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LC-DAD-ESI-MS/MS characterization of soybean phenolics extracted with various solvents

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Abstract

Phenolics are broadly distributed in the plant kingdom and the most abundant secondary metabolites of plants. Over the past few years, preparation of phenolic extracts from different plants, purification of phenolic compounds and their identification have become a major area of health- and medical-related research. In this study, the effect of extraction solvents (methanol, ethanol, acetonitrile and acetone), on total phenolic content, antioxidant activity, and the composition of the phenolic compounds in soybean extracts were studied. The antioxidant activity was determined using two different methods: DPPH and ABTS assays. Liquid chromatography coupled to diode array detection and electrospray ionisation tandem mass spectrometry (LC-DAD-ESI-MS/MS) was used for identification and quantification of phenolic compounds. All the parameters analyzed were found to be affected by the extraction method, especially by the solvent used, and the best results were obtained in the methanolic extract. The methanolic and ethanolic extracts exhibited strong antioxidant activity. Genistein-7-diglucoside, daidzin, genistin, 4' 7 dihidroxiflavan, daidzein, and genistein were found as major isoflavonoids in all extracts.

Keywords: Antioxidant capacity, Extraction methods, LC-MS/MS, Soybean phenolics.

Çeşitli çözgenlerle ekstrakte edilen soya fasulyesi fenoliklerinin LC-DAD-ESI MS/MS ile karakterizasyonu

Özet

Fenolik bileşikler bitkiler aleminde en yaygın ve en fazla bulunan sekonder metabolitlerdir. Geçtiğimiz birkaç yıl içinde, çeşitli bitkilerden fenolik ekstraktların hazırlanması, saflaştırılması ve tanımlanması sağlık üzerine yapılan araştırmalar arasında önemli bir alan oluşturmuştur. Bu çalışmada, ekstraksiyon çözgenlerinin (metanol, etanol, asetonitril ve aseton) soya fasulyelerinin toplam fenolik içeriği, antioksidan aktivitesi ve fenolik bileşik profili üzerine etkisi araştırılmıştır. Ekstraktların antioksidan aktiviteleri DPPH ve ABTS yöntemleriyle belirlenmiştir. Diyot array detektörüne sahip sıvı kromatografisi ve elektrosprey iyonizasyon tandem kütle spektrometresi (LC-DAD-ESI-MS/MS), fenolik bileşikleri tanımlamak ve kantifikasyon amacıyla kullanılmıştır. Tüm parametrelerin ektraksiyon yönteminden özellikle çözgen türünden etkilendiği ve en iyi sonuçların metanol ekstraktı ile elde edildiği belirlenmiştir. Metanol ve etanol ekstraktlarının kuvvetli antioksidan aktiviteye sahip olduğu gözlemlenmiştir. Genistein-7-diglukozit, daidzin, genistin, 4'7 dihidroksiflavan, daidzein, ve genistein bütün ekstraktlardaki önemli izoflavonoidler olarak saptanmıştır.

Anahtar Kelimeler: Antioksidan kapasite, ekstraksiyon yöntemleri, LC-MS/MS, Soya fasulyesi fenolikleri

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1. Introduction

Soybean (*Glycine max* cv. Merr.) is a good source of proteins and bioactive compounds, such as vitamins, carotenoids, saponins and phenolics. The benefits of soybean-based foods for human health are well known, and nowadays the demand for soybean products has increased because of the renewed interest in functional foods. Soybeans contain phenolic compounds (phenolic acids and flavonoids) in high concentrations. Among the phenolic compounds found in soybeans, the most abundant phenolics are isoflavones whose health benefits are well recognized in the world [1-3]

The main isoflavones present in the whole soybean include the following glycosides: daidzin, genistin, and glycitin. Furthermore, the major isoflavones in soybean can be classified as either malonyl or acetyl glycosides, depending on their conjugated functional groups. There are 12 kinds of isoflavones in soybeans that are divided into 4 subgroups: aglycones (daidzein, genistein, and glycitein), glycosides (daidzin, genistin, and glycitin), malonyl glycosides (malonyldaidzin, malonylgenistin, and malonylglycitin), and acetyl glycosides (acetyldaidzin, acetylgenistin, and acetylglycitin) [4].

Various studies have shown the positive effects of isoflavones on health, for example, reduction in the risk of cardiovascular disease and cancer [5, 6]. The consumption of isoflavones results in the reduction of the incidence of heart diseases, menopausal symptoms, bone resorption and etc. In addition, isoflavones exhibits anti-carcinogenic, anti-atherogenic, anti-fungal and anti-oxidant properties [7, 8].

In this study, extraction efficiencies of commonly used solvents (methanol, ethanol, acetone, acetonitrile) were compared. The effects of extraction solvents, on the total phenolic content, the anti-oxidant activity and the composition of phenolic compounds of the soybean extracts were studied. Liquid chromatography coupled to diode array detection and electrospray ionisation tandem mass spectrometry (LC-DAD-ESI-MS/MS) was used for identification and quantification of phenolic compounds. Antioxidant activities of soybean extracts were determined by DPPH and ABTS assays.

2. Material and methods

2.1. Preparation of soybean seed extracts

Soybean seeds were purchased from a local market in Adana. Immediately, after purchase, the material was ground in a home style coffee grinder. The ground material was then passed through a standard 20-mesh sieve (particle size <0.825 mm). The analytical conditions were based on the method described by Luthria et al [8]. Extractions were carried out using eight different solvent mixtures 1) Methyl alcohol:water (90/10, v/v), 2) Methyl alcohol:water (70/30, v/v), 3) Ethyl alcohol:water (90/10, v/v), 4. Ethyl alcohol:water (70/30, v/v); 5. Acetonitrile:water (90/10, v/v); 6. Acetonitrile:water (70/30, v/v); 7. Acetone:water (90/10, v/v); 8. Acetone:water (70/30, v/v). Soybeans flour (10 g) was mixed with different solution, and sonicated for 3 hours; this procedure was performed three times. The supernatants were then separated by centrifuging for 10 min. at 3,000 xg and 5 ºC and combined. The extracts were filtered through a 0.45 μm cellulose acetate filter (Millipore), before the analyses by high-performance liquid chromatography (HPLC). The extraction was performed in duplicate.

2.2. Extraction of phenolic compounds

The ground seed samples were extracted with five different solvent mixtures: 50 or 80% acetone, 50 or 70%ethanol, or 80%methanol (v/v). The extraction was conducted at the sample mass/solvent ratio of 1:20 (g/mL) under shaking in a dark room at ambient temperature for 15 h. This sample mass to solvent volume ratio is selected since the previous experiments showed that the soybeen seed/solvent ratio of 1:10 (g/mL) for 15 h is sufficient to extract most phenolic compounds by 50% acetone [8-11]. Considering the different extraction efficiencies of the solvent mixtures used in this

experiment. The seed/solvent ratio is reduced to 1:20 to ensure that the maximal extraction of soybean anti-oxidants can be achieved. After filtration (Whatman no. 2 filter paper), the extracts were centrifuged by an optima L-90K ultracentrifuge (Beckman Coulter Inc., Brea,CA) at 1500g and 4 °C for 10 min. The supernatant was collected and further filtered with a 0.45 µm syringe filter (Acrodisc, Gelmen Science). The clear filtrate was kept in the refrigerator for further antioxidant analysis.

2.3. LC-DAD-ESI-MS/MS analysis of phenolic compounds

Extractions of soybean polyphenols were carried out according to the method reported by Kelebek [10]. Samples were filtered through a 0.45-µm pore size membrane filter before injection. An Agilent 1100 HPLC system equipped with a DAD was used. The system consisted of a binary pump, degasser, and auto sampler. The column was a Phenomenex Luna reversed-phase C-18 column (4.6 mm×250 mm, 5 µm) (Torrance, California, USA). The mobile phase consisted of two solvents: Solvent A, water/formic acid (99:1; v/v) and Solvent B, acetonitrile/solvent A (60:40; v/v). The identification and assignation of each compound was performed by comparing their retention times and UV spectra with those of authentic standards. The identity of each compound were also confirmed by LC-MS/MS analysis. An Agilent 6430 LC-MS/MS spectrometer equipped with an electrospray ionization source was used. The electrospray ionization mass spectrometry detection was performed in negative and positive ion mode with the following optimized parameters: capillary temperature 400°C; drying gas N_2 12 L/min; nebulizer pressure, 45 psi. Individual compounds were quantified using calibration curves obtained by using corresponding standard compounds. When reference compounds were not available, the calibration of structurally related substances was used, including a molecular weight correction factor. The stock solution was diluted to a series of different concentrations with the methanol, and 10 µL of sample was injected into the HPLC apparatus for analysis.

2.4. Antioxidant assays

The DPPH assay was performed according to the method of Kelebek et al. [11]. Three different dilutions of each extract (1, 20 and 50 mg/mL) were prepared in ethanol/water (v/v). An aliquot of 0.1 mL of diluted extracts was added to 3.9 mL of DPPH solution in methanol (6×10^{-5} M). The mixture was shaken vigorously and left standing at room temperature for 30 minutes. The absorbance of the resulting solution was then measured at 515 nm by a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, California, USA).

The ABTS assay was performed according to the method of Kelebek et al. (2013). The ABTS radical cation was prepared by the reaction of 7 mM ABTS with 2.54 mM potassium persulfate, after incubation at room temperature for 12-16 hours. Prior to the assay, the ABTS solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. A total of 3.9 mL of the diluted ABTS solution was added to 0.1 mL of each sample. The reaction mixture was allowed to stand at room temperature for 30 minutes and the absorbance at 734 nm was immediately recorded.

2.5. Statistical analysis

One way analysis of variance (ANOVA) was applied to indicate the differences among the samples combined with the Fisher's least significant difference test at $p<0.05$ significance level. In addition, Pearson's correlation coefficient, r was calculated to determine the correlation between these parameters. All results were analyzed using Minitab® 17 program (Minitab Inc., State College, USA).

3. Results and discussion

3.1. Identification of phenolic compounds

Daidzein 7-*O*-glucoside (daidzin), daidzein derivative, genistein 7-*O*-glucoside (genistin), genistein malonylglycoside, genistein derivative, daidzein, genistein acetylglycoside and genistein were identified in soybean samples (Table 1). A typical HPLC chromatogram of the isoflavones extracted from soybean sample is shown in Figure 1.

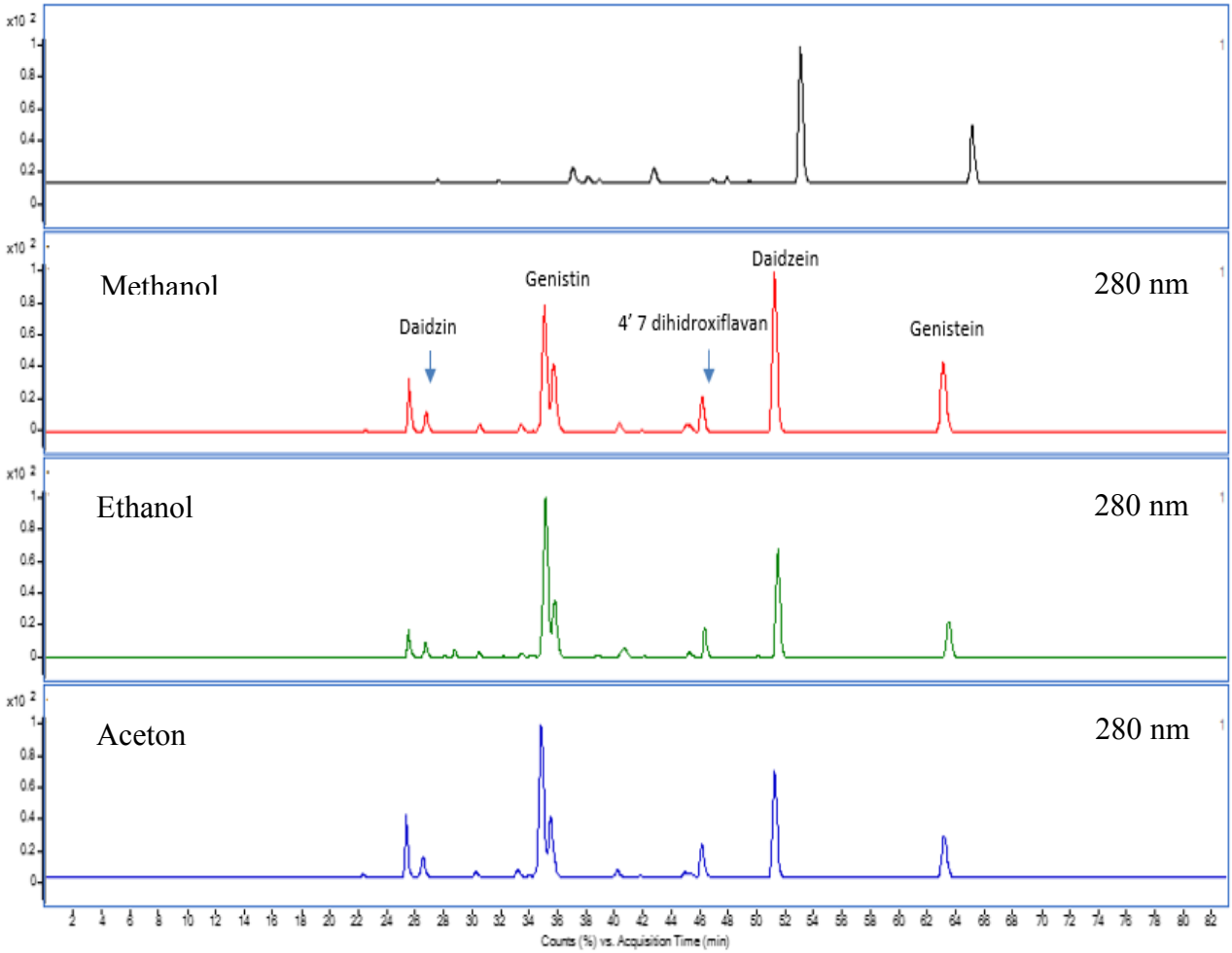

Figure 1. HPLC chromatogram of soybean extracts obtained using different solvents

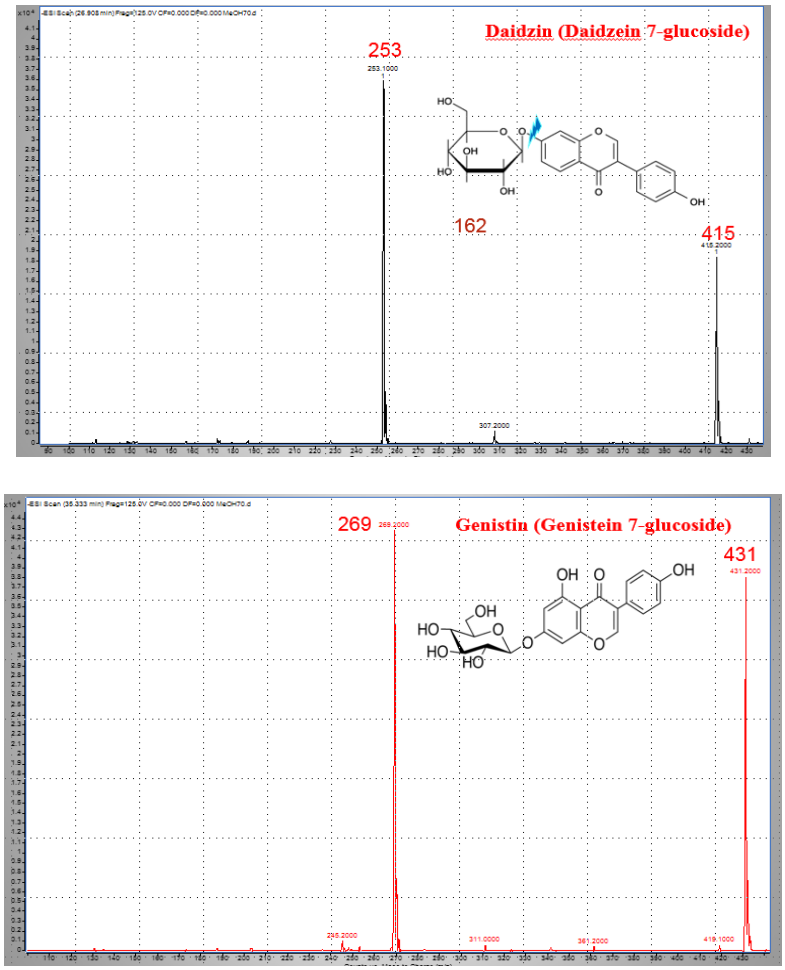

Figure 2. LC-MS/MS spectrum of daidzin and genistin

In DAD detection the spectra of the individual peaks were obtained for the range of wavelength from 200 to 800 nm. The profiles clearly indicated flavonoid-like character of the individual signals (Table 1). Isoflavones are readily distinguished from flavones and flavonols by their UV spectra and by their LC-MS/MS spectrum (Figure 2).

Five peaks were identified as daidzein derivatives according to their UV spectra and MS fragmentation leading to the daidzein aglycone at *m/z* 269 in negative mode. Within the isoflavones, the major peak detected in the extracts was genistin ($[M-H]^-$ at *m/z* 431). Three peaks were identified as daidzein derivatives according to their MS fragmentation leading to the aglycone form at *m/z* 253 in negative mode. The molecular ion fragmentation yielded fragment ions corresponding to daidzin (*m/z* 415) following the loss of the hexose moiety, and finally to daidzein (*m/z* 253) after losing a hexose moiety (162 amu).

Table 1. Retention times, mass spectral characteristics, and identity of isoflavones present in extracts

	Rt (min)	λmax (nm)	[M-H]–	Fragments
Daidzin (Daidzein 7-glucoside)	26.79	256, 313	415	253
Daidzein derivative	27.65	250, 301	253	
Genistin (Genistein 7-glucoside)	35.19	260, 327	431	269
Genistein malonylglycoside	35.99	258, 320	518	269
Genistein derivatives	46.31	258, 330	269	
Daidzein	51.20	250, 298	253	
Genistein acetylglycoside	51.35	259, 320	473	269
Genistein	62.91	260, 326s	269	

3.2. Effect of extraction solvents on isoflavones from soybeans

Individual peak concentrations and HPLC profiles for different isoflavones were significantly dissimilar with different solvent mixtures. There were significant differences ($p < 0.001$) in the isoflavones contents of extracts obtained from a soybean sample using different mixtures. The highest total isoflavones content in the extracts as measured by HPLC was obtained when the extraction was performed using methanol:water (70:30, v/v) mixture. The lowest contents of total isoflavones were obtained with acetonitrile in water (Table 2). Interestingly, the isoflavones content decreased with increasing methanol content in solvent mixture. The similar trend was also observed for ethanol, acetone and acetonitrile solvents.

Table 2. Isoflavone contents (μg/g ± standard deviation) of soybean extracts obtained with different solvents

	Methanol		**Ethanol**		**Acetone**		**Acetonirile**	
	70	**90**	**70**	**90**	**70**	**90**	**70**	**90**
Daidzin (Daidzein 7-glucoside)	77.83±1.09	54.19±0.76	73.31±1.03	36.89±0.52	50.59±0.71	23.20±0.32	25.57±0.36	17.42±0.24
Daidzein derivatives	11.22±0.16	7.45±0.10	3.94±0.06	3.40±0.05	3.34±0.05	2.60±0.04	2.23±0.03	1.46±0.02
Genistin (Genistein 7-glucoside)	103.04±1.44	93.80±1.31	86.18±1.21	71.14±1.00	112.36±1.57	51.16±0.72	80.47±1.13	34.79±0.49
Genistein malonylglycoside	45.12±0.63	24.71±0.35	25.80±0.36	7.18±0.10	23.31±0.33	6.32±0.09	21.55±0.30	16.61±0.23
Genistein derivatives	71.10±1.00	49.15±0.69	64.20±0.90	31.19±0.44	40.72±0.57	22.68±0.32	0.00±0.00	16.58±0.23
Daidzein	7.36±0.10	5.91±0.08	7.07±0.10	4.50±0.06	5.79±0.08	5.03±0.07	4.72±0.07	6.84±0.10
Genistein acetylglycoside	4.65±0.07	3.82±0.05	4.71±0.07	3.00±0.04	3.86±0.05	3.35±0.05	3.25±0.05	4.56±0.06
Genistein	13.71±0.19	9.44±0.13	11.34±0.16	7.94±0.11	11.62±0.16	9.04±0.13	14.37±0.20	11.93±0.17
Total	***334.02±4.68***	***248.47±3.48***	***276.56±3.87***	***165.25±2.31***	***251.58±3.52***	***123.37±1.73***	***152.17±2.13***	***110.21±1.54***

3.3. Antioxidant activity

In this study, antioxidant activity of soybean extracts changed between 15.9 and 49.5 μmol of Trolox/g according to DPPH assay while the values ranged from 22.8 to 73.1 μmol of Trolox equiv/g with respect to ABTS assay (Figure 3). In accordance with phenolic composition results, the antioxidant activity decreased with increasing methanol content in solvent mixture. The similar changes were observed for ethanol, acetone and acetonitrile solvents. Among the all solvents tested, 70 % methanol yielded the extracts with the highest anti-oxidant values which were obtained using the both antioxidant assays.

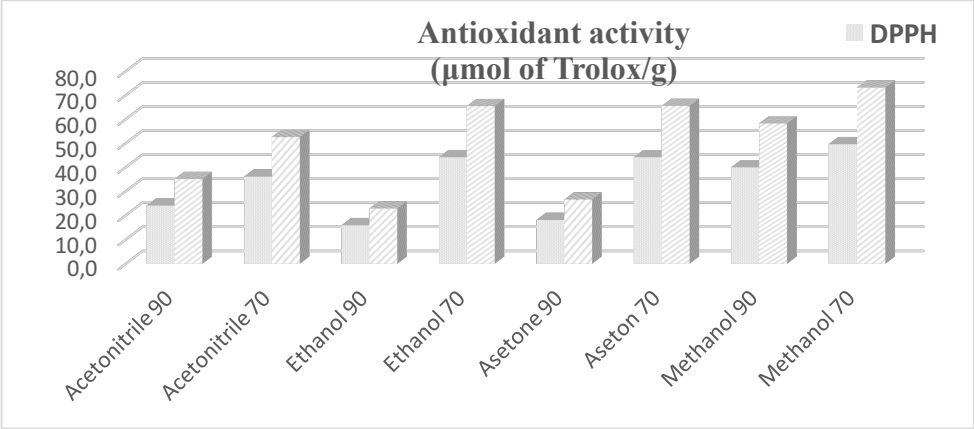

Figure 3. Antioxidant potential of soybean extracts

Table 3. Pearson's correlation coefficients among the phenolic compounds and antioxidant capacity

	DPPH	ABTS
Daidzin	0.791**	0.793**
Daidzein derivatives	0.611	0.606
Genistin	0.807**	0.807**
Genistein malonylglyc	0.902**	0.900**
Genistein derivatives	0.630*	0.635*
Daidzein	0.612	0.616
Genistein acetylglycoside	0.592	0.597
Genistein	0.624*	0.622*
Total	0.864**	0.866**

*. Correlation is significant at the 0.05 level.
**. Correlation is significant at the 0.01 level.

Daidzin and genistin were found to be the major isoflavonoids accounted for the largest proportion of the total phenolic contents. Overall, 70% methanol is recommended for extracting soybean

phenolics on the basis of the antioxidant activity results. Also, the significant correlation between the isoflavanoid contents and antioxidant activities of the soybean extracts were also obtained (Table 3).

References

[1] Lee J.H., Jeon J.Y., Kim S.G., Kim S.H., Chun T., Imm J.Y., (2011).Comparative analyses of total phenols, flavonoids, saponins and antioxidant activity in yellow soy beans and mung beans. Int. J. Food Sci. Tech.,46, 2513–2519.

[2] Nagata C., Takatsuka N., Inaba S., Kawakawi N., Shimizu H., (1998). Effect of soymilk consumption on serum estrogen concentrations in premenopausal Japanese women. J. Natl. Cancer Inst., 90, 1830–1835.

[3] Dueñas, M., Hernández, T., Robredo, S., Lamparski, G., Estrella, I., Muñoz, R. (2012). Bioactive phenolic compounds of soybean (Glycine max cv. Merit): modifications by different microbiological fermentations. Pol. J. Food Nutr. Sci., 62, 4, 241-250.

[4] Wang, H. J., Murphy, P. A. (1994). Isoflavone content in commercial soybean foods. J. Agric. Food Chem. 42, 1666−1673.

[5] Li, Y., Kong, D., Bao, B., Ahmad, A., Sarkar, F. H. (2011). Induction of cancer cell death by isoflavone: the role of multiple signaling pathways. Nutrition ,3, 877−896.

[6] Albertazzi, P., Purdie, D. W. (2002). The nature and utility of the phytoestrogens: a review of the evidence. Maturitas, 42, 173−185.

[7] Lee, S.J., Seguin, P., Kim, J. J., Moon, H.I., Ro, H.M., Kim, E. H., Seo, S.H., Kang, E.Y., Ahn, J.K., Chung, I.M. (2010). Isoflavones in Korean soybeans differing in seed coat and cotyledon color. J. Food Compos. Anal. 23, 160−165.

[8] Jeng, T.L., Shih, Y.J., Wu, M.T., Sung, J.M. (2010). Comparisons of flavonoids and anti-oxidative activities in seed coat, embryonic axis and cotyledon of black soybeans. Food Chem., 123, 1112−1116.

[9] Luthria, D.L., Biswas, R., Natarajan, S. (2007). Comparison of extraction solvents and techniques used for the assay of isoflavones from soybean. Food Chemistry, 105(1), 325-333.

[10] Kelebek, H. 2016. LC-DAD-ESI-MS/MS characterization of phenolic constituents in Turkish black tea: Effect of infusion time and temperature. Food Chem, 204: 227–238.

[11] Kelebek, H., Jourdes, M., Selli, S., and Teissedre P-L, (2013). Comparative evaluation of the phenolic content and antioxidant capacity of sun-dried raisins. J Sci Food Agric 93(12): 2963–72.