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Stability of saponins from chickpea, soy and faba beans in vegetarian, broccoli-based bars subjected to different cooking techniques

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ABSTRACT

Recently, saponins have been controversially discussed due to increasing evidence on their health promoting impacts. The present study aimed to determine the stability of saponins in vegetarian, broccoli-based bars (BBBs) incorporating chickpea (cp), soy (sb) and faba beans (fb) as protein sources after being subjected to different cooking methods. Commonly domestic ways of BBB preparation were microwaving, frying, frying and microwaving, steaming and baking. Saponins were analyzed by high-performance thin-layer chromatography (HPTLC) coupled to mass spectrometry (MS). Results indicated that HPTLC analysis with post-chromatographic derivatization and coupling to ESI-MS was capable of separating, identification and quantification of two saponin bands in chickpeas and faba beans, i.e. saponin B and 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyranone (DDMP) saponin. Defatted soy bean flour exhibited four bands (saponin B, DDMP saponin, derivatives of soyasaponins A and B). The total saponin content was 297, 4446, and 113 $\mu g \cdot g^{-1}$ dw in chickpea, defatted soy bean flour, and faba beans, respectively. Pretreatments, for instance soaking and peeling of chickpeas and faba beans reduced the total amount of saponins by 8 and 35%, respectively. Subsequently, different cooking conditions significantly reduced the saponin content by 23–32%, 18–59% and 26–36% in sb-BBBs, cp-BBBs and fb-BBB, respectively. Particularly, the DDMP saponin/saponin B ratio was affected. Apparently, conversion of unstable DDMP saponin to saponin B has been observed during the treatments. However, percentile concentration of the different saponins in the processed BBB does not vary compared to the untreated BBB. Soy beans seem not only to be an adequate source of vegetative proteins, but might be also used as a source of valuable saponins. Finally, an efficient determination method was presented providing evidence for predicting the thermal impact on saponins in innovative vegetarian BBBs. In this regard, optimization of cooking conditions considering the retained saponin amounts is recommended, especially for designing new functional foods.

1. Introduction

Saponins are widely distributed secondary metabolites in the plant kingdom. They act as a chemical barrier or shielding compounds against pathogens and herbivores in the plant defense system (Augustin, Kuzina, Andersen, & Bak, 2011). The name of these compounds derives from the ability to form stable, soap-like foams in aqueous solutions (Francis, Kerem, Makkar-Becker, & Becker, 2002; Kerem, German-Shashoua, & Yarden, 2005; Shi et al., 2004). They are divided into two major classes: triterpenoid and steroid glycosides. Structures greatly vary because of the number of attached sugar units at different positions in the molecule. One of the major sources for saponins is legumes. Soy bean and chickpea seeds comprise saponin contents of 1.0–5.6 g 100 g^{-1} dry weight (Kerem et al., 2005; Shi et al., 2004). Also, Sharma and Sehgal (1992) found saponin content in faba bean of about 1.3–1.5 $g \cdot 100\ g^{-1}$ dw. The chemical structures of soy and chickpea saponins (for both so-called "soyasaponins") have been described previously. Soyasaponins are triterpenoidal glycosides structurally divided into two groups. Based on the individual aglycones (soyasapogenol) and the amount of attached sugar moieties they are classified in group A and B soyasaponins, respectively. Group A soyasaponins are bidesmosidic (two sugar moieties at C3 and C22) and are sub-divided in two further groups known as acetylated and de-acetylated types. Whereas group B soyasaponins have only one glycosylation site at C3 (monodesmosidic) and are categorized into two sub-groups based on the conjugation with a 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyranone (DDMP) unit at the carbon atom C22. DDMP conjugated soyasaponins are named as soyasaponin αg, βa, βg, γa and γg while non-DDMP conjugated saponins are called soyasaponin I (Bb), II (Bc), III (Bb′), IV (Bc′) and V (Ba). Group E soyasaponins (Bd, Be) usually formed as artifacts during saponin extraction are also reported (Zhang & Popovich, 2009). Soy comprises four

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different saponin types: groups A, B, E and DDMP (Price, Fenwick, & Jurzysta, 1986; Yoshiki, Kudou, & Okubo, 1998). Chickpeas contain mainly soyasaponin βg and lower amounts of Bb and Be (Kerem et al., 2005; Serventi et al., 2013). According to Amarowicz, Yoshiki, Pegg, and Okubo (1997) faba bean saponins are similar to group B soyasaponins which are also previously recorded by Shiraiwa, Harada, and Okubo (1991).

In recent years, saponins are attracting considerable interest as a result of their diverse properties, both deleterious and beneficial. Obviously, some saponins were recognized as antinutrients, because of possessing strong hemolytic activity (Price, Johnson, & Fenwick, 1987). Sparg, Light, and van Staden (2004) summarized the biological activities of different saponins from various plant families. In the same context, clinical studies suggested saponins being health-promoting components, because of lowering cholesterol levels, blood lipids, and blood glucose response. Güçlü-Üstündağ and Mazza (2007) highlighted the potential reduction of cardiovascular diseases and obesity risk in humans consuming a diet rich in legume-food containing saponins. However, saponins exhibit antioxidant activity by binding to cholesterol and preventing its oxidation. High antioxidant fractions rich in phenolic compounds and saponins might be a potential active ingredient that could be applied in nutraceuticals, functional foods as well as in natural food preservation (Chan, Iqbal, Khong, Ooi, & Ismail, 2014).

A strategy involving the incorporation of legume ingredients into commonly consumed food products in developing countries represents a viable alternative for complementing the protein deficiency and increasing the health benefits as well. Recently, legume-based ingredients are being used to develop breads as a potential means of decreasing the risk of cardiovascular disease (Nilufer, Boyacioglu, & Vodovotz, 2008; Vittadini & Vodovotz, 2003). However, processing conditions such as heat, pH, matrix, and solvents can affect saponin content and their profile in foods (Güçlü-Üstündağ & Mazza, 2007). For example, during food processing the DDMP saponins are almost hydrolyzed to B saponins and maltol (Heng, Vincken, Hoppe, et al., 2006; Reim & Rohn, 2015; Shi et al., 2004). For all that, the amount of saponin retention in processed food has not been studied sufficiently and might depend on different processing parameters and techniques.

Therefore, the objective of the present study was to assess the stability of saponins from different legumes as protein sources in innovative vegetarian, broccoli-based bars (BBB) under different cooking procedures. Saponins were analyzed by high-performance thin-layer chromatography (HPTLC) and post-chromatographic derivatization in combination with mass spectrometry (MS) for substance assignment.

2. Materials and methods

2.1. Plant materials

Broccoli florets (*Brassica oleracea* var. *italica*), chickpea (*Cicer arietinum* L.), faba bean (*Vicia faba* L.) sweet potato (*Ipomoea batatas* L.), naked barley (*Hordeum vulgare* L. var. *nudum*), carrot (*Daucus carota* L.), onion (*Allium cepa* L.), sweet red pepper (*Capsicum annuum* L.), fresh garlic (*Allium sativum* L.), fresh coriander leaves (cilantro; *Coriandrum sativum* L.), fresh dill (*Anethum graveolens* L.), fresh parsley (*Petroselinum crispum* Mill.), and edible salt of prime fresh quality were purchased from a local supermarket in Hamburg, Germany. Otherwise, defatted soy bean flour (*Glycine max* L.) with 48% protein and 6% fat was obtained from Food Technology Research Institute (FTRI), Agricultural Research Centre (ARC), Cairo, Egypt. In addition, the traditional seasoning species were bought in Ragab El-Attar's local spices supermarket, Egypt.

2.2. Preparation of different broccoli-based bars (BBBs) ingredients

The green leaves of fresh broccoli plants were removed; the florets were cut into 1.5–2.0 cm parts prior to blanching under live steam for 3 min. Unpeeled chickpeas and faba beans were washed and soaked in water for 12 h (1:2, w/v). The excessive water was drained and the seeds were peeled and ground for 3 min using a conventional kitchen machine. Soy bean flour was rehydrated with water (1:2, w/v) to produce soy bean flour dough. Sweet potato and carrots were peeled, washed, chopped in 1 cm slices and blanched using a live steam blancher for 7 and 5 min, respectively. Subsequently, the blanched materials were immediately cooled down and homogenized to a puree. The whole naked barley kernels were milled twice to obtain homogeneous and fine barley flour. Sweet red peppers were washed and chopped in small cubes. Peeled fresh onions and garlic were added before preparing the vegetarian bars.

A green leafy mixture of herbs, i.e. fresh coriander, dill, and parsley leaves were mixed (2:1:1). Also, the spices were ground and mixed [25 g black pepper (*Piper nigrum* L.), 20 g cumin (*Cuminum cyminum* L.), 20 g relish ('Baharat'; ready-mix of specific spices), 10 g dried coriander seeds (*C. sativum* L.), 10 g ginger (*Zingiber officinale* R.), 10 g paprika (*C. annuum* L.) and 5 g hot chili (*Capsicum chinense* L.)] to prepare 100 g of traditional spices mix for immediate use.

2.3. Preparation of ready-to-use and ready-to-eat BBBs

Broccoli-based vegetarian bars were prepared from the previously described ingredients according to the recipes in Table 1. About 1 kg from each recipe was mixed using a kitchen machine. The whole experiment was done in triplicate. Initially, raw BBBs served as control samples. Appropriate amounts of each BBB mixture were cut to BB bars of 10.0 × 0.8 × 0.6 cm prior to exposing them to various domestic cooking methods. Food preparation devices applied were microwaving, frying, frying/microwaving, steaming, and baking. For microwaving, the BB bars were treated at 590 W for 5 min. Before frying, sunflower oil was preheated to 160 °C in a deep-frying skillet. The BB bars were fried at 180 °C for 5 min. For the combination of frying and microwaving, the BB bars were similarly fried for 3 min prior to microwaving for 2 min. Excessive oil was removed by kitchen papers. Steaming was conducted by wrapping the BB bars with aluminum foil and cooking over a live steam for 5 min in a cooking pot. At the end of steaming, the bars reached a temperature of 80–85 °C which is also sufficient to inhibit undesired enzymes. Baking of BBBs was carried out on aluminum foil sheets brushed with little amount of sunflower oil in a pre-heated electric oven at 200 °C (fan assisted) and left for 5 min at constant temperature. After all, the unprocessed BBBs and cooked samples were immediately frozen overnight (−20 °C) until freeze-drying for at least 96 h (−20 °C, vacuum: 0.1 bar) using an Alpha 1-4 LSC system (Martin Christ GmbH, Osterode, Germany).

Table 1
Composition of vegetarian broccoli-based bars incorporating different protein sources.

Ingredients	Fresh vegetarian bars ingredients (%)		
	cp-BBB	sb-BBB	fb-BBB
Blanched broccoli	25	25	25
Peeled soaked chickpea	25	–	–
Hydrated soy bean flour (1:2)	–	25	–
Peeled soaked faba bean	–	–	25
Blanched sweet potato	12	12	12
Whole barley flour	10	10	10
Blanched carrot puree	8	8	8
Green leafy herbs mix[a]	7	7	7
Red pepper paste	5	5	5
Fresh onion	5	5	5
Salt	1.25	1.25	1.25
Fresh garlic	0.75	0.75	0.75
Traditional spices mix[b]	1	1	1

BBB: vegetarian broccoli-based bars formulated with soaked chickpea (cp), dehydrated soy bean flour (sb), soaked faba bean (fb).
[a] Green leafy vegetables herbs (coriander, dill, and parsley; 2:1:1).
[b] See Materials and methods.

2.4. Saponin extraction and analysis by high-performance thin-layer chromatography

Saponin extraction was carried out following the protocol of Reim and Rohn (2015) with slight modifications. About 2 g from freeze-dried samples were extracted in 8 mL methanol (HPLC grade) in water bath for 4 h at 50 °C. Afterwards, the samples were centrifuged at 3.225 ×*g* for 30 min, re-extracted with 5 mL methanol and re-centrifuged similarly again. To precipitate non-saponin constituents the collected raw extract was mixed with equal volume of 0.4 M ammonium sulfate and shaken overnight. Subsequently, the clear extracts were dried under stream of gaseous nitrogen (N_2).

The residue was redissolved in ddH_2O and used for solid phase extraction (SPE). After appropriate preparation of the C18 adsorbent (500 mg/6 mL, Macherey-Nagel GmbH & Co. KG, Düren, Germany), saponins were eluted as described previously (Reim & Rohn, 2015). The collected fractions I–V were concentrated by gaseous nitrogen (N_2) and re-suspended in 0.25 mL of corresponding eluent previously used for SPE.

Analysis of the saponin extracts was performed by high-performance thin-layer chromatography (HPTLC) already described by Reim and Rohn (2015). For this purpose all saponin containing fractions were combined in equal parts and 10–20 μL of the pooled sample was applied as 8 mm bands on silica gel 60 $_{F254}$ plates (Merck KGaA, Darmstadt, Germany). Sample application was carried out automatically using a TLC autosampler (ATS4, CAMAG AG, Muttenz, Switzerland). Development was performed in twin trough chambers by a solvent system consisting of chloroform:acetic acid:methanol:ddH_2O (6.4:3.2:1.2:0.8; v/v/v/v). Subsequently, the plates were air dried for 2 h at room temperature ensuring evaporation of solvent excess. Post-chromatographic derivatization with *p*-anisaldehyde sulfuric acid was obligatory for detection. To this, the developed plate was immersed once for 2 s with the staining solution, air dried for 10 min at room temperature and heated for 5 min at 70–74 °C.

Quantitative analysis was carried out densitometrically by scanning the derivatized plate at 545 nm using a TLC scanner 3 (CAMAG AG, Muttenz, Switzerland). Soyasaponin I (saponin B) from soy bean (*G. max*, Sigma-Aldrich GmbH, Steinheim, Germany) served as standard in a concentration range of c = 0.125–0.625 mg/mL. A TLC visualizer (CAMAG AG, Muttenz, Switzerland) was applied for photo documentation. The densitograms were evaluated with the software winCats (CAMAG AG, Muttenz, Switzerland).

2.5. High-performance thin-layer chromatography mass spectrometry

In accordance to Reim and Rohn (2015), saponin identification was performed by hyphenation of HPTLC with mass spectrometry (MS) via a TLC–MS interface (CAMAG AG, Muttenz, Switzerland). The samples were applied twice on the plate and developed as mentioned before with minor modifications. Depending on the purpose separation was carried out with two different solvent mixtures providing an appropriate band resolution, (1) chloroform:acetic acid:methanol:ddH_2O (6.4:3.2:1.2:0.8; v/v/v/v) and (2) chloroform:methanol:ddH_2O (6.5:3.5:0.9; v/v/v). One part of the plate was derivatized with *p*-anisaldehyde staining solution serving as template for highlighting the bands of interest on the non-derivatized measuring plate. The labeled zones were eluted semi-automatically by a piston using 0.1% formic acid in ddH_2O and acetonitrile (40:60, v/v). With a flow rate of 0.25 mL min^{-1}, the extracted substances were assigned online into an ion trap mass spectrometer (amazon ETD, Bruker Daltonik GmbH, Bremen, Germany). The target compounds were recorded at positive ion mode within a mass range of m/z 100–3000. Evaluation was performed by the software DataAnalysis v. 4.0 (Bruker Daltonik GmbH, Bremen, Germany). Finally, the obtained hypothetical molecular weights were compared with results from appropriate literature.

2.6. Statistical analysis

The statistical analysis was carried out using SPSS program with multi-function utility regarding to the experimental design under significance level of 0.05 for the whole results and multiple comparisons were carried out applying LSD with a Duncan test according to Steel, Torrie, and Dickey (1997).

3. Results and discussion

3.1. Detection and identification of saponins

The sample preparation procedure was successful for cleaning-up chickpea, soy and faba been saponins. Due to the absence of strong chromophores, a post-chromatographic derivatization with *p*-anisaldehyde sulfuric acid reagent was necessary for the detection of saponins (Oleszek & Bialy, 2006). Distinction of non-UV-active compounds is ensured immediately by different colored bands resulting from the staining (Spangenberg, 2008). In all legume based samples, few characteristic bands (named as “soyasaponins 1–4”, “SS 1”–“SS 4”) with specific rf-values were detected. Four different saponins (“SS 1”–“SS 4”) were present in defatted soy bean flour (Fig. 1A). In chickpeas and faba beans only two saponins (“SS 1”, “SS 2”) occurred (Fig. 1B and C). When applying UV-light (366 nm) following the derivatization, saponin B (rf = 0.44–0.46) appears as an orange band while the DDMP saponin (rf = 0.48–0.50) has a violet–blue color on a dark blue background (Fig. 1A–C, left TLC stripe). Documentation at white light yielded violet bands (Fig. 1A–C, right TLC stripe). Besides, substance zones at the solvent front or below the saponins were detected. Those might be representing degradation products or matrix components but did not have any influence on the results.

Likewise, assigning saponins chromatographically with the standard substance saponin B (soyasaponin I/Bb), identification of the bands was also carried out mass spectrometrically by hyphenation of HPTLC via a commercially available TLC–MS interface (Reim & Rohn, 2015). The bands of interest were extracted semi-automatically from the plate and sensitive mass spectrometric signals were gained within a few minutes (Tuzimski, 2011). Molecular ion species cationized by sodium dominated the spectra in the positive ion mode. However, di-sodium, potassium, ammonium, and/or acetonitrile adducts were also determined sporadically. The most prominent m/z values recorded in soy bean, chickpea, and faba bean flour (raw materials) are summarized in Table 2.

According to Fig. 1A–C the band labeled as “SS 1” in soy bean flour and raw chickpeas was identified as saponin B (soyasaponin I/Bb, m/z 965.6 $[M + Na]^+$). The second band “SS 2” was determined as DDMP saponin (soyasaponin βg, m/z 1091.8 $[M + Na]^+$). Exemplarily, corresponding mass spectra from soy bean flour are shown in Fig. 2A. Similarly, saponin B was obviously detected in faba beans by m/z 981.6 $[M + K]^+$ or m/z 987.6 $[M + 2Na–H]^+$. Documentation under UV-light displayed a DDMP saponin comparable colored band (violet–blue, Fig. 1C, left TLC stripe, “SS 2”). Unfortunately, the concentration might have been too low to be measured by TLC–ESI-MS with high intensity.

In addition, HPTLC–MS analysis provided further saponins (“SS 3”, “SS 4”) in soy bean flour which is in agreement with previously published data (Serventi et al., 2013; Zhang & Popovich, 2009). In fact, although the saponin bands are adequately separated (Fig. 1A–C), they elute quite closely spaced why a slight contamination during the elution for MS analysis is unavoidable. Along with saponin B and DDMP saponin it might be assumed that “SS 3” correspond to further group B soyasaponins, for instance soyasaponin II/Bc (MW 912 g/mol) or Be (MW 940 g/mol). Whereas “SS 4” might belong to group A soyasaponins, e.g. A1/Ab or Ac with an average molecular weight of 1436 g/mol and 1420 g/mol, respectively (Serventi et al., 2013). The latter higher molecular weight saponins were generally assigned in terms of double charged

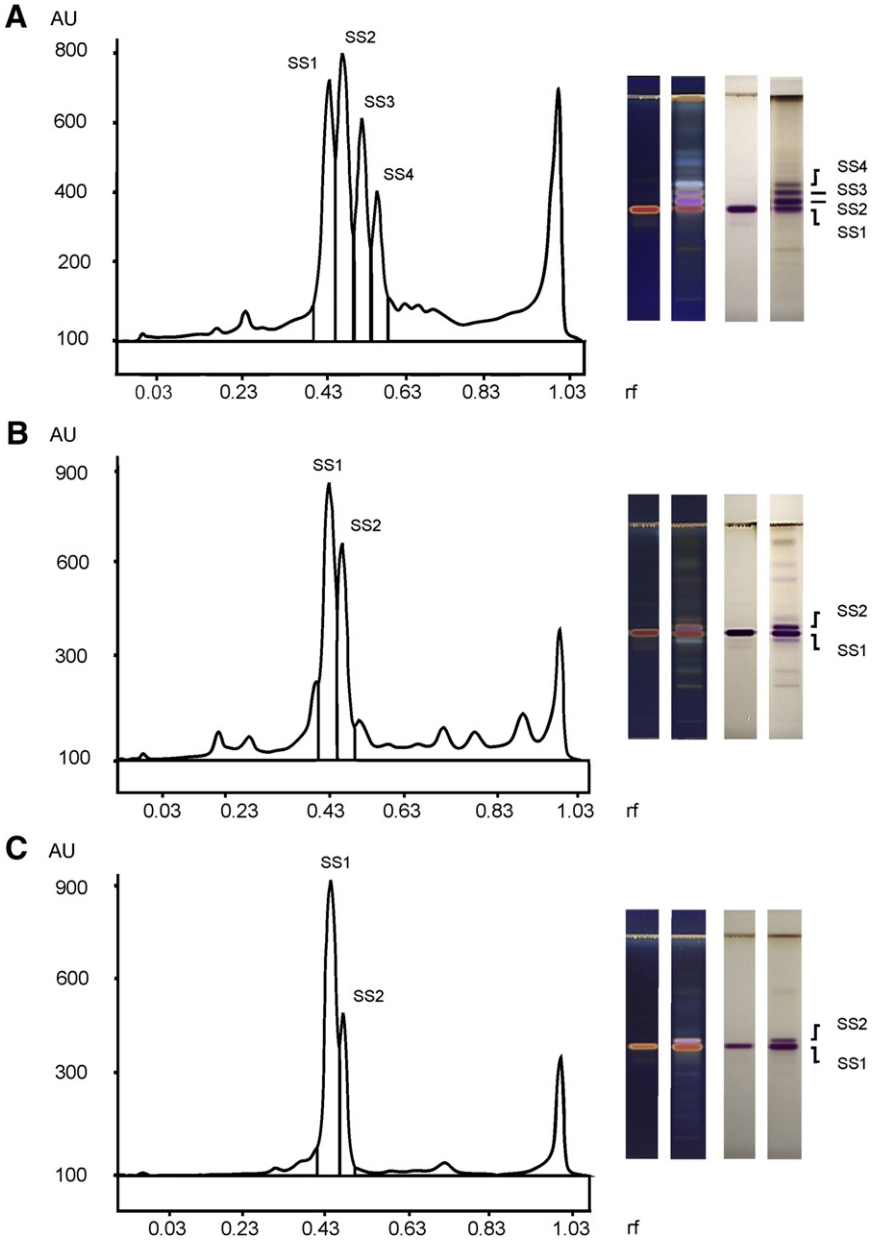

Fig. 1. Separation of saponins from soy bean flour (A), chickpea (B), and faba bean (C) by HPTLC. Post-chromatographic derivatization with *p*-anisaldehyde sulfuric acid reagent was carried out. Documentation of silica gel plates at WL (white light) and UV (ultra violet light 366 nm) was done. "SS 1"–"SS 4": soyasaponins 1–4, "SS 1": saponin B (soyasaponin I/Bb) "SS 2": DDMP saponin (soyasaponin βg), "SS 3": soyasaponin II/Bc; Be, "SS 4": soyasaponin A1/Ab; A.

fragment ions with m/z 729.4 $[2\ M + Na]^+$ or $[2\ M + K]^+$ and 737.4 $[2\ M + K]^+$, respectively (Fig. 2B, Table 2). Minor intensities of soyasaponin A2/Af (MW 1274 g/mol) and A3/Ah (MW 1244 g/mol) were also detected but were not the subject of the present study (Table 2, "SS 5").

Alternatively, combining HPTLC with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) might also provide a new technique for high resolution molecular analysis directly from the plate. This method does not only focus just on the analytes of interest. It can also monitor the complete area of the sample lane. Therefore, HPTLC–MALDI-TOF-MS is suitable, particularly for narrow-banded compounds. Nevertheless, the illustrated results confirmed that saponins in soy beans, chickpeas, and faba beans are structurally similar as mentioned previously (Hu, Lee, Hendrich, & Murphy, 2002; Price et al., 1987; Reim & Rohn, 2015; Serventi et al., 2013; Zhang & Popovich, 2009).

3.2. Saponins in raw legume ingredients

The main objective was to identify and quantify saponins in raw ingredients used as protein sources in differently cooked vegetarian, broccoli-based bars (BBBs). Soyasaponin I (saponin B) from soy beans was used as a standard (Fig. 1A–C; rf = 0.44–0.46). Due to the instability of the DDMP conjugate, an adequate standard compound is not commercially available. Besides the DDMP saponin, further detected saponins, e.g. in soy beans were only determined tentatively using the calibration curve of saponin B, as well. Total saponins were shown as the sum of saponin B ("SS 1"), DDMP ("SS 2"), group B ("SS 3") and group A soyasaponins ("SS 4"). The total saponin content of raw chickpeas, defatted soy bean flour and raw faba beans was 297, 4446, and 113 $\mu g \cdot g^{-1}$ dw, respectively (Table 3). Pretreatment of raw materials, for instance soaking and peeling of chickpeas and faba beans reduced

Table 2
Saponin identification in soy bean flour, chickpea and faba bean raw materials by HPTLC–ESI-MS.

Raw material	SS	m/z	Ion species	MW[a]	Substance	MW[b]
sb	1	965.6	$[M + Na]^+$	942.6	I/Bb/B	942
		981.6	$[M + K]^+$	942.6	I/Bb/B	942
	2	1091.8	$[M + Na]^+$	1068.8	βg	1068
	3	819.6	$[M + Na]^+$	796.6	III/Bb′	796
		935.6	$[M + Na]^+$	912.6	II/Bc	912
		951.6	$[M + K]^+$	912.6	II/Bc	912
		963.6	$[M + Na]^+$	940.6	Be	940
	4	729.4	$[2\,M + Na]^+$	1435.8	A1/Ab	1436
		737.4	$[2\,M + K]^+$	1435.8	A1/Ab	1436
		729.4	$[2\,M + K]^+$	1419.8	Ac	1420
	5	646.3	$[2\,M + H + NH_4]^+$	1274.6	A2/Af	1274
		654.4	$[2\,M + ACN + Na]^+$	1244.8	A3/Ah	1244
cp	1	965.7	$[M + Na]^+$	942.7	I/Bb/B	942
	2	1091.8	$[M + Na]^+$	1068.8	βg	1068
fb	1	965.7	$[M + Na]^+$	942.7		
		981.6	$[M + K]^+$	942.6	I/Bb/B	942
		987.6	$[M + 2Na–H]^+$	942.6		
	2	1091.8	$[M + Na]^+$	1068.8	βg	1068

sb: soybean flour, cp: chickpeas, fb: faba beans,SS: soyasaponin; abbreviation analogue to labeled bands in Fig. 1, saponins belonging to group A (A1/Ab, Ac, A2/Af, A3/Ah); group B (I/Bb/B, II/Bc, III/Bb′, Be); DDMP group (βg) soyasaponins.
[a] Calculated molecular weight.
[b] Mentioned molecular weight in literature.

Table 3
Saponin content in raw and prepared materials as protein sources.

Saponin types	Saponin content in raw materials [μg g^{-1} dw]				
	Chickpea (cp)		Soy bean (sb)	Faba bean (fb)	
	Raw	Soaked	Defatted flour*	Raw	Soaked
SS 1	171 ± 18^b	155 ± 5^b	921 ± 30^a	39 ± 05^c	24 ± 01^c
SS 2	126 ± 7^b	119 ± 7^b	1800 ± 87^a	74 ± 07^c	50 ± 02^c
SS 3	nd	nd	1064 ± 25	nd	nd
SS 4	nd	nd	661 ± 17	nd	nd
Total	297 ± 21^b	274 ± 11^b	4446 ± 48^a	113 ± 12^c	74 ± 04^c
SS 1/SS 2/SS 3/SS 4	58/42/–/–	57/43/–/–	21/41/24/15	35/65/–/–	32/68/–/–

[a, b, c]: means with the same letter in the same row are not significantly different at ($p > 0.05$), nd: not detected. "SS 1"–"SS 4": soyasaponin types 1–4, "SS 1": saponin B (soyasaponin I/Bb), "SS 2": DDMP saponin (soyasaponin βg), "SS 3": soyasaponin II/Bc; Be, "SS 4": soyasaponin A1/Ab; Ac.
* Defatted flour contains (48% protein and 6% fat).

the total amount of saponins by 8% and 35%, respectively. The relative ratio between saponin B ("SS 1") and DDMP saponin ("SS 2") in raw chickpeas was 58:42 and does not differ noticeably to the soaked samples (57:43). Likewise, the percentile distribution in faba beans was unaltered prior to (35:65) and after (32:68) soaking and peeling (Table 3). In comparison to equally treated chickpeas the percentage ratio of saponin B and DDMP saponin in faba beans was the opposite. Above all, DDMP saponin was more abundant than saponin B in soy bean as described before (Heng, Vincken, Hoppe, et al., 2006). Additional saponin types quantified in soy bean flour were further group B soyasaponins ("SS 3" (Bc/Be), 1064 μg·g^{-1} dw) and group A soyasaponins ("SS 4" (Ab/Ac), 661 μg·g^{-1} dw).

Among all foods of plant origin legumes are the main sources of saponins presented as triterpene glycosides. It is already known that soy bean, chickpea and faba bean varieties differ in their saponin content and the reported yields greatly depend on genotype, ecophysiological factors, and storage conditions. Previous data report 56 mg·g^{-1} DM of total saponins in chickpeas, followed by soy beans (43 mg·g^{-1} DM), whole faba bean meal (4.3 mg·g^{-1} DM), or lentils with an average of 4.2 mg·g^{-1} DM (Fenwick & Oakenfull, 1983). With regard to the raw materials, concentrations of saponins in chickpeas, soy, and faba beans are in agreement as described previously (Alajaji & El-Adawy, 2006; Kerem et al., 2005; Ruiz, Price, Fenwick, & Rhodes, 1996; Serventi et al., 2013; Sharma & Sehgal, 1992; Shiraiwa et al., 1991; Vittadini & Vodovotz, 2003).

Pertaining to the mostly undesired bitter flavor of saponins, processing of dry legumes, e.g. soaking or boiling may reduce the bitterness by leaching out these compounds or converting the DDMP saponin to the less bitter-tasting saponin B (Duhan, Khetarpaul, & Bishnoi, 2001; Heng, Vincken, van Koningsveld, et al., 2006). Saponin distribution is depending on the plant part, too. In general, hulls comprise more saponins compared to the whole seeds as shown for soybeans (Shi et al., 2004). Soaking and dehulling of chickpeas reduced the total saponin content by 8% which seemed to be slightly higher than

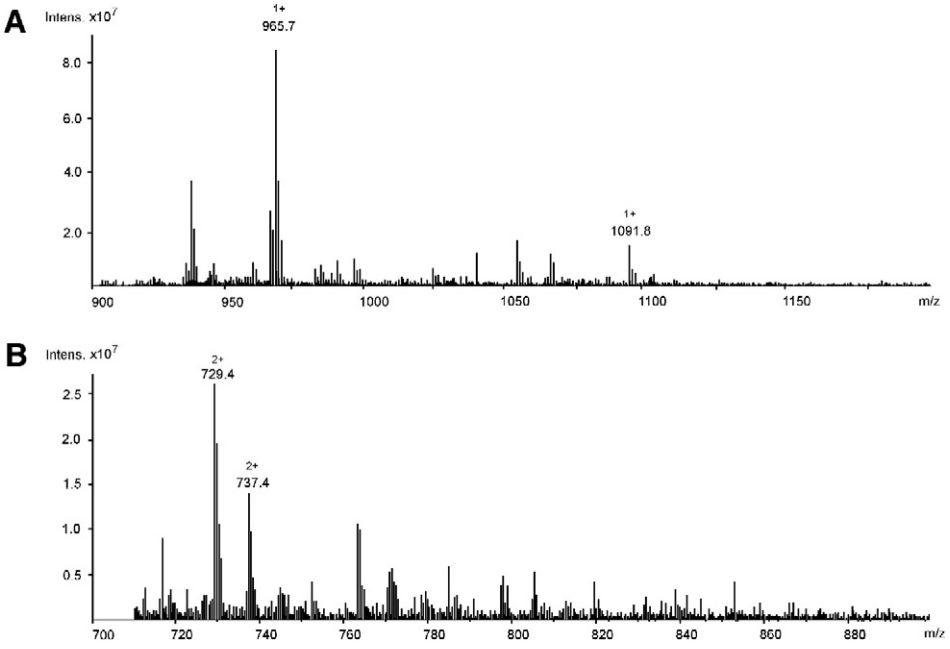

Fig. 2. HPTLC–ESI-MS analysis of saponins in soy bean flour. Mass spectra of (A) DDMP saponin (m/z 1091.5 $[M + Na]^+$) and saponin B (m/z 965.5 $[M + Na]^+$); (B) soyasaponin A1/Ab (m/z 729.4 $[2\,M + Na]^+$, 737.4 $[2\,M + K]^+$).

mentioned by Ruiz et al. (1996) who described 1–6% loss of saponins in two chickpeas varieties. Almost in agreement with Sharma and Sehgal (1992) who reported 30% reduction of saponins in soaked and dehulled faba beans. However, a loss of 35% was observed in this study (Table 3). Saponin loss during soaking occurs via a leaching process. Particularly, a synergistic effect of soaking time and seed-to-water ratio have a noticeable impact on the quantity of saponins leached out from the beans' matrix as described by Shi et al. (2009). First, the dry peas/beans absorb the water to soften their hard coats. Prolonged soaking favors further water absorption. Hence, dissolving of sugars leads to an increased cell membrane permeability of the seed coat. The moisten tissue promotes penetration of water to a greater extend into the matrix releasing water soluble saponins by diffusion. Therefore, short soaking times and limited amount of soaking media do not complete the hydration of the beans and might have a stabilizing effect on the saponins in the seed's matrix. Otherwise, after the bean tissue is completely hydrated persistent soaking (>12 h) do not influence saponin diffusion any further.

Thus, in this study a seed-to-water ratio of 1:2 (w/v) and 12 h soaking time resulted in a complete hydration of the seeds. So, the water soluble saponins leached out through diffusion (Duhan et al., 2001; Shi et al., 2009). Moreover, saponins were additionally removed by dehulling the soaked seeds. It is remarkable that the loss of saponins in faba beans was more pronounced compared to chickpeas. This may be due to increasing the surface area, structure and geometrical shape of the faba beans as hypothesized by Sharma and Sehgal (1992) and Ruiz et al. (1996). Nevertheless, soaking of chickpeas and faba beans did not modify the percentile distribution of saponin B and DDMP saponin in the seeds. It can be assumed that hydrolysis of DDMP saponin into saponin B did not take place meanwhile the soaking period supposing a stabilizing matrix effect. No transformation occurred not before all feasible saponins leached into the water where they are much more susceptible for conversion. In case that this reaction is favored in solvents with a high dielectric constant and more oxygen is present in the soaking water (Heng, Vincken, Hoppe, et al., 2006; Shi et al., 2009).

3.3. Stability of saponins during cooking of formulated BBBs

Especially, the effect of domestic cooking methods on saponin profile and its content in the different legume-based bars have been considered by HPTLC (Table 3). Recovery of similar saponin types was successful in different cooked BBBs illustrating the effect of preparation and cooking conditions (Table 4). However, the saponins in BBBs quantified were much more than expected. In all formulated BBBs, significant difference ($p < 0.05$) in total saponin content was mostly found between unprocessed and differently cooked BBBs. The highest retaining level was observed in sb-BBB, where saponin content was retained by 77%, 68%, 73%, 74%, and 76% in microwaved, fried, fried/microwaved, steamed and baked BBBs, respectively. In similarly treated fb-BBBs saponins retained in comparable amounts as 64%, 74%, 69%, 69%, and 73%. Likewise, a moderate retaining of saponins by 52% (microwaved), 54% (fried), 51% (fried/microwaved), and 41% (baked) cp-BBB, was recorded. Steaming of cp-BBB provided significantly the lowest loss of saponins by 18%, while frying/microwaving and baking reduced total saponins, dramatically. Concerning cp-BBBs, a markedly loss during the latter preparations may be attributed to the thermolabile nature of saponins. Whereas steaming did not exceed temperatures higher than 80–85 °C, frying and/or baking was carried out at 180–200 °C. In addition, excessive oil used for frying might maintain a higher temperature in the cooking media enhancing its permeability into the matrix and promoting saponin degradation (Shi et al., 2009). Generally, the different cooking conditions significantly reduced the saponin content by 23–32% in sb-BBBs, 18–59% in cp-BBBs, and 26–36% in fb-BBB. Compared to previously published data by Sharma and Sehgal (1992) cooking of soaked faba beans reduced the saponin content by 35%, while 36–37% saponin reduction was recorded in soaked, dehulled and cooked faba beans. Moreover, the saponin decline was even more pronounced to 48–50% and 81–84% when soaked and dehulled seeds were autoclaved for 15 and 25 min, respectively (Sharma & Sehgal, 1992). A comparable saponin decrease with 47% was observed by microwaving of chickpeas (Alajaji & El-Adawy, 2006). On the contrary, lower reduction with approx. 4–5% was obtained when cooking chickpeas seeds (Ruiz et al., 1996). In case of soy bean, recovery of total saponins was 42–83% in soy and chickpeas-soy breads (Serventi et al., 2013).

Considering the different processed legume-based diets there was an increase of saponin B concentration in the cooked samples compared to the raw and/or soaked material while DDMP content decreased (Tables 3 and 4). Apparently, the content of DDMP saponin in raw legumes is particularly higher than that of saponin B and a conversion of unstable DDMP saponin to saponin B took place during the different treatments. Exceptionally, the ratio of saponin B to DDMP saponin in processed fb-BBBs did not differ markedly compared with the uncooked fb-BBB samples (Table 4). In this case, a transformation into soyasaponin Be (group E soyasaponins) can be an explanation. The latter is considered to be a phyto-oxidation product of group B soyasaponins comprising soyasapogenol E as the corresponding aglycone. Besides, further degradation of saponin B into the aglycone soyasapogenol B due to the heating treatment is possible (Sagratini

Table 4
Saponin content in formulated vegetarian broccoli-based bars (BBB).

BBB	Saponin content in BBBs [μg g^{-1} dw]						
	Saponin types	Uncooked	Microwaved	Fried	Fried/microwaved	Steamed	Baked
cp	SS 1	188 ± 13^{a}	96 ± 08^{cd}	100 ± 13^{c}	94 ± 11^{cd}	158 ± 11^{b}	71 ± 24^{d}
	SS 2	61 ± 17^{a}	34 ± 10^{b}	34 ± 08^{b}	32 ± 09^{b}	48 ± 11^{ab}	33 ± 11^{b}
	Total	249 ± 30^{a}	130 ± 18^{c}	134 ± 19^{c}	126 ± 20^{c}	206 ± 23^{b}	104 ± 30^{c}
	SS 1/SS 2	76:24	74:26	75:25	75:25	77:23	68:32
sb	SS 1	525 ± 34^{a}	479 ± 61^{a}	417 ± 108^{a}	468 ± 60^{a}	452 ± 41^{a}	447 ± 36^{a}
	SS 2	658 ± 70^{a}	503 ± 59^{b}	438 ± 102^{b}	470 ± 62^{b}	481 ± 28^{b}	510 ± 31^{b}
	SS 3	513 ± 49^{a}	378 ± 36^{b}	329 ± 80^{b}	355 ± 29^{b}	357 ± 20^{b}	369 ± 24^{b}
	SS 4	374 ± 56^{a}	241 ± 51^{b}	223 ± 63^{b}	225 ± 32^{b}	241 ± 12^{b}	254 ± 11^{b}
	Total	2070 ± 194^{a}	1601 ± 202^{b}	1407 ± 349^{b}	1518 ± 180^{b}	1531 ± 63^{b}	1580 ± 63^{b}
	SS 1/SS 2/SS 3/SS 4	25:32:25:18	30:31:24:15	30:31:23:16	31:31:23:15	30:31:23:16	28:32:23:16
fb	SS 1	53 ± 12^{a}	33 ± 04^{b}	34 ± 06^{b}	35 ± 05^{b}	36 ± 08^{b}	37 ± 09^{b}
	SS 2	35 ± 06^{a}	23 ± 03^{c}	$31 \pm 01b^{a}$	26 ± 05^{bc}	25 ± 03^{bc}	27 ± 01^{bc}
	Total	88 ± 16^{a}	56 ± 04^{b}	65 ± 05^{b}	61 ± 07^{b}	61 ± 07^{b}	64 ± 09^{b}
	SS 1/SS 2	60:40	59:41	52:48	57:43	59:41	58:42

BBB: vegetarian broccoli-based bars formulated with soaked chickpea (cp), dehydrated soy bean flour (sb), soaked faba bean (fb), SS: soyasaponin; abbreviation analogue to labeled bands in Fig. 1A–C; "SS 1": saponin B (soyasaponin I/Bb), "SS 2": DDMP saponin (soyasaponin βg), "SS 3": soyasaponin II/Bc; Be. "SS 4": soyasaponin A1/Ab; Ac, [a, b, c]: means with the same letter in the same row are not significantly different at ($p > 0.05$).

et al., 2013). With regard to chemical structure, aglycones are heat stable and do not completely decompose during domestic cooking. Although no obvious breakdown compounds were detected in the saponin rich fractions (fr II, III), the non-polar aglycones might be retained by the apolar C18-adsorbent and eluted in fraction V (MeOH), subsequently. Exemplarily, aglycones from fb-BBBs 1–4 (uncooked, microwaved, fried, fried/microwaved) were analyzed by HPTLC–ESI-MS. It turned out that fraction V exhibited a characteristic band with rf = 0.42 below the glycosidic saponin B (rf = 0.46). Mass spectrometric analysis revealed an outstanding peak (m/z 520.4) most likely being an adduct of soyasapogenol B (MW = 458.7 g/mol), acetonitrile, and ammonia ($[M + ACN + NH_4]^+$). Subsequently, MS/MS analysis of the target compound revealed two fragment ions with m/z 502 and m/z 459. Based on the parent ion (m/z 520), an elimination of one molecule of water took place. Likewise, removing of acetonitrile and ammonia occurred so that finally the aglycone soyasapogenol B (m/z 459, $[M + H]^+$) might be generated. Saponins in faba beans are structurally similar to those from soybeans why same results were obtained for soyasapogenol B in sb-BBBs. According to Kamo, Suzuki, and Sato (2014) the majority of aglycones detected in processed soy products was soyasapogenol B rather than soyasapogenol A. The carbohydrate structure of group A saponins is more complex than that of group B saponins. Therefore, a complete cleavage of the sugar moieties is rather uncommon during ordinary cooking (Kamo et al., 2014). Nevertheless, soyasapogenol E (MW = 456.7 g/mol) may have been formed due to appreciable processing, especially frying and microwaving, as well as applied extraction conditions according to the pinacol rearrangement of soyasapogenol A (Rupasinghe et al., 2003).

Loss of DDMP saponin during the preparation of the BBBs is attributed to the high reactivity of the maltol moiety. Heng, Vincken, Hoppe, et al. (2006) indicated that the maltol moiety of DDMP saponin was easily released by temperatures exceeding 30 °C, slight acidity, and strong polar solvents such as water, all being more or less the typical conditions for making BBBs. Degradation of DDMP saponin has been proposed to follow the subsequent pathways. Hydrolysis in media with a high dielectric constant such as water containing starch favors generation of an ionized intermediate that undergoes molecular rearrangement with subsequent release of maltol and saponin B. The conversion rate of DDMP saponin in formulated BBBs was also correlated to the protein source. In the majority of BBBs treated, saponin reduction was lower in soy bean followed by faba beans and markedly declined in chickpeas. Hence, retaining more saponins may depend on further components. For instance, the several ingredients in the sb-BBBs and the fb-BBBs may have stabilized the acetal linkage. Therefore, formation of the intermediate for subsequent DDMP hydrolysis might have been minimized in the differently cooked sb- and fb-BBBs. In addition, the dielectric constant for soy bean oil is much lower than that of common food starches (Cannon & Honary, 1999; Miller, Gordon, & Davis, 1991). As there was a slight increase in the formation of type B soyasaponins associated with the conversion of DDMP, degradation appeared to be in accordance with Heng, Vincken, Hoppe, et al. (2006) and Reim and Rohn (2015). In contrast, Serventi et al. (2013) suggested that degradation of DDMP saponin might occur by a process, namely a reduction of the ketone group that is characteristic for DDMP saponin. They also observed low recovery of DDMP saponin in soy/chickpea breads.

The absolute recovery of further group B ("SS 3") and A ("SS 4") soyasaponins in sb-BBB was reduced compared to the uncooked sb-samples. The average loss ranged between 26–31% and 32–40% for type B and A soyasaponins, respectively. The percentile distribution of type B and A soyasaponins in the processed sb-BBB did not vary evidently compared to the untreated sb-diet and the defatted soy bean flour (Tables 3 and 4). Generally, in sb-BBBs detected soyasaponins A (A1/Ab or Ac) and B (II/Bc or Be) seemed to be relatively stable under the applied cooking conditions as already described by Serventi et al. (2013).

4. Conclusion

Common domestic cooking of different ready-to-use and ready-to-eat BBBs including microwaving, frying, microwaving and frying, steaming, and baking were applied and the processed BBBs were screened for saponin profile and quantitative composition. The content of saponins in BBBs significantly altered depending on the legume source, the different cooking methods and the diet matrix as well. HPTLC analysis seems to be an efficient determination technique assuring a valid estimation of the impact of processing on saponin stability. Notably, in the context of developing innovative functional food with considering human nutrient requirements, formulations as well as processing techniques have to be optimized. Particularly, time-saving but also gentle cooking treatments, not exceeding temperature might release and/or retain the most nutritive value. Hence, the bioavailability of saponins is still influenced by many factors. Therefore, specific studies on saponin consumption and bioavailability are needed.

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