc(2,3,4,6-tetra-*O*-acetyl)	1244	65.1
Ba	glc(1→2)gal(1→2)glcUA(1→3)**B**	958	57.0
Bb	rha(1→2)gal(1→2)glcUA(1→3)**B**	942	58.1
Bc	rha(1→2)ara(1→2)glcUA(1→3)**B**	912	61.1
Bb′	gal(1→2)glcUA(1→3)**B**	796	60.7
Bc′	ara(1→2)glcUA(1→3)**B**	766	n.d.[d]
Bd	glc(1→2)gal(1→2)glcUA(1→3)**E**	956	60.1
Be	rha(1→2)gal(1→2)glcUA(1→3)**E**	940	61.5
αg	glc(1→2)gal(1→2)glcUA(1→3)**B**(22→2′)DDMP	1084	68.5
βg	rha(1→2)gal(1→2)glcUA(1→3)**B**(22→2′)DDMP	1068	71.0
βa	rha(1→2)ara(1→2)glcUA(1→3)**B**(22→2′)DDMP	1038	n.d.
γg	gal(1→2)glcUA(1→3)**B**(22→2′)DDMP	922	n.d.
γa	ara(1→2)glcUA(1→3)**B**(22→2′)DDMP	892	n.d.

[a] glc, β-D-glucopyranosyl; gal, β-D-galactopyranosyl; glcUA, β-D-glucoronopyranosyl; ara, α-L-arabinopyranosyl; rha, α-L-rhamnopyranosyl; xyl, β-D-xylopyranosyl; for **A**, **B** and **E**, see above.
[b] MW: molecular weight.
[c] RT: retention time (min).
[d] n.d.: not detected.

Soyasaponins are considered as important bioactive components contributing to the beneficial health effects of soy consumption. Biological effects of especially group B soyasaponins have been reported extensively, and include immunostimulatory, anti-viral, hypocholesterolaemic, hepatoprotective, haemolytic and antitumourigenic activities [9–16]. The group A soyasaponins are considered to be responsible for the astringent and bitter taste of soy-based food products, mainly because of the presence of the acetyl groups [17]. The exact mechanisms behind the biological properties of soyasaponins are yet to be revealed, due to the absence of purified test compounds and limited information on the contents and the compositions of soyasaponins in soybean and soy-based products.

Quantification of individual soyasaponins has always been a difficult issue, partly due to the difficulties in isolating authentic standards and the structural complexity of this group of phytochemicals. Moreover, the covalent bonds connecting the acetyl, and particularly the DDMP groups, to the saponin molecule are relatively weak, even under relatively mild extraction conditions, which makes it difficult to obtain the saponins in their native form [12,18]. Historically, the total saponin content has been determined with very low accuracy by colorimetric [19] and biological methods (such as the haemolytic activity of the saponins towards erythrocytes [20]). Recently, Gurfunkel and Rao [21] developed a rapid and accurate densitometric method using TLC for the quantification of total soyasaponins.

Many of the methods developed so far include a treatment prior to quantitative analysis to reduce the complexity of the soyasaponin mixture originally present in soybeans, and hence to simplify the separation of the individual components. Acid hydrolysis converts the saponins into their corresponding sapogenols, which are subsequently quantified by liquid chromatography [22] or gas chromatography after derivatisation to trimethylsilyl ethers [23]. Gu et al. proposed an alkaline treatment, which converts the group A soyasaponins into their non-acetylated forms and deconjugates DDMP saponins, facilitating separation by liquid chromatography. Other authors include a pre-separation in group A and B saponins [24,25], or focus only on one of the two groups [26]. All these methods, although accurate, are often laborious and do not reflect the exact composition of the native soyasaponin mixture. Until now, no methods have been

reported which determine the native soyasaponin composition in soybeans, including the different acetylated forms of group A soyasaponins, and the DDMP-conjugated and non-conjugated forms of group B soyasaponins.

Reverse-phase high-performance liquid chromatography (RP-HPLC) seems to be the most powerful tool for separation of soyasaponins. However, absence of a chromophore in triterpene saponins hampers detection with ultraviolet light, except for the DDMP-conjugated saponins which have an UV absorption maximum around 295 nm. A good alternative is evaporative light scattering detection (ELSD), which is based on mass detection by light scattering after evaporation of the mobile phase. ELSD has already been used successfully for the detection and quantification of ginseng saponins [27,28] and soyasapogenols [22,29] but not for the detection of all soyasaponins.

Soy hypocotyls exhibit the largest variation in soyasaponin species compared to the other parts of the soybean, as they are the main source of group A soyasaponins and contain both DDMP-conjugated and non-DDMP-conjugated group B soyasaponins [25]. In this paper, the soyasaponin composition of the hypocotyls of eight different soybean varieties is determined using a novel method to analyse and quantify all soyasaponins, including the relatively unstable (partially) acetylated and DDMP-conjugated ones, in a single run by RP-HPLC with ELSD.

2. Experimental

2.1. Extraction of soyasaponins from soybean hypocotyls

Hypocotyls of eight different industrial soybean samples (Table 2) were analysed in this study. Prior to isolation of the hypocotyls, the soybeans were subjected to a hot-air heat treatment as follows: 20 min at 120 °C for samples A–D; 30 min at 60 °C for samples G and H; mild drying with not specified conditions for samples E and F. The hypocotyls samples were provided by Acatris Holding (Giessen, The Netherlands). The hypocotyls were ground manually to a fine powder with a mortar and pestle to ca. 0.75 mm particle size. One gram of powder was subsequently extracted with 100 mL hexane for 6 h under reflux conditions for defatting and air-dried afterwards. In order to assess the effect of the hexane extraction on soyasaponin content, sample A was analysed with and without hexane extraction and the soyasaponin content was compared. One hundred milligrams of the defatted powder was then extracted with 100 mL of 70% (v/v) aqueous ethanol in an Erlenmeyer by shaking at 150 rpm for 3 h at room temperature. The efficiency of this extraction was assessed after comparing the soyasaponin content of sample A extracted with, respectively, 100 and 200 mL of 70% (v/v) aqueous ethanol. After filtration over a paper filter (type 597½; Schleicher & Schuell, Ghent, Belgium), 50 μL of a 1000 mg/L ethanol stock solution of the internal standard, equilenin (Fluka, Bornem, Belgium), was added to the aqueous ethanol extract. After evaporation under vacuum at 37 °C of the ethanol, the aqueous phase was applied on a SepPak® Plus tC18 cartridge (Waters, Etten-Leur, The Netherlands). The cartridges were preconditioned with 10 mL methanol, followed by 10 mL water. After sample application, the cartridges were washed with 10 mL of water and eluted with 10 mL of methanol. The flow was maintained at approximately 2 mL/min. The methanol eluate was air-dried under a stream of air, resolubilized in 1 mL of 50% (v/v) aqueous ethanol, and stored at −20 °C for HPLC analysis. In order to evaluate potential losses of soyasaponins during the SPE step, the washing water was resubjected to SPE, and analysed. We also verified whether all soyasaponins were recovered from the SPE column after elution with 10 mL of methanol by applying a second aliquot of 10 mL of methanol, and analysing this for their presence. These two experiments were performed in triplicate on sample A. Furthermore, 100 μg of purified standards of soyasaponins Ab and βg (corresponding to the lowest amounts that were included in the standard curve) were subjected to this extraction procedure in order to trace potential losses of possibly labile acetylated or DDMP-conjugated saponin species, respectively.

Table 2
Soygerms tested in this study, and their geographical origin and year of harvest

Sample	Variety	Geographical origin	Year of harvest
A	HP204 USA	Iowa or Michigan, USA	2002
B	FTE-24-40	Vinton, Iowa, USA	2002
C	SI 2398 USA	Iowa, USA	2002
D	Northrup King var S20F8	Iowa, USA	2002
E	Unknown	North-east China	2002
F	Unknown	North-east China	2002
G	Unknown	Mato Grosso, Goias, Minas Gerais, Brazil	2003
H	Unknown	Mato Grosso, Goias, Minas Gerais, Brazil	2003

2.2. Chromatographic analysis of soyasaponins

A Spectra HPLC system (Thermo Separations, Amersfoort, The Netherlands), consisting of an autosampler, a gradient pump and an AS3000 (Thermo Separations) diode array detector was used. The eluate of the diode array detector was split (5:95), using a splitter, so that 50 μL/min was delivered to an electrospray ionisation mass spectrometer (ESI-MS) and the rest to an ELS detector. Sample separation was carried out with an Aquasil RP C18 column (150 mm × 4.6 mm, 3 μm) (Thermo Hypersil, Amersfoort, The Netherlands) with a solvent system consisting of 0.001% (v/v) acetic acid in water (A) and 0.001% (v/v) acetic acid in acetonitrile (B). Twenty microlitres of sample was injected and the flow rate was 1 mL/min and the elution program was as follows: 0 → 10 min, 10% B (isocratic); 10 → 30 min, 10 → 30% B (linear gradient); 30 → 80 min, 30 → 40% B

(linear gradient); 80 → 90 min, 40 → 100% B (linear gradient); 90 → 110 min, 100% B (isocratic); 110 → 120 min, 10% B (isocratic).

A Sedex 55 ELSD system (Sedere, Alfortville, France) was used. The nebulizer temperature of the ELSD system was set at 40 °C, the gas pressure at 2.3 bar, and the gain was 12. Data acquisition was performed using ChromQuest 4.1 (Thermo Separations) software.

2.3. Mass spectroscopic analysis of soyasaponins

An LCQ ion-trap mass spectrometer (Finnigan MAT 95, San Jose, USA) was used in the positive mode with a spray voltage of 5.5 kV, a capillary voltage of 15 V, and a capillary temperature of 200 °C. A full scan mass spectrum over an *m*/*z* (mass to charge ratio) range of 150–1500 was obtained. The mass spectra were recorded and analysed with the use of Xcalibur® software (Thermo, Amersfoort, The Netherlands).

2.4. Isolation of soyasaponin standards from soy hypocotyls

The starting material for the isolation of original soyasaponin standards was from the same origin as sample A. A detailed description of the purification method will be provided elsewhere. Briefly, 10 g of defatted soygerm powder (see Section 2.1) was suspended in 1 L of 70% (v/v) aqueous ethanol and shaken at 25 °C and 150 rpm for 3 h. After removal of the ethanol by vacuum drying at 30 °C, the aqueous extract was applied on a Supelpak XAD-2 (Supelco, Bellefonte, PA, USA) column (210 mm × 26 mm), which had been conditioned with 200 mL of 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 of 96% (v/v) aqueous ethanol. One hundred milligrams of the vacuum-dried (at 30 °C) eluate, containing mainly isoflavones and soyasaponins, was redissolved in 5 mL of 50% (v/v) aqueous methanol and applied on a Source 15 RPC (Amersham Biosciences, Uppsala, Sweden) column (600 mm × 26 mm). The column was eluted with a solvent system consisting of water with 0.0001% (v/v) acetic acid (A) and acetonitrile with 0.0001% (v/v) acetic acid (B). The flow rate was 20 mL/min and the following gradient 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 → 130 min, 35 → 100% B (linear gradient); 130 → 150 min, 100% B (isocratic). Eluting peaks were detected using an UV detector at 205 nm and fractions corresponding to the peaks in the chromatogram were collected. After removing the acetonitrile by vacuum drying, the sample was freeze-dried. The freeze-dried powder was redissolved in 50% (v/v) aqueous methanol reapplied on the Source 15 RPC column and eluted in the same way as the crude extract. Again peaks were collected and the fractions were dried as described for the crude extract (see above). Pure soyasaponins Ab, Bb, αg and γg with a purity >96%, as determined by RP-HPLC and full scan MS, were obtained.

2.5. Quantification of the soyasaponins

The concentration of the internal standard was 50 μg equilenin for 100 mg of sample. Nominal peak areas were calculated by dividing the absolute area of the individual soyasaponin peaks by the area of the equilenin peak.

Authentic standards for saponins Ab, Bb, αg and βg were used for calibration of the ELSD system. Dilution series of the soyasaponin standards of a concentration ranging from 0.1 to 1.5 mg/mL in 50% (v/v) aqueous ethanol were analysed. Calibration curves were calculated by linear regression of the double logarithmic plots of the nominal peak area versus the amount of soyasaponin injected. These calibration curves were used to calculate the amount of soyasaponins present in the original sample. The calibration curve for soyasaponin Ab was used for the quantification (partially) acetylated and non-acetylated group A soyasaponins; the one for soyasaponin Bb for the non-DDMP-conjugated group B and E soyasaponins. For soyasaponins αg and βg, individual calibration curves were constructed.

2.6. Reproducibility of the method

To investigate the accuracy of the method, five replicates of sample A were extracted and analysed independently within 24 h to evaluate intra-day variation. The sample was extracted and analysed over five separate days over a 2-week period to evaluate the inter-day variation. One extracted sample was injected five times to evaluate the intra-sample variation. For the determination of the different coefficients of variance, only the most abundant soyasaponins in the sample were taken into account, i.e. differently acetylated soyasaponin Ab and soyasaponins Ba, Bb, Be, αg and γg.

3. Results and discussion

3.1. Separation of soyasaponins by HPLC

A typical chromatogram obtained upon analysis of a 70% (v/v) ethanol extract derived from soygerm material is presented in Fig. 1, in which the traces of the ELSD response and the UV signal at 295 nm are shown. The ELS detector produced a stable baseline, which did not shift during gradient elution. Roughly, the chromatogram could be divided in two parts: a region of 15–35 min and one of 37–71 min, in which isoflavones and soyasaponins eluted, respectively.

Isoflavones, which were co-extracted with the soyasaponins, have UV absorption maxima between 248 and 259 nm [30], but still showed a relatively high signal at 295 nm; they also had an ELSD response. Interpretation of the mass spectra (no further data shown) revealed that the glycosyl conjugates (*m*/*z* of $[M+H]^+ = 417$, 447 and 433; RT

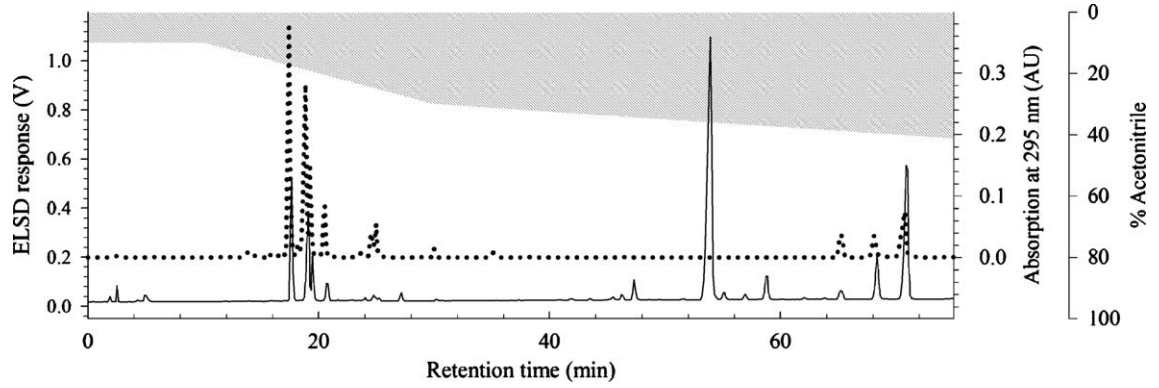


Fig. 1. Representative separation profile of soygerm components using RP-HPLC combined with ELS and UV detection. The gradient of acetonitrile used during elution is indicated by the hatched area. Solid line: ELSD signal; dotted line: UV signal at 295 nm.

(retention time) = 17.5, 17.8 and 19.5 min) eluted first, followed by malonylated glycosides (*m/z* of $[M + H]^+$ = 503, 533 and 519; RT = 18.6, 19.1 and 20.5 min), acetylated glycosides (*m/z* of $[M + H]^+$ = 459, 489 and 475; RT = 24.3, 24.8 and 30.0 min), and finally the aglycones (*m/z* of $[M + H]^+$ = 255, 285 and 271; RT = 34.1, 34.7 and 40.5 min). This order of elution of the different isoflavone forms is consistent with earlier reported methods using RP-HPLC for the analysis of soy isoflavones [30,31]. As the focus of this method was set on detection and quantification soyasaponins, no further efforts were made to optimize the separation of isoflavones.

The compounds of interest in this study, soyasaponins, absorb UV light poorly. The only soyasaponins that could be detected at 295 nm were DDMP-conjugated (Fig. 1), as they show an UV absorption maximum around 295 nm [3]. Based on the peaks in the chromatogram recorded at 295 nm, it was possible to identify peaks of DDMP-conjugated soyasaponins in the ELSD chromatogram. Genuine soyasaponins were efficiently detected using ELSD and appeared as separate peaks (Fig. 2) of which the exact retention times are indicated in Table 1. The peaks were identified by the *m/z* ratio of their molecular ions $[M + H]^+$, as well as their sodium adducts $[M + Na]^+$, found in the mass spectra of the peaks. All of the soyasaponins that are described until present, except for soyasaponins Bc′, βa, γg and γa, were detected in at least one of the different samples analysed. Soyasaponins Ad, Ag, Ah and Bb′ were not present in any of the samples in sufficient amounts to be detected accurately (signal/noise (S/N) <3) by ELSD and were, therefore, not quantified.

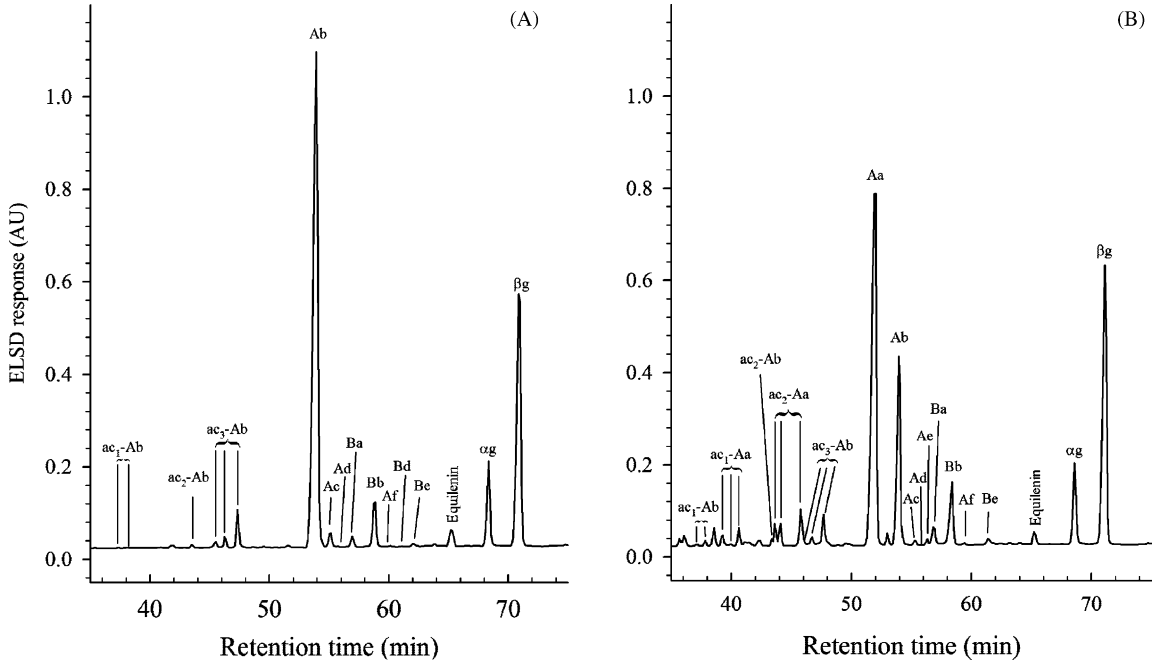


Fig. 2. RP-HPLC elution profiles of soyasaponins of sample A (A) and sample E (B) recorded by the ELS detector. The following prefixes indicate partially acetylated forms of soyasaponins Aa and Ab: ac_1, monoacetyl; ac_2, diacetyl; ac_3, triacetyl. The fully acetylated forms, Aa and Ab, are indicated without a prefix. Amounts of compounds corresponding to the peaks are presented in Table 5.

Table 3
Theoretical and observed number of differently acetylated forms of soyasaponins Aa and Ab, their observed molecular weight (MW) and retention times in the chromatogram

Mother compound	Species	Theoretical number of positional isomers	Detected number of positional isomers	Retention time (min)	MW of $[M+H]^+$
Aa	Triacetyl	1	1	52.0	1364.1
	Diacetyl	3	3	43.8, 44.1, 46.1	1323.1
	Monoacetyl	3	3	39.2, 40.0, 40.6	1281.1
	Non-acetylated	1	1	37.5	1239.5
Ab	Tetra-acetyl	1	1	54.0	1436.1
	Triacetyl	4	3	45.7, 46.2, 47.5	1395.1
	Diacetyl	6	1	43.8	1353.1
	Monoacetyl	4	2	37.8, 38.2	1311.5
	Non-acetylated	1	1	34.0	1269.5

Soyasaponins with different degree of acetylation were well separated on the Aquasil RP C18 column. Soyasaponins Aa and Ab, as well as (some of) their partially acetylated forms, were observed. Partially acetylated forms of the other group A soyasaponins were not found, possibly because of their low abundance in the samples. The retention times of the various acetylated soyasaponin species are summarized in Table 3. Theoretically, incomplete acetylation of the terminal glycosyl residue of the C-22 sugar chain can lead to different positional isomers. The number of isomers depends on the number of positions available for acetylation; i.e. four if the terminal sugar is a glucosyl residue (as in soyasaponin Ab), three if it is a xylosyl residue (as in soyasaponin Aa). The observed number of peaks representing different species of partially acetylated forms corresponded to the theoretically possible number for the partially acetylated forms of soyasaponin Aa. For the partially acetylated forms of soyasaponin Ab, however, only 7 of the theoretically 15 peaks were observed. This might be explained by co-elution of species with the same number of acetylated positions, or differences in abundance of the different isomers, causing only certain species to be detected. Determination of the precise position of deacetylation is not possible by mass spectroscopy and was not further pursued.

The internal standard equilenin (MW = 266.3), a steroid compound, eluted at retention time of 65.2 min, separately from the soyasaponins.

3.2. Quantification of soyasaponins

Authentic standards for soyasaponins Ab, Bb, αg and βg with a purity of >95% were used for the calibration of the ELSD response. The concentration of each standard was chosen in such a way that the ELSD signals covered the range between the minimum and maximum response for the used set of detector parameters. The ELS detector response is given by $y = am^b$ [32], where y is the peak area, m the sample amount, a the response factor, and b the slope. Plots of peak area versus sample amount in double logarithmic coordinates were linear for the four soyasaponins, as well as for equilenin (Fig. 3). The linear regression correlation coefficients ranged from 0.9975 to 0.9998. The slopes b were 1.657, 1.557, 1.590 and 1.597, and the response factors a were 3.617, 4.712, 3.444 and 3.456 for soyasaponin Ab, Bb, αg and βg, respectively. For equilenin, b was 1.713 and a was 10.655. The slopes were quiet similar for all the compounds tested, suggesting that this parameter is not correlated to the molecular structure of the tested compounds. The observed differences could be due to small differences in purity of the compounds. The values for the slopes in this study were on average 20% higher than those reported earlier for ginsenosides [28]. The values for the response factors varied more.

Calibration curves for soyasaponin αg and βg, which differ very little in chemical structure and molecular weight, were identical. Based on this and the overall similarity in ELSD responses of the soyasaponin standards, the soyasaponins for which no authentic standards were available were quantified using the calibration curves of the available standard compound closest in chemical structure and molecular weight. The detection limits for quantification of

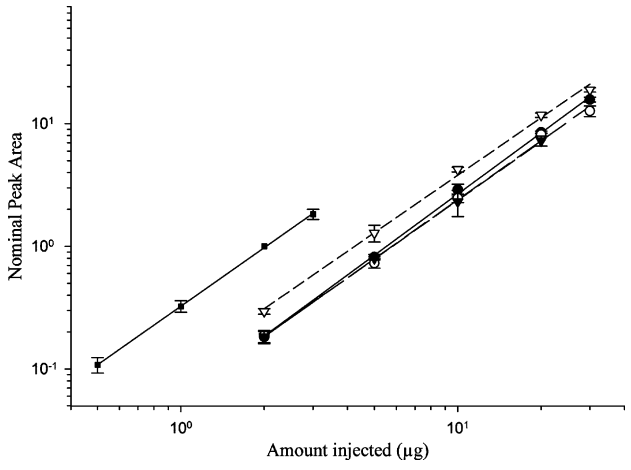


Fig. 3. Calibration curves for the different standards used in this study. Inset shows the relationship of response factors of the different standards and their molecular weight (MW). The response factors are calculated from the regression of the calibration curves. (■) Equilenin; (●) soyasaponin Ab; (○) soyasaponin Ba; (▼) soyasaponin αg; (▽) soyasaponin βg. Error bars indicate SD ($n = 2$ for the soyasaponin standards; $n = 4$ for equilenin).

soyasaponins in the original samples were 0.025, 0.016 and 0.023 mg/100 mg soygerm for, respectively, group A, group B, and DDMP-conjugated soyasaponins, based on the criterion that the signal to noise ratio should be >3, for quantification purposes.

For soyasaponins αg and βg, calibration curves based on UV detection were made, as they contain the chromophore DDMP, which absorbs UV light well. The molar extinction coefficient at a wavelength of 295 nm (ε) was determined as 4851 M^{-1} cm^{-1} for soyasaponin αg and 4286 M^{-1} cm^{-1} for soyasaponin βg. These values correspond well to the extinction coefficients at 292 nm, reported by Hu et al. [26] (4838 and 4504 M^{-1} cm^{-1}, respectively).

3.3. Validation of the method

No difference in soyasaponin content between samples with and without hexane pretreatment was observed ($p \geq 0.21$, $n = 3$). This indicated that no soyasaponins were lost with the hexane defatting step, and that no chemical degradation of labile soyasaponins occurred. When 100 mg of sample A was extracted with 200 mL of 70% (v/v) aqueous ethanol, the same amount of the individual soyasaponins was obtained as when only half of this volume was used ($p \geq 0.16$, $n = 3$); this indicated that 100 mL is sufficient to have a maximal extraction efficiency. Soyasaponins could neither be detected in the washing water after solid phase extraction, nor in the second 10 mL methanol elution. When the purified soyasaponin standards were subjected to the SPE procedure, neither loss of DDMP-conjugated soyasaponins nor of acetylated soyasaponin species was observed (of, respectively, soyasaponins βg and Ab), indicating that the soyasaponins were quantitatively recovered during SPE extraction. The overall recovery of the internal standard, equilenin, which was added prior to extraction with 70% (v/v) aqueous ethanol, was determined as 97.7% ($n = 20$). All together, these results showed that practically no soyasaponins were lost during the extraction protocol.

Analytical accuracy of the method was determined by repeated analysis of sample A. The calculated coefficients of variance for the most abundant soyasaponins in this sample are presented in Table 4. The intra-sample, intra- and inter-day variation was less then 3.7, 6.4 and 9.6%, respectively; for equilenin, this was 1.3, 3.9 and 7.0%, respectively.

Table 4
Coefficients of variance (%) for the intra-sample, intra- and inter-day of the most abundant soyasaponins in sample A[a]

Component	Intra sample	Intra-day	Inter-day
Monoacetyl Ab	3.1	6.2	9.3
Diacetyl Ab	3.7	5.4	9.6
Triacetyl Ab	1.0	3.4	5.4
Tetra-acetyl Ab	1.2	3.6	4.5
Ba	3.0	4.1	7.3
Bb	0.3	1.9	7.5
Be	2.2	6.4	9.0
αg	2.6	5.2	6.6
βg	3.5	4.0	5.2
Equilenin	1.3	3.9	7.0

[a] The coefficients of variance are determined as: (SD/mean) × 100.

3.4. Analysis and quantification of soyasaponins in the samples

The results of the analysis of the different soygerm samples are summarized in Table 5. The total soyasaponin content varied between 3.3 ± 0.2 mg/100 mg (sample D) and 4.4 ± 0.3 mg/100 mg dry weight soygerm (sample H). The total group A soyasaponins varied from 1.5 ± 0.1 to 2.8 ± 0.2 mg/100 mg, and total group B soyasaponins from 1.2 ± 0.1 to 2.0 ± 0.1 mg/100 mg on dry weight soygerm basis. Shiraiwa et al. [6] investigated the variation in soyasaponin composition and content of soygerms among 457 different soybean varieties, measuring the fully acetylated group A soyasaponins and non-DDMP-conjugated group B and E soyasaponins. They reported values ranging 0.62–6.16, 0.36–3.14 and 0.26–2.75 mg/100 mg dry weight for total, group A, and group B soyasaponin content, respectively. The values obtained in our study fall within this range. Other values reported for soygerms are 1.707 and 3.040 mg/100 mg for, respectively, group A and group B soyasaponins [33] and 2.877 mg/100 mg for only group B soyasaponins [26]. The total soyasaponin content of each sample in our study consisted on average of 56.8 ± 6.5% group A, 42.5 ± 6.5% group B and 0.7 ± 0.1% group E soyasaponins.

The main variation observed among the different samples was the composition of the group A soyasaponins, which only occur in soy hypocotyls [24]. In particular, the concentrations of the predominant soyasaponins Aa and Ab were highly variable, depending on the soybean origin. Shiraiwa et al. [6] defined three predominant types of soybean varieties, based on the composition of group A soyasaponins: varieties with only soyasaponin Aa, only Ab, and varieties where both species are present. In our study, two types were prevailing: four varieties contained only soyasaponin Ab (samples A–D) and four contained both Aa and Ab (samples E–H). The ratio of Aa to Ab was variable; in samples E and F, Ab was more abundant than Aa, whereas this was the opposite for samples G and H. The difference between these two types can be seen clearly in Fig. 4, representing a total mass spectrum for samples A and E; only the peaks for the group A saponins and their partially acetylated forms ($m/z > 1200$) differ obviously. Soyasaponin Ae was only found in the samples where also soyasaponin Aa was present. The only difference between these two soyasaponins is the terminal glucosyl residue of the C-3 side chain of Aa, which is absent in Ae. Soyasaponin Ae is thought to be the precursor of Aa in biosynthesis, and depending on the expression level of the glucosyl transferase these soyasaponin species are likely to coexist. The differences in the composition of group A soyasaponins among varieties is considered to be genetically determined [34].

Table 5
Concentrations of soyasaponins in soygerms from different varieties (mg/100 mg dry weight of the soygerms)[a]

Component	Sample							
	A	B	C	D	E	F	G	H
Aa								
Triacetyl	n.d.[b]	n.d.	n.d.	n.d.	0.92 ± 0.01	0.90 ± 0.02	0.57 ± 0.01	0.74 ± 0.02
Diacetyl	n.d.	n.d.	n.d.	n.d.	0.26 ± 0.02	0.27 ± 0.03	0.22 ± 0.1	0.26 ± 0.02
Monoacetyl	n.d.	n.d.	n.d.	n.d.	0.16 ± 0.01	0.16 ± 0.02	0.095 ± 0.004	0.11 ± 0.02
Non-acetylated	n.d.	n.d.	n.d.	n.d.	0.057 ± 0.009	0.086 ± 0.005	0.14 ± 0.04	0.13 ± 0.01
Ab								
Tetra-acetyl	1.4 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	0.58 ± 0.01	0.64 ± 0.01	1.1 ± 0.1	1.2 ± 0.1
Triacetyl	0.34 ± 0.02	0.37 ± 0.04	0.32 ± 0.04	0.22 ± 0.04	0.14 ± 0.01	0.16 ± 0.01	0.27 ± 0.01	0.28 ± 0.04
Diacetyl	0.061 ± 0.006	0.059 ± 0.003	0.047 ± 0.003	0.040 ± 0.003	0.026 ± 0.006	0.025 ± 0.003	0.050 ± 0.002	0.051 ± 0.009
Monoacetyl	0.041 ± 0.006	tr.[c]	0.040 ± 0.009	tr.	tr.	0.064 ± 0.011	0.059 ± 0.020	0.056 ± 0.020
Non-acetylated	0.047 ± 0.005	tr.	0.048 ± 0.006	tr.	tr.	0.056 ± 0.019	tr.	tr.
Ac	0.085 ± 0.006	0.072 ± 0.004	0.071 ± 0.005	0.059 ± 0.003	0.077 ± 0.004	0.067 ± 0.008	0.049 ± 0.003	0.051 ± 0.004
Ae	n.d.	n.d.	n.d.	n.d.	0.068 ± 0.004	0.075 ± 0.007	0.038 ± 0.004	0.056 ± 0.003
Af	0.032 ± 0.004	0.031 ± 0.006	0.029 ± 0.002	tr.	0.040 ± 0.011	0.037 ± 0.008	tr.	0.033 ± 0.002
Ba	0.070 ± 0.006	0.087 ± 0.003	0.088 ± 0.002	0.050 ± 0.003	0.082 ± 0.003	0.073 ± 0.001	0.092 ± 0.001	0.087 ± 0.006
Bb	0.20 ± 0.01	0.20 ± 0.01	0.25 ± 0.01	0.23 ± 0.01	0.25 ± 0.01	0.23 ± 0.01	0.30 ± 0.01	0.17 ± 0.02
Bd	tr.	tr.	tr.	tr.	tr.	0.013 ± 0.002	tr.	tr.
Be	0.015 ± 0.003	0.018 ± 0.002	0.026 ± 0.001	0.022 ± 0.002	0.023 ± 0.002	0.022 ± 0.002	0.028 ± 0.001	0.030 ± 0.002
DDMP Ba (αg)	0.38 ± 0.02	0.30 ± 0.01	0.55 ± 0.01	0.37 ± 0.02	0.34 ± 0.01	0.35 ± 0.02	0.36 ± 0.01	0.37 ± 0.02
DDMP Bb (βg)	0.82 ± 0.01	0.60 ± 0.02	1.1 ± 0.1	1.1 ± 0.1	0.85 ± 0.02	0.82 ± 0.05	0.84 ± 0.02	0.85 ± 0.03
Total	3.5 ± 0.1	3.4 ± 0.1	4.1 ± 0.2	3.3 ± 0.2	3.7 ± 0.1	3.9 ± 0.3	4.2 ± 0.1	4.4 ± 0.3

[a] Data are presented as mean ± SD ($n = 2$).
[b] n.d.: not detected.
[c] tr.: traces, below detection limit of ELS detector.

In the samples containing soyasaponin Ab and not Aa (sample A–D), 21 ± 4% of the group A soyasaponins appeared not fully acetylated; for the samples containing both soyasaponins Aa and Ab, this was 33 ± 2% (27 ± 6% on average over all the samples). It has been suggested that partially acetylated forms of the group A soyasaponins may be result of soybean processing [7]. However, the germs that were analysed in this study did not undergo any processing but a short dry heat treatment with hot air, and the extraction conditions applied were very mild. Still, a considerable amount of partially acetylated soyasaponins Ab and Aa were observed in the samples. From the data obtained in this study, it could not be concluded whether the presence of partially acetylated group A soyasaponins is the result of deacetylation during sample preparation, or incomplete acetylation during biosynthesis. Quantitative data on the content of partially acetylated group A saponins are not available in literature for comparison.

The composition of the group B and E soyasaponins and DDMP-conjugated soyasaponins did not vary much among the different varieties. As can be seen from Fig. 4 ($m/z < 1200$) and Table 5, the same species were present in all the varieties analysed, and concentrations were independent of the soyasaponin A profile. This was also reported by Shiraiwa et al. [6].

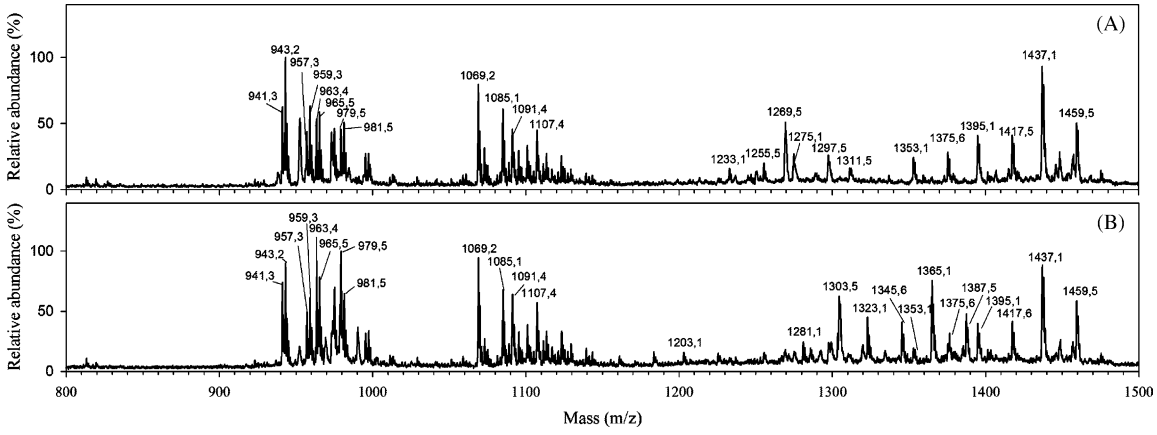


Fig. 4. Mass spectrum (m/z 800–1500) in positive mode of sample A (A) and sample E (B).

However, in that study no data about the proportion of native DDMP conjugates were presented. DDMP-conjugated soyasaponins are thought to be the genuine constituents of group B soyasaponins in soygerms [3]. As DDMP soyasaponins are not stable, the DDMP moiety is easily lost when heat-treated or in aqueous environment, e.g. during extraction procedures [26]. In the soygerm samples investigated in the present study, it was found that 75–84% of the group B soyasaponins was DDMP-conjugated. This is higher than the 63% reported by Hu et al. [26]. The extraction conditions in our method were kept mild, in order to preserve the DDMP saponins as much as possible. The germs were subject to a heat treatment with hot air before grinding, which might explain the presence of the non-DDMP-conjugated group B soyasaponins. It remains to be investigated whether the deconjugated soyasaponins were present in the original sample (and represent the natural ratio of DDMP-conjugated and non-conjugated species in the plant), or whether the DDMP soyasaponins were deconjugated during the drying step or the extraction. Experiments on the stability of DDMP soyasaponins are currently going on.

4. Conclusion

A rapid and accurate HPLC method was developed to determine the soyasaponin content in soy hypocotyls. Using ELS detection and an in-line ESI mass spectrometer allowed the detection and quantification of group A soyasaponins, their partially acetylated forms, as well as group B and E soyasaponins and DDMP-conjugated forms, in a single chromatographic run.

Different group A soyasaponin compositions were found in the hypocotyls of different soybean origins, whereas that of the group B soyasaponins was more uniform. Also, partially acetylated group A soyasaponins, were found in considerable amounts ($27 \pm 6\%$ of total group A soyasaponins) in raw soy hypocotyls. On average, considering all samples investigated, $80 \pm 3\%$ of the group B soyasaponins was DDMP-conjugated.

Acknowledgements

The authors would like to thank Dr. Dayun Zhao, Dr. Willemiek van Oostveen-van Casteren, and Dr. Gerrit van Koningsveld for their help in establishing protocols for soyasaponin extraction and analysis in the Laboratory of Food Chemistry. K.D. benefits an IWT-scholarship.

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