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Comprehensive Phytochemical Profile of Soy Protein Isolate

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Although an FDA health claim for soy protein has been issued, the potential health benefits of soy foods remain controversial among scientists, especially with regard to soy infant formula. The UV detectable isoflavones have been the focus of the majority of studies concerning health-related effects of soy protein isolate (SPI). However, the chemical identities and health effects of other SPI phytochemicals without UV absorption properties are less well-studied. In the current study, we employed liquid chromatography−tandem mass spectrometry methods to reveal a complicated phytochemical profile for SPI consisting of 136 phytochemicals. Also, we have quantitated many of these SPI phytochemicals so that dietary intakes can be estimated for foods containing SPI. On a weight/weight basis, fatty acids are the largest group of phytochemicals in the extract (64.13% total fat), followed by saponins (21.48%), and then isoflavones at 6.82%. Of the 56 lysophospholipids identified in SPI, 0.50% was lysophosphatidylcholines and 0.23% was lysophosphatidylethanolamines.

KEYWORDS: Soy protein; isoflavones; soysaponins; lysophospholipids; fatty acids; LC-MS/MS; folates

INTRODUCTION

Asian countries have lower incidences of several diseases, and this has been attributed to healthier lifestyles that include diets higher in vegetables, grains, and fish and diets lower in red meats and fat (*1*). Although the soybean is believed to be one of the foods responsible for lower incidence of some diseases in Asian countries as compared with Western countries, it is a somewhat controversial food in the West. Several human and animal studies suggest significant health benefits of soybeans, including the prevention of heart disease and certain cancers (*1*−*3*). Contrary to health benefits, however, dietary soy intake has also been associated with increased bladder cancer incidence (*4*), thyroid disorders (*5*), breast cell hyperplasia (*6*, *7*), and dementia (*8*). Some studies have raised concerns about potential adverse effects from soy-based formula intake in infants as the result of high circulating phytoestrogen (soy isoflavone) concentrations (*9*) and potential for early and premature estrogenic effects in infants (*10*, *11*).

The majority of the studies of physiological activities of SPI have focused on the UV detectable isoflavones, genistein and daidzein, and the daidzein metabolite equol. Far fewer studies have considered the other nonisoflavone phytochemicals associated with SPI, such as the major bioactive phytochemical component, the saponins (*12*−*17*). This is partly because less data are available on the phytochemical profile of SPI. The increasing use of soy foods and the continued feeding of soy infant formula have sparked a greater interest in research into the health effects of SPI, and this line of research requires more complete identification and quantification of phytochemicals associated with this protein.

We previously identified 56 lyso-PL that were associated with SPI, but no quantification was available (*18*). The aim of the current study was to more completely determine the phytochemical profile of SPI by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and to provide sufficient quantification of the major phytochemicals that could be used to estimate phytochemical intake from foods containing SPI.

MATERIALS AND METHODS

Materials. Nine isoflavone standards, including genistein, genistin (genistein 7-*O*-*β*-D-glucopyranoside), 6″-*O*-acetylgenistin, 6″-*O*-malonylgenistin, daidzein, daidzin (daidzein 7-*O*-*β*-D-glucopyranoside), 6″-*O*-acetyldaidzin, glycitein, glycitin (glycitein 7-*O*-D-*β*-glucopyranoside), and internal standard (IS) biochanin A were purchased from LC Laboratories (Woburn, MA). Two lipid standard mixtures were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), namely, L-α-lysophosphatidylcholine mixture (prepared by the action of phospholipase A_2 on soybean L-α-phosphatidylcholine) containing primarily C-18 unsaturated fatty acids and L-α-lysophosphatidylethanolamine mixture (prepared from egg yolk) containing primarily stearic and palmitic acids. Steryl glucoside (soybean) was purchased from Indofine Chemical Company (Hillsborough, NJ). SPI product (SPI 670) was a kind gift from Protein Technologies International, DuPont (St. Louis, MO) and is essentially the same product used in most soy infant formulas marketed in the United States.

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Preparation of a Phytochemical Concentrate of SPI. SPI (4 g) was extracted with 100 mL of 50% aqueous methanol at 5 °C for 24 h with occasional stirring. The slurry was filtered through a Büchner funnel with a no. 4 Whatman filter paper. The extraction process was repeated with 80% (100 mL) aqueous methanol and followed with a third extraction with 100% methanol (100 mL). The three extracts were combined and concentrated on a rotary evaporator under reduced pressure at room temperature until the methanol was removed. The concentrated extract was passed through a C18 (60 cm^3/10 g) Mega Bond Elute cartridge from Supelco (Bellefonte, PA) after activation with 2 volumes of methanol and then 2 volumes of water. The cartridge was then rinsed free of the sugars and some simple organic acids with 2 volumes of water. The phytochemicals retained by the cartridge were eluted with 50% aqueous methanol (50 mL), followed by 100% methanol (50 mL), and then acetone (50 mL). All three effluents were combined to give the fraction of interest, which was rotary evaporated under reduced pressure at room temperature followed by drying in a freeze dryer.

LC-MS/MS Analysis. The phytochemical concentrate of SPI and standards were dissolved in 80% aqueous methanol and directly analyzed by LC-MS/MS with a 20 μL injection. LC-MS/MS was performed using a Bruker Esquire-LC multiple ion trap mass spectrometer equipped with an Agilent 1100 series liquid chromatograph. An HP ChemStation was used for data collection and manipulation. A 150 mm × 4.6 mm i.d. Eclipse XDB-C8 column (Agilent Technologies, Wilmington, DE) was used with LC solvent at a flow rate of 0.8 mL/min. The LC gradient was 0.1% formic acid/acetonitrile (solvent B) in 0.1% formic acid/water (solvent A) as follows: 10−15% in 15 min; 15−18% from 15 to 16 min; 18−22% from 16 to 33 min; 22−40% from 33 to 40 min; held at 40% from 40 to 45 min; 40−42% from 45 to 49 min; 42−45% from 49 to 50 min; 45−50% from 50 to 65 min; 50−65% from 65 to 75 min; 65−85% from 75 to 76 min; 85−100% from 76 to 80 min; held at 100% from 80 to 85 min and finally back to 10% at 90 min. Phytochemicals in the eluate were monitored by a diode array detector and analyzed by automatic MS/MS with both negative and positive ion modes. For optimum MS analysis, 10 mM ammonium acetate (for negative ion mode) or 2% formic acid (for positive ion mode) in methanol was used as the ionization reagent and added at a flow rate of 0.2 mL/min via a tee in the eluant stream of the high-performance liquid chromatography (HPLC) just prior to the mass spectrometer. Conditions for ESI-MS analysis of HPLC peaks in both negative and positive ion modes included a capillary voltage of 3200 V, a nebulizing pressure of 33.4 psi, a drying gas flow of 8 mL/min, and a temperature of 250 °C. Parameters that control the API interface and the mass spectrometer were set via Smart Tune with a compound stability of 50% and a trap drive level of 50%. ICC was "on" including: target, 5000; maximum accumulation time, 50.00 ms; scan, *m*/*z* 80.00−850.00; averages, 10; and rolling averaging, off. Conditions for automatic MS/MS were as follows: width of the isolation, 4.0; fragmentation amplitude, 1.00 V; and number of parents, 1.

Quantitative Determination. Phytochemicals were separated by LC and monitored by a diode array detector and MS. Isoflavones were quantified on the basis of the areas of UV peaks (UV 265), and lyso-PLs were based on the areas of EIC from MS with positive ion mode. SPI sample (40 μg) or standards were dissolved in 20 μL of 80% aqueous methanol, which contained 200 ng of biochanin A (10 ng/μL) as IS. The concentrations of individual components in SPI samples were determined from calibration curves of the corresponding standard. Compounds were quantified by calibration curves of their structure-related standards when their corresponding standards were not available. Calibration curves of each standard were created with peak areas from seven concentrations using Microsoft Excel software. The recovery rates of IS in SPI extract and standard samples were used to monitor the recovery of analytes, and the final data were corrected using the recovery rates obtained from IS. All samples were analyzed in triplicate, and the calculated results were expressed as mean amounts ($n = 3$) after normalization with IS biochanin A.

RESULTS AND DISCUSSION

Extraction and Analysis Strategy. The methanol extraction process was considered nearly complete, because there were no detectable phytochemicals in a fourth extraction with 100% methanol. The phytochemical concentrate (168.2 mg from 4 g of SPI) represented 4.21% of SPI (w/w). Five wavelengths (200, 265, 315, 360, and 420 nm) were set on the diode array detector to monitor phytochemicals with UV absorption properties in LC-MS/MS analysis. LC-MS/MS analyses were very reproducible based on the recovery rates of IS and all standards. For example, the mean of UV peak areas (265 nm) of IS biochanin A from seven analyses was 942.72 ± 11.73 (average ± SD, $n = 7$). There were several peaks at 200 nm that were identified as unsaturated fatty acids by comparison with corresponding standards and lack of significant peaks detected at 420 nm. UV profiles at 265 (**Figure 1C**,**D**), 315, and 360 nm suggested that major phytochemicals in SPI extracts were isoflavones. Other phytochemicals, if present, were not present at significant levels based on UV profiles of phytochemicals in SPI (except for fatty acids). Mass spectrometry was used to detect phytochemicals with and without UV absorption properties. In contrast to the results from UV profiles, the TICs of SPI extract shown in **Figure 1A** (positive ion mode) and **1B** (negative ion mode) reveal a much more complicated composition for phytochemicals associated with SPI. **Figure 2** shows the basic chemical structures discussed in this study.

Table 1. Identification of Isoflavones in SPI

no. in Figure 1	ESI-MS negative[a]	ESI-MS positive[a]	chemical structure
1	**431**, 269	433, **271**	genistein-glycoside[b]
2	415, **253**	417, **255**	daidzein 7-glucoside
3	445, **283**	447, **285**	glycitein 7-glucoside
4	457, **253**	503, **255**	daidzein-malonylglycoside[b]
5	**431**, 269	433, **271**	genistein 7-glucoside
7	457, **253**	503, **255**	daidzein 7-malonylglucoside
8	487, **283**	533, **285**	glycitein 7-malonylglucoside
10	**457** 253,	459, **255**	daidzein 7-acetylglucoside
12	487, **283**	489, **285**	glycitein 7-acetylglucoside
13	473, **269**	519, **271**	genistein-malonylglycoside[b]
14	473, **269**	519, **271**	genistein 7-malonylglucoside
15	**473**, 268	475, **271**	genistein-acetylglycoside[b]
16	253	255	daidzein
17	283	285	glycitein
18	**473**, 269	475, **271**	genistein 7-acetylglucoside
24	269	271	genistein

[a] Numbers in bold are base peaks in the MS spectrum. [b] The positions of sugar (glucosyl or galactosyl) could not be determined by MS data in this study.

Identification and Quantification of Isoflavones Associated with SPI. Sixteen isoflavones in SPI extract were identified and quantitated by direct comparison with their corresponding standards or their structure-related standards (**Figure 1D** and **Table 1**). Isoflavones made up 6.82% (w/w) of the SPI phytochemical extract and 2.87 mg per g of SPI determined by calibration curves from seven concentrations of isoflavones ($n = 3$). This was in good agreement with analyses reported by the manufacturer (**Table 2**).

Quantification of Lyso-PLs. Fifty-six lyso-PL were identified in extracts from SPI in a previous report from our laboratory (*18*). In the present study, lyso-PC and lyso-PE, two major subclasses of lyso-PLs, were quantitated by direct comparison with their corresponding standards or their structure-related standards. Because lyso-PC and lyso-PE do not have UV absorption properties and yield simple and characteristic mass spectra, quantification was based on the areas of extracted major ions from MS in the positive ion mode. Concentrations of lyso-PCs and lyso-PEs were 0.50 and 0.23% (w/w) of the SPI phytochemical extract, respectively (**Table 2**).

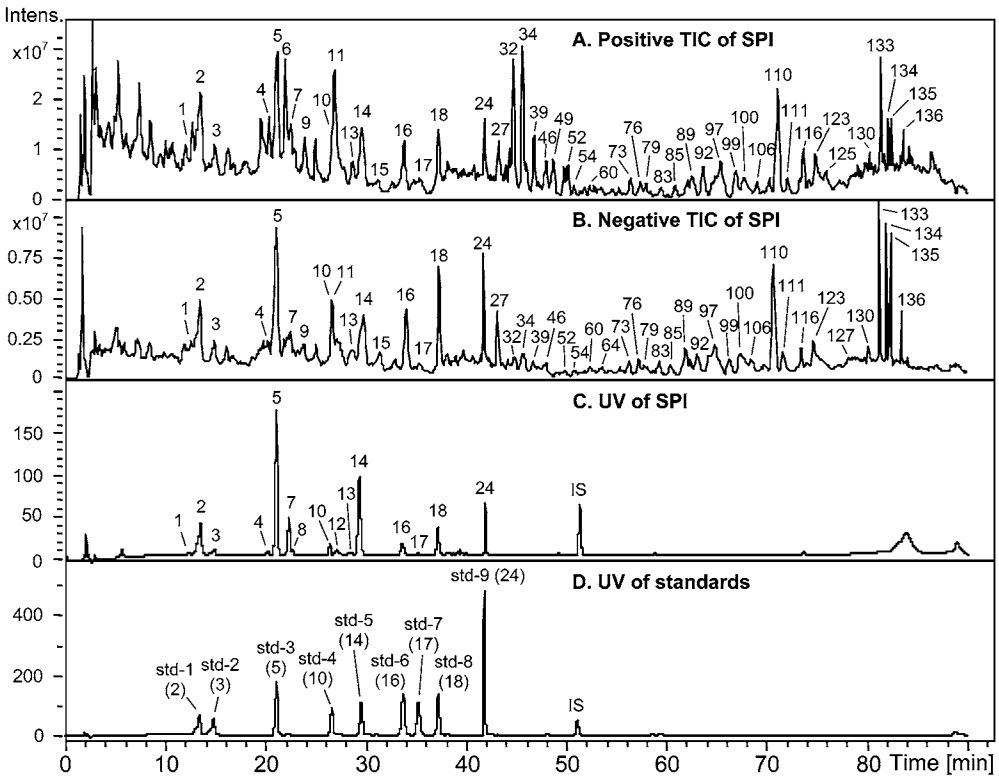
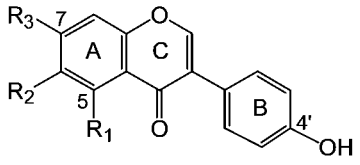


Figure 1. LC-MS/MS chromatograms: (**A**) positive TIC of SPI extract; (**B**) negative TIC of SPI extract; (**C**) UV (265 ± 10 nm) chromatogram of SPI extract; (**D**) UV (265 ± 10 nm) chromatogram of isoflavone standards; IS, biochanin A. Isoflavones: **1**–**5**, **7**, **8**, **10**, **12**–**18**, and **24** (**Table 1**). Folates: **6**, **9**, and **11** (**Figure 3**). Soyasaponins: **21**–**23**, **30**–**32**, **34**, **35**, **37**, **38**, **41**, **44**, **45**, **47**, **49**, **51**–**54**, **56**, **58**, and **63** (**Table 3**). Fatty acids: **25**–**29**, **33**, **36**, **42**, **57**, **59**, **60**, **62**, **66**, **68**, **70**, **73**, **76**, **79**, **81**, **83**, **84**, **86**, **88**, **94**, **96**, **103**, **110**, **112**, **114**, **116**, **118**, **122**, and **130**–**136** (**Table 4**). Lyso-PLs: **80**, **87**, **89**, **92**, **95**, **97**, **99**, **109**, and **125** (**Table 2**). Compounds that were not shown in the tables and identified in ref *18*: **19**, triOH-18:1a/lyso-PC; **20**, triOH-18:1a/lyso-PA; **38**, lyso/OH-18:2a-PC; **40**, lyso/OH-18:2a-PE; **43**, OH-18:2a/lyso-PC; **46**, OH-18:2a/lyso-PI; **48**, OH-18:2a/lyso-PC; **50**, OH-18:2a/lyso-PC; **55**, OH-18:2a/lyso-PC; **61**, OH-lyso/18:2a-PA; **64**, OH-18:2a/lyso-PA; **65**, OH-lyso/18:2a-PA; **67**, lyso/14:0a-PC; **69**, OH-18:2a/lyso-PA; **71**, 14:0a/lyso-PE; **72**, lyso/18:3a-PE; **74**, 14:0a/lyso-PC; **75**, lyso/18:3a-PC; **77**, lyso/18:3a-PI; **78**, 18:3a/lyso-PE; **82**, 18:3a/lyso-PI; **85**, lyso/18:2a-PE; **90**, lyso/18:2a-PI; **91**, lyso/16:0a-PE; **93**, 18:2a/lyso-PI; **98**, lyso/16:0a-PI; **100**, lyso/18:1a-PE; **101**, 16:0a/lyso-PI; **102**, lyso/18:3a-PA; **104**, lyso/18:1a-PI; **105**, lyso/18:1a-PC; **106**, 18:1a/lyso-PE; **107**, 18:3a/lyso-PA; **108**, lyso/18:2a-PG; **111**, 18:1a/lyso-PI; **113**, 18:2a/lyso-PG; **115**, lyso/18:0a-PE; **117**, lyso/18:2a-PA; **119**, lyso/16:0a-PG; **120**, 18:0a/lyso-PE; **121**, lyso/18:0a-PC; **123**, 18:2a/lyso-PA; **124**, 16:0a/lyso-PG; **126**, lyso/18:0a-PI; **127**, lyso/16:0a-PA; **128**, 18:0a/lyso-PI; **129**, 16:0a/lyso-PA.

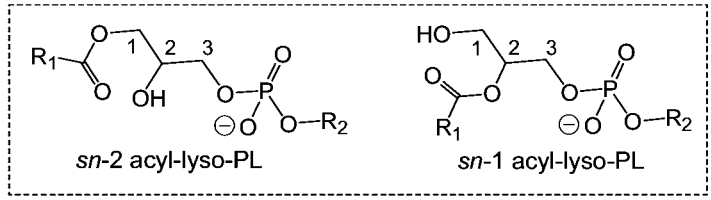
Identification of Soyasaponins Associated with SPI. Saponins are triterpenoid or steroid glycosides. Saponins in soybeans (soyasaponins) are divided into three groups on the basis of their aglycone structures: group A, B, and E soyasaponins (*19*) (**Figure 2**). Group B soyasaponins have been reported as the major soyasaponins in soybeans (*20*). Five 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP)-conjugated group B soyasaponins were considered the naturally occurring saponins of the soybean, and several non-DDMP counterparts were products formed during treatment (*19*). In the present study, all five DDMP-conjugated soyasaponins and their non-DDMP counterparts were found in SPI on the basis of their MS data (**Table 3**). The DDMP-conjugated soyasaponins were compounds **45** (soyasaponin αg), **49** (soyasaponin *β*g), **53** (soyasaponin *β*a), **58** (soyasaponin *γ*g), and **63** (soyasaponin *γ*a), and their non-DDMP counterparts were **30** (soyasaponin V), **32** (soyasaponin I), **34** (soyasaponin II), **37** (soyasaponin III), and **39** (soyasaponin IV). MS data suggested that compounds **31** and **35** were isomers of **34** and **39**, respectively (**Table 3**), but their exact structures could not be determined only by LC-MS/MS in this study. A further degradation product (**54**) of DDMP-conjugated soyasaponin, soyasapogenol B monoglucuronide (*21*), was also found in SPI. Soyasapogenol E and soyasapogenol A are two hydrogen atoms (2 Da) less and one oxygen (16 Da) more than soyasapogenol B, respectively. Under this MS/MS condition, the product ion patterns of group A, B, and E soyasaponins were dominated by the same CID pathway (**Table 3**). Six group E soyasaponins (**41**, **44**, **47**, **51**, **52**, and **56**) were identified in SPI by comparison of MS data between these compounds and their corresponding group B soyasaponins (**Table 3**). Soyasapogenol A (aglycone of group A soyasaponins) has one more hydroxyl group at C-21 than soyasapogenol B, and group A soyasaponins are either bis-desmosides at C-3 and C-22 or mono-desmosides (*22*). Compounds **21**−**23** were identified as group A soyasaponins based on comparison of MS data with that of group E soyasaponins, but MS data failed to provide evidence for determination of positions of ether-linked sugar chains. Therefore, compounds **21**−**23** were tentatively named as group A soyasaponins 1, 2, and 3, respectively, in this study. Compounds **32** (soyasaponin I) and **34** (soyasaponin II) were two of the most abundant peaks in the positive MS TIC profile (**Figure 1A**). Five soyasaponin components (**32**, **34**, **45**, **49**, and **53**) have been previously reported in the same material that we used in the present study, and the concentrations of soyasaponins in the SPI (9.51 *μ*mol/g) were significantly higher than that in the raw soybean flour (3.31 *μ*mol/g) (*12*).

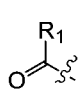
Structures of isoflavones:



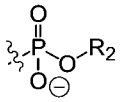
No.	name	R_1	R_2	R_3
16	daidzein	H	H	OH
2	daidzin	H	H	OGlc
10	6"-*O*-acetyldaidzin	H	H	OacetylGlc
7	6"-*O*-malonyldaidzin	H	H	OmalonylGlc
24	genistein	OH	H	OH
5	genistin	OH	H	OGlc
18	6"-*O*-acetylgenistin	OH	H	OacetylGlc
14	6"-*O*-malonylgenistin	OH	H	OmalonylGlc
17	glycitein	H	OH	OH
3	glycitin	H	OH	OGlc
12	6"-*O*-acetylglycitin	H	OH	OacetylGlc
8	6"-*O*-malonylglycitin	H	OH	OmalonylGlc

Structures of lysophospholipids:*

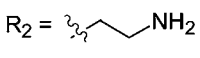




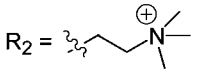
fatty acyl groups



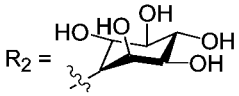
polar headgroups



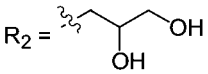
phosphatidyl ethanolamine



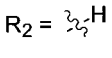
phosphatidyl choline



phosphatidyl inositol



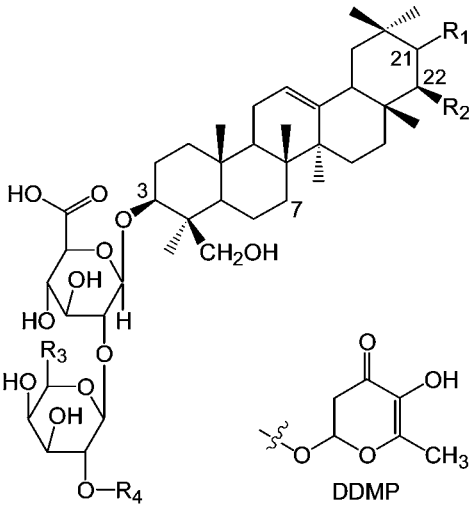
phosphatidyl glycerol



phosphatidic acid

* see ref. 18 for structures of 56 lysophospholipids associated with SPI.

Structures of soyasaponins:*



No.	name	R_1	R_2	R_3	R_4
	Group B soyasaponins:				
	Aglycone	H	OH	OH at C-3	
49	soyasaponin βg	H	O-DDMP	CH_2OH	Rha
53	soyasaponin βa	H	O-DDMP	H	Rha
58	soyasaponin γg	H	O-DDMP	CH_2OH	H
63	soyasaponin γa	H	O-DDMP	H	H
45	soyasaponin αg	H	O-DDMP	CH_2OH	Glc
32	soyasaponin I	H	OH	CH_2OH	Rha
34	soyasaponin II	H	OH	H	Rha
37	soyasaponin III	H	OH	CH_2OH	H
39	soyasaponin IV	H	OH	H	H
30	soyasaponin V	H	OH	CH_2OH	Glc
	Group E soyasaponins:				
	Aglycone	H	=O	OH at C-3	
47	soyasaponin I	H	=O	CH_2OH	Rha
51	soyasaponin II	H	=O	H	Rha
52	soyasaponin III	H	=O	CH_2OH	H
56	soyasaponin IV	H	=O	H	H
41	soyasaponin V	H	=O	CH_2OH	Glc
	Group A soyasaponins:				
	Aglycone	OH	OH	OH at C-3	

* **54** is soyasapogenol B monoglucuronide (B M).

Figure 2. Structures of phytochemicals associated with SPI.

Identification of Fatty Acids Associated with SPI. The SPI studied in this report contained 2.7% fat (data from manufacturer), and we identified 39 fatty acids in SPI (**Figure 1** and **Table 4**). These consisted of typical fatty acids, such as linolenic acid, myristic acid, palmitoleic acid, linoleic acid, palmitic acid, oleic acid, and stearic acid, as well as 32 oxygenated fatty acids. While fatty acids with one extra oxygen atom were clearly designated as monohydroxylated fatty acids, structures of fatty acids with two or three extra oxygen atoms could be either di- or trihydroxylated fatty acids or hydroperoxy fatty acids. The identification of 32 oxygenated fatty acids could not be completed by LC-MS/MS in this study (**Table 4**). A hydroxyl-

Table 2. Phytochemical Content in SPI

no. in **Figure 1**	mg/g ext.[a]	% in ext.	μmol/g SPI	μg/g SPI	% in SPI	mg/25 g SPI[b]
			isoflavones[c]			
1. genistein-glucoside	0.36 ± 0.03	0.036	0.03 ± 0.003	15.01 ± 1.41	0.002	0.38 ± 0.04
2. daidzein 7-glucoside	9.89 ± 0.11	0.989	1.00 ± 0.012	416.40 ± 4.79	0.042	10.41 ± 0.12
3. glycitein 7-glucoside	1.86 ± 0.14	0.186	0.18 ± 0.013	78.45 ± 5.73	0.008	1.96 ± 0.14
4. daidzein-malonylglucoside	1.37 ± 0.08	0.137	0.13 ± 0.007	57.88 ± 3.21	0.006	1.45 ± 0.08
5. genistein 7-glucoside	15.58 ± 0.25	1.558	1.52 ± 0.024	655.96 ± 10.52	0.066	16.40 ± 0.26
7. daidzein 7-malonylglucoside	7.69 ± 0.10	0.769	0.65 ± 0.008	323.93 ± 4.22	0.032	8.10 ± 0.11
8. glycitein 7-malonylglucoside	1.36 ± 0.12	0.136	0.13 ± 0.011	57.33 ± 4.89	0.006	1.43 ± 0.12
10. daidzein 7-acetylglucoside	2.60 ± 0.06	0.260	0.24 ± 0.005	109.57 ± 2.45	0.011	2.74 ± 0.06
12. glycitein 7-acetylglucoside	2.39 ± 0.09	0.239	0.21 ± 0.008	100.65 ± 3.75	0.010	2.52 ± 0.09
13. genistein-malonylglucoside	0.97 ± 0.09	0.097	0.09 ± 0.008	40.83 ± 3.88	0.004	1.02 ± 0.10
14. genistein 7-malonylglucoside	14.54 ± 0.09	1.454	1.18 ± 0.007	612.03 ± 3.74	0.061	15.30 ± 0.09
16. daidzein	2.04 ± 0.06	0.204	0.34 ± 0.010	85.71 ± 2.49	0.009	2.14 ± 0.06
17. glycitein	0.29 ± 0.01	0.029	0.04 ± 0.001	12.28 ± 0.40	0.001	0.31 ± 0.01
18. genistein 7-acetylglucoside	4.80 ± 0.09	0.480	0.43 ± 0.008	201.90 ± 3.73	0.020	5.05 ± 0.09
24. genistein	2.47 ± 0.03	0.247	0.39 ± 0.004	103.98 ± 1.13	0.010	2.60 ± 0.03
subtotal	68.22 ± 0.43	6.822	6.54 ± 0.042	2871.90 ± 18.04	0.287	71.80 ± 0.45
			soyasaponins[d]			
32. soyasaponin B I	125.08	12.508	5.59	5265.78	0.527	131.64
34. soyasaponin B II	54.16	5.416	2.50	2280.00	0.228	57.00
45. soyasaponin B αg	1.80	0.180	0.07	75.88	0.008	1.90
49. soyasaponin B βg	25.62	2.562	1.01	1078.68	0.108	26.97
53. soyasaponin B βa	8.14	0.814	0.33	342.54	0.034	8.56
subtotal	214.80	21.48	9.51	9042.88	0.904	226.07
			lyso-PC[c]			
80. 18:3a/lyso-PC	0.11 ± 0.019	0.011	0.009 ± 0.002	4.71 ± 0.796	0.0005	0.12 ± 0.02
87. lyso/18:2a-PC	0.30 ± 0.012	0.030	0.024 ± 0.001	12.53 ± 0.513	0.0012	0.31 ± 0.01
92. 18:2a/lyso-PC	2.13 ± 0.100	0.213	0.173 ± 0.008	89.55 ± 4.199	0.0090	2.24 ± 0.10
95. lyso/16:0a-PC	0.15 ± 0.015	0.015	0.013 ± 0.001	6.51 ± 0.644	0.0007	0.16 ± 0.02
99. 16:0a/lyso-PC	1.45 ± 0.058	0.145	0.123 ± 0.005	61.05 ± 2.448	0.0061	1.53 ± 0.06
109. 18:1a/lyso-PC	0.58 ± 0.013	0.058	0.046 ± 0.001	24.35 ± 0.534	0.0024	0.61 ± 0.01
125. 18:0a/lyso-PC	0.30 ± 0.024	0.030	0.024 ± 0.002	12.76 ± 0.989	0.0013	0.32 ± 0.02
subtotal	5.02 ± 0.075	0.502	0.413 ± 0.006	211.45 ± 3.143	0.0211	5.29 ± 0.08
			lyso-PE			
89. 18:2a/lyso-PE	1.15 ± 0.09	0.115	0.101 ± 0.008	48.40 ± 3.69	0.005	1.21 ± 0.09
97. 16:0a/lyso-PE	1.14 ± 0.10	0.114	0.106 ± 0.009	48.15 ± 4.12	0.005	1.20 ± 0.10
subtotal	2.29 ± 0.18	0.229	0.207 ± 0.017	96.55 ± 7.78	0.010	2.41 ± 0.19
subtotal of fat including 39 fatty acids[e]	641.33	64.13		27 000	2.7	675
subtotal of three folates and unknown compounds	unknown					

[a] The phytochemical extract (168.2 mg/4 g SPI) represented 4.21% of SPI (w/w). [b] The FDA approved a health claim for soy protein that states "25 g of soy protein a day may reduce the risk of heart disease" (*47*). [c] Data represent means (± SD) obtained from three replicate LC-MS/MS analyses of a set of samples including SPI extract and standards. [d] The μmol/g SPI data of five soyasaponins were quoted from ref *12*, and other data for soyasaponins were converted from original μmol/g data by authors. The other 17 soyasaponins that we identified in SPI were not reported in ref *12*. [e] The total amount of fat by acid hydrolysis is 2.7% of SPI reported by the manufacturer, and other data were converted from original data by authors.

ated octadecadienoic acid (OH-18:2, **110**) appeared as a prominent peak in both positive and negative TIC of SPI (**Figure 1A**,**B**).

Identification of Folates Associated with SPI. Folates are derivatives of tetrahydrofolic acid, a water soluble B vitamin. The major peak **11** (**Figure 1**) was identified as folic acid, and peaks **6** and **9** were characterized as folates (**Figure 3**). MS data indicated that compound **6** is (1-carboxy-ethylamino)folic acid, and structural elucidation of **9** could not be completed by LC-MS/MS in this study.

Potential Health Benefits of Phytochemicals Bound to SPI. Clinical and animal studies suggest significant putative health benefits, as well as possible adverse health effects, of consuming soybeans and components of soybeans, including SPI. Isoflavones are the most widely studied of the soy phytochemicals and are considered major bioactive constituents associated with SPI. Actually, isoflavones accounted for only 16 of 136 phytochemicals that we identified in SPI, and the total amount of isoflavones was only 6.82% of the phytochemical extract or 0.29% of SPI (w/w). However, isoflavones in foods containing SPIs are known to be both bioavailable and bioactive, whereas less is known about other SPI phytochemicals (*23*−*31*).

Little attention had been paid to nonisoflavone phytochemicals found in SPIs, such as the oxygenated fatty acids, saponins, or lyso-PL. This is primarily because isoflavones have been known for decades to have powerful biological effects in vivo and in vitro, and investigators have focused on high profile metabolic and endocrinologic systems involving the estrogen receptor and which have many health-related implications; there are very few published reports on the identity of other phytochemicals bound to SPI, mainly because standards for these compounds are not readily available to allow for easy identification and quantitation. Thus, less is known about other SPI phytochemicals and, other than isoflavones, soy phytochemicals or their metabolites have not been found in high concentrations in blood or urine of people or animals after consuming soy foods.

Although the total fat content of SPI is low, typically ranging from 2.5 to 5%, the fatty acid group makes up the majority of the phytochemicals in SPI, representing almost 64% of the total weight of the methanol extract studied in this report. Many of the typical fatty acids were found in SPI, with linoleic acid being the most prominent. Although individual fatty acids were not quantitated, it is clear that the majority of the SPI fatty acids

Table 3. ESI-MS Data for Soyasapogenol Associated with SPI

DDMP-Conjugated Group B Soyasaponins

		product ions in negative CID spectra, *m/z* (relative intensity, %)[a]														
no. in **Figure 1**	R_t (min)	$[M-H]^-$ (parent)	$[M-H-H_2O]^-$	$[M-H-62]^-$	$[M-H-100]^-$	$[M-H-126]^-$	$[M-H-sugar]^-$	$[M-H-188]^-$	$[M-H-2sugar]^-$	$[759-H_2O]^-$	$[M-H-sugar-188]^-$	$[759-2H_2O]^-$	$[759-108]^-$	$[Agly]^-$	$[Agly-32]^-$	$[Agly-126]^-$
45	47.7	1083	1065 (56)	1021 (8)	983 (100)		921 (19)	895 (81)	759 (79)	741 (30)	733 (98)	723 (92)	651 (93)		551 (12)	
49	48.5	1067	1049 (82)	1005 (59)	967 (59)	941 (7)	921 (74)	879 (84)	759 (32)	741 (52)	733 (100)	723 (42)	651 (28)	583 (80)	551 (16)	457 (16)
53	50.3	1037	1019 (62)	975 (4)	937 (100)		891 (19)	849 (16)		741 (40)	703 (12)	723 (10)	651 (15)	583 (22)		457 (15)
58	51.7	921			821 (100)					741 (38)		723 (10)	651 (11)	583 (31)		457 (32)
63	52.7	891			791 (18)	765 (57)	759 (39)			741 (100)	703 (28)		651 (14)			457 (18)

Group B Soyasaponins

		product ions in negative CID spectra, *m/z* (relative intensity, %)[a]												
no. in **Figure 1**	R_t (min)	$[M-H]^-$ (parent)	$[M-H-H_2O]^-$	$[M-H-62]^-$	$[M-H-sugar]^-$	$[M-H-sugar-62]^-$	[Glu Agly]$^-$	[Glu Agly – 18]$^-$	[Glu Agly – 36]$^-$	[Glu Agly – 62]$^-$	[Glu Agly – 108]$^-$	$[Agly]^-$	[Glu Agly – 196]$^-$	other ions
30	44.0	957	939 (66)	895 (42)	795 (40)	733 (25)	633 (7)	615 (68)	597 (78)	571 (12)	525 (100)	457 (15)	437 (16)	
31	44.3	911	893 (100)	849 (16)	765 (28)	703 (13)		615 (30)	597 (31)	571 (54)	525 (19)	457 (8)		425 (21)
32	44.5	941	923 (100)	879 (62)	795 (8)	733 (20)	633 (2)	615 (8)	597 (64)	571 (6)	525 (94)	457 (17)	437 (13)	751 (16), 425 (14)
34	45.4	911	893 (100)	849 (11)	765 (2)	703 (26)	633 (16)	615 (32)	597 (48)	571 (7)	525 (13)	457 (21)	437 (14)	867 (20), 721 (22), 553 (3)
35	45.6	765					633 (56)	615 (58)		571 (32)		457 (64)	437 (43)	517 (100)
37	45.8	795		733 (20)			633 (45)	615 (100)	597 (17)	571 (6)	525 (21)	457 (35)	437 (8)	553 (13), 479 (25), 465 (17)
39	46.5	765	747 (60)				633 (100)	615 (13)			525 (24)	457 (34)	437 (47)	
54	50.6	633	615 (53)									457 (74)	437 (89)	573 (100), 499 (25)

Group E Soyasaponins

		product ions in negative CID spectra, *m/z* (relative intensity, %)[a]													
no. in **Figure 1**	R_t (min)	$[M-H]^-$ (parent)	$[M-H-H_2O]^-$	$[M-H-62]^-$	$[M-H-Rha]^-$	$[M-H-Rha-62]^-$	[Glu Agly]$^-$	[Glu Agly – 18]$^-$	[Glu Agly – 36]$^-$	[Glu Agly – 62]$^-$	[Glu Agly – 108]$^-$	$[Agly]^-$	[Glu Agly – 196]$^-$	$[Agly-48]^-$	other ions
41	47.0	955	937 (29)					613 (27)	595 (100)		523 (36)	455 (14)		407 (74)	851 (20), 849 (47)
44	47.6	909	891 (100)	847 (39)	763 (12)	701 (42)			595 (25)	569 (9)	523 (18)	455 (35)	435 (24)		495 (14), 423 (8)
47	47.9	939	921 (100)	877 (92)	793 (52)	731 (51)	631 (13)	613 (16)	595 (31)	569 (23)	523 (44)	455 (17)	435 (5)	407 (21)	749 (15), 775 (10)
51	49.6	909	891 (100)	884 (59)	763 (2)	701 (62)	631 (3)	613 (45)	595 (20)	569 (4)	523 (25)	455 (88)		407 (59)	719 (15), 495 (47)
52	49.9	793						613 (100)			523 (83)	455 (14)		407 (100)	477 (49)
56	51.2	763						613 (99)		569 (100)		455 (34)			391 (32)

Group A soyasaponins:b

		product ions in negative CID spectra, *m/z* (relative intensity, %)[a]												
no. in **Figure 1**	R_t (min)	$[M-H]^-$ (parent)	$[M-H-H_2O]^-$	$[M-H-62]^-$	$[M-H-Rha]^-$	$[M-H-Rha-62]^-$	[Glu Agly]$^-$	[Glu Agly – 18]$^-$	[Glu Agly – 36]$^-$	[631 – 62]$^-$	[Glu Agly – 108]$^-$	$[Agly]^-$	$[Agly-H_2O]^-$	other ions
21	40.5	957	939 (100)	895 (15)	811 (10)	749 (54)		631 (52)	613 (52)	569 (59)	541 (60)	473 (25)	465 (29)	909 (43), 823 (32), 767 (70)
22	40.7	927	909 (100)	865 (28)				631 (70)	613 (45)		541 (21)	473 (16)		737 (23), 509 (45)
23	40.9	811						631 (68)			541 (100)	473 (16)		686 (30), 483 (18), 425 (46)

[a] Sugar, rhamnopyranosyl, or glucopyranosyl; Agly, aglycone; Glu Agly, glucuronopyranosyl aglycone; Rha, rhamnopyranosyl. [b] Structural elucidation of compounds **21**–**23** could not be completed in this study because MS data failed to provide evidences for the determination of positions of ether-linked sugar chains.

are oxidized (**Table 4**). The origin of the oxygenated fatty acids (hydroxylated and/or hydroperoxidated fatty acids) is unknown, but they probably develop during processing. The chemical structures and biological actions of these compounds are not well-known, and future studies in our laboratory will chemically elucidate their structure and investigate their bioavailability and bioactivity.

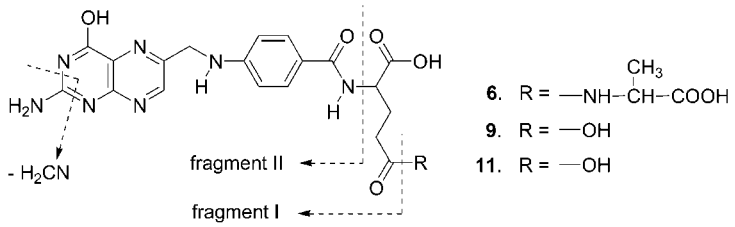
Soyasaponins are present in SPI at three times the levels of isoflavones (9.04 vs 2.8 mg/g). LC-MS/MS analysis revealed 22 soyasaponins in SPI, and two major peaks (**32** and **34**) were soyasaponins (**Figure 1**). While these compounds have been reported to have several bioactivities, including effects on cholesterol reduction, colon cancer, protection against chemical hepatotoxicity, estrogen actions, and cell proliferation (*13*−*17*, *32*), they are not well-absorbed; thus, their abilities to reach target tissues at biologically relevant levels is questionable. However, saponins are present in high concentrations in the gastrointestinal (GI) tract after a soy meal, and thus, they could be biologically important in GI tract development, GI function/health by interacting with the endothelial cell lining or through actions on gut microflora, or other physiologic or metabolic systems by acting indirectly through other mediators. It is also possible that saponin absorption in the developing GI tract of infants could be greater from that of adults, and greater levels

Table 4. Negative-ion ESI MS/MS Data for Free Fatty Acids Associated with SPI

no. in **Figure 1**	R_t (min)	$[M-H]^-$ (parent)	product ions in negative CID spectra, *m*/*z* (relative intensity, %)												structure
fatty acids with two oxygen atoms (nonhydroxy-fatty acids)															
130	79.9	277	259 (30.8)		233 (100)								linolenic acid		std: 18:3 (*cis*-9,12,15)
131	80.0	227											myristic acid		std: 14:0
132	80.6	253	235 (100)										palmitoleic acid		std: 16:1
133	81.0	279	261 (100)										linoleic acid		std: 18:2 (*cis*-9,12)
134	81.8	255	237 (100)										palmitic acid		std: 16:0
135	82.1	281	263 (100)										oleic acid		std: 18:1 (*cis*-9)
136	83.3	283	265 (100)										stearic acid		std: 18:0
fatty acids with three oxygen atoms (monohydroxy-fatty acids)															
94	64.7	293	275 (100)	257 (13)		231 (23)	205 (5)	195 (88)			177 (18)	171 (15)			OH-18:3
96	65.1	293	275 (87)	257 (6)		231 (43)	205 (36)	195 (100)		179 (58)					OH-18:3
103	68.3	295	277 (100)	259 (5)		233 (7)		195 (55)							OH-18:2
110	70.5	295	277 (100)		251 (4)	233 (5)		195 (59)		179 (13)		171 (7)		113 (4)	OH-18:2
112	71.5	295	277 (100)	259 (3)		233 (5)						171 (89)			OH-18:2
114	72.8	293	275 (13)		249 (61)			195 (15)		179 (13)	177 (22)			113 (100)	OH-18:3
116	73.3	293	275 (12)		249 (86)					179 (11)	177 (11)			113 (100)	OH-18:3
118	73.8	293	275 (44)	257 (7)	249 (38)		205 (32)	195 (33)	185 (100)				167 (83)		OH-18:3
122	74.5	293	275 (20)	257 (7)					185 (100)		177 (8)	171 (14)	167 (16)		OH-18:3
fatty acids with four oxygen atoms (hydroxylated or hydroperoxidated-fatty acids)															
57	51.6	309	291 (100)		221 (33)	209 (27)			195 (60)			185 (28)			C18 derivative
59	52.1	309	291 (100)		221 (41)	209 (16)			195 (12)			179 (8)			C18 derivative
60	52.2	311	293 (79)	275 (14)	223 (100)				195 (8)			177 (25)			C18 derivative
62	52.6	309	291 (100)		221 (15)				195 (52)		171 (10)				C18 derivative
66	54.3	313	295 (56)	277 (18)					195 (49)	183 (100)					C18 derivative
68	55.0	311	293 (50)	275 (14)			201 (100)			183 (8)	171 (21)	209 (16)			C18 derivative
70	55.4	313	295 (68)	277 (23)			201 (100)				171 (7)				C18 derivative
73	56.1	313	295 (100)	277 (39)					195 (34)	183 (97)					C18 derivative
76	57.0	313	295 (45)	277 (57)			201 (100)				171 (68)				C18 derivative
79	57.6	311	293 (100)	275 (18)		211 (46)		197 (70)				181 (18)	169 (20)	129 (28)	C18 derivative
81	58.5	311	293 (100)	275 (26)		211 (34)	201 (60)	197 (45)			171 (67)				C18 derivative
83	59.2	311	293 (100)	275 (11)		211 (53)		197 (32)				129 (17)			C18 derivative
84	60.1	315	297 (99)	279 (92)							171 (100)	143 (39)			C18 derivative
86	60.7	315	297 (100)	279 (41)						183 (53)		199 (19)	169 (26)	155 (64)	C18 derivative
88	62.1	315	297 (100)	279 (27)							171 (46)	141 (20)			C18 derivative
fatty acids with five oxygen atoms (hydroxylated and/or hydroperoxidated-fatty acids)															
25	41.7	327	309 (14)	291 (85)	273 (13)	239 (15)	229 (65)	211 (23)					171 (100)		C18 derivative
26	42.0	327	309 (72)	291 (71)		239 (56)	229 (53)	211 (98)	201 (48)				171 (100)		C18 derivative
27	42.9	329	311 (33)	293 (41)			229 (95)	211 (100)					171 (26)		C18 derivative
28	43.2	329	311 (19)	293 (29)				211 (17)					171 (100)	139 (13)	C18 derivative
29	43.5	327	309 (21)	291 (81)	273 (7)	239 (100)					197 (9)			221 (13)	C18 derivative
33	44.7	329	311 (83)	293 (26)	275 (10)			211 (100)		199 (63)		181 (44)		169 (43)	C18 derivative
36	45.6	329	311 (100)	293 (38)	275 (15)			211 (49)	201 (45)	199 (14)	197 (35)	181 (48)	171 (53)	169 (29)	C18 derivative
42	47.1	329	311 (100)	293 (42)	275 (39)			211 (61)	201 (72)	199 (13)		181 (20)	171 (64)	125 (26)	C18 derivative

of saponins may reach target cells. Furthermore, reported in vivo effects, such as retardation of kidney disease (*33*), depressor and bradycardia actions (*34*), enhancement of immune function (*35*), and protection against liver injury (*36*) suggest that soyasaponins may be bioavailable but circulate at undetectable levels and must be very potent, metabolized to other bioavailable bioactive components that have not been detected, or able to exert their biological effects by acting indirectly through other mediators.

Fifty-six lyso-PL were identified, and the total content of nine major lyso-PL components in SPI was 0.31 mg/g SPI. Lyso-PL are normal constituents of cellular membranes (*37*, *38*), body fluids, and lipoproteins (*39*, *40*). Lyso-PL have been known for decades to have diverse effects on growth and cellular functions in multiple organ systems implicated in pathophysiological conditions, including cardiovascular disease, cancer, neurological disease, and immune function (*41*−*43*). Lyso-PL can influence membrane permeability and integrity (*44*) and exogenous lyso-



No. in Figure	Rt. (Min.)	Positive Ionization mode, m/z (rel. intens. %)							Negative Ionization mode, m/z (rel. intens. %)						Structure
		Major ions in MS		Product ions in CID spectra					Product ions in CID spectra						
		$[M+H]^+$	$[II]^+$	parent	$[I]^+$	$[II]^+$	$[II-H_2CN]^+$	Other ions	Parent[a]	[parent-44]$^-$	[parent-106]$^-$	$[II]^-$		Other ions	
6	21.8	513 (100)	311 (69)	513	424 (100)	311 (42)	283 (23)	495 (7), 203 (13)	511	467 (3)	405 (2)		258 (100)	422 (18), 378 (6), 214 (11), 171 (4)	Folate
9[b]	23.8	524 (100)	393[b] (46)	524	506 (100)	393 (59)	365 (5)	375 (34), 292 (20)	522	478 (100)				448 (3), 460 (3), 317 (3)	Folate
11	26.8	442 (78)	311 (100)	442	424 (9)	311 (100)	283 (53)		440	396 (8)	334 (31)	309 (100)	232 (18)	217 (32), 203 (13), 130 (39)	Folic acid

a. $[M-1]^-$ are base peaks in MS.
b. An unknown part of structure (82 Da) substitute on pteroyl (fragment II of structure) based on MS data, but final structural elucidation of compound **9** could not be achieved only by MS data in this study.

Figure 3. MS data and proposed diagnostic mass spectral fragmentation pathways of folates.

PL can enter the neutrophil membrane (*45*). In an in vitro study, lyso-PC was found to increase the DNA binding activity of NF-*k*B in cultured human umbilical vein endothelial cells (*46*), and cytotoxic effects of lyso-PC were observed in MCF-7 cells in our laboratory (data not shown). There are no published data on the effects of lyso-PLs from dietary intake.

Much less is known about the bioavailability and potency of nonisoflavone SPI phytochemicals. These other phytochemicals may be most important in infants fed soy formula, as these children are clearly exposed to the highest level of these compounds. However, the low levels of other less abundant phytochemicals bound to SPI make it less likely that any one phytochemical would have significant health consequences, unless it had extremely high bioavailability and potency or acted in synergy with other bioactive compounds. Further studies in our laboratory will focus on determining the bioavailability and bioactivity of some of these compounds.

It is important to note that there are several different SPI products and their phytochemical profiles are significantly variable depending on their SPI processing. While water washing concentrates the protein by removing very polar phytochemicals and oligosaccharides, ethanol washing substantially decreases the concentrations of these polar lipophilic phytochemicals that we discussed in this study.

ABBREVIATIONS USED

SPI, soy protein isolate; ESI-MS, electrospray ionization mass spectrometry; API, atmospheric pressure interface; API-ES, atmospheric pressure interface-electrospray; ICC, ion charge control; TIC, total ion chromatogram; EIC, extracted ion chromatogram; CID, collision-induced dissociation; lyso-PL, lysophospholipids; lyso-PC, lysophosphatidylcholines; lyso-PE, lysophosphatidylethanolamines; lyso-PI, lysophosphatidylinositols; lyso-PA, lysophosphatidic acids; lyso-PG, lysophosphatidylglycerols.

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