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ARTICLE

Quantification of Soyasaponins I and βg in Italian Lentil Seeds by Solid-Phase Extraction (SPE) and High-Performance Liquid Chromatography−Mass Spectrometry (HPLC-MS)

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Lentil saponins are triterpene glycosides, mainly soyasaponins I and βg (also known as VI), with multiple health-promoting properties. This paper reports the isolation of soyasaponins I and βg from soybeans as analytical standards and the development of a new analytical procedure for quantification of their content in various cultivars of Italian lentils, by SPE-HPLC-MS. Soyasaponins I and βg were isolated from soybeans at a purity of >90% and characterized by MS/MS (ion trap) experiments. The determination of soyasaponins in lentils was performed by extraction, SPE purification, and HPLC-MS (single quadrupole) analysis; results were confirmed by MALDI-TOF experiments. Calibration curves for soyasaponin I and βg showed correlation coefficients of 0.998 and 0.997, respectively. LOD and LOQ values were 0.02 and 0.2 mg kg^{-1} for soyasaponins I and 0.1 and 1 mg kg^{-1} for soyasaponin βg. Recoveries calculated at a 100 mg kg^{-1} fortification level ranged from 85 to 97%, with n = 10 and RSDs of <12%. In the 32 lentil samples, contents of soyasaponin I ranged from 28 to 407 mg kg^{-1}, whereas that of soyasaponin βg ranged from 110 to 1242 mg kg^{-1}.

KEYWORDS: Legumes; soybeans; lentils; soyasaponins; HPLC-MS

INTRODUCTION

The Mediterranean diet is characterized by low animal fat intake, moderate to high fish intake, high olive oil consumption, moderate to high cereal intake, moderate wine consumption, and high intakes of legumes, vegetables, and fruit (*1*). Legumes are the edible seeds of some leguminous plants including lentils (*Lens culinaris* L.), bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.), and chickpea (*Cicer arietinum* L.). It has been reported that inclusion of legumes in the daily diet has many beneficial effects in controlling and preventing various chronic metabolic diseases, such as diabetes mellitus and coronary heart disease (*2*). Legumes, eaten after cooking, are considered to be an excellent source of vegetable proteins and are also rich in starch, dietary fiber, minerals, and vitamins (*3*). Legumes are a primary dietary source of food saponins (triterpenoidal or steroidal glycosides), which are naturally occurring in plants. They are bioactive compounds that have been demonstrated to possess multiple health-promoting properties, such as lowering of cholesterol level, anticarcinogenic and antihepatotoxic properties, and antireplicative effects against HIV (*4*). In particular, recent research works have shown that soybean saponins induce apoptosis and macroautophagy in cultured cancer cells (*5*), prevent hypercholesterolemia and aortic atherosclerosis in rats (*6*), and show antimutagenic activity in mammalian cells (*7*).

Among legumes, soybean contains the highest level of soyasaponins (6500 mg kg^{-1}), whereas in haricot and kidney beans the soyasaponin contents are 4100 and 3500 mg kg^{-1}, respectively (*8*). Lentils and peas show comparable levels of soyasaponins (about 1100 mg kg^{-1}) (*9*), which is higher than in lupins (from 379 to 740 mg kg^{-1}) (*10*).

Soyasaponins are triterpenoidal glycosides structurally divided into two groups, called "A" and "B" (*11*). Group A represents the bidesmosidic saponins, having two glycosylation sites on the aglycone moiety (soyasapogenol A), whereas group B includes the monodesmosidic saponins, having a single glycosylation site on two different aglycons (soyasapogenols B and E) (*12*). Lentils mainly contain soyasaponin I (soyasaponin βb) and soyasaponin βg (also called soyasaponin VI), both belonging to the group B soyasaponins (**Figure 1**) (*3*). Soyasaponin βg contains the DDMP group (5-hydroxy-6-methyl-2*H*-pyran-4(3*H*)-one) at the C-22 position of soyasaponin I and may be the natural precursor of soyasaponin I. Because of the DDMP moiety (*13*), soyasaponin βg displays an interesting efficacy as a scavenger of reactive oxygen species (ROS). A recent study indicates that dietary soyasaponins can be metabolized by human gut microflora and that the sugar moieties of soyasaponins seem to be hydrolyzed sequentially to yield smaller and more hydrophobic metabolites (*14*).

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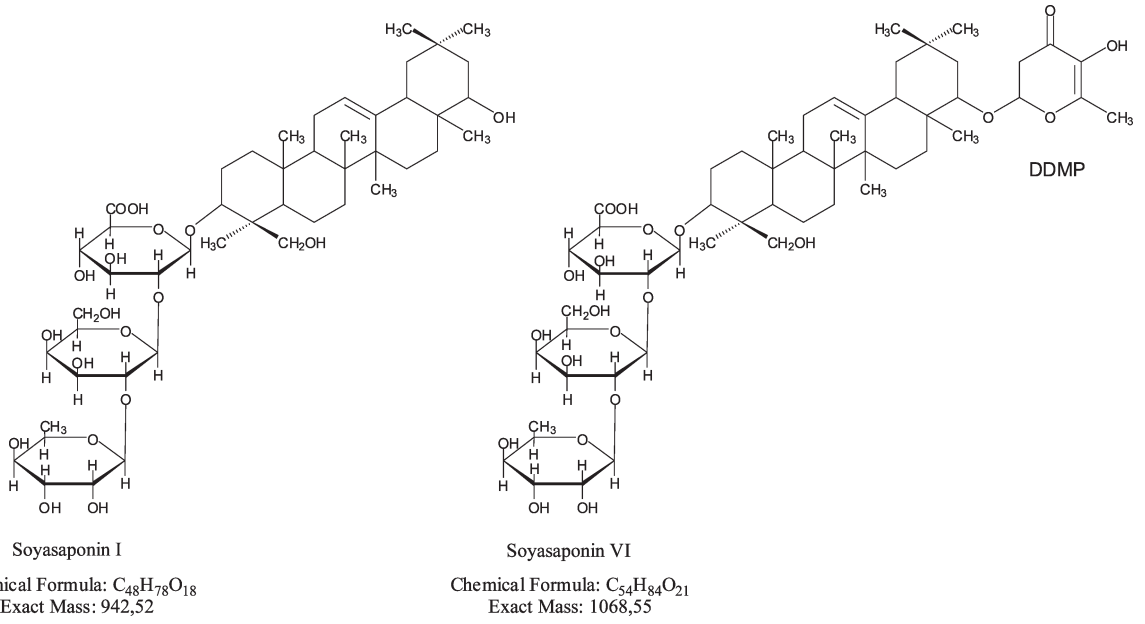

Figure 1. Chemical structures of soyasaponins I and βg.

The accurate quantification of soyasaponins in vegetable matrices is tied to the difficulty of isolating authentic standards, especially the thermolabile DDMP-containing soyasaponins, such as soyasaponin βg, and of detecting of triterpene saponins, which, in the absence of DDMP moiety, do not contain relevant chromophore groups absorbing radiation in the near-UV field (*15*, *16*).

To promote lentils as a "healthy food" and a typical component of the Mediterranean diet, it is important to define the presence not only of the most abundant constituents, such as fibers and proteins, but also of the bioactive compounds, such as soyasaponins, that are an important added value to the already known dietary properties of this legume. Analytical methods used for the quantification of soyasaponin I and βg content in lentils showing efficacy and robustness are needed. Only a few papers have described analytical methodologies to evaluate soyasaponins in lentils. The detection method proposed by Ruiz et al. (*8*), based on Soxhlet extraction and FAB-MS analysis, showed a total soyasaponin content in lentils ranging from 654 to 1269 mg kg^{-1}. In addition, the same authors demonstrated the transformation of soyasaponin βg in soyasaponin I during the common soaking and cooking process of lentils by aqueous ethanol extraction and HPLC-UV analysis (*3*).

The aim of the present work was the isolation of soyasaponins I and βg as pure standards from soybeans by a semipreparative HPLC-DAD technique and the development of a new analytical procedure for the quantification of soyasaponin I and βg content in various cultivars of Italian lentils, using an SPE-HPLC-MS system. Mass spectrometry was used both for identification and structure confirmation of the extracted saponins from soybeans (tandem mass spectrometry) and for quantitative determination of saponins in lentils.

MATERIALS AND METHODS

Materials and Standards. Pure standard of soyasaponin I, used as reference compound, was purchased from the research group of Prof. W. Oleszek (Institute of Soil Science and Plant Cultivate, State Research Institute, Pulawy, Poland). Individual stock solutions were prepared by dissolving 5 mg of soyasaponin I in 5 mL of methanol and storing this solution in glass-stoppered bottles at 4 °C. Standard working solutions, at various concentrations, were prepared when needed by appropriate dilution with methanol of stock solution aliquots.

HPLC-grade methanol, ethanol, and acetonitrile were purchased from Sigma-Aldrich (Milano, Italy), HPLC-grade acetic acid 99−100% was from J. T. Baker B.V. (Deventer, Holland). Deionized water (> 8 MΩ cm resistivity) was obtained from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA). All solvents and solutions were filtered through a 0.45 μm PTFE filter from Supelco (Bellefonte, PA) before use. Cartridges Strata C_{18}-E SPE Tube (6 mL, 1 g) were purchased from Phenomenex (Bologna, Italy). Octadecyl-functionalized silica gel for flash chromatography was supplied by Sigma-Aldrich (Steinheim, Germany).

Sample Collection. Soybeans were bought in a local market of the Camerino community. The 32 samples of lentils, showing various testa color and size (red, black, brown, and green; giant and mignon), were either bought from local markets of the Marche region or provided from Italian producers localized in central and southern Italy.

Isolation of Soyasaponins I and βg from Soybeans. The method used for isolation of soyasaponins I and βg from soybean was modified from that reported by Hu et al. (*4*). One hundred grams of finely ground soybeans was extracted, for 3 h under magnetic stirring, with 960 mL of 70% aqueous ethanol at room temperature. The mixture was then filtered under vacuum and the solution concentrated to 100 mL with a rotary evaporator (Büchi R200, Labortechnik, Flawil, Switzerland) at $T < 30$ °C under reduced pressure (60 mbar). The extract was purified by flash chromatography using an octadecyl-functionalized silica gel as stationary phase and acetonitrile/water as eluting mixture using a gradient from 30 to 100% acetonitrile. The obtained fractions were injected in HPLC-MS to control the presence of soyasaponins I and βg, and the appropriate fractions were collected and evaporated under vacuum up to 10 mL of residual volume. Purification of soyasaponins I and βg was performed in a semipreparative HPLC system with a photodiode array detector using a Symmetry Prep C18 column in reverse phase. The eluted fractions containing the individual soyasaponins were collected, the acetonitrile esd evaporated, and then the aqueous residue was freeze-dried to obtain dry, pure soyasaponins. Soyasaponin identification was obtained by known HPLC retention times, UV spectra, and electrospray ionized (ESI) mass spectra (*4*). Important for the soyasaponin βg was the maximal absorbance at 292 nm, which is specific for the DDMP-conjugated system (*17*). Structure characterization of isolated soyasaponins was performed using an ion trap mass spectrometer, performing MS^n experiments. The chemical purity of both extracted soyasaponins was evaluated by injecting a methanolic solution of samples in a HPLC-DAD, in reverse phase.

Lentil Sample Preparation. One gram of finely ground lentil seeds was extracted, for 3 h under magnetic stirring, with 10 mL of 70% aqueous ethanol at room temperature. The mixture was then filtered under vacuum, and the solution was evaporated to 0.5 mL with a rotary

evaporator at $T < 30$ °C under reduced pressure (60 mbar). The aqueous residue was purified on a Strata C_{18}-E cartridge (6 mL, 1 g). The cartridge was activated with 2 × 2 mL of methanol, conditioned with 2 × 2 mL of water, and then the aqueous residue was loaded onto the cartridge at a flow rate of < 0.5 mL min^{-1}; the cartridge was then washed with 2 × 2 mL of water and thoroughly dried, and then elution was performed using 150 mL of methanol. The eluate was directly injected in HPLC-MS without further concentration.

Recovery studies were performed by spiking the ground lentil seeds with a standard mixture of soyasaponins to obtain a final concentration of 100 mg kg^{-1}. In detail, the aqueous residue obtained after evaporation of extracting solution was loaded onto a C_{18} SPE cartridge, and the volume of methanol used for the elution of soyasaponins was optimized. To define the volume of methanol needed for complete removal of soyasaponins from the stationary phase of the cartridge, we used 30, 50, 100, 120, and 150 mL of solvent, controlling after each elution by HPLC-MS the eventual saponin residue after a cartridge washing with 2 × 2 mL of methanol. The volume needed to completely elute both saponins from the cartridge was 150 mL.

LC-DAD, LC-MS, and LC-MS/MS Analyses. *Semipreparative*. Purification of individual soyasaponins was performed in a semipreparative HPLC system Waters 600 Controller equipped with a Waters 2996 photodiode array detector, using a Symmetry Prep C_{18} column (300 × 19 mm i.d., 7 μm) by Waters (Milford, MA). The mobile phase was a mixture of (A) water with 0.25% acetic acid (v/v) and (B) methanol with 0.25% acetic acid (v/v), flowing at 5 mL/min in isocratic conditions: 17% A, 83% B. The selected wavelengths were 206 nm for soyasaponin I and 292 nm for soyasaponin VI. The volume of a single injection was 500 μL.

Analytical. The separation of soyasaponins was achieved on an analytical column Gemini C_{18} (150 × 4.6 mm i.d., 5 μm) from Phenomenex (Chesire, U.K.). The mobile phase for LC-DAD and LC-ESI-MS (single quadrupole) analyses was a mixture of (A) water with 0.25% acetic acid (v/v) and (B) methanol with 0.25% acetic acid (v/v), flowing at 1 mL min^{-1} in isocratic conditions: 20% A, 80% B. The mobile phase for FIA/ESI-MS/MS (quadrupole ion trap) analysis was (A) water with 0.25% acetic acid (v/v) and (B) methanol with 0.25% acetic acid (v/v), in isocratic conditions: 20% A, 80% B.

LC-DAD and LC-MS studies were performed using a Hewlett-Packard (Palo Alto, CA) HP-1090 series II, made of an autosampler and a binary solvent pump, with a diode array detector (DAD) and a mass spectrometer detector (MSD) equipped with an ESI interface in negative ionization (NI) mode. LC-DAD analyses were performed by monitoring two different wavelengths: 206 nm for soyasaponin I and 292 nm for soyasaponin VI.

Optimization of the LC-MS conditions was carried out by varying them in flow injection analysis (FIA) of the analytes (20 μL of a 100 μg mL^{-1} individual standard solutions). The optimized parameters of the ESI interface were as follows: vaporizer temperature, 325 °C; nebulizer gas (nitrogen) pressure, 60 psi; drying gas (nitrogen) flow rate, 13 mL min^{-1}; temperature, 350 °C; capillary voltage, 3500 V. Time-scheduled conditions for monitoring soyasaponins are reported in **Table 1**.

LC-MS/MS studies were performed using an Agilent 1100 series (Santa Clara, CA) and MSD Trap SL equipped with an ESI source operating in negative ionization mode. The mass spectrometer was tuned for each compound, optimizing ionization source parameters, voltage of the lenses, and trap conditions in the ExpertTune mode of the Daltonic Esquire Control software, while infusing a standard solution (100 μg mL^{-1}), via a syringe pump at a flow rate of 5 μL min^{-1}, which was mixed with the mobile phase at 0.5 mL min^{-1} by means of a T piece. Operating ESI conditions were the same as mentioned before for the single quadrupole.

The mass spectrometer was run in full scan and MRM modes. Negative ions were detected using the standard scan at normal resolution (scan speed 10300 m/z/s; peak with 0.6 fwhm/m/z). The trap parameters were set in ion charge control (ICC) using rolling averaging set at 2 with a target of 20000 and maximum accumulation time of 50 ms at m/z range from 50 to 1500 u. Fragment m/z values, fragmentation, and time conditions are reported in **Table 1**.

MALDI-TOF Analysis. MALDI-MS measurements were performed using an Ultraflex II MALDI time of flight (TOF) instrument (Bruker Daltonics, Bremen, Germany), operating in reflectron negative ion mode. Ions were formed by a pulsed UV laser beam ($\lambda = 337$ nm). The instrumental conditions were as follows: IS1 = 20.00 kV; IS2 = 17.32 kV; reflectron potential = 21.0 kV; delay time = 0 ns. The matrix employed was dihydroxybenzoic acid (DHB) [saturated solution in H_2O/acetonitrile (50:50; v/v) containing 0.1% trifluoroacetic acid]. Five milligrams of the soyasaponin lyophilized ethanolic extract from lentil samples under study (1−32) were dissolved in 1 mL of H_2O. Five microliters of the obtained soyasaponin solutions and 5 μL of matrix solution were mixed. One microliter of the resulting mixture was deposited on the stainless steel sample holder and allowed to dry before introduction into the mass spectrometer. External mass calibration was done using the peptide calibration standard (Bruker Daltonics), based on the monoisotopic values of $[M - H]^-$ of angiotensin II, angiotensin I, substance P, bombesin, ACTH clip (1−17), ACTH clip (18−39), and somatostatin 28 at "mass/charge" (m/z) 1044.5272, 1294.6702, 1345.7208, 1617.8077, 2091.0716, 2463.1837, and 3145.4564, respectively.

Table 1. Conditions and Transitions Used in Mass Spectrometry Experiments[a]

LC-MS

compound	SIM ion (m/z)	fragmentor (eV)	time window (min)
soyasaponin I	941 $[M - H]^-$	200	0−12
soyasaponin βg	1067 $[M - H]^-$	200	12−20

Ion Trap MS^n

compound	MS experiments	mass	cutoff	amplitude
soyasaponin I	MS/MS	941	254	1.20
	MS^3	923	249	1.20
	MS^4	879	238	1.20
	MS^5	733		
soyasaponin βg	MS/MS	1067	288	0.90
	MS^3	1049	284	0.80
	MS^4	879	238	1.00
	MS^5	733		

[a] In MS^n experiments (quadrupole ion trap) the width parameter was in all cases equal to 4.0.

RESULTS AND DISCUSSION

Isolation and Characterization of Soyasaponins I and βg from Soybeans. Soyasaponins I and βg for standard use were isolated from soybeans because of their high content of these saponins. The yields of soyasaponins I and βg, calculated as the amount of individual soyasaponins isolated from 100 g of starting material, were 2 and 3.2 mg, respectively. In both cases the percentage of purity was >90%, as revealed by HPLC-DAD analysis.

During the vacuum evaporation of extraction solvent (70% aqueous ethanol), the temperature was set at < 30 °C because of the high thermolability and easy degradation of the DDMP conjugated group present in soyasaponin βg (*5*). After the purification step with a chromatographic column, the eluted fractions were collected on the basis of the presence of soyasaponins I and βg and determined by HPLC-MS analysis. SIM chromatograms were obtained by monitoring the principal ions $[M - H]^-$ at m/z 941 and at m/z 1067 for soyasaponins I and βg, respectively (**Table 1**).

A further purification of both saponins was carried out using a semipreparative HPLC system equipped with a DAD, using 206 and 292 nm wavelengths for no-DDMP (I) and DDMP−soyasaponin (βg), respectively. The chromatograms obtained at 206 nm present a weak signal for soyasaponin I, because of the cutoff of acetic acid (230 nm) used in the mobile phase.

For the structural characterization, the purified soyasaponins I and βg were analyzed in mass spectrometry in fluid injections analysis (FIA) at a concentration of 200 mg kg^{-1} using both a

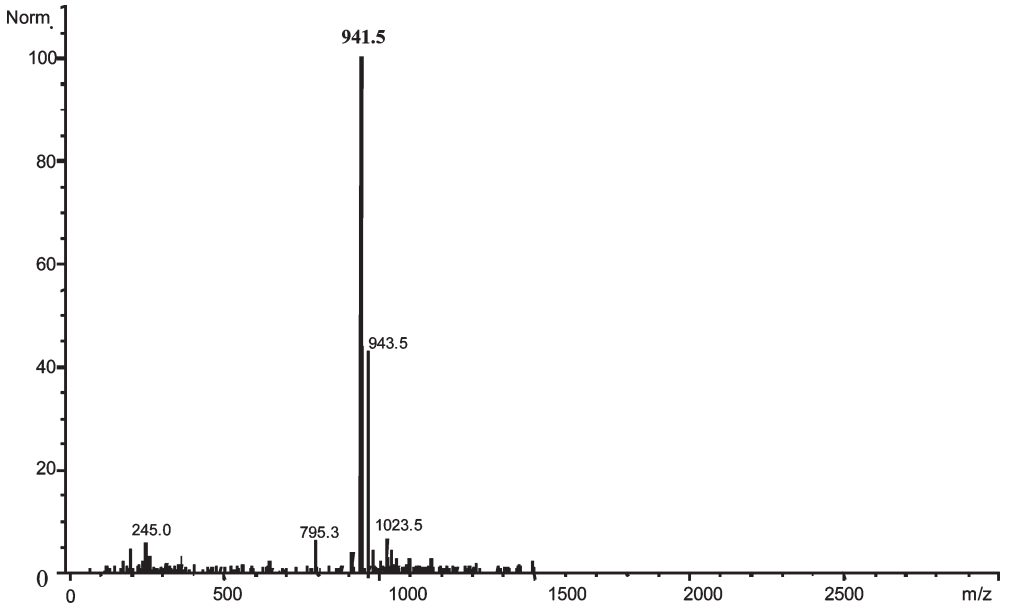

Figure 2. ESI full scan mass spectrum in negative ionization mode of soyasaponin I (200 ng injected) using single quadrupole.

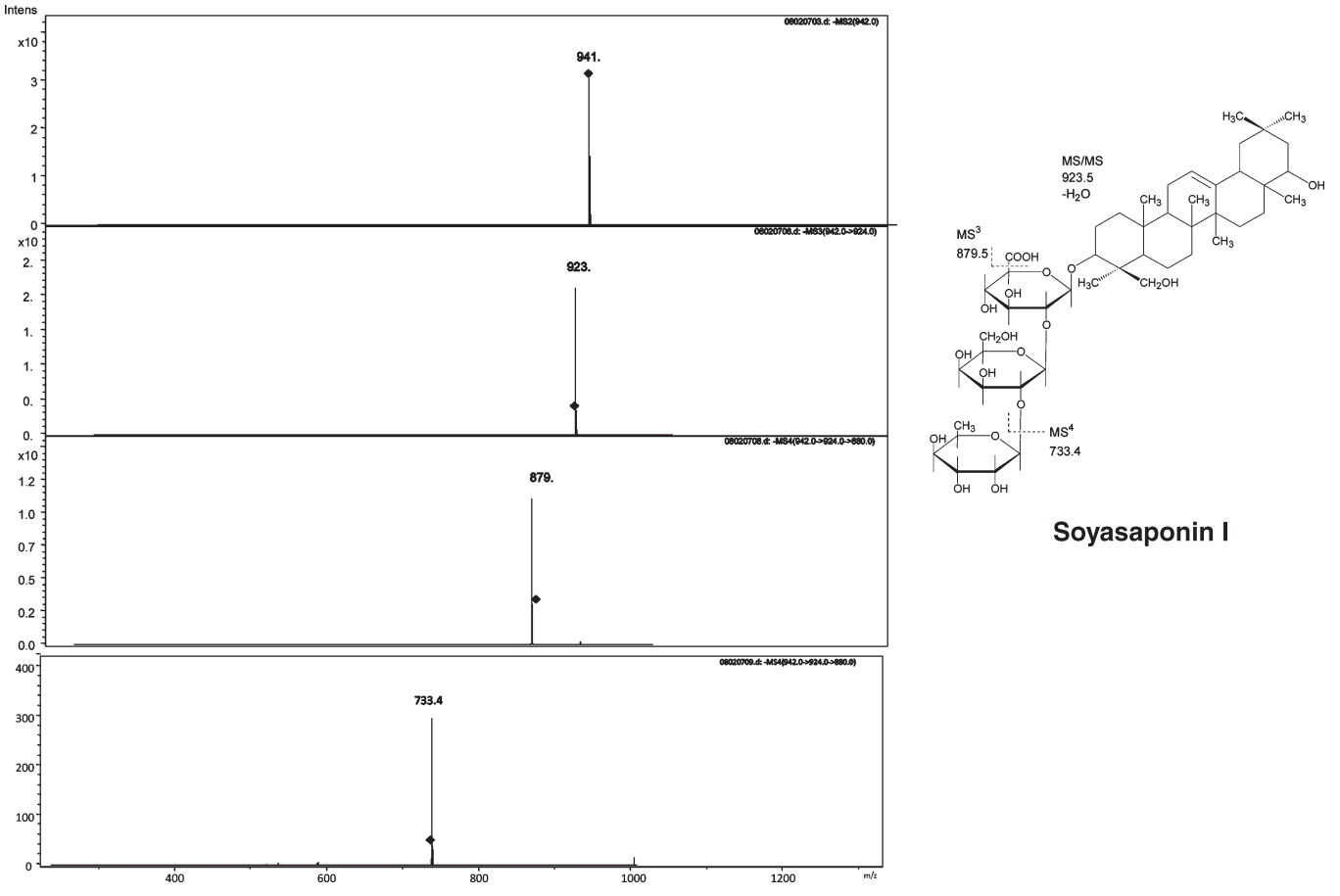

Figure 3. Precursor and product (MS^2, MS^3, and MS^4) ion scan mass spectra of isolated soyasaponin I obtained in the ion trap (amount injected = 200 ng).

single quadrupole and an ion trap as analyzers, equipped with an ESI source operating in negative mode. As an example, **Figure 2** illustrates the full scan ESI^- mass spectrum of soyasaponin I, in which the most abundant fragment was the ion $[M - H]^-$ at m/z 941.5.

The soyasaponin samples were analyzed by MS/MS spectra by injecting solutions of pure saponins in the ion trap mass spectrometer. **Figures 3** and **4** show the ESI^- soyasaponin I and βg precursor ions and product ion mass spectra (MS^2, MS^3, and MS^4) obtained by quadrupole ion trap. Both soyasaponins I and βg produce as precursor ions the $[M - H]^-$ at m/z 941.5 and 1067.5, respectively, according to the literature (*18*, *19*). The MS/MS spectra of both $[M - H]^-$ show intense signals at m/z 923.5, for soyasaponin I, and 1049.5, for soyasaponin βg, which

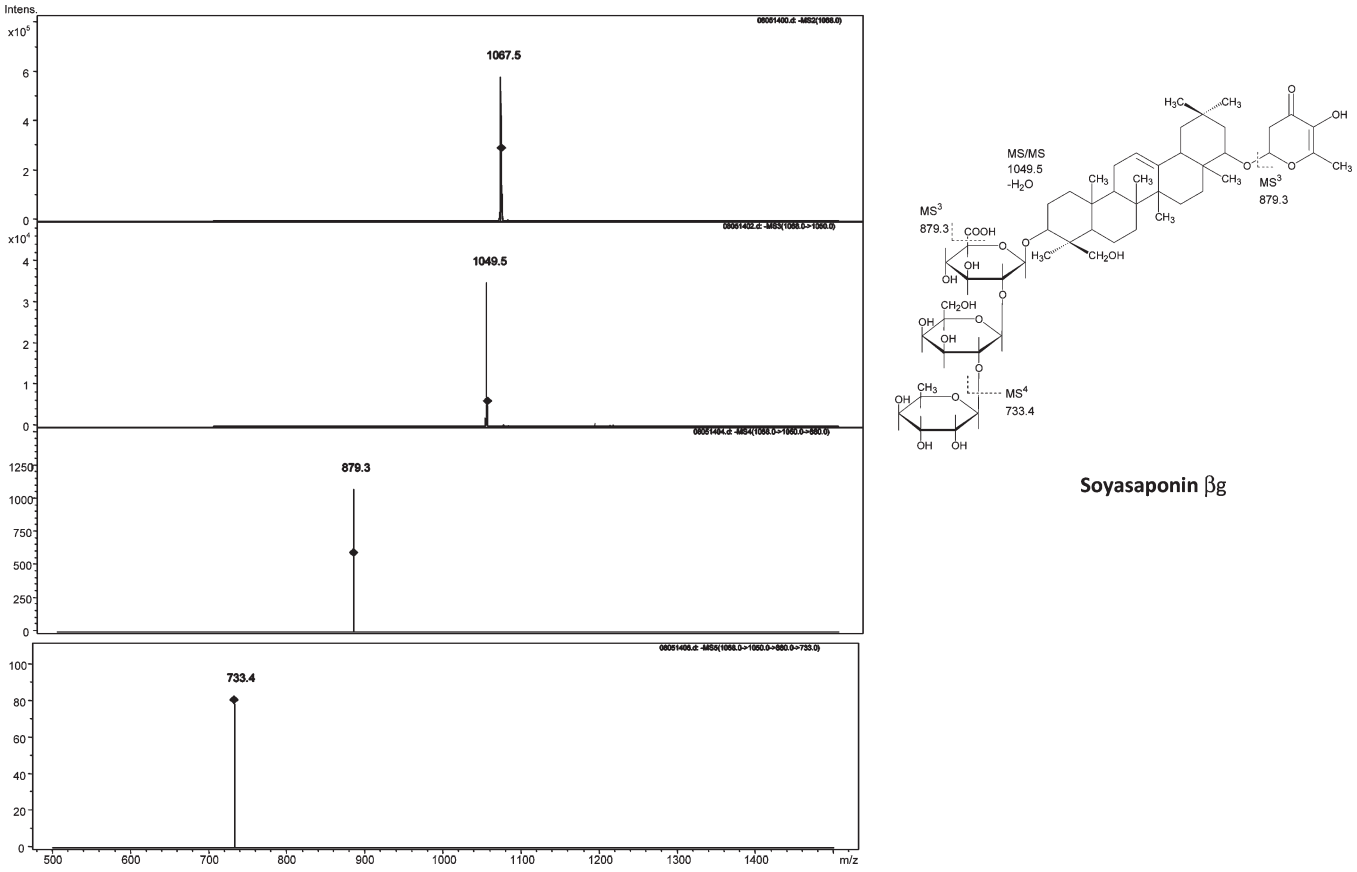

Figure 4. Precursor and product (MS^2, MS^3, and MS^4) ions scan mass spectra of isolated soyasaponin βg obtained in the ion trap (amount injected = 200 ng).

correspond, according to the proposed fragmentation, to the ions produced by the loss of a molecule of water (m/z 18). For soyasaponin I the following fragmentation produced an MS^3 spectrum characterized by a signal at m/z 879.5, generated by loss of a carbon dioxide molecule (m/z 44). Similarly, the MS^3 spectrum of soyasaponin βg gave a signal at m/z 879.3 generated by loss of both carbon dioxide (m/z 44) and the DDMP group (5-hydroxy-6-methyl-2*H*-pyran-4(3*H*)-one) (m/z 128). The weak most abundant signal present in the MS^4 spectra of both saponins was that at m/z 733.4, corresponding to the loss of a rhamnosium (m/z 147) glycosidic bond (*20*).

The purity of both soyasaponins was evaluated in HPLC-DAD, in reverse phase, by injecting methanolic solutions of samples at a concentration of 100 mg kg^{-1}. From the obtained chromatograms, monitoring soyasaponin I and βg at 205 and 292 nm, respectively, a percentage of purity > 90% was calculated for both saponins.

Quantifications of Soyasaponins I and βg in Italian Lentil Seeds. For quantification of soyasaponins I and βg in lentils, we developed a new, rapid, and specific analytical procedure based on the extraction of saponins from matrix using an ethanolic solution, purification, and concentration step by C_{18} SPE cartridge in reverse phase and quantification by injection of the sample in HPLC-MS.

In **Figure 5** is reported an HPLC-MS (SIM mode) chromatogram of the methanolic eluate of a lentil sample. The high chromatographic resolution and the absence of the interferences in the chromatogram indicate the high specificity of the proposed method.

The method was applied to the analysis of 32 samples of lentil seeds, from central and southern Italy. The analytical data obtained in LC-MS from all analyzed samples are reported in **Table 2**, where the number of the samples, origin, weight of 50 seeds, testa seed color, and content of soyasaponins I and βg and total content expressed in milligrams per kilogram are shown. The quantification of soyasaponins was obtained by comparing the peak areas of compounds identified in the extracts of lentil samples with those of pure standards extracted from soybeans. For most samples, area of cultivation was exactly known, whereas for some of them (13, 19, 20, 22, 23, 27, 28, 30, and 31), all bought in local markets, only packing location was available.

Sample 9, lentil from Castelluccio di Norcia, displayed the highest content of total soyasaponins (1600 mg kg^{-1}) and of soyasaponin βg (1240 mg kg^{-1}); however, also samples 1, 2, 4, 14, and 24 showed total amounts higher than 1000 mg kg^{-1} and corresponding high levels of soyasaponin βg. Soyasaponin I showed its highest quantity (410 mg kg^{-1}) in lentils from Ustica (sample 1) and the lowest (28 mg kg^{-1}) in Giuseppe Vesuviano sample (23). In all cases the content of soyasaponin βg was superior to that of soyasaponin I. In particular, in samples 7, 10, 19, 23, 25, and 28, the content was > 10-fold higher, whereas the minimal ratios (1.6−2.8) were shown by samples 1, 8, 12, 13, 16, 18, 20, and 29. The lentils with highest content of soyasaponins were those with mainly small size seeds (meanly 1.20 g/50 seeds), whereas lentils with higher size seeds, ranging from 1.55 to 3.65 g/50 seeds (samples 17, 18, 20−23, 26−28, 31, and 32), displayed a mean/low content of total saponins (< 1000 mg kg^{-1}). As an exception, sample 29, represented by hulled Colfiorito red lentil, showed the lowest content of total soyasaponins (180 mg kg^{-1}), although constituted of small seeds. This seems to indicate that an important amount of soyasaponins in lentils is located in the seed hull.

With regard to the relationship between soyasaponin quantity and brown, green, red, or black seed testa color, the data do not

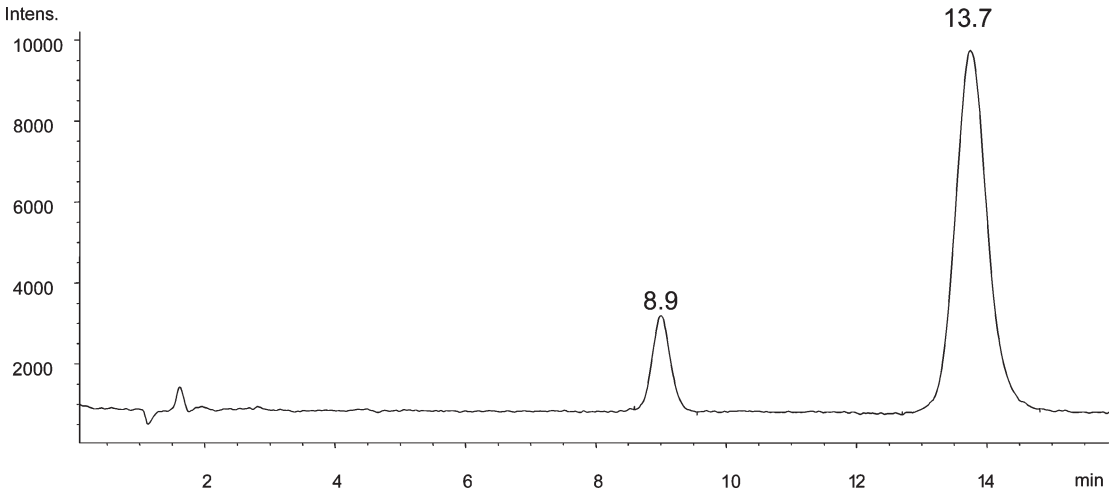

Figure 5. HPLC-ESI-MS chromatogram of a final methanolic eluate of a lentil sample. Soyasaponin I, t_R = 8.9 min. Soyasaponin βg, t_R = 13.7 min.

Table 2. Soyasaponin Content in Samples of Italian Lentils (*Lens culinaris*)[a]

no.	origin[b]	50-seed wt (g)	seed testa color	soyasaponin I (mg kg^{-1})	soyasaponin βg (mg kg^{-1})	total (mg kg^{-1})
1	Ustica	1.16	brown	407	680	1087
2	Forcatura di Foligno	1.16	brown	124	1055	1179
3	Forcatura di Foligno	1.18	brown	94	650	744
4	Selvapiana di Montecavallo	1.18	brown	170	1123	1293
5	Castelluccio di Norcia	1.19	brown	116	702	818
6	Foligno 2006	1.25	brown	109	700	809
7	Foligno 2007	1.25	brown	52	787	839
8	S. Maria in Selva	1.26	brown	182	344	526
9	Castelluccio di Norcia	1.26	brown	353	1242	1595
10	Colfiorito	1.27	brown	42	597	639
11	Castelluccio di Norcia	1.28	brown	137	818	955
12	Morrovalle	1.33	brown	335	546	871
13	Colfiorito	1.33	brown	194	551	745
14	Castelluccio di Norcia	1.33	brown	136	1057	1193
15	Pievetorina	1.35	brown	158	743	901
16	Recanati	1.49	brown	209	442	651
17	Monte Castello	1.55	brown	85	384	469
18	Ventotene	1.56	brown	94	263	357
19	Colfiorito	1.61	brown	65	690	755
20	Molvena	1.70	brown	97	213	310
21	Onano	2.46	brown	68	298	366
22	Colfiorito	2.77	brown	36	322	358
23	Giuseppe Vesuviano	2.90	brown	28	299	327
24	Altamura	1.16	green	150	1017	1167
25	Colfiorito	1.18	green	50	790	840
26	Colfiorito	1.59	green	83	339	422
27	Molvena	2.60	green	69	531	600
28	Molvena	3.65	green	61	708	769
29	Colfiorito[c]	0.77	red	70	110	180
30	Molvena	1.60	red	101	714	815
31	Molvena	1.84	red	94	337	431
32	Enna	2.44	black	96	387	483

[a] Each sample was analyzed in triplicate. [b] For samples 13, 19, 20, 22, 23, 27, 28, 30, and 31, origin refers to packing factory location; for all others, it indicates the area where lentils were cultivated. [c] Lentil 29 is hulled.

show relevant correlations. Fundamentally, the lentils displaying the highest saponin contents are those having a brown seed testa color related to a small seed size, with the exception of the green seeds of Altamura lentil (24). However, the data indicated that several other samples of brown lentils contain low amounts of soyasaponins as well the lentils with green, red, and black seeds. This can signify that many other parameters may influence the content of soyasaponins in lentils, such as the different physical−chemical characteristics of the soil or tillage and the climatic conditions that are related to the territory geography. In this connection, an evaluation can be attempted of the data obtained from lentil samples from very different geographical tillage: insular, low altitude, and uplands. The lentils cultivated in small islands, at sea level, such as Ventotene and Ustica (samples 18 and 1), or in proximity of sea lands at low altitude, such as Recanati (altitude 293 m) and Morrovalle (altitude 245 m) (samples 16 and 12), showed a content of soyasaponin βg lower than that of lentils cultivated in uplands, such as Forcatura di Foligno (altitude 781 m) and Castelluccio di Norcia (altitude 1452) (samples 2 and 9), ranging from 263 to 680 mg kg^{-1} and from 1055 to 1242 mg kg^{-1},

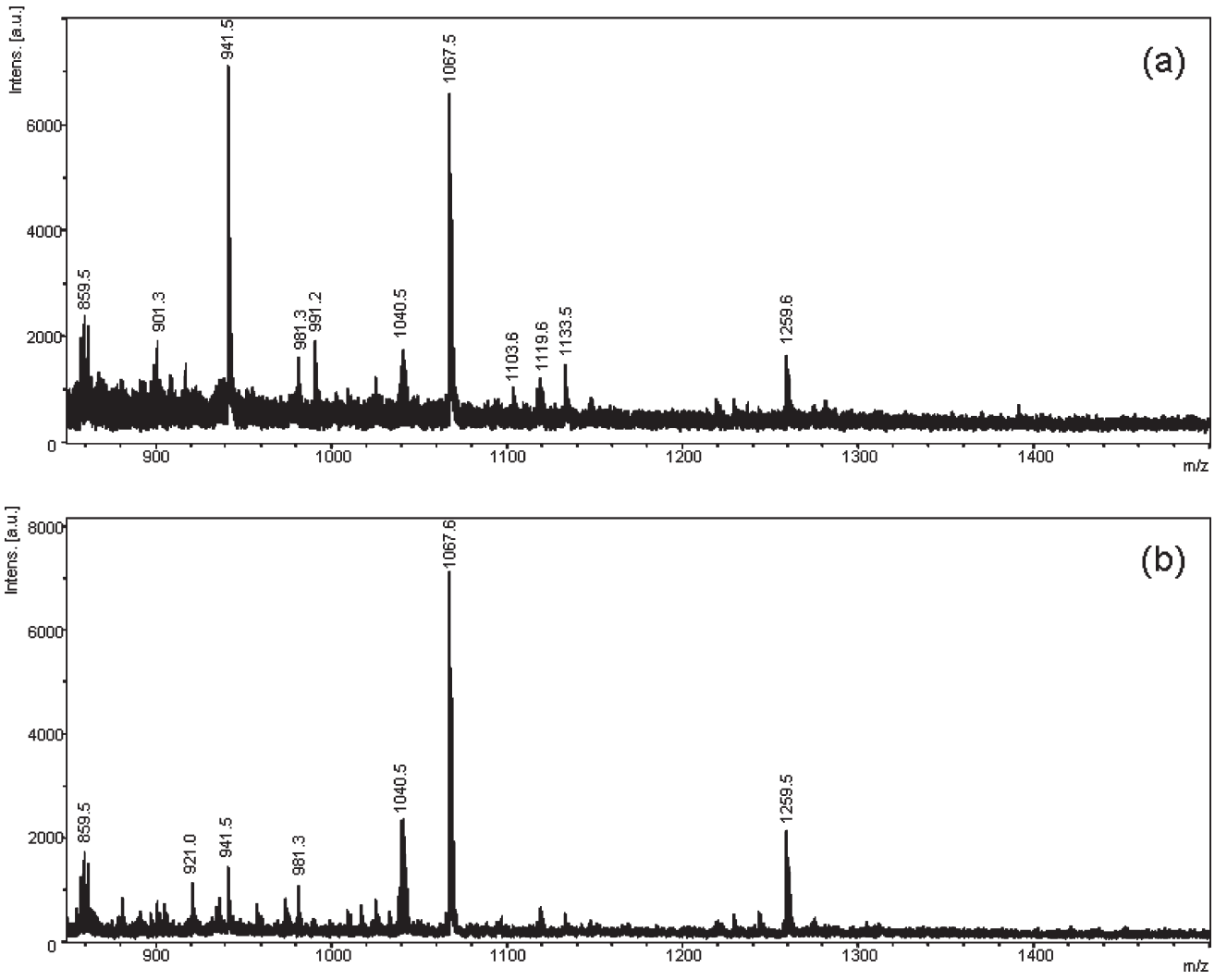

Figure 6. MALDI negative ion spectra of Colfiorito lentil extract (**a**) and Castelluccio di Norcia lentil extract (**b**). Dihydroxybenzoic acid was used as matrix.

respectively. In comparison, also the soyasaponin VI/soyasaponin I ratio was higher for the uplands with respect to the low-altitude- and insular-cultivated lentils.

The data of entries 6 and 7, concerning the saponins quantity in seed samples of the same cultivar (Foligno) cropped on two successive years, 2006 and 2007, respectively, show a minor content of soyasaponin βg and an higher content of soyasaponin I for the 2006 sample with respect to the 2007 one. This could be the consequence of a natural degradation of soyasaponin βg into soyasaponin I through a hydrolytic process of the DDMP group during storage. This transformation process has already been observed during the lentil soaking operation (*3*).

As a confirmatory assay of the LC-MS analytical approach, MALDI-MS data were obtained for each lentil sample considered. As an example, the MALDI negative ion spectra of two lentil extracts, Colfiorito (29) and Castelluccio of Norcia (14), are reported in **Figure 6**, panels **a** and **b**, respectively. In both spectra the peaks at m/z 941.5 and 1067.5, corresponding to soyasaponin I and βg, respectively, were detectable. However, clear differences in the relative abundances of the two soyasaponins are observed. Whereas MALDI analysis of Colfiorito extract (29) yields signal abundance quite similar for the two soysaponins (5923 au for soyasaponins I and 5709 au for soyasaponin βg), in the case of Castelluccio di Norcia extract (14) the signal intensity of soyasaponin βg (7233 au) is 7 times higher than that of soyasaponin I (1068 au), confirming the data already obtained by LC-MS and reported in **Table 2**. An analogous trend (i.e., a good agreement between MALDI and LC-MS abundance data) has been found for all of the samples under study.

Method Validation. Calibration curves of the analyzed soyasaponins were constructed by injecting 20 μL of mix standard solutions at six different concentrations, that is, 1.375, 13.75, 27.5, 55, 110, and 220 mg kg^{-1} in LC-MS technique. Three replicates for each concentration were performed, and the relative standard deviations (RSDs) ranged from 1 to 2% for run-to-run precision and from 8 to 14% for day-to day precision. The calibration curves of the analyzed soyasaponins showed a correlation coefficient equal to 0.998 (soyasaponin I) and 0.997 (soyasaponin VI).

The repeatability of the method, evaluated three times on each kind of lentil sample, was expressed by percent CV, which was $< 19\%$.

The limits of detection (LODs) and the limits of quantification (LOQs) of the studied soyasaponins, expressed in milligrams per kilogram, were calculated by injecting in LC-MS standard solutions of both saponins at various concentrations. LOD values for soyasaponins I and βg were 0.02 and 0.2 mg kg^{-1}, respectively. LOQ values for soyasaponins I and βg were 0.1 and 1 mg kg^{-1}, respectively.

The recovery percentages of soyasaponins I and βg were investigated by spiking with 50 μL of standard soyasaponins mixtures the ground lentils before ethanolic extraction, for a final concentration level of 100 mg kg^{-1}. Mean recoveries of the two

Table 3. Soyasaponin Content in the Same Sample of Lentil Seeds Extracted at Room Temperature and at $T < 0$ °C[a]

cxtraction condition	soyasaponin I (mg kg^{-1})	soyasaponin βg (mg kg^{-1})	soyasaponin total	I/βg ratio	I/total ratio
room temperature	116	702	818	0.1652	0.1418
$T < 0$ °C	55	460	515	0.1196	0.1068

[a] Each result is the mean value of three determinations, with RSD of <8%.

compounds ranged from 85 to 97% with $n = 10$ and RSDs of < 12%.

Stability of DDMP-Conjugated Soyasaponin in Extraction Process. To assess the influence of temperature on the stability of soyasaponin βg during the extraction process, a set of experiments was conducted with the temperature maintained at < 4 °C. In particular, three samples of the same cultivar of lentils were extracted at room temperature described before, whereas three other identical samples were analyzed using the following procedure. The lentil samples were ground in a mortar using a pestle with the addition of liquid nitrogen to obtain a more friable matrix. The extraction process, using 70% aqueous ethanol, was performed in a water bath at 0 °C for 3 h. The heterogeneous mixture was filtered and then freeze-dried for one night; after the addition of water, the purification was performed on an SPE C_{18} cartridge according to the same previously described procedure but maintaining the system at 4 °C. Finally, the eluate was injected in HPLC-MS to quantitate the content of soyasaponins I and βg.

The results are shown in **Table 3**. As expected, extractions performed in conditions of lower energy gave as a result a total level of saponins lower than in normal operating conditions (515 vs 818 mg kg^{-1}). It is interesting to compare, though, the ratio soyasaponin I/soyasaponin βg. In fact, comparison of that ratio in the samples analyzed at $T < 0$ °C with those tested at room temperature by using the classical conditions shows that in the low-temperature-treated samples the content of soyasaponin I was 38% lower than for samples extracted in classical conditions. This result clearly demonstrates the dependence on temperature of the transformation of soyasaponin βg into soyasaponin I, through the hydrolysis of its DDMP group. It also indicates the necessity of low-temperature usage when the specific content of soyasaponin βg and soyasaponin I on lentil samples has to be evaluated.

In conclusion, the present work allows us to achieve two main goals: (a) the isolation from soybeans of high-purity (>90%) samples of soyasaponins I and βg for the use as analytical standards and (b) the development of a new analytical method for the qualitative and quantitative detection of soyasaponins I and βg in 32 samples of Italian lentils. The evaluation of these bioactive compounds is a useful way to get proper knowledge on some healthy properties of these legumes.

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LITERATURE CITED

(1) Lairon, D. Intervention studies on Mediterranean diet and cardiovascular risk. *Mol. Nutr. Food Res.* **2007**, *51*, 1209–1214.

(2) Chung, H. J.; Liu, Q.; Hoover, R.; Warkentin, T. D.; Vandenberg, B. *In vitro* starch digestibility, expected glycemic index, and thermal and pasting properties of flours from pea, lentil and chickpea cultivars. *Food Chem.* **2008**, *111*, 316–321.

(3) Ruiz, R. G.; Price, K. R.; Arthur, A. E.; Rose, M. E.; Rhodes, M. J. C.; Fenwick, R. G. Effect of soaking and cooking on the saponin content and composition of chickpeas (*Cicer arietinum*) and lentils (*Lens culinaris*). *J. Agric. Food Chem.* **1996**, *44*, 1526–1530.

(4) Hu, J.; Lee, S. O.; Hendrich, S.; Murphy, P. A. Quantification of the group B soyasaponins by high-performance liquid chromatography. *J. Agric. Food Chem.* **2002**, *50*, 2587–2594.

(5) Berhow, M. A.; Kong, S. B.; Vermillion, K. E.; Duval, S. M. Complete quantification of group A and group B soyasaponins in soybeans. *J. Agric. Food Chem.* **2006**, *54*, 2035–2044.

(6) Oakenfull, D. G.; Topping, D. L.; Illman, R. J.; Fenwick, D. E. Prevention of dietary hypercholosterolaemia in the rat by soyabean and Quillaja saponins. *Nutr. Rep. Int.* **1984**, *29*, 1039–1046.

(7) Berhow, M. A.; Wagner, E. D.; Vaughn, S. F.; Plewa, M. J. Characterization and antimutagenic activity of soybean saponins. *Mutat. Res.* **2000**, *448*, 11–22.

(8) Ruiz, R. G.; Price, K. R.; Rose, M. E.; Fenwick, G. R. Effect of seed size and testa color on saponin content of Spanish lentil seed. *Food Chem.* **1997**, *58*, 223–226.

(9) Price, K. R.; Curl, C. L.; Fenwick, G. R. The saponin content and sapogenol composition of the seed of 13 varieties of legume. *J. Sci. Food Agric.* **1986**, *37*, 186–191.

(10) Ruiz, R. G.; Price, K. R.; Rose, M. E.; Arthur, A. E.; Petterson, D. S.; Fenwick, G. R. The effect of cultivar and environment on saponin content of Australian sweet lupin seed. *J. Sci. Food Agric.* **1995**, *69*, 347–351.

(11) Dalluge, J. J.; Eliason, E.; Frazer, S. Simultaneous identification of soyasaponins and isoflavones and quantification of soyasaponin Bb in soy products, using liquid chromatography/electrospray ionization−mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 3520–3524.

(12) Gu, L.; Tao, G.; Gu, W.; Prior, R. L. Determination of soyasaponins in soy with LC-MS following structural unification by partial alkaline degradation. *J. Agric. Food Chem.* **2002**, *50*, 6951–6959.

(13) Yoshiki, Y.; Okubo, K. Active oxygen scavenging activity of DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one) saponin in soybean seed. *Biosci., Biotechnol., Biochem* **1995**, *59*, 1556–1557.

(14) Hu, J.; Zheng, Y. L.; Hyde, W.; Hendrich, S.; Murphy, P. A. Human fecal metabolism of soaysaponin I. *J. Agric. Food Chem.* **2004**, *52*, 2689–2696.

(15) Hubert, J.; Berger, M.; Daydè, J. Use of a simplified HPLC-UV analysis for soyasaponin B determination: study of saponin and isoflavone variability in soybean cultivars and soy-based health food products. *J. Agric. Food Chem.* **2005**, *53*, 3923–3930.

(16) Kite, G. C.; Porter, E. A.; Simmonds, M. S. J. Chromatographic behaviour of steroidal saponins studied by high-performance liquid chromatography−mass spectrometry. *J. Chromatogr., A* **2007**, *1148*, 177–183.

(17) Kudou, S.; Tonomura, M.; Tsukamoto, C.; Uchida, T.; Yoshikoshi, M.; Okubo, K. Structural elucidation and physiological properties of genuine soybean saponins. In *Food Phytochemicals for Cancer Prevention I: Fruits and Vegetables*; Huang, M.-T., Osawa, T., Ho, C.-T., Rosen, R. T., Eds.; American Chemical Society: Washington, DC, 1994; pp 340−347.

(18) Jin, M.; Yang, Y.; Su, B.; Ren, Q. Rapid quantification and characterization of soyasaponins by high-performance liquid chromatography coupled with electrospray mass spectrometry. *J. Chromatogr., A* **2006**, *1108*, 31–37.

(19) Determination of soaysaponins βa and βb in human serum by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *J. Chromatogr., B* **2007**, *846*, 169−175.

(20) Lee, M. R.; Chen, C. M.; Hwang, B. H.; Hsu, L. M. Analysis of saponins from black bean by electrospray ionization and fast atom bombardment tandem mass spectrometry. *J. Mass Spectrom.* **1999**, *34*, 804–812.

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