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Profiling and quantification of isoflavones in soymilk from soy protein isolate using extracted ion chromatography and positive ion fragmentation techniques

Daniel O. Otieno [a], Harry Rose [b], Nagendra P. Shah [a,*]

[a] School of Molecular Sciences, Victoria University, Werribee Campus, P.O. Box 14428, Melbourne, Victoria 8001, Australia
[b] Defence Science and Technology Organisation (DSTO), CB Degradation Research, CBRN Defence Centre, Platform Sciences Laboratory, Melbourne, Victoria 3032, Australia

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Abstract

Dietary supplements on soy based foods and beverages are increasingly gaining prominence all over the world. In this study, liquid chromatography coupled with positive electrospray ionisation tandem mass spectrometry (LC-ESI-MS) and diode array detection was used for the quantitation and characterisation of isoflavones in fermented and unfermented soymilk made from soy protein isolate SUPRO 590. *Bifidobacterium animalis* ssp. *lactis* Bb12 was used for the fermentation of soymilk. The isoflavones were found to produce characteristic radical ions as well as molecules of H_2O, CO_2, a sugar unit, and an alcohol through collision-induced fragmentation. Product ion fragments revealed unique fragmentation pathways for each isoflavone compound. Characteristic fragmentation of different isoflavones were unequivocally identified and differentiated. The occurrence of aldehydes such as pentanal, ethanal and methanal was shown to be specifically linked with isoflavone aglycones, daidzein, genistein and glycitein, respectively. Main glycosides such as genistin, daidzin and glycitin as well as the acetyl-, and malonyl forms also showed respective aglycone ions in their spectra fragmentation. Thus positive ion fragmentation was important in the absolute confirmation of isoflavones and to reveal the occurrence of other related compounds such as aldehydes in soymilk.

Keywords: Soy isoflavones; Biotransformation; LC MS/MS; Product ions; Ion spectra; Electrospray ionisation chromatogram (EIC)

1. Introduction

Isoflavones as a class of isoflavonoids are distinct in their structure, sources and activities which have come under increasing scrutiny due to their estrogen-like properties (Kurzer, 2000). They have polyphenolic structure in which the B ring is attached at C-3 position of phenolic C ring instead of the more typical C-2 position as in most flavonoids and are found in large amounts in soybeans among legumes consumed by humans. They have been reported to prevent the oxidation of low-density lipoprotein (LDL), thus reducing atherogenesis (Yamakoshi et al., 2000), to decrease bone reabsorption in a manner similar to the activity of estrogen (Gao & Yamaguchi, 1999), to lower the prevalence of breast and prostate cancers in societies where consumption of soy is common (Watson, Cai, & Jones, 2000), and to reduce the risk of atherosclerosis (de Kleijn, van der Schouw, Wilson, Grobbee, & Jacques, 2002), neuro-degeneration (Kim, Xia, Li, & Gewin, 2000) and osteoporosis (Chiechi et al., 2002). The extent and nature of isoflavone glycoside configuration whether aglycone, simple glycoside, malonyl-, or acetyl-glycoside, may influence bioavailability upon consumption (King, Broadbent, & Head, 1996). Thus it is important to know the total amount of each of the isoflavone compounds in a food, and the relative proportions of the bioactive aglycone components, daidzein, genistein and

* Corresponding author. Tel.: +61 3 9919 8289; fax: + 61 3 9919 8284.
E-mail address: Nagendra.Shah@vu.edu.au (N.P. Shah).

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glycetein and their various glycosides (King & Bignell, 2000). A number of studies have shown that the isoflavone content of soybean is strongly influenced by the genetic and environmental factors (Tsukamoto et al., 1995). In addition, preparation of soy protein isolate, while important in the removal of flatulence factors and beany flavours (Rackis, 1979), is known to substantially reduce the amount of total isoflavones (upto 53%) through extraction process using organic solvents (Wang & Murphy, 1994, 1996). The intake level of isoflavones required to provide an optimal health benefit is still not known. However, intake of soy food products in daily diets in such countries as China and Japan, has been estimated to average about 30–40 mg per day (Chen et al., 1999; Wakai et al., 1999), expressed as aglycone equivalent. Interestingly, there is low mortality from a number of cancers and from cardiovascular diseases in these countries. This contrasts with estimates for the USA of approximately 0.1 mg per day (Strom et al., 1999), which would also be expected to apply to the entire western world including Australia. There are very few published values for the isoflavone content of Australian soy foods (Dalais, Wahlqvist, & Rice, 1997; Knight, Eden, Huang, & Waring, 1998). One report (Knight et al., 1998) related only to soymilk and soy infant formula for daidzein and genistein only. Values for total isoflavone content of soy milk and other soy based foods such as soy bread, soyflour, tofu and soy sauce in Australia have been reported by King and Bignell (2000). Probiotic micro-organisms including *Lactobacillus* and *Bifidobacterium* have been known to possess endogenous β-glucosidases which can play an important role in altering the profile of isoflavones during fermentation (Otieno, Ashton, & Shah, 2005). Although each group of probiotics has varying potential in the hydrolysis of isoflavones during fermentation (Otieno, Ashton, & Shah, 2006a), the hydrolytic action has been found to cause major increases in the concentration of bioactive isoflavone aglycones and concomitant decrease in the concentration of isoflavone glycosides (Otieno et al., 2006a).

It is critical that the isoflavone content and their concentration in soy protein isolate are determined as it is the major ingredient in the preparation of unfermented and fermented soymilk. Accordingly, there is a need to develop a method for the rapid and accurate identification and quantification of these isoflavonoids. Most methods for the analysis of isoflavones are based on high performance liquid chromatography (HPLC)- or capillary electrophoresis (CE) separation with UV-, fluorescence- or electrochemical detection (Chen, Zhang, & Ye, 2001; Wang, Prasain, & Barnes, 2002). These methods however are limited to the detection of a limited number of known compounds and are not applicable for the characterisation of unknown isoflavonoids in a crude mixture (Prasain et al., 2003). Mass spectrometry (MS) combined with LC-MS/MS is currently the most sensitive and selective analytical method for the rapid qualitative and quantitative analysis of known compounds as well as for the identification of unknown compounds from purified samples of natural products (Barnes, Kirk, & Coward, 1994; Prasain, Ueki, Stefanowicz, & Osada, 2002). Its unique ability to filter and isolate molecular ions with specific mass-to-charge (m/z) ratios from a complex mixture makes MS a valuable tool for analysis. Generally, electrospray ionization mass spectrometry (ESI-MS) provides a mass spectrum with little or no fragmentation, and this technique is suitable for the characterization of a single compound as well as complex mixture of natural products. We report an integrated approach consisting of LC-MS/MS, including neutral ion-scan mass spectrometry for the quantification and identification of isoflavones in fermented and unfermented soymilk made from soy protein isolate (SPI; SUPRO 590). To the best of our knowledge, we for the first time also report an intricate link between the occurrence of isoflavone aglycones and specific aldehydes in soymilk.

2. Experimental

2.1. Bacteria

Pure culture of *B. animalis* ssp. *lactis* Bb12 was obtained from Chr. Hansen Pty. Ltd. (Bayswater, Vic, Australia). The purity of the culture was checked with Gram staining and the stock culture was propagated and stored at −80 °C in 40% glycerol for further use.

2.2. Bacterial growth media

Rehydrated de Mann Rogosa Sharpe (MRS) broth (pH adjusted to 6.7 using 5.0 M sodium hydroxide) (de Mann, Rogosa, & Sharpe, 1960) was prepared according to manufacturer instructions (Oxoid Ltd., West Heidelberg, Vic, Australia) and autoclaved at 121 °C for 15 min.

2.3. Soymilk manufacture

Soy protein isolate (SPI; SUPRO 590), supplied by The Solae Co. (Chatswood, NSW, Australia), was used in the production of soymilk at 40 g per litre according to our previous method (Otieno et al., 2006a) resulting into a homogenised milk-like product devoid of any protein suspensions. After reconstitution, the soymilk was dispensed into a glass bottle at 250 mL, then autoclaved at 121 °C for 15 min. After cooling to room temperature, the pH was adjusted in a laminar flow to 6.7 using 5 M sodium hydroxide.

2.4. Fermentation of soymilk with *B. animalis ssp. lactis Bb12*

The micro-organism was activated in MRS broth at 37 °C for 20 h successively 3 times followed by a fourth activation in sterile soymilk. An inoculum level of 5% (v/v) and the incubation at 37 °C for 20 h were used for activation. In order to conduct studies in the changing profile

and concentration of isoflavones, a 250 mL of sterile soymilk was inoculated (in duplicate) with the active culture (5% v/v), and incubated at 37 °C for 24 h to achieve peak β-glucosidase activity thus transforming the predominant isoflavone glycosides to the aglycone forms as found in our earlier study (Otieno et al., 2005). Aliquots of 50 mL were withdrawn aseptically at 12 h intervals and then stored immediately at −80 °C for analysis of isoflavones. The frozen aliquot was freeze-dried using a Dynavac® FD300 freeze drier (Rowville, Vic, Australia) for extraction and analysis of isoflavone using reverse-phase HPLC and liquid chromatography mass spectrometry (LC-MS).

2.5. Extraction of isoflavones for LC-MS/MS analysis

The extraction of isoflavones, including malonyl-, acetyl-, and β-glycosides, and aglycones from fermented and non-fermented soymilk was performed in triplicate using a modified version of a method described by Tsangalis, Ashton, McGill, and Shah (2002). A 1-g freeze-dried sample was added to 50 mL of methanol in a 150 mL round bottom flask and refluxed on a heating mantle for 1 h. The mixture was then filtered through a Whatman No. 1 filter paper into a 100 mL volumetric flask. The remaining dried soy matter was washed with the filtered portion and then refiltered into the same flask. A 5 mL aliquot was taken and after adding 60 μL of internal standard (ISTD) flavone solution (10 mg/50 mL), the sample was dried under a stream of nitrogen using a Techne sample concentrator (Pearce Biotechnology Inc., Rockford, IL, USA). The resultant dried matter was then resuspended in 1 mL of 10 mM ammonium acetate buffer (containing 0.1% trifluoro-acetic acid) and methanol (50:50) solution and centrifuged (14,000*g* for 30 min) using an Eppendorf centrifuge (model 5415C; Crown Scientific Pty. Ltd., Vic, Australia), then filtered through a 0.5-μm FH membrane prior to transferring to HPLC vials.

2.6. Isoflavone standards

All the aglycone standards of genistein, daidzein and glycitein as well as flavone (ISTD) were purchased from Sigma Chemicals (Castle Hill, NSW, Australia) while the β-glycoside standards of genistin, daidzin, puerarin and glycitin as well as daidzein metabolite equol were purchased from Indofine Chemical Co. (Sommerville, NJ, USA). Genistein, genistin, flavone, daidzein, and equol were prepared in HPLC grade methanol, and daidzin, glycitein and glycitin in ethanol due to their varied solubility characteristics.

2.7. LC-MS and LC-MS/MS instrumentation

Components of the isoflavone extract were separated by HPLC using a 150 mm × 2.1 mm internal diameter (i.d.), C18 aquapore reversed-phase column pre-equilibrated using a gradient elution system with 0.05% methanol aqueous solution and 0.05% formic acid aqueous solution. The mobile phase was initially composed of 10:90 acetonitrile/water (both containing 0.05% methanol and formic acid), followed by a linear gradient to 40% methanol over 30 min at a flow rate of 0.95 mL/min. The column eluate was passed into the ionspray ionization interface operating in the positive mode of a PE Sciex (Concord, ON, Canada) API III triple-quadrupole mass spectrometer. The voltage on the ionspray interphase was 121 V, and the orifice potential was set at 40 V. Positive ion mass spectra were recorded over an m/z range of 200–600. Selected $[M + H]^+$ were analysed by collision-induced dissociation with 90% argon/ 10% nitrogen and the daughter ion mass spectra were recorded. Neutral loss scanning (a tandem mass spectrometric mode to obtain an array of all parent ions that lose a common neutral fragment) of the isoflavone extract were acquired in the positive ion mode with a dwell time of 5 ms and a step size of 1 m/z.

The MS/MS analyses of isoflavonoid glycosides were performed using a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source. Product ion spectra were obtained in the positive and negative ion modes. Product ion spectra were obtained by selecting the protonated and deprotonated ions for collision (energy = 40 eV), using argon as a collision gas.

2.8. Statistical analysis

To find the difference in isoflavone concentrations in soymilk before and after 12 h fermentation, means were analysed using one-way analysis of variance (ANOVA) and 99% confidence levels using Microsoft® Excel Statpro® as described by Albright, Winston, and Zappe (1999). ANOVA data with a $P < 0.01$ was classified as statistically significant.

3. Results and discussion

3.1. Compositional variations of isoflavones during fermentation

The profiles of isoflavone glycosides and aglycones in unfermented soymilk ($h = 0$) is shown in Fig. 1a while that after 12 h of fermentation at 37 °C using *B. animalis* ssp. *lactis* Bb12 is shown in Fig. 1b. We reported earlier (Otieno et al., 2006a), that unfermented soymilk contained higher amounts of isoflavone glycosides than the aglycone components. During fermentation, there was an increase in the aglycone concentration with a concomitant reduction of the isoflavone glycosides. The concentration of isoflavones compounds was influenced by the β-glucosidase activity and the growth of the micro-organism in the soymilk (results not shown). The increase in isoflavone aglycones was due to the hydrolytic ability of endogenous β-glucosidases (Otieno et al., 2006a) and possibly by the crude β-galactosidase enzymes within the bacterial cell-wall. It is interesting to note that 11 of the 13 known isoflavones in

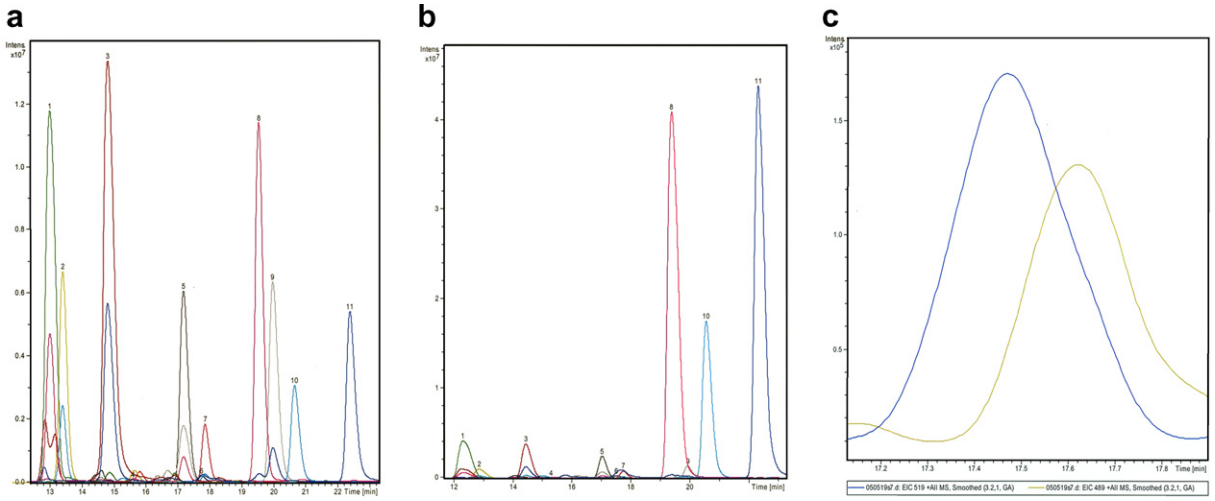

Fig. 1. Electro-spray ionisation chromatogram using LC-MS of isoflavones in unfermented soymilk (0 h) (a), after fermentation with *B. animalis* ssp. *lactis* Bb12 for 12 h at 37 °C (b), and separation and quantitation of malonyl-genistin (bigger peak-EIC 519) and acetyl-glycitin (smaller peak-EIC 489) in fermented soymilk using LC-MS (c). Elution order and peak identification in (a) and (b). (1) Daidzin (EIC; 417), (2) glycitin (EIC; 447), (3) genistin (EIC; 433), (4) malonyl-daidzin (EIC; 503), (5) acetyl-daidzin (EIC; 459), (6) malonyl-genistin (EIC; 519), (7) acetyl-glycitin (EIC; 489), (8) daidzein (EIC; 255), (9) acetyl-genistin (EIC; 475), (10) glycitein (EIC; 285) and (11) genistein (EIC; 271).

soymilk were detected in the soymilk made from SPI (SUPRO 590). Malonyl-glycitin and equol were not detected in the unfermented and fermented soymilk as shown in Fig. 1a and b, respectively. According to Wang and Murphy (1994) soybeans have variable isoflavone contents depending on the variety and environmental conditions (location and/or crop year). Equol is a daidzein metabolite and occurs mostly as a result of enzyme-induced reduction in the intestinal tract. Equol may never be detected in an *in vitro* system, but is detectable from physiological fluids such as blood plasma and urine using most analytical techniques including HPLC and LC-MS (Jenkins et al., 2002; King & Bursill, 1998). This was confirmed using several analytical procedures of soy isoflavones in unfermented and fermented soymilk (Otieno et al., 2006a; Otieno, Ashton, & Shah, 2006b).

3.2. Identification and quantitation of isoflavone compounds

The peak heights in Fig. 1a and b show the intensity or the concentration of each compound in the soymilk. Peak 3 (EIC 433) representing glycoside genistin was the tallest peak followed by peak 1 (EIC 417) representing glycoside daidzin in Fig. 2. The malonyl forms, that is, peaks 4 (EIC 503) and 6 (EIC 519) represented by malonyl-daidzin and malonyl-genistin, respectively, were the least in concentration in unfermented soymilk as compared with three other classes of isoflavones. Acetyl-genistin (peak 9, EIC 475) was the highest in concentration amongst the acetylated forms (Fig. 1a). Unlike the malonyl forms, there were three acetylated isoflavones detected in the soymilk with the other two being peaks 5 (EIC 459) and 7 (EIC 489) represented by acetyl-daidzin and acetyl-glycitin, respectively. In general, isoflavone glycosides (genistin, daidzin and glycitin) were the most predominant forms followed by the aglycones. In unfermented soymilk, the major peaks were without question those of the isoflavone glycosides such as daidzin (peak 1) and genistin (peak 3). Acetyl-daidzin (peak 5) also appeared to be in high concentration relative to the rest of the isoflavones. Interestingly, aglycone daidzein (peak 8) also occurred in relatively high concentration in the soymilk before fermentation.

There was a noticeable shift in the concentration of the classes of isoflavones in the soymilk after 12 h of fermentation with probiotic *B. animalis* ssp. *lactis* Bb12 as shown in Fig. 1b. The breaking down of β-1, 6 glycoside bonds in the isoflavone glycosides was due to the activity of endogenous β-glucosidase enzyme and could be in part due to the presence and activity of crude β-galactosidase enzyme (Tochikura, Sakai, Fujiyoshi, Tachiki, & Kumagai, 1986). The products of hydrolysis were the sugar moiety as well as the aglycones in the soymilk which provided a source of nutrients for the growth of the micro-organism (Ballongue, 1993). As shown, there was a reduction in the concentration of isoflavone glycosides of peaks 1, 2 and 3 represented by daidzin (EIC 417), glycitin (EIC 447), and genistin (EIC 433), respectively. In contrast, there was a considerable increment in the intensity of aglycones, daidzein, glycitein and genistein represented by peaks 8 (EIC 255), 10 (EIC 285) and 11 (EIC 271), respectively. An important change in the isoflavone profile in the soymilk during fermentation was the occurrence of major peaks (with higher concentration) of mainly aglycones. The highest peaks were those of 11, 8 and 10 representing aglycones genistein, daidzein and glycitein, respectively. Genistein, daidzein and glycitein increased in their intensities after 12 h fermentation (Fig. 1b) to 4.7×10^7, 4.2×10^7, and 1.8×10^7 from 0.75×10^7, 1.2×10^7 and 0.375×10^7, respectively before fermentation (Fig. 1a) in the soymilk. The increment of aglycone components during fermentation is important in

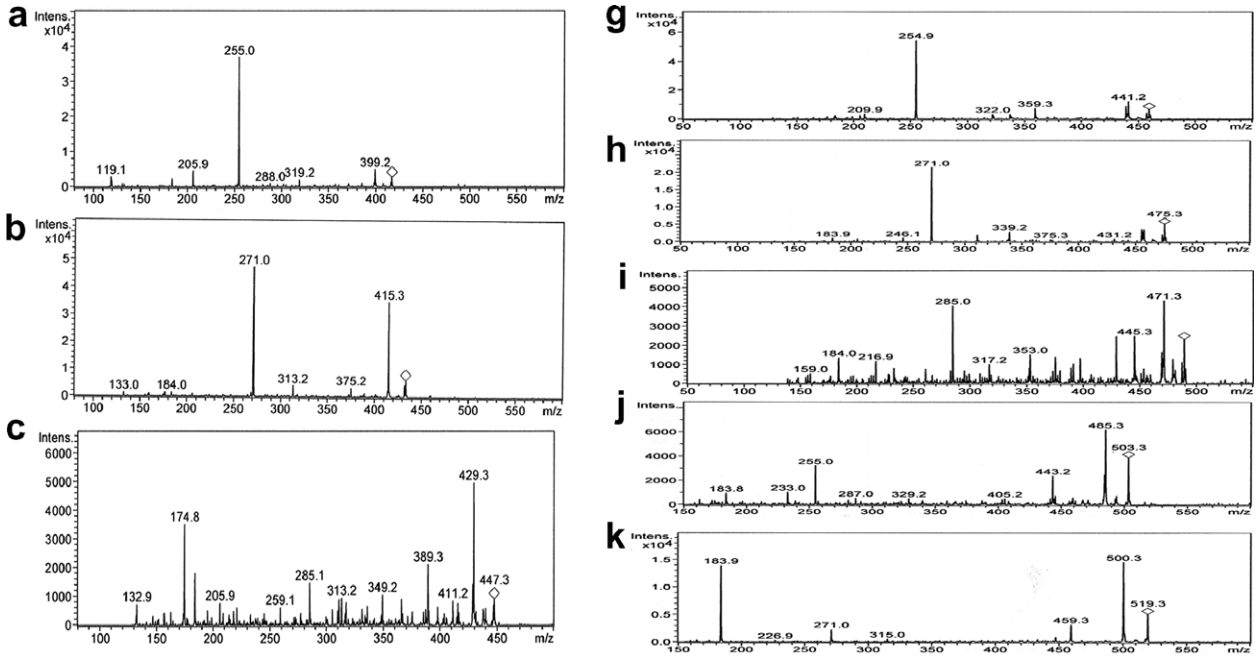

Fig. 2. Products ions obtained in ESI-MS/MS experiments of protonated isoflavone glycosides (M + H) daidzin, genistin and glycitin (a, b, and c), respectively and of acetyl-daidzin, acetyl-genistin, acetyl-glycitin, malonyl-daidzin and malonyl-genistin (g, h, i, j, and k) product ions, respectively in soymilk. Parent ions are indicated by the diamond mark.

enhancing the biological activity of the soymilk due to their structural similarity to the human estrogen. This structural similarity to human estrogen enables their possession of varying estrogen-like activities. Genistein has been found to be the most estrogenically potent aglycone followed by daidzein and glycitein (Valachovicova, Slivova, Bergman, Shuherk, & Sliva, 2004).

Table 1 shows the quantitation of isoflavones and the changes in concentration of glycosides and aglycones occurring during fermentation with *B. animalis* ssp. *lactis* Bb12. Using LC-MS, the total amount of isoflavones detected in the soymilk was about 56.08 μg/mL. The isoflavone profile consisted predominantly of upto 84.8% glycosides before fermentation. However, after 12 h of fermentation, the total glycoside concentration reduced by a factor of 4.95 times than the initial concentration. Concomitantly, the amount of isoflavone aglycones instead increased by a factor of 5.46 times than the initial concentration due to the hydrolytic action of crude endogenous β-glucosidase and β-galactosidase within the microbial cell wall. The hydrolytic action did not alter the profile *in vitro* but affected the concentration of the detected isoflavone glycosides and aglycones.

Table 1
Concentration of isoflavones (μg/mL) in fermented soymilk using *Bifidobacterium animalis* ssp. *lactis* Bb12

Isoflavone compounds	0 h	6.0 h	12.0 h	*P*-value
Glycitin	$15.95^{a} \pm 1.3$	$5.31^{a} \pm 0.5$	$5.56^{a} \pm 0.6$	0.0000*
Daidzin	$6.57^{a} \pm 0.6$	$0.87^{ab} \pm 0.1$	$1.11^{ab} \pm 0.2$	0.0000*
Genistin	$20.44^{a} \pm 2.0$	$1.45^{b} \pm 0.4$	$1.01^{b} \pm 0.1$	0.0000*
β-glycosides[1]	$37.13^{a} \pm 2.8$	$7.63^{bc} \pm 0.6$	$7.68^{bc} \pm 0.6$	0.0000*
Glycitein	$0.37^{a} \pm 0.1$	$1.48^{a} \pm 0.3$	$1.58^{a} \pm 0.2$	0.0000*
Daidzein	$4.15^{a} \pm 1.0$	$19.39^{b} \pm 0.6$	$19.04^{b} \pm 0.3$	0.0000*
Genistein	$3.99^{a} \pm 0.8$	$25.21^{bc} \pm 1.1$	$25.86^{bc} \pm 0.3$	0.0000*
Aglycones[1]	$8.52^{a} \pm 1.6$	$46.1^{bc} \pm 1.2$	$46.5^{bc} \pm 0.3$	0.0000*
Malonyl-glycitin	ND	ND	ND	N/A
Malonydaidzin	$0.99^{a} \pm 0.3$	$0.23^{a} \pm 0.0$	$0.15^{a} \pm 0.1$	0.0000*
Malonyl-genistin	$3.02^{a} \pm 0.2$	$1.15^{a} \pm 0.2$	$1.10^{a} \pm 0.2$	0.0002*
Malonyl-glycosides[1]	$4.01^{a} \pm 0.5$	$1.38^{a} \pm 0.3$	$1.25^{a} \pm 0.2$	0.0000*
Acetyl-glycitin	$2.25^{a} \pm 1.8$	$0.46^{a} \pm 0.1$	$0.35^{a} \pm 0.0$	0.0573*
Acetyl-daidzin	$1.09^{a} \pm 0.2$	$0.15^{a} \pm 0.1$	$0.03^{a} \pm 0.0$	0.0000*
Acetyl-genistin	$3.08^{a} \pm 0.3$	$0.38^{b} \pm 0.2$	$0.28^{b} \pm 0.1$	0.0000*
Acetyl-glycosides[1]	$6.42^{a} \pm 0.9$	$0.99^{a} \pm 0.6$	$0.67^{a} \pm 0.1$	0.0000*
Equol	ND	ND	ND	N/A
Total Isoflavones[2]	$56.08^{a} \pm 0$	$56.08^{a} \pm 0$	$56.08^{a} \pm 0$	0.0000*

Results expressed as means ± standard error (SE) in μg/mL of soymilk ($n = 6$).
Statistical analysis by means of one-way ANOVA.
[a–c] Means in the same row with different lower case scripts are significantly different ($P < 0.01$).
[1] Mean total of three respective isomers.
[2] Mean total of malonyl-, acetyl-, β-glycoside and aglycone isomers.

3.3. Separation of existing isoflavone compounds in fermented and unfermented soymilk

Based on molecular weight and polarity of the isoflavone compounds in the soymilk, separation occurred in the stationary phase of the column. High performance liquid chromatography-mass spectrometry (HPLC-MS)

offers the distinct advantage of identification of compounds based on molecular weight and ion charge separation. The degree of polarisation of two out of eleven compounds could not allow for a clear separation of the isoflavones which were identified as malonyl-genistin (EIC 519) and acetyl-glycitin (EIC 489) (Fig. 1c). As a result of the near equal polarity, they appeared to have similar charges and consequently near equal affinity to the stationary phase of the C_{18} column used. In our previous studies, the use of ammonium acetate plus trifluoroacetic acid and acetonitrile in the mobile phase rather than formic acid gave better separation of the two compounds (manonyl genistin and acetyl-glycitin), although we had encountered difficulties in the separation of genistein and equol (Otieno et al., 2006a, 2006b). The two compounds appear as peaks 6 and 7, respectively (Fig. 1a and b). Fig. 1c shows the enlarged manifestation of the protonated peaks of the two compounds. Using LC-MS/MS, the protonated ions of the isoflavone compounds enabled their identification despite the inability to clearly separate them.

3.4. Product ion analysis of isoflavone glycosides in soymilk

To understand the mass spectrometric behaviour of isoflavones, LC-MS analysis of authentic daidzin, genistin and glycitin was performed using ESI in the positive ion mode. The molecular mass and structural and elemental formulae of these glycosides were shown in Fig. 2. Product ion spectra of isoflavone glycosides, glycitin, genistin and daidzin represented by a, b and c, respectively (Fig. 2) revealed several diagnostic product ions. There are similarities and differences in the fragmentation patterns of the three isoflavone glycosides. An important similarity was the loss of 18 Da from the parent peaks of *m*/*z* 447–428, 433–415 and 417–399 for glycitin, genistin and daidzin, respectively, representing the neutral loss of water (H_2O). Peaks of *m*/*z* 417 and 433 appear as parent peaks in daidzin (a) and genistin (b), respectively. The other similarity was the occurrence of respective protonated aglycone ions in each mass spectrum of all three respective glycosides represented by a loss of 162 Da. The loss of ions from the parent peaks of an isoflavone glycoside leading to emergence of a protonated Y_o^+ aglycone is associated with the loss of an entire sugar unit. The occurrence of the respective Y_o^+ aglycone ions at *m*/*z* 285, 271 and 255 in a, b and c, respectively is a further confirmation of the identity of the isoflavone glycosides determined in the soymilk.

3.5. Comparison of product ions obtained in ESI-MS/MS of the malonyl-, and acetyl-glycosides detected in soymilk

The analysis of the ion spectra also involved that of malonyl and acetyl-glycosides in the soymilk and is shown in Fig. 2. Malonyl-glycitin was not detected in the soymilk. There was no commonality in the fragmentation pattern of the acetyl- and malonyl-glycosides except the non successive neutral loss of 18 Da represented by water (H_2O) in all the glycosides. However, the occurrences of respective Y_o^+ aglycone ions in the fragment spectrum of each of the acetyl- and malonyl-glycoside were detected. For example, the aglycone ions of daizein (*m*/*z* 255), genistein (*m*/*z* 271) and glycitein (*m*/*z* 285) appeared on the product ion spectra of acetyl- and malonyl-daidzin (g and j), acetyl- and malonyl-genistin (h and k), and acetyl-glycitin (i), respectively. Interestingly, there was no occurrence of respective main glycoside ions such as genistin, daidzin and glycitin in the spectra of fragmented acetyl- and malonyl-glycosides. A summary of product ions and molecules formed during ESI-LC-MS fragmentation of isoflavones in soymilk is shown in Table 2 and a summary of protonated fragments from each isoflavone detected is shown in Table 3.

3.6. Comparison of product ions obtained in ESI-MS/MS of the isoflavone aglycones and effect of structural differences in isomeric compounds

The fragmentation pathway of any molecule is therefore unequivocal confirmation of the uniqueness of the molecule. For instance isomeric compounds like puerarin and diadzin, both of equal molecular weight of 416 and of almost similar molecular structure (Fig. 3) can be confirmed to be different using LC-MS/MS positive ion fragmentation. On the other hand, (b) in positive mode showed fragments of ions at *m*/*z* 399, 255 and 205, puerarin (a) showed two prominent diagnostic ions of *m*/*z* 297 and 267, respectively indicating neutral losses of 120 and 150 Da, respectively. The loss of 120 Da was indicative of the occurrence of C-glycosides (Waridel et al., 2001). A series of ions at *m*/*z* 381 and 363 in puerarin were obtained due to the successive neutral losses of water molecules. Two types of typical isoflavone product ions existed namely *C*- and *O*-glycosides. By definition, *C*-glycosides are compounds in which the inter-glycosidic oxygen atom has been replaced by a carbon atom to produce a stable glycoside derivative that is not prone to enzymatic or chemical hydrolysis (Postema, Piper, & Betts, 2006). To be suitable mimic of the *O*-glycoside, *C*-glycoside must possess conformation similar to that of the parent *O*-glycoside or adopt that of the parent *O*-glycoside conformation that still elicits a biological response (Postema et al., 2006). The *O*-glycosides have sugar substituents bound to a 7-hydroxy group of the aglycone, where as *C*-glycosides have sugar substituents bound to a carbon of the aglycone, generally at positions C-6 and C-8. Glycosides such as glycitin, genistin and glycitin undergo facile losses of the sugar moiety on MS-MS fragmentation and this is probably been due to the weaker C–O bond between the sugar and the aglycone (Prasain et al., 2003). Puerarin did not show similar pattern as that of daidzin due to the C–C bond linking the sugar and the aglycone (Fig. 3). Several studies (Tsuruta, Yuasa, Kurono, & Hashimoto, 1999; Wang, Kovac, Sinay, & Glaudemans, 1998; Wei, Boy, & Kishi, 1995; Yang, Franck, Bitman, Samadder, & Arthur, 2001) have shown that the

Table 2
Summary of product ion data for protonated Isoflavones in soymilk during fragmentation of using ESI-LC MS/MS

Compound	Parent ion $[M+H]^+$	Methyl $[M+H-CH_3]^+$	Carbon hydride $[M+H-CH]^+$	Carbonmonoxide $[M+H-CO]^+$	Carbonmonoxide $[M+H-2CO]^+$	Carbonmonoxide $[M+H-3CO]^+$	Methanol $[M+H-CH_3OH]^+$	[a]Unknown Compound	[b]Unknown Compound	Methanal $[M+H-HCHO]^+$	Water $[M+H-H_2O]^+$	Carbondioxide $[M+H-CO_2]^+$	Sugar $[M+H-S_o]^+$
Daidzein	255			227	199				145				
Genistein	271			243	215	187		153					
Glycitein	285	270	257	229			196			166			
Daidzin	417										399		255
Genistin	433										415		271
Glycitin	447										428		285
Acetyl-daidzin	459										441		255
Acetyl-genistin	475											431	271
Acetyl-glycitin	489										471		285
Malonyl-daidzin	503										485		255
Malonyl-genistin	519										501		271

Parent ion – the initial peak ion being subjected to MS/MS fragmentation.

[a] Unknown compound – fragment ion of 34 Da from isoflavone genitein. Could be identified using nuclear magnetic resonance (NMR).

[b] Unknown compound – fragment ion of 54 Da from isoflavone daidzein. Could be identified further using nuclear magnetic resonance (NMR).

Table 3
Summary of the *m*/*z*, peaks of fragments and the molecular weights of isoflavones in the soymilk using ESI-MS/MS on positive fragmentation

[a]Isoflavones identity	MW	MS	MS/MS fragments
Daidzein	254	255	227, 199, 137, 93
Genistein	270	271	243, 215, 187, 153, 103
Glycitein	284	285	270, 257, 229, 197, 167, 145, 124, 109, 95
Daidzin	416	417	399, 319, 288, 255, 206, 119
Genistin	432	433	415, 375, 313, 271, 184, 133
Glycitin	446	447	429, 411, 389, 349, 313, 285, 259, 206, 175, 133
Acetyl-daidzin	458	459	441, 359, 322, 255, 210
Acetyl-genistin	474	475	431, 375, 339, 271, 246, 184
Acetyl-glycitin	488	489	471, 445, 353, 317, 285, 217, 184, 159
Malonyl-daidzin	502	503	485, 443, 405, 329, 287, 255, 233, 184
Malonyl-genistin	518	519	500, 459, 315, 271, 227, 184

[a] Isoflavone identity – Identification were aided by revelation of molecular weights and fragments from LC-MS/MS and is the list of isoflavones in order of molecular weights detected in the soymilk made from soy protein isolate SUPRO 590.

substitution of the inter-glycosidic oxygen atom with a carbon atom did not greatly alter biological activity.

Fig. 3 showed the comparison of the product ions obtained in ESI-MS/MS of the isoflavone aglycones, daidzein (d), genistein (e) and glycitein (f) when subjected to positive ion fragmentation on MS/MS. Glycitein (f) showed a series of ions beginning with parent peak at *m*/*z* 285, 270, 257, 229, 196 and 166 represented by losses of 15, 13, 28, 32 and 30 Da, respectively. The radicals represented by the successive losses during glycitein fragmentation are methyl (CH_3), carbon hydride (CH), carbon monoxide (CO), methanol (CH_3OH) and a methanal (HCHO). Aglycone genistein (e) on the other hand showed a series of ions at *m*/*z* 271, 243, 215, 187 and 153 represented by three successive losses of 28 and 34 Da. The fragmentation pattern of genistein therefore involved 3 successive losses of carbon monoxide (CO), followed by an unidentified radical. Daidzein (d) on fragmentation showed ions at *m*/*z* 255, 227, 199, and 145 represented by 2 successive losses of 28 Da, followed by an unidentifiable radical of 54 Da. These unidentifiable compounds could possibly be known using nuclear magnetic resonance (NMR) techniques. Aldehydes such as pentanal and hexanal have been known to occur in soybased matrices (Tsangalis & Shah, 2004) and act as a prebiotic to support the growth of probiotic micro-organisms in the soymilk. Accordingly, SPI with higher oligosaccharide content also had higher levels of pentanal and hexanal. The occurrence and variations in the levels of pentanal and hexanal in SPI could be influenced by the extent of formation of these volatiles in the defatted soy flour from which SPI is prepared. Fujimaki, Arai, Kirigaya, and Sakurai (1965) reported that hexanal and some other volatile components were formed by simple autoxidation of lipids remaining in defatted soybean. Thus the occurrence of aldehydes during fragmentation of isoflavone aglycones could be due to autoxidative process in lipids but the link of specific aldehydes to specific aglycone parent ions on fragmentation remains to be investigated further. According to Tsangalis and Shah (2004), fermentation of soymilk with strains of *Bifidobacterium* also decreased the levels of hexanal and pentanal. Thus it appears that the aldehydes could be metabolised for growth and viability of micro-organisms in soymilk which in the process causes biotransformation of isoflavone glycosides.

4. Conclusions

Since there was no similarity in the fragment product ions, this indicates the uniqueness of each isoflavone compound.

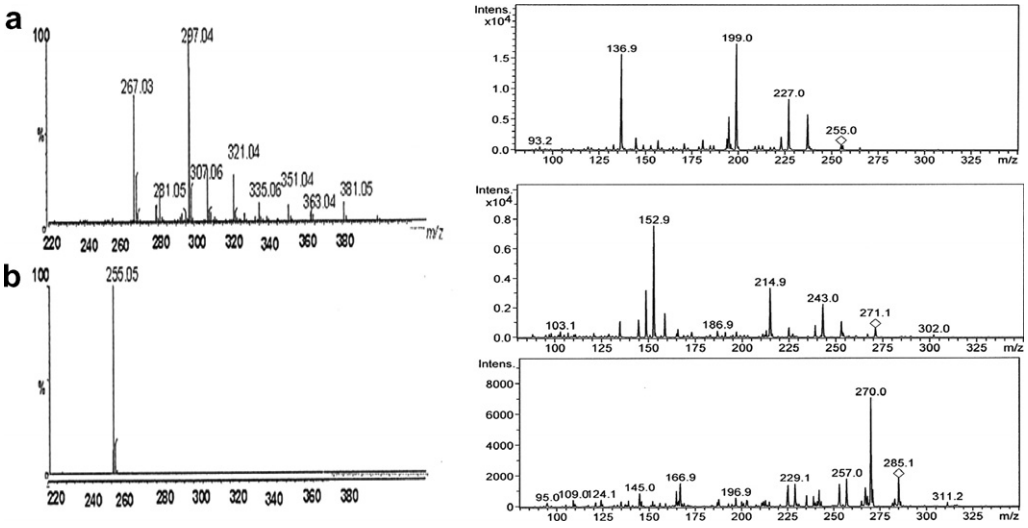

Fig. 3. Comparison of the products ions obtained in ESI-MS/MS experiments of protonated standards of isomeric compounds puerarin (a; *C*–glycosylated) and daidzin (b; *O*–glycosylated), respectively and products ions obtained in ESI-MS/MS of protonated isoflavone aglycones daidzein, genistein and glycitein (d, e and f), respectively in soymilk.

Although there were differences in the spectra of product ions from individual isoflavones in the soymilk, the fragmentation patterns of classes of isoflavone i.e. aglycones, glycosides, acetyl-, and malonyl-forms followed a similar trend. There was no similarity of particular peaks amongst the isoflavone aglycones but there was a similarity in the trend of loss of carbon monoxide, at least successively with daidzein and genistein. All isoflavone glycosides including the malonyl and acetyl forms detected in the soymilk had respective aglycone ions as major peaks in the spectra. Glycosides such as daidzin and glycitin had a similar fragment ion of m/z 206 while genistin, acetyl-genistin, acetyl-glycitin, malonyl-daidzin and malonyl-genistin had a common occurrence of fragment ion of 184 m/z. Even though there were similarities in the fragments amongst the isoflavone glycosides and in the trend of losses ions and molecules during fragmentation of each class of isoflavones, each compound however, had a unique fragmentation pattern leading to their unequivocal identification. The identification of isoflavones in fermented and unfermented soymilk is necessary to generate information for *in vivo* and *in vitro* studies and for the authentification of isoflavones in soy based drinks and beverages.

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