PHYTOESTROGEN CONTENTS
OF
SELECTED FOODS

ESRA GÜLTEKİN

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PHYTOESTROGEN CONTENTS OF SELECTED FOODS

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Approval of the Graduate School of Natural and Applied Sciences

_____

Prof. Dr. Canan Özgen
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

_____

Prof. Dr. Levent Bayındırlı
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

_____

Prof. Dr. Fatih Yıldız
Supervisor

Examining Committee Members

Prof. Dr. Ufuk Gündüz	(METU, BIO)	_____
Prof. Dr. Fatih Yıldız	(METU, FDE)	_____
Prof. Dr. Sedat Velioğlu	(AU, FDE)	_____
Assoc. Prof. Dr. Esra Yener	(METU, FDE)	_____
Assoc. Prof. Dr. Fatih İzgü	(METU, BIO)	_____

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ABSTRACT

PHYTOESTROGEN CONTENTS OF SELECTED FOODS

Gültekin, Esra

M. Sc., Department of Food Engineering

Supervisor : Prof. Dr. Fatih Yıldız

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Phytoestrogens are naturally occurring chemicals of plant origin that have the ability to cause estrogenic and/or anti-estrogenic effects due to their structural similarities to the human hormone oestradiol. It has been proposed that phytoestrogens protect against a wide range of ailments, including breast and prostate cancers, cardiovascular disease, osteoporosis, and menopausal symptoms. Daidzein, biochanin A and especially genistein which has been reported to be the most biologically active dietary phytoestrogen attract great deal of interest in today's researches.

In this study, twenty different food items, including legumes, fruits, nuts and herbs, (haricot beans, chickpeas, green lentils, red lentils, soybeans, licorice root, yarrow, dried chestnuts, prunes, raisins, currants, black cumin, dried apricots, dried parsley, dried dates, dried figs, sage (from Aegean region), sage (from Mediterranean region), grapevine leaves, gilaburu) were selected. Following an extraction procedure employing acid hydrolysis and heating; they were analysed for their daidzein, genistein and biochanin A contents using a reversed-phase C_{18} column with linear gradient elution on a high-performance liquid chromatography (HPLC) coupled with diode-array detector (DAD).

Soybeans were found to contain high amounts of daidzein (91.36 mg/100 g) and genistein (85.57 mg/100 g). Chickpeas were found to contain much less amount of genistein (0.89 mg/100 g) compared with that of soybeans and also biochanin A (0.95 mg/100 g) which was not detected in soybeans. None of daidzein, genistein and biochanin A was detected in the remaining eighteen food items.

Keywords : Phytoestrogen; daidzein; genistein; biochanin A; HPLC-DAD

ÖZ

SEÇİLMİŞ GIDALARIN FİTOÖSTROJEN İÇERİKLERİ

Gültekin, Esra
Yüksek Lisans, Gıda Mühendisliği Bölümü
Tez Yöneticisi : Prof. Dr. Fatih Yıldız

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Fitoöstrojenler; insan hormonu östradiole yapısal benzerlikleri dolayısıyla östrojenik ve/veya anti-östrojenik etkiler gösteren, bitki kökenli doğal kimyasal bileşiklerdir. Fitoöstrojenlerin; göğüs ve prostat kanseri, kalp-damar hastalıkları, osteoporoz ve menopoz belirtileri dahil pekçok hastalığa karşı koruyucu etkisi bulunduğu düşünülmektedir. Daidzein, biochanin A ve özellikle de biyolojik olarak en aktif fitoöstrojen olduğu belirtilen genistein, günümüz araştırmalarında büyük ilgi çekmektedir.

Bu çalışmada; yirmi farklı gıda maddesi (kuru fasulye, nohut, yeşil mercimek, kırmızı mercimek, soya fasulyesi, meyankökü, civanperçemi, kuru kestane, kuru erik, kuru üzüm, kuş üzümü, çörekotu, kuru kayısı, kuru maydonoz, kuru hurma, kuru incir, adaçayı (Ege Bölgesi), adaçayı (Akdeniz Bölgesi), asma yaprağı, gilaburu) belirlenmiştir. Asit hidrolizi ve ısıtma aşamalarını içeren bir ekstraksiyon prosedürünü takiben; yüksek performanslı sıvı kromatografisi (HPLC) ve diyot-array dedektör (DAD) kullanılarak, ters-fazlı C_{18} kolonda, lineer gradient yöntemle, bu gıdaların daidzein, genistein ve biochanin A içerikleri araştırılmıştır.

Soya fasülyesinin yüksek miktarlarda daidzein (91.36 mg/100 g) ve genistein (85.57 mg/100 g) içerdiği saptanmıştır. Nohutta, soya fasulyesindeki genistein miktarından çok daha düşük miktarda genistein (0.89 mg/100 g) ve ayrıca soya fasulyesinde tespit edilmeyen biochanin A (0.95 mg/100 g) saptanmıştır. Diğer onsekiz gıdada ise, daidzein, genistein ve biochanin A bileşiklerinin hiçbiri tespit edilmemiştir.

Anahtar Kelimeler : Fitoöstrojen; daidzein; genistein; biochanin A; HPLC-DAD

To My Family and Gürkan

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RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
UV	Ultra-Violet Detector
DAD	Diode-Array Detector
ER	Estrogen Receptor
HRT	Hormone Replacement Therapy
GC	Gas Chromatography
LC	Liquid Chromatography
MS	Mass Spectrometry

CHAPTER 1

INTRODUCTION

1.1. Phytoestrogens

Phytoestrogens are naturally occurring chemicals of plant origin that have the ability to cause estrogenic and/or anti-estrogenic effects due to their structural similarities to the human hormone oestradiol (17β-estradiol). (Setchell and Cassidy, 1999)

1.1.1. Classes of Phytoestrogens

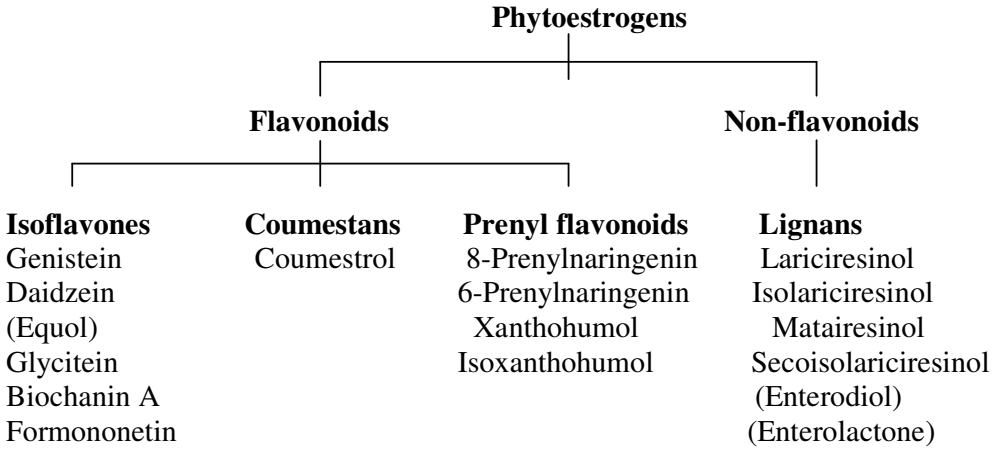


Figure 1.1 The relationship between various groups of phytoestrogens (given in bold) and members of each group. (The compounds in paranthesis are not inherently present in plants but are oestrogenic products resulting from metabolism of members of that class of phytoestrogens.)

The majority of phytoestrogens belong to a large group of substituted phenolic compounds known as flavonoids. There are several groups of flavonoids with estrogenic properties. Of these, "coumestans" and "isoflavones" possess the greatest estrogenic activity (Collins *et. al.*, 1997). A class of prenylated flavonoids with estrogenic activities intermediate to those of the coumestans and isoflavones has recently been identified (Milligan *et. al.*, 1999). Lignans, a class of non-flavonoid phytoestrogens, have also been shown to exert estrogenic effects (Setchell and Adlercreutz, 1988). The relationship between these types of phytoestrogens and the names of the compounds most commonly found in food from these four groups are summarized in Figure 1.1.

1.1.2. Structural Similarities of Phytoestrogens to Oestradiol

Similarity of phytoestrogens to estrogens at the molecular level provides them the ability to mildly mimic and in some cases act as an antagonist to estrogen (Oomah, 2002). Common features of phytoestrogens and oestradiol are listed in Table 1.1 (Mazur and Adlercreutz, 2000).

Table 1.1. Key structural elements crucial for estradiol-like action

- Presence of the phenolic ring indispensable for binding to estrogen receptors (ERs)
- Role of the ring of isoflavones mimicking the ring of estrogens at receptor binding
- Low molecular weights, similar to that of estradiol ($C_{18}H_{24}O_2$) (MW = 272)
- Distance between two aromatic hydroxyl groups in the nucleus of the isoflavones almost identical to the distance between two hydroxyl groups of estradiol
- Optimal pattern of hydroxylation, i.e., hydroxyl substituents at 4', 5, and 7 positions (e.g., genistein)

The structural similarities between members of the four main groups of phytoestrogens identified in Figure 1.1 and oestradiol are shown in Figure 1.2 (Food Standards Agency, 2003)

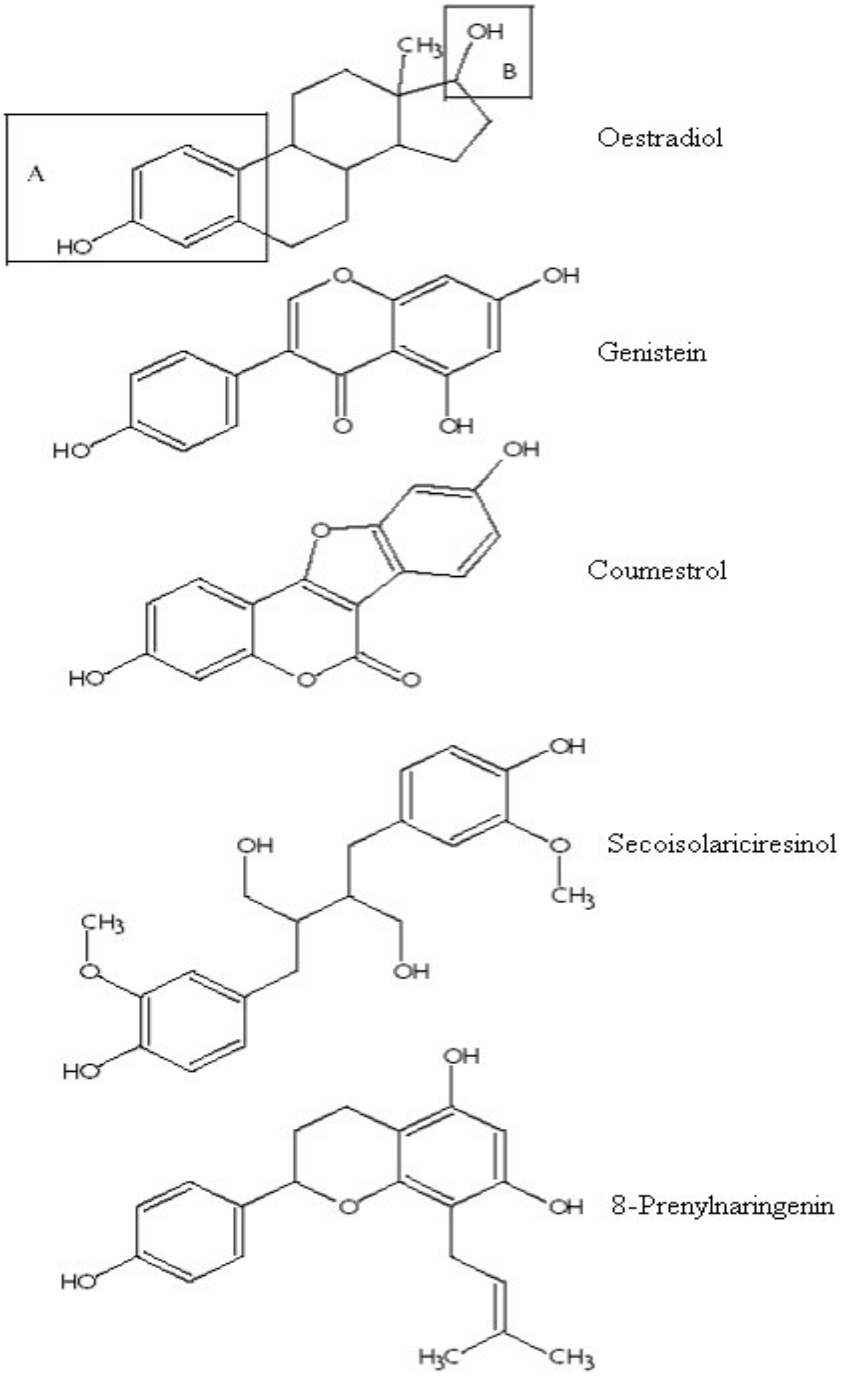


Figure 1.2 The structural similarities of phytoestrogens to oestradiol.

(The similarity of the structure of oestradiol and examples from the four classes of phytoestrogens from Figure 1.1. All the structures possess the phenolic (A) and hydroxyl (B) moieties outlined in boxes on the oestradiol structure and the distances between the two groups in each compound are similar.)

1.1.3. Oestrogenic Properties of Phytoestrogens

In 1940s, it was first realized that some plant-derived compounds could cause estrogenic effects in animals (Bennetts *et. al.*, 1946). Sheep grazing on pastures containing red clover had multiple fertility problems. The clover in these pastures had high amounts of the isoflavones, formononetin and biochanin A (Rossiter and Beck, 1966). The phytoestrogens, daidzein and genistein were responsible for the infertility of some captive cheetahs fed a soybean enriched diet subsequently found to contain high quantities of these compounds (Setchell *et al.*, 1987).

Evidence is beginning to accrue that phytoestrogens may begin to offer protection against a wide range of human conditions, including breast, bowel, prostate, and other cancers; cardiovascular disease; brain function; alcohol abuse; osteoporosis; and menopausal symptoms (Bingham *et. al.*, 1998). The basis for these effects has not been established, but the weak estrogenic activity of isoflavones may be a factor in conferring these properties (Oomah, 2002).

The incidence of a number of cancers, including those of the breast and prostate, has been found to be much higher in Western populations compared with that in countries such as Japan and China. Epidemiological and migrant studies have suggested that racial characteristics and other factors including lifestyle, diet and fat or fibre intake may play a role in the aetiology of these diseases. One notable dietary difference is the relatively high consumption of soy and soy-based foods amongst Asian populations. Comparison of estimated dietary isoflavone intakes in Western and Eastern (e.g. Japanese and China) populations illustrate that Eastern populations have a significantly higher intake of phytoestrogens. Estimates suggest that the average Japanese consumer is exposed to approximately 25-100 mg isoflavones/day, while an average United Kingdom consumer ingests approximately 1 mg isoflavones/day (Food Standards Agency, 2003). As such; soy, which has been known to be the richest source of isoflavones, has attracted much attention as a potential chemoprotective factor (Bingham *et al.*, 1998; Cassidy and Faughnan, 2000).

1.1.4. Oestrogenic Potencies of Phytoestrogens

In general, phytoestrogens are relatively weak oestrogens, requiring much higher concentrations than oestradiol to produce an equivalent biological response. Since potency values can vary significantly between methods, relative absolute estrogenic potency of phytoestrogens is difficult to determine. However, taking the results of both *in vitro* and *in vivo* studies together, a single rank order of oestrogenic potency of phytoestrogens may be estimated: oestradiol ≥ coumestrol > genistein, equol > glycitein > 8-prenylnaringenin > daidzein > formononetin, biochanin A, 6-prenylnaringenin, xanthohumol, isoxanthohumol. (Food Standards Agency, 2003)

Since coumestans, being reported to be the most potent phytoestrogen (Pelissero *et. al.*, 1991), are found predominantly in clover and alfalfa plants (Miksicek, 1993) and so are rare components of the human diet (Adlercreutz, 1997); isoflavones attract great deal of interest in today's studies due to wider range of foods containing them.

1.2. Isoflavones

Isoflavones are polyphenolic phytoestrogens that occur mainly as gluco-conjugates (glucosides) of genistein, daidzein, and glycitein (Clarke *et al.*, 2002). They enjoy a restricted distribution in the plant kingdom and are predominantly found in leguminous plants (Dixon and Ferreira, 2002). The main dietary sources of isoflavones are soybeans and soyfoods (Lapcik *et. al.*, 1998).

1.2.1. Classes of Isoflavones

The most prevalent isoflavones present in plant-based foods are as follows (Bingham *et al.*, 1998) (Figure 1.3) :

- genistein
- daidzein
- glycitein
- biochanin A (methylated derivative of genistein)
- formononetin (methylated derivative of daidzein)

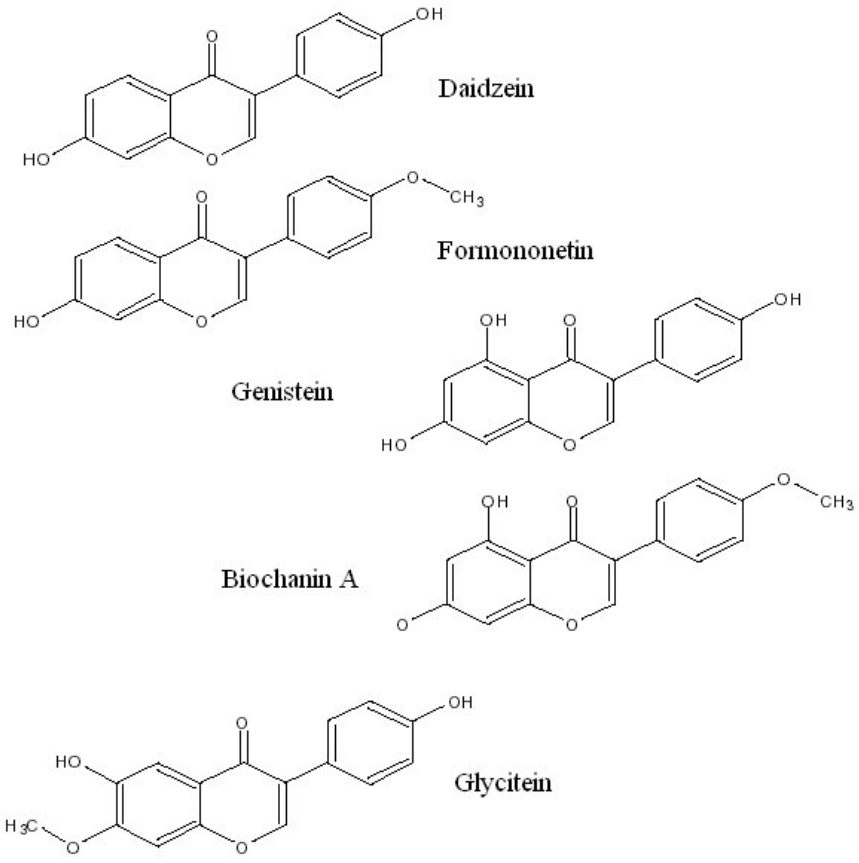


Figure 1.3 Isoflavone aglucones: daidzein, formononetin, genistein, biochanin A and glycitein

1.2.2. Physical and Chemical Properties of Isoflavones

1.2.2.1. Water Solubility

Isoflavones are low molecular weight (MW of daidzein = 254, MW of genistein = 270, MW of biochanin A = 284) hydrophobic compounds. The aqueous solubilities of the isoflavone aglucones are low and due to the acidic nature of the phenolic groups are pH dependent. The methylated derivatives, biochanin A and formononetin are less soluble than genistein and daidzein, respectively. (Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment, 2000) Conjugation to glucose, glucuronide or sulphate groups increases the solubility (Setchell and Cassidy, 1999).

1.2.2.2. Chemical Stability

The isoflavone aglucones are stable under physiological conditions. Under acidic conditions, the glucosides can be deconjugated to give aglucones. In the body, enzymes in the gut and liver can carry out these reactions during metabolism. (Food Standards Agency, 2003)

1.2.3. Absorption, Distribution, Metabolism and Excretion of Isoflavones

In plants, isoflavones are present as glucosides. Processing reduces the isoflavone content and can partially convert them to aglucones. So, isoflavones are ingested mainly as glucosides and undergo hydrolysis by gut bacterial and mammalian enzymes prior to absorption. (Wang *et. al.*, 2002) Following absorption of the aglucones, these compounds are reconjugated with sulphate and glucuronide and excreted in the bile or urine (Bingham *et al.*, 1998). Gut microflora can also modify estrogenic isoflavones into more active forms. The methylated isoflavones, formononetin and biochanin A are demethylated by gut microflora to daidzein and genistein, respectively.

1.3. Analysis of Phytoestrogens

1.3.1. Isolation of Phytoestrogens

Isoflavones are often present as glucosides in plants. Since acidic conditions make glucosides to deconjugate into aglucones, most extraction procedures involve acid hydrolysis. Hydrolysis with 1-2 M hydrochloric acid at 100 ºC or refluxing with acid in the presence of ethanol has been used to form the aglucones (Wilkinson *et. al.*, 2002). However, there are some reports indicating genistein to be unstable under acid hydrolysis conditions (Franke *et. al.*, 1994; Garrett *et. al.*, 1999).

For analysis of soy foods, typical extraction conditions that have been used are stirring-freeze-dried powdered samples with methanol-water (80:20, v/v) at room temperature or 4 ºC, or with a mixture of acetonitrile - hydrochloric acid (0.1 M) -

water (Wang and Murphy, 1994; Murphy *et. al.*, 1997; Song *et. al.*, 1998; Murphy *et. al.*, 1999; Griffith and Collison, 2001). Using acidified solvents has been highly recommended by the compilers of the US Department of Agriculture – Iowa State University Database (1999); on the other hand, alcohol extraction has been reported to reduce the isoflavone contents of soy products significantly.

In the case of non-soy foods; since the exact nature and composition of isoflavone glucosides present in foods other than soy is unknown, none of the methods employed for isoflavone detection in non-soy foods have been used to determine glucosides. So all the reported sample preparation protocols utilise a hydrolysis step to form aglucones. (Wilkinson *et. al.*, 2002)

Meksem *et. al.* (2001) and Lee *et. al.* (2003) employed the following sample preparation method : 2 grams of ground soybean seeds were mixed with 2 mL of 0.1 N HCl and 10 mL of acetonitrile, stirred for 2 hours at room temperature and filtered. The filtrate was dried under vacuum at temperatures below 30 ºC and then redissolved in 10 mL of 80% HPLC grade methyl alcohol in distilled water.

Hutabarat *et. al.* (2001) investigated the optimum extraction of daidzein, genistein, formononetin and biochanin A in soybeans. 1 gram of ground food was diluted with 40 mL of 96% ethanol and extracted with or without hydrolysis by refluxing and heating on a water bath at 80 or 100 ºC at varying pH of phosphoric acid or hydrochloric acid (10 mL) for 0, 1, 2, 3, 4 or 6 h. The optimum extraction was by hydrolysis with 2 M hydrochloric acid with refluxing on a water bath at 100 ºC.

Pandjaitan *et. al.* (2000) evaluated genistin and genistein contents of soy protein concentrates prepared by three basic methods (acid, alcohol and hot-water leach). The acid leach method gave the highest total genistin+genistein content (0.742 mg/g) compared to soy protein concentrates prepared with the hot-water leach method (0.671 mg/g), and the alcohol leach method (0.070 mg/g). The acid leach, hot-water leach and alcohol leach methods had 20.3%, 24.2% and 91.2% losses of total genistin+genistein, respectively.

1.3.2. Analytical Methods

The most widely used techniques for measurement of phytoestrogens are :

- reversed-phase high-performance liquid chromatography (RP-HPLC) with ultraviolet (UV) or diode-array detection (DAD)
- gas chromatography with mass spectrometric detection (GC-MS)
- liquid chromatography with mass spectrometric detection (LC-MS) (Food Standards Agency, 2003)

HPLC with UV/DAD is a relatively rapid way of measuring phytoestrogens compared to MS-based methods (Coward *et. al.*, 1998; Murphy *et. al.*, 1999; Nakamura *et. al.*, 2000). The advantage of this method is that only hydrolysis and extraction of the samples are needed before analysis (Adlercreutz, 1999).

For soy foods, the main analytical techniques are HPLC with UV or DAD using reversed-phase C_{18} stationary phases with gradient elution (Wilkinson *et. al.*, 2002). On the other hand; since isoflavonoid levels in non-soy foods are much lower than in soy; UV detection has been considered not sufficiently sensitive for the analysis of these expected low levels of isoflavonoids (Mazur *et. al.*, 1996; Mazur and Adlercreutz, 1998). In general, if food samples containing phytoestrogen concentrations that are greater than 50 ppm (this is largely restricted to soybean or red clover products) are to be analysed, HPLC with DAD-UV detection is the method of choice. But, in case of concentrations less than 50 ppm, HPLC-UV is not adequate. (Wang *et. al.*, 2002)

In general, the mobile phases employed with RP-HPLC columns have been acetonitrile and/or methanol in combination with water containing small amounts of an acid (Wang *et. al.*, 2002). Merken and Beecher (2000) reported that most of the different classes of phytoestrogens and their metabolites are separated by RP-HPLC using elution with a gradient of methanol or acetonitrile in an acidic (0.1-1 % acetic, formic, or trifluoroacetic acids) or neutral (10 mM ammonium acetate or ammonium formate) solvent.

Franke *et. al.* (1994; 1995) quantified daidzein, genistein and biochanin A in legumes by HPLC-DAD on a C_{18} reversed-phase column using a gradient solvent system consisting of A : acetic acid-water (10:90, v/v), B : acetonitrile; 23-70 B% in 8 min followed by holding at 23 B% for 12 min. Meksem *et. al.* (2001) and Lee *et. al.* (2003) conducted an HPLC-DAD analysis on a YMC-Pack column according to the method of Wang and Murphy (1994a; 1994b). Solvent A was 0.1% glacial acetic acid in distilled water, and solvent B was 0.1% glacial acetic acid in acetonitrile. Solvent B was increased from 15% to 35% over 50 min and then held at 35% for 10 min. The USDA-Iowa State University Isoflavones Database (1999) used the analytical method described by Murphy *et. al.* (1997) as the reference method for evaluating analytical methodologies for isoflavones in soy products. A linear gradient was composed of 0.1% aqueous acetic acid and 0.1% acetic acid in acetonitrile with a total elution time greater than 45 min.

All the phytoestrogens and their metabolites contain at least one aromatic ring. This means that they absorb UV light with a maximum wavelength in the range from 250 to 270 nm. (Wang *et. al.*, 2002). Maximum absorption of daidzein was achieved at a wavelength of 249 nm with a shoulder at 302 nm, genistein at 259 nm and biochanin A at 260 nm (Hutabarat *et. al.*; 1998, 2000 and 2001). Franke *et. al.* (1994 and 1995) monitored daidzein, genistein, formononetin and biochanin A at or very near their absorption maximum with DAD at 260 nm. Hsu *et. al.* (2001) detected daidzein, genistein and biochanin A in *Psoralea corylifolia* at 260 nm. Griffith and Collison (2001) monitored isoflavones at 260 nm. Lee *et. al.* (2003) measured elutions of daidzein and genistein at an absorbance of 254 nm, while Mitani *et. al.* (2003) selected a wavelength of 259 nm for detection of these compounds.

1.4. Reported Phytoestrogen Contents of Foods

The major dietary sources of isoflavonoids for humans are soybeans and soy-based foods (Dixon and Ferreira, 2002; Cornwell *et. al.*, 2004). Mitani *et. al.* (2003) reported that daidzein and genistein were detected at high concentrations from dried soybeans. Mazur *et. al.* (1998) stated that soybeans proved to be the richest source of genistein.

Table 1.2. Daidzein, genistein and biochanin A contents of soybeans (mg/100 g)

Soybean Type	Daidzein	Genistein	Biochanin A	Reference
Soybeans "Centennial"	25.2	34.3	0.0147	Mazur et. al., 1998
Soybeans "INIAP Bolivia"	10.5	26.8	nd	
Soybeans "Santa rosa"	56.0	84.1	0.015	
Soybeans "Chapman"	41.3	46.4	tr	
Soybeans (Brazil, raw)	20.16	67.47		USDA-Iowa State University, 1999
Soybeans (Japan, raw)	34.52	64.78		
Soybeans (Korea, raw)	72.68	72.31		
Soybeans (Taiwan, raw)	28.21	31.54		
Soybeans (green, mature seeds, raw)	67.79	72.51		
Soybeans (mature seeds, dry roasted)	52.04	65.88		
Soybeans (mature seeds, raw) (US, food quality)	46.64	73.76		
Soybeans (mature seeds, raw) (US, commodity grade)	52.20	91.71		
Soybeans			0.01	
Soybeans	49	71.3		Kim and Kwon, 2001
Dried soybean (USA)	30.8	72.3		Hutabarat *et. al.*,2001
Dried soybeans (Indonesia)	127.7	83.4		
Dried soybean (McKenzie's, Australia)	96.4	61.4		
Bowyer dried soybeans (Riverina,NSW,Australia)	65.4	72.0		
Fresh soybeans, Indonesia	19.8	7.6		
Dried soybeans	7.45	26.77		Mitani *et. al.*, 2003
Dried soybeans	15.82	29.56		Klejdus *et. al.*, 2004
Soybeans (Oriental diet)	10.5-85.0	26.8 – 102.5		Mazur and Adlercreutz, 2000
Soybean seeds	6.8 – 100.6	1.8 -138.2		Food Standards Agency, 2003
Soybeans (mature)	0.5-91	1.1 – 150		Cornwell *et. al.*, 2004
Powdered soybean chips	80	50		Dixon and Ferreira, 2002

Isoflavone content data of soybeans from previous studies are summarized in Table 1.2. The variety, the crop year and the location affect the isoflavone contents of the soybeans (Wang and Murphy, 1994) and contribute to the large variability in the isoflavone contents of soybeans (USDA – Iowa State University, 1999).

Isoflavone content data of chickpeas (Table 1.3), lentils (Table 1.4), beans (Table 1.5) and fruits and nuts (Table 1.6) from previous studies are collected and summarized below.

Table 1.3. Daidzein, genistein and biochanin A contents of chickpeas (mg/100 g)

Chickpea Type	Daidzein	Genistein	Biochanin A	References
Chickpeas (Bengal gram) Goya "Garbanzo"	0.0342	0.0693	1.42	Mazur et. al., 1998
Chickpeas (Garbanzo bean)	0.192	0.214	3.08	
Chickpeas	0.0114	0.0763	0.838	
Chickpeas (garbanzo bean,bengal gram,raw	0.04	0.06		USDA-Iowa State University, 1999
Chickpeas	0.011-0.192	0.069-0.214		Mazur and Adlercreutz, 2000
Chickpeas	0.01-0.2	0.07-0.2		Cornwell et. al., 2004

Table 1.4. Daidzein, genistein and biochanin A contents of lentils (mg/100 g)

Lentil Type	Daidzein	Genistein	Biochanin A	References
Lentils "Jack Rabbit"	0.0104	0.0188	tr	Mazur et. al., 1998
Lentils "Masoor dahl"	0.0033	0.0071	0.0071	
Lentils, raw	0.00	0.00	0.00	USDA-Iowa State University, 1999

Table 1.5. Daidzein, genistein and biochanin A contents of beans (mg/100 g)

Bean Type	Daidzein	Genistein	Biochanin A	References
Navy beans (Haricot)	0.0137	0.408	0.0044	Mazur et. al., 1998
Navy beans (dry)			0.00	USDA-Iowa State University, 1999
Navy beans (raw)	0.01	0.2		
Beans, great northern, raw	0.00	0.00		
Beans, small, white, raw	0.00	0.74		
Kidney, navy and pinto beans	0.008-0.04	0.007-0.5		Cornwell et. al., 2004

Table 1.6. Daidzein and genistein contents of fruits and nuts (mg/100g)

Fruit and Nut Type	Daidzein	Genistein	Reference
Apricots, dry	0.005	nd	Liggins et. al., 2000
Currants	0.056	0.2167	
Dates, dried	0.0018	0.0054	
Figs, dried	0.0019	0.0045	
Prunes (dried, raw)	0.0052	0.0104	
Raisins (California)	0.069	0.1458	
Chestnuts, raw	0.0079	0.0059	

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Food Materials

Table 2.1 Food materials analysed

Food Material	Botanical Name
Haricot beans	*Phaseolus vulgaris*
Chickpeas	*Cicer arietinum*
Green lentils	*Lens culinaris*
Red lentils	
Soybeans	*Glycine max*
Licorice root	*Glycyrrhiza glabra*
Yarrow	*Achillea millefolium*
Dried chestnuts	*Aesculus hippocastanum*
Prunes	*Prunus domestica*
Raisins	*Vitis vinifera*
Currants	*Ribes nigrum*
Black cumin	*Nigella sativa*
Dried apricots	*Pelargonium "Apricot"*
Dried parsley	*Petroselinum crispum*
Dried dates	*Hovenia dulcis*
Dried figs	*Ficus carica*
Sage (Aegean region)	*Salvia officinalis*
Sage (Mediterranean region)	
Grapevine leaves	*Vitis vinifera*
Gilaburu	*Viburnum opulus*

Legumes, fruits, nuts and herbs, listed in Table 2.1, were used in the experiments. Haricot beans, chickpeas, green lentils, red lentils, soybeans were purchased from a local supermarket. Licorice root, yarrow, prunes, raisins, currants, black cumin, dried apricots, dried parsley, dried dates, dried figs, sage (from the Aegean region of Turkey) and sage (from the Mediterranean region of Turkey) were purchased from a local herbalist. Dried chestnuts, grapevine leaves and gilaburu were provided by Food Engineering Department of Ankara University. Gilaburu and grapevine leaves were stored at less than 0 ºC in the freezer for one night, and rest of the food materials were kept at ambient temperatures for one day, prior to the experiments.

2.1.2. Standards

Daidzein (4',7-Dihydroxyisoflavone, $C_{15}H_{10}O_4$, MW=254.2, Synthetic : Minimum 98%), Genistein (4',5,7-Trihydroxyisoflavone, $C_{15}H_{10}O_5$, MW=270.2, Minimum 98%) and Biochanin A (5,7-Dihydroxy-4'-Methoxyisoflavone, $C_{16}H_{12}O_5$, MW=284.3) were purchased from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany). Daidzein was stored at less than –20 ºC and in dark. Genistein was stored at less than 0 ºC. Biochanin A was stored at ambient temperatures.

2.1.3. Solvents and Reagents

Hydrochloric acid (HCl), acetonitrile (CH_3CN) (gradient grade for liquid chromatography) and sodium acetate ($CH_3COONa.3H_2O$) were purchased from Merck KGaA (Darmstadt, Germany). Ultra pure water was used throughout the analysis and was generated by an Easypure UV-Compact Ultra Pure Water System (Barnstead/Thermolyne). All are stored at ambient temperatures.

2.2. Methods

2.2.1. Experimental Plan

An outline of the experimental procedure is given in Figure 2.1.

2.2.2. Preparation of Samples

Food materials were ground using a Turbo Blender (Black&Decker). Where necessary, they were also sieved using an Endecotts Test Sieve of 425 microns (Endecotts Limited, London, England). A sample of 2 grams was weighed from each ground food material. 18 mL of 2 M HCl and then 20 mL of acetonitrile were added to each of those samples. Each of the mixtures was homogenized using a Janke&Kunkel VF2 Vortex (IKA-Labortechnik) and was heated at 80 ºC for 2.5 hours in a GFL Model water bath.

After heating, they were centrifuged at 8000 rpm for 10 minutes in a centrifuge model HN-S Centrifuge (International Equipment Company, U.S.A.). The clear supernatant was filtered through a RC 0.45 μm syringe filter (AYSET). The filtrates were stored at less than 0 ºC until HPLC analysis was carried out.

2.2.3. Preparation of Standard Solution

First, separate standard solutions of daidzein (111 ppm), genistein (60 ppm) and biochanin A (75 ppm) were prepared by weighing the three standards accurately using a calibrated analytical balance (Shimadzu) and dissolving them in HPLC grade acetonitrile. Then 1 mL was taken from each standard solution; and the mixture was adjusted to 10 mL, diluting each standard to 1/10. Dilutions were done using a Brand model micropipette (Transferpette). The standard mixture, containing daidzein (11.1 ppm), genistein (6 ppm) and biochanin A (7.5 ppm), was prepared just before HPLC analysis in order to avoid decomposition of standards and stored at –20 ºC after use.

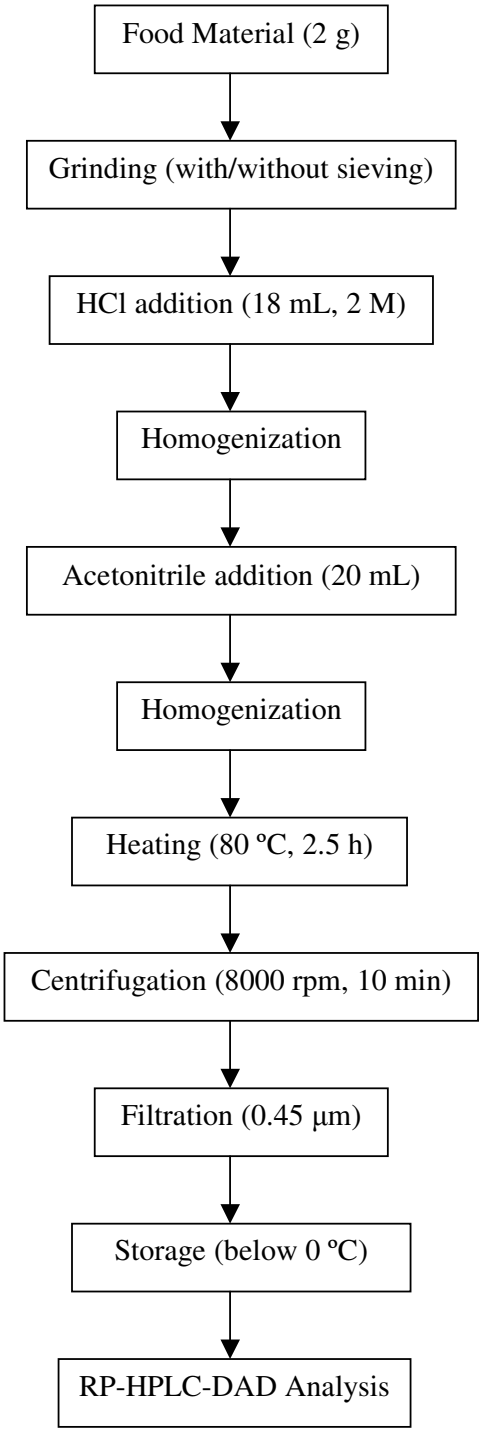


Figure 2.1 Outline of the experimental procedure

2.2.4. HPLC-DAD Analysis

An Agilent 1100 Series HPLC system equipped with a UV-DAD detector, an autosampler and a HP ChemStation Software Programme (Agilent ChemStation for LC and LC-MS Systems) was utilized. The analysis was performed on a LiChrospher 100 Reversed-Phase-C18 (5 μm, LiChroCART 250 HPLC Cartridge) (Hewlett Packard) column. Column temperature was maintained at 25 ºC.

Standard solution and samples were transferred into screw cap vials (Agilent Technologies, Germany) and vials were closed with blue screw caps (Agilent, US) for HPLC analysis. Injection volume was 20 μL; and after each injection, vials were immediately covered with Parafilm "M" Laboratory Film (American National Can, Chicago) and returned to refrigerator for next use.

Mobile phase consisted of solvent A (2 mM sodium acetate) and solvent B (acetonitrile) at a flow rate of 1 mL/min with the following gradient solvent system:

Retention time (min)	Solvent B (%)
0	20
50	100

Running time was 50 minutes with a post-running time of 6 minutes. Air in the mobile phase was drawn out using a Vacuubrand Diaphragm Vacuumpump (Wertheim, Germany). Pressure was constant at 141 bar.

First; the standard mixture, containing daidzein (11.1 ppm), genistein (6 ppm) and biochanin A (7.5 ppm) was injected to the HPLC system. The wavelength ranged from 225 to 440 nm throughout the chromatogram. The spectrum of each compound was examined. The wavelengths, at which the compounds achieved their maximum absorbances, were determined. According to this, the rest of the analysis was performed and the analytes were monitored at a single wavelength of 259 nm. Then the retention times of daidzein, genistein and biochanin A were determined.

Second, twenty samples were injected to the system with one injection of standard mixture after each five injections of samples; in order to control whether there is a deviation in the retention times of standards or not. By comparing the retention times of standard peaks with that of the sample peaks; possible daidzein, genistein and biochanin A peaks in chromatograms of the samples were determined. Chromatogram of the sample was laid over chromatogram of standard mixture; so whether the retention time of a peak of the sample fits to the retention time of an analyte was observed more clearly.

Then; spectrum of each peak at chromatogram of sample was wieved and compared with spectrum of the analyte eluted within the same minute. Spectrum of peak was laid over the spectrum of the analyte to control if they are same compounds.

If spectrum of peak and analyte also fit each other; to be sure about the result, sample was spiked with the standard (Figure 3.6) and analysed again employing another gradient system (solvent B increasing from 15% to 30% in 30 minutes, from 30% to 50% between 30th and 50th minutes, and then staying at 15% for a post-run of 10 minutes). If the peak area increased, the presence of analyte in the sample was confirmed.

Having qualitatively determined the analytes in samples; quantitative analysis was performed. For the samples containing one or more of the analytes; comparing the peak area of the analyte at chromatogram of the standard mixture with that of the peak area of the analyte at chromatogram of the sample and using direct proportionation between two areas, concentration of the analyte in the sample was determined. Lastly, analyte content in the original food material was calculated as “milligrams of analyte per 100 grams of food material”. All analysis and measurements were done on triplicate set of samples.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Results for Standard Mixture

3.1.1. Chromatogram of Standard Mixture

The prepared standard mixture, containing 11.1 ppm daidzein, 6 ppm genistein and 7.5 ppm biochanin A, was injected to the RP-HPLC system. The chromatogram obtained is given in Figure 3.1.

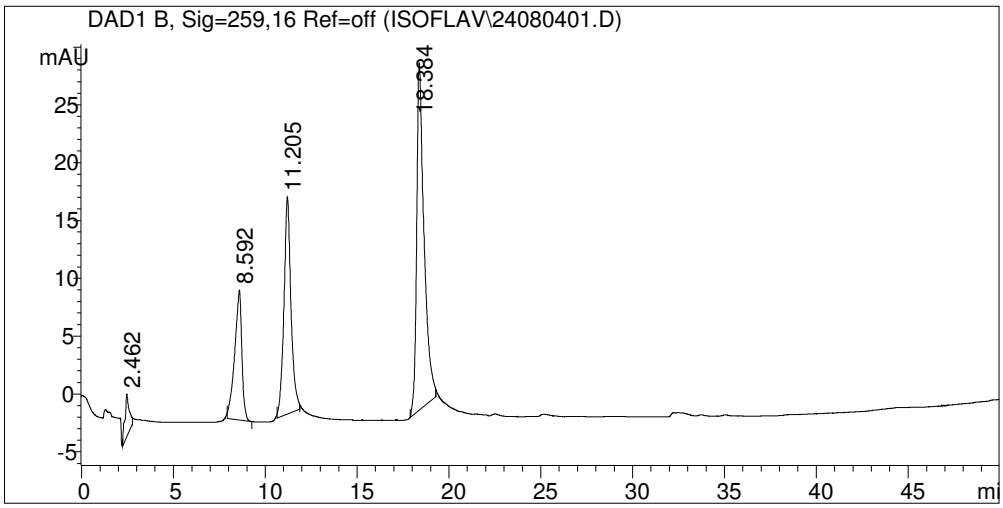


Figure 3.1. Chromatogram of the standard mixture

3.1.2. Determination of the Proper Wavelength

The spectra of daidzein, genistein and biochanin A are shown in Figure 3.2.

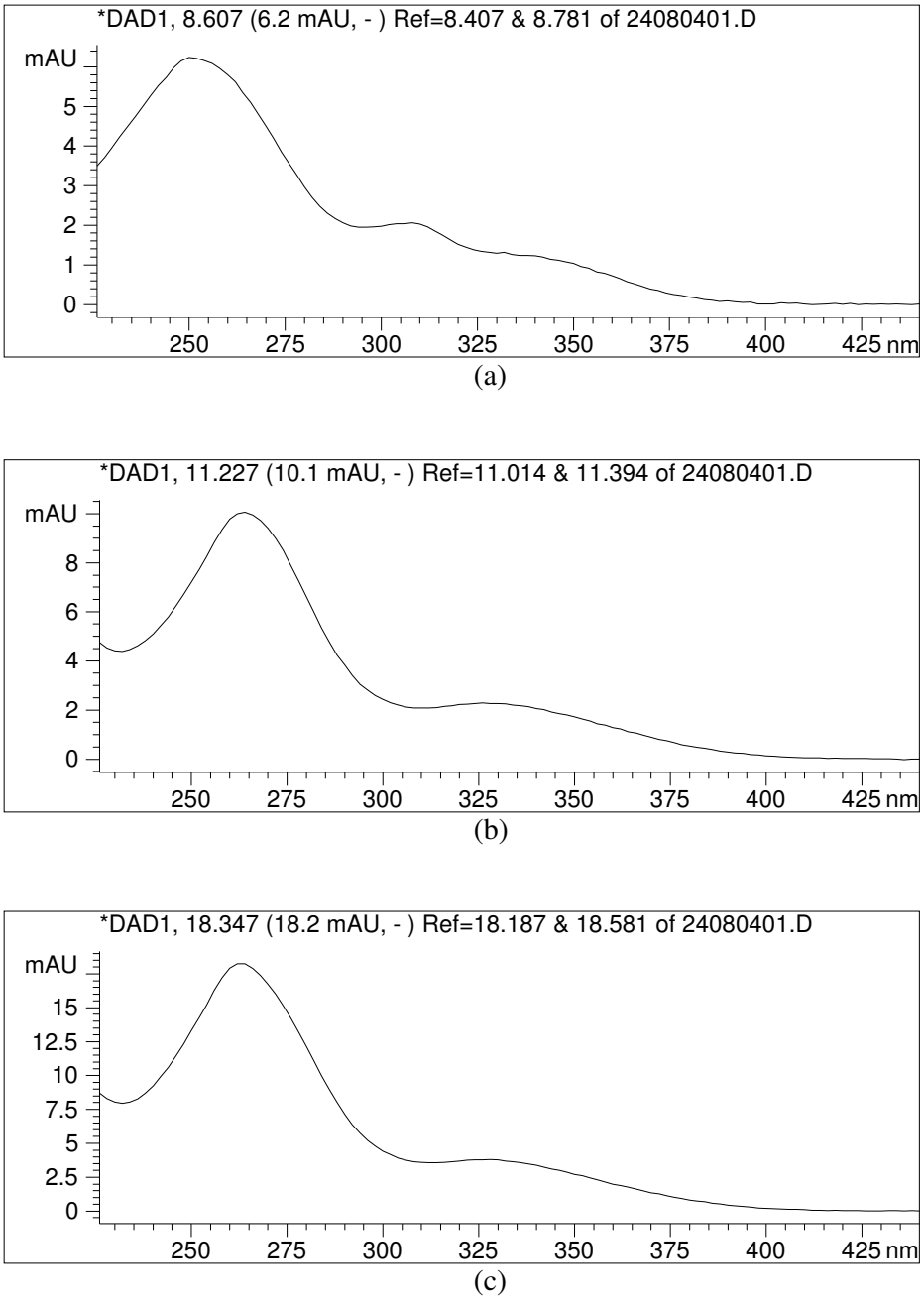


Figure 3.2. Spectra of (a) daidzein, (b) genistein, (c) biochanin A

The wavelengths, at which maximum absorption of daidzein, genistein and biochanin A was achieved, are given in Table 3.1.

Table 3.1. Absorbance maxima of daidzein, genistein and biochanin A

Analyte	λ_{max} (nm)
Daidzein	250, 302 shoulder
Genistein	260, 325 weak band
Biochanin A	262, 327 weak band

Since absorption maxima of all analytes are in the range of 250 to 262 nm, a single wavelength of 259 nm was used throughout the analysis.

3.1.3. Retention Times of the Analytes

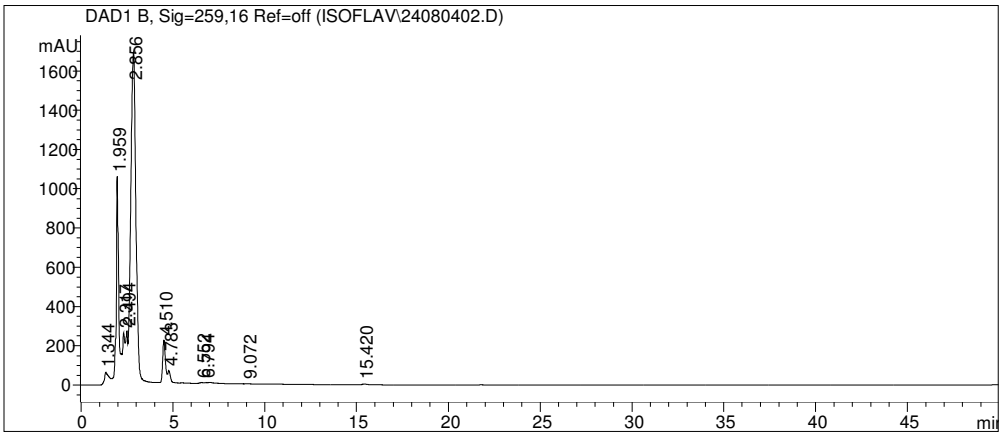
Analytes eluted in the order of daidzein, genistein and biochanin A; daidzein and genistein appearing close to each other in the early part of the chromatogram and biochanin A eluting much later (Figure 3.1). The retention times of the analytes are given in Table 3.2.

Table 3.2. Retention times of daidzein, genistein and biochanin A

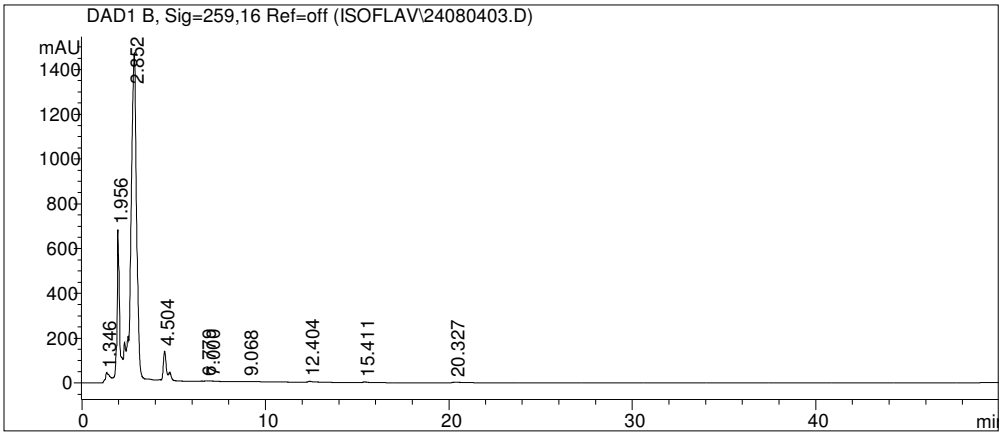
Analyte	Retention time (min)
Daidzein	8.592
Genistein	11.205
Biochanin A	18.284

3.2. Screening of 20 Samples

20 different samples, prepared according to the extraction method explained in Chapter 2, were injected to the RP-HPLC system. Chromatograms of the samples are given in Figure 3.3.

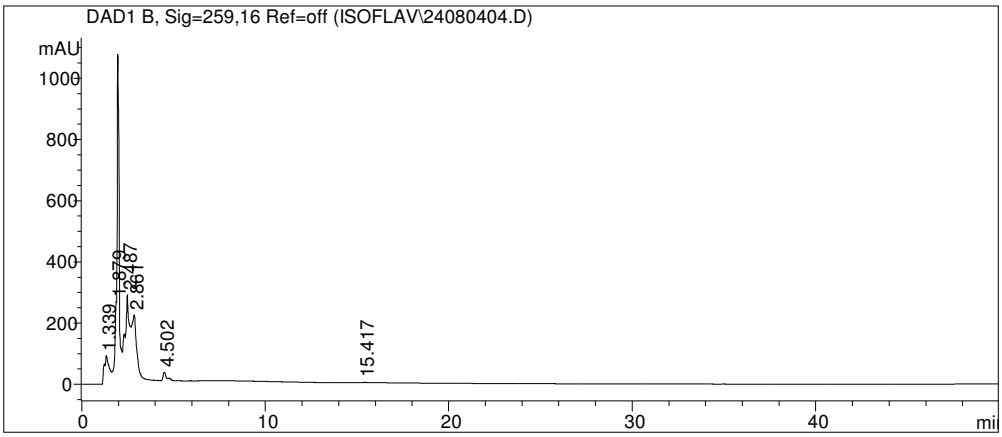


(a)

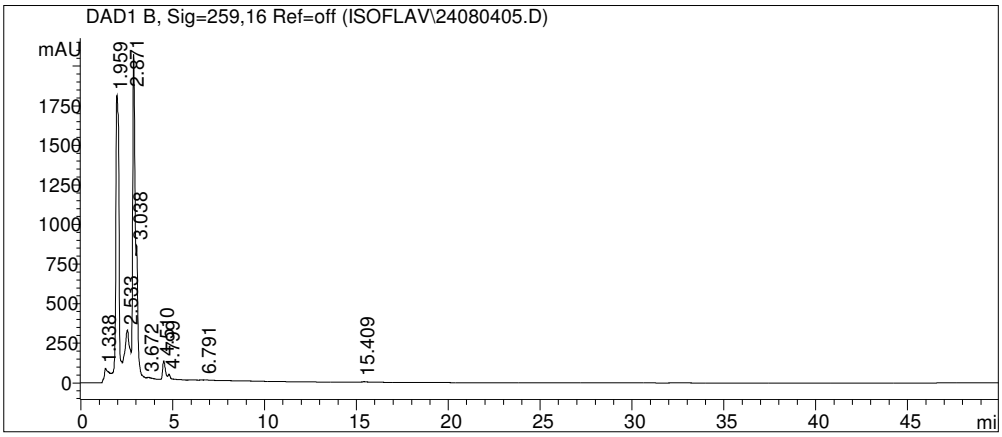


(b)

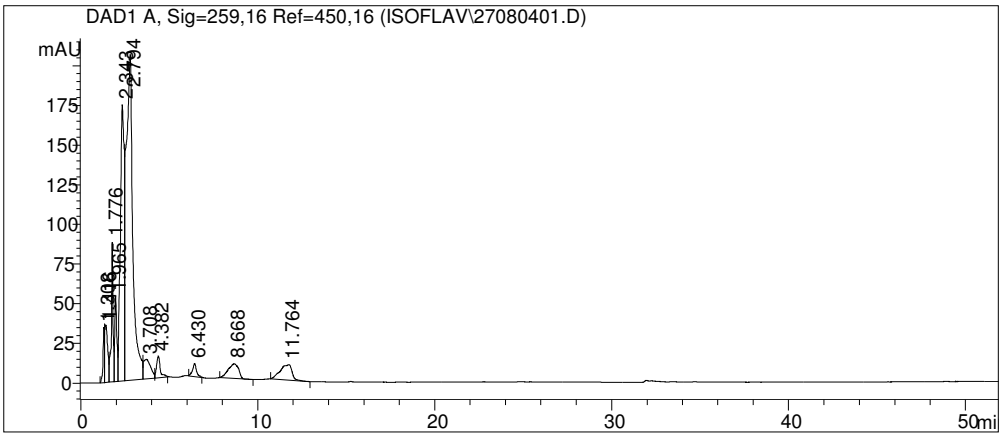
Figure 3.3. Chromatograms of (a) haricot bean, (b) chickpea



(c)

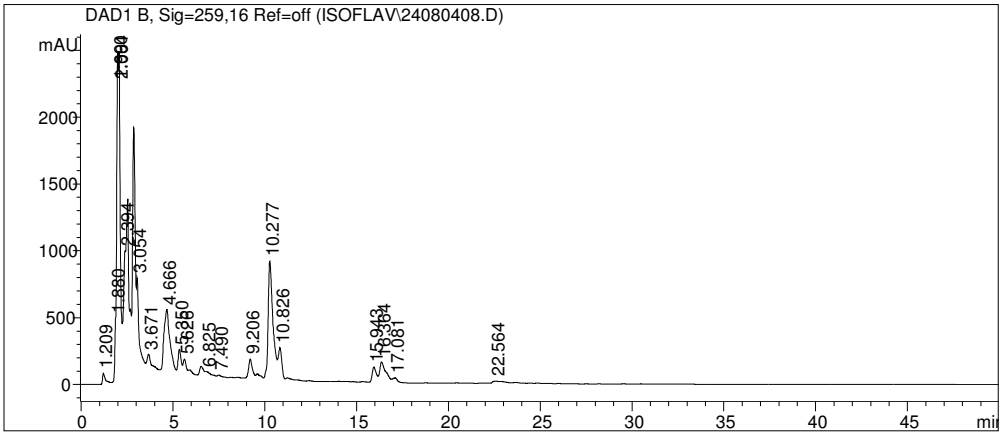


(d)

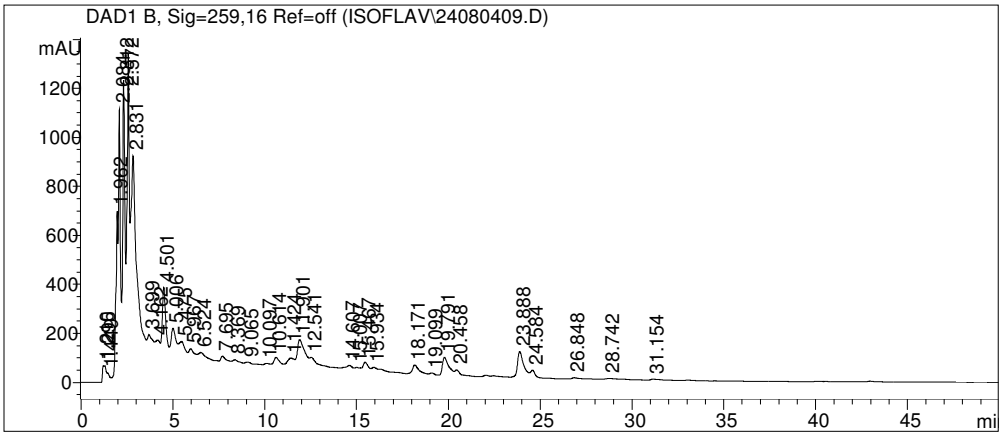


(e)

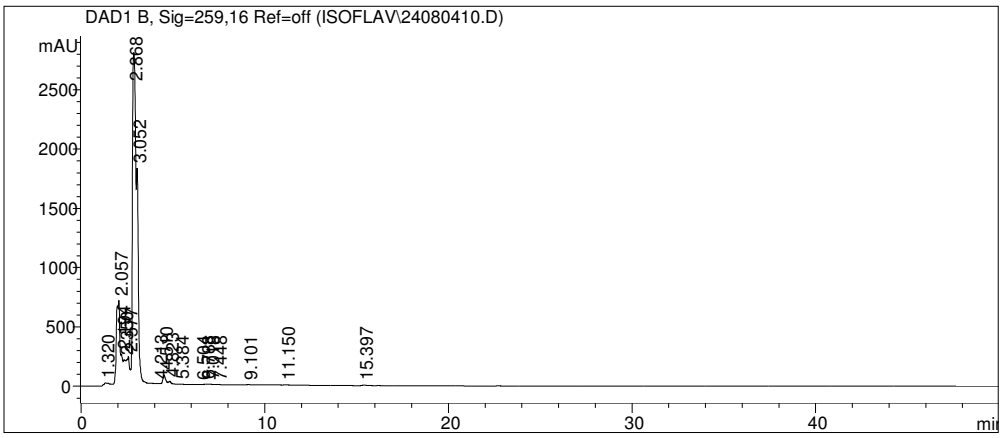
Figure 3.3. Chromatograms of (c) green lentil, (d) red lentil, (e) soybean (cont'd)



(f)

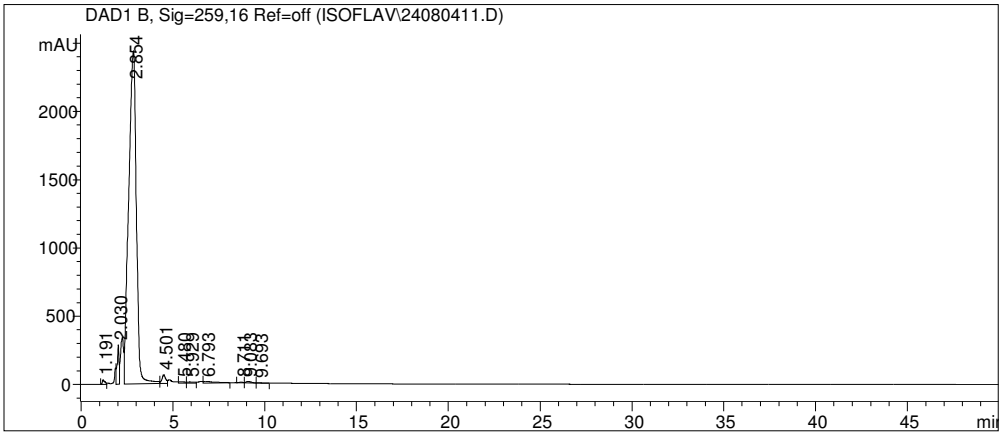


(g)

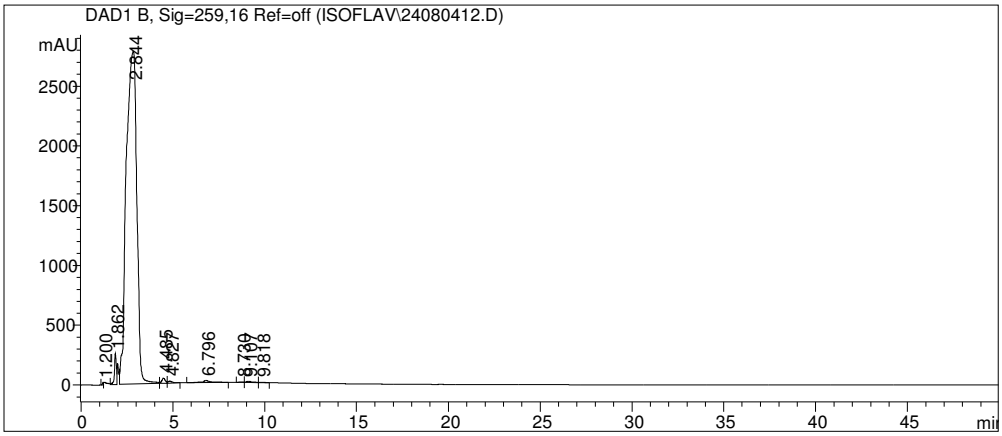


(h)

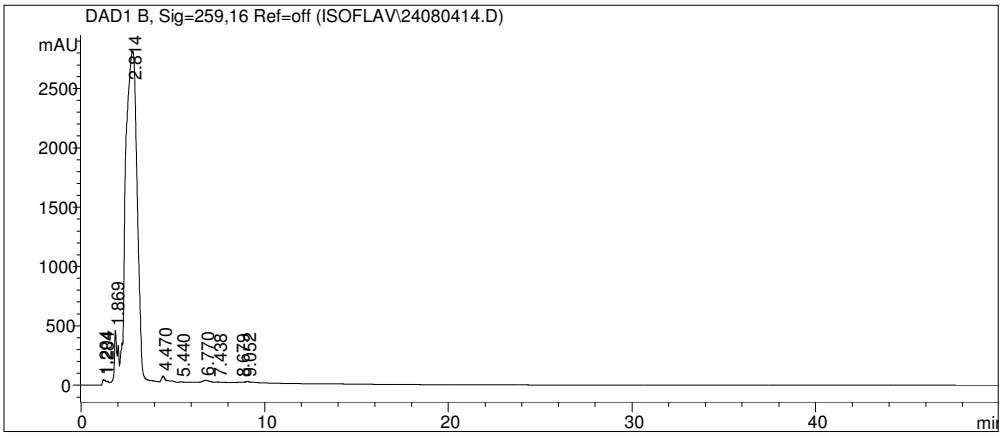
Figure 3.3. Chromatograms of (f) licorice root, (g) yarrow, (h) chestnut (cont'd)



(i)

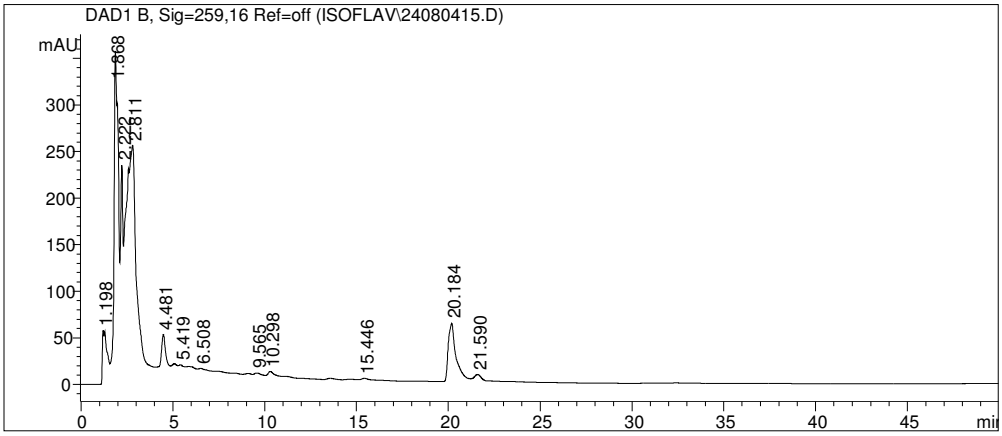


(j)

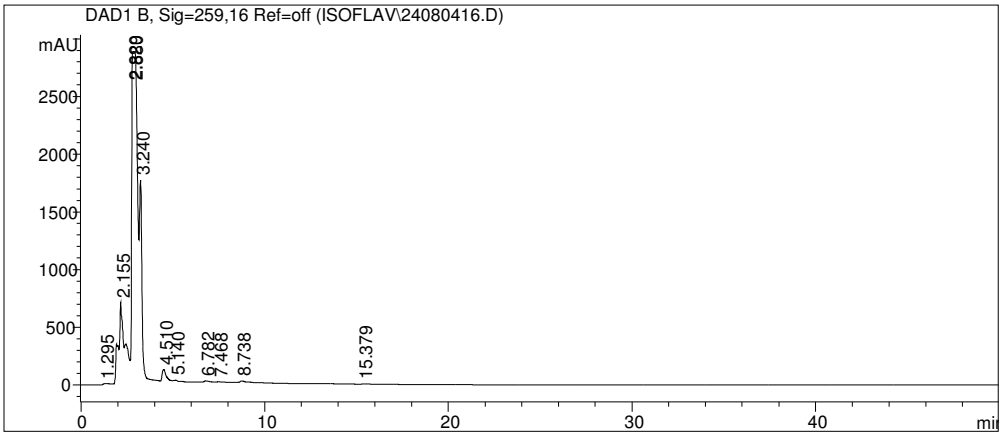


(k)

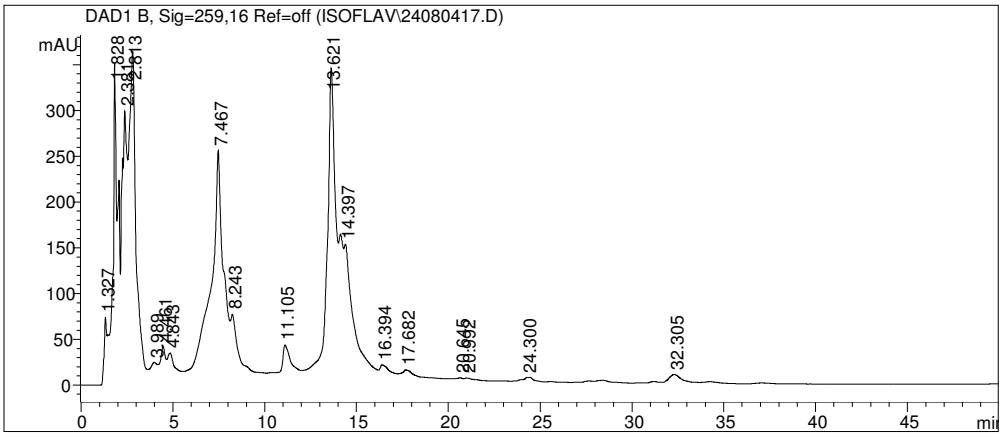
Figure 3.3. Chromatograms of (i) prunes, (j) raisins, (k) currants (cont'd)



(l)

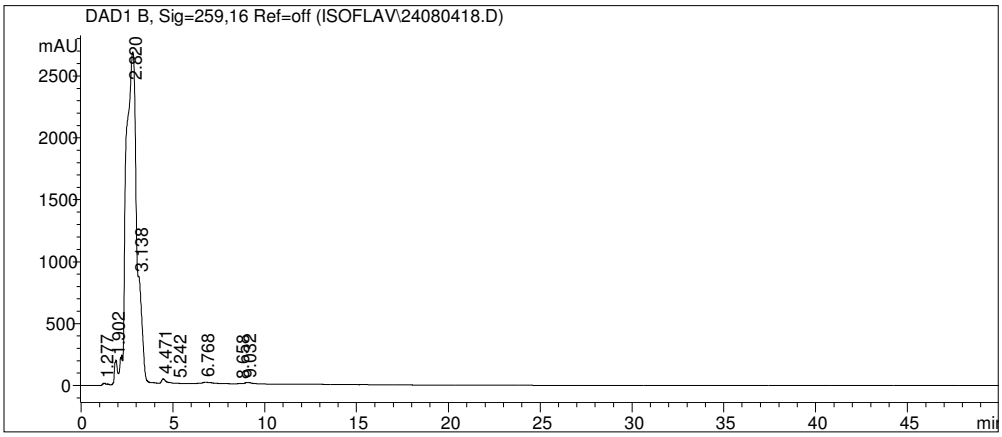


(m)

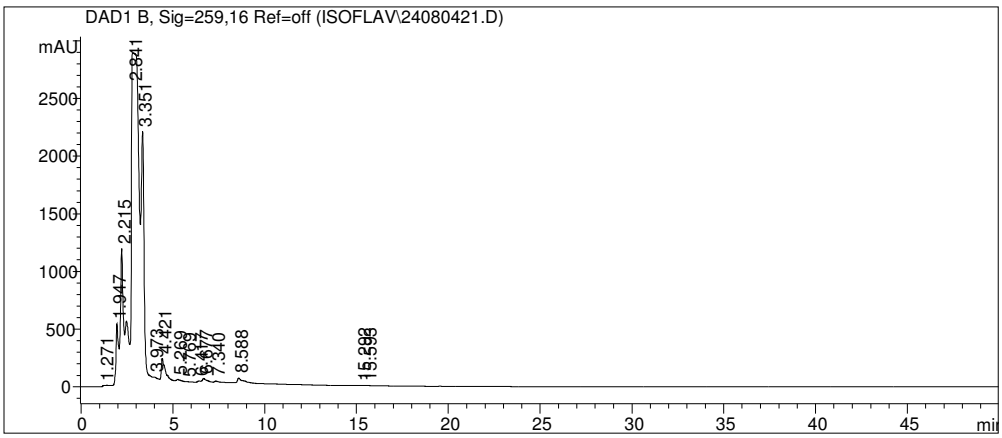


(n)

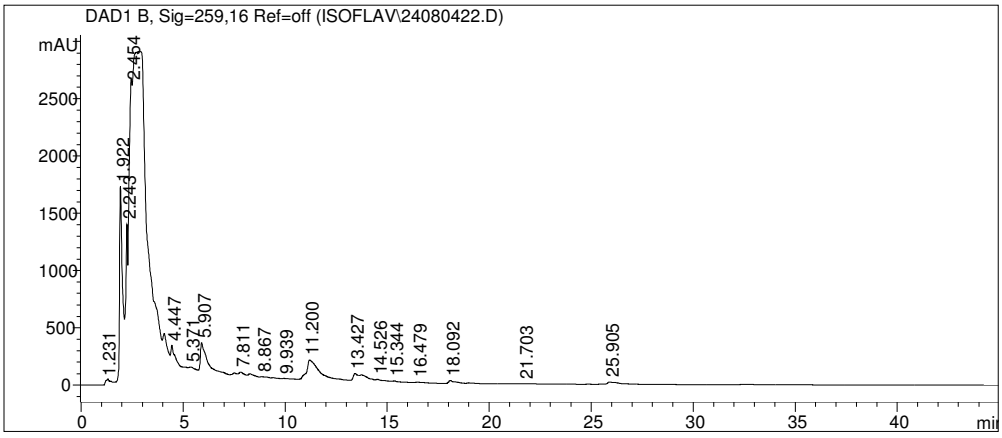
Figure 3.3. Chromatogram of (l)black cumin,m)dried apricot,(n)dried parsley(cont'd)



(o)

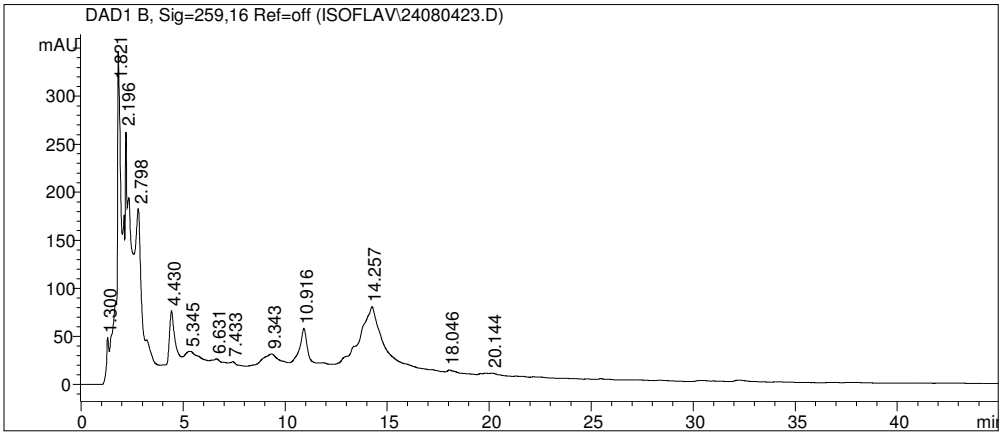


(p)

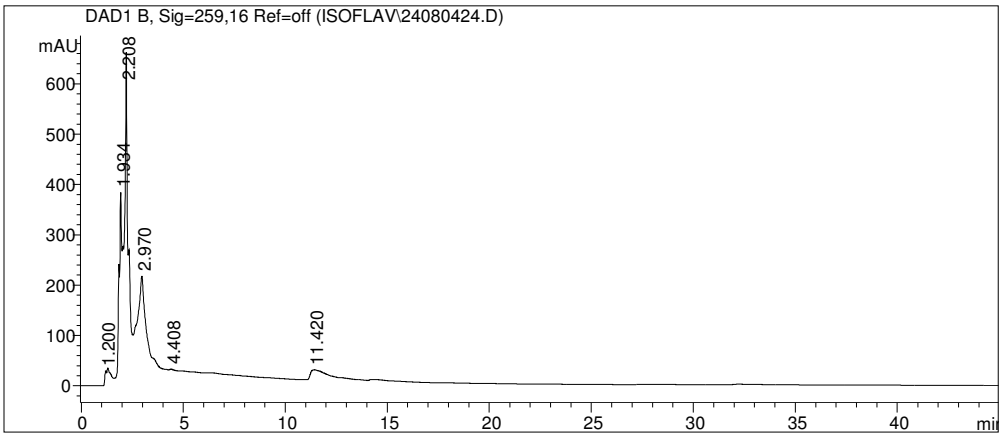


(r)

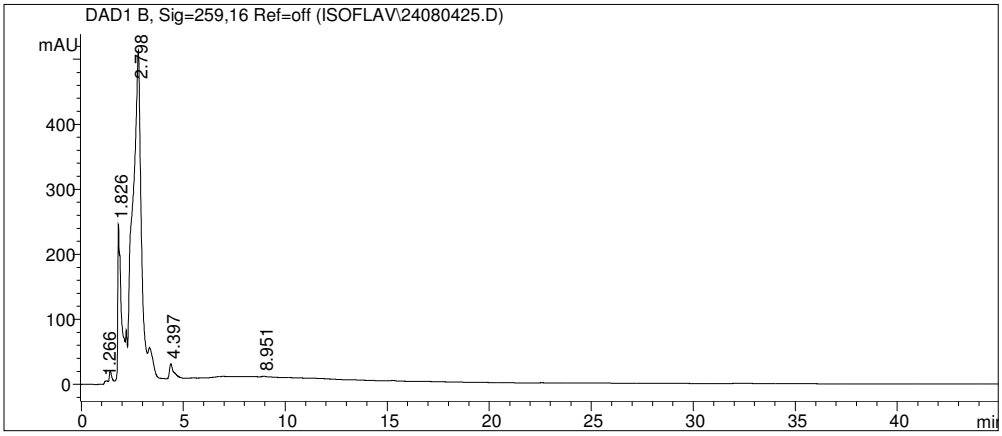
Figure 3.3. Chromatogram of (o)dried dates, (p)dried figs, (r)sage (Aegean region) (cont'd)



(s)



(t)



(u)

Figure 3.3. Chromatograms of (s) sage (Mediterranean region), (t) grapevine leaves, (u) gilaburu (cont'd)

3.3. Evaluation of Chromatograms of 20 Samples

In order to determine whether there is a peak/peaks at the chromatogram of the sample, eluting at or very near the retention time(s) of one/more of the analytes, the chromatogram of sample was laid over the chromatogram of standard mixture. If a peak coincided with or appeared very close to one of the analyte peaks, spectrum of the peak was then wieved with a suspect of its being one of daidzein, genistein or biochanin A. If spectrum of the suspected peak fit to spectrum of the analyte peak, it was concluded that suspected peak was, with high probability, peak of the analyte.

Examining twenty chromatograms in this respect, none of the analyte peaks was observed at chromatograms of twenty samples excluding soybeans and chickpeas, meaning that none of the compounds were identified in these eighteen food materials at detectable levels. Even if there was a peak almost coinciding with one of the analyte peaks; when overlaying of the two spectra was done, it was clearly observed that they were certainly different compounds.

As for soybeans; two peaks eluting at 8.668 min and 11.764 min were investigated, whether they were peaks of daidzein and genistein, respectively (Figure 3.4a). When overlaid; spectrum of the peak eluting at 8.668 min perfectly fit to the spectrum of daidzein (Figure 3.4b) and spectrum of the peak eluting at 11.764 min perfectly fit to the spectrum of genistein (Figure 3.4c). These are shown in Part 3.4. Since there was no peak at the retention time of biochanin A, it was concluded that there was no biochanin A at detectable levels in soybeans.

As for chickpeas; two peaks eluting at 12.404 min and 20.327 min were investigated, whether they were peaks of genistein and biochanin A, respectively (Figure 3.5a). When overlaid; spectrum of the peak eluting at 12.404 min perfectly fit to the spectrum of genistein (Figure 3.5b) and spectrum of the peak eluting at 20.327 min perfectly fit to the spectrum of biochanin A (Figure 3.5c). In order to be sure, the chickpea sample and the chickpea sample spiked with genistein and biochanin A standards were also analyzed (Figure 3.6); resulting in higher sample peaks in the latter chromatogram. These are shown in Part 3.5.

3.4. Qualitative Determination of Daidzein and Genistein in Soybeans

Chromatograms of standard mixture and soybean (overlaid), spectra of daidzein and peak at 8.668 min (overlaid) and spectra of genistein and peak at 11.764 min (overlaid) are given in Figure 3.4

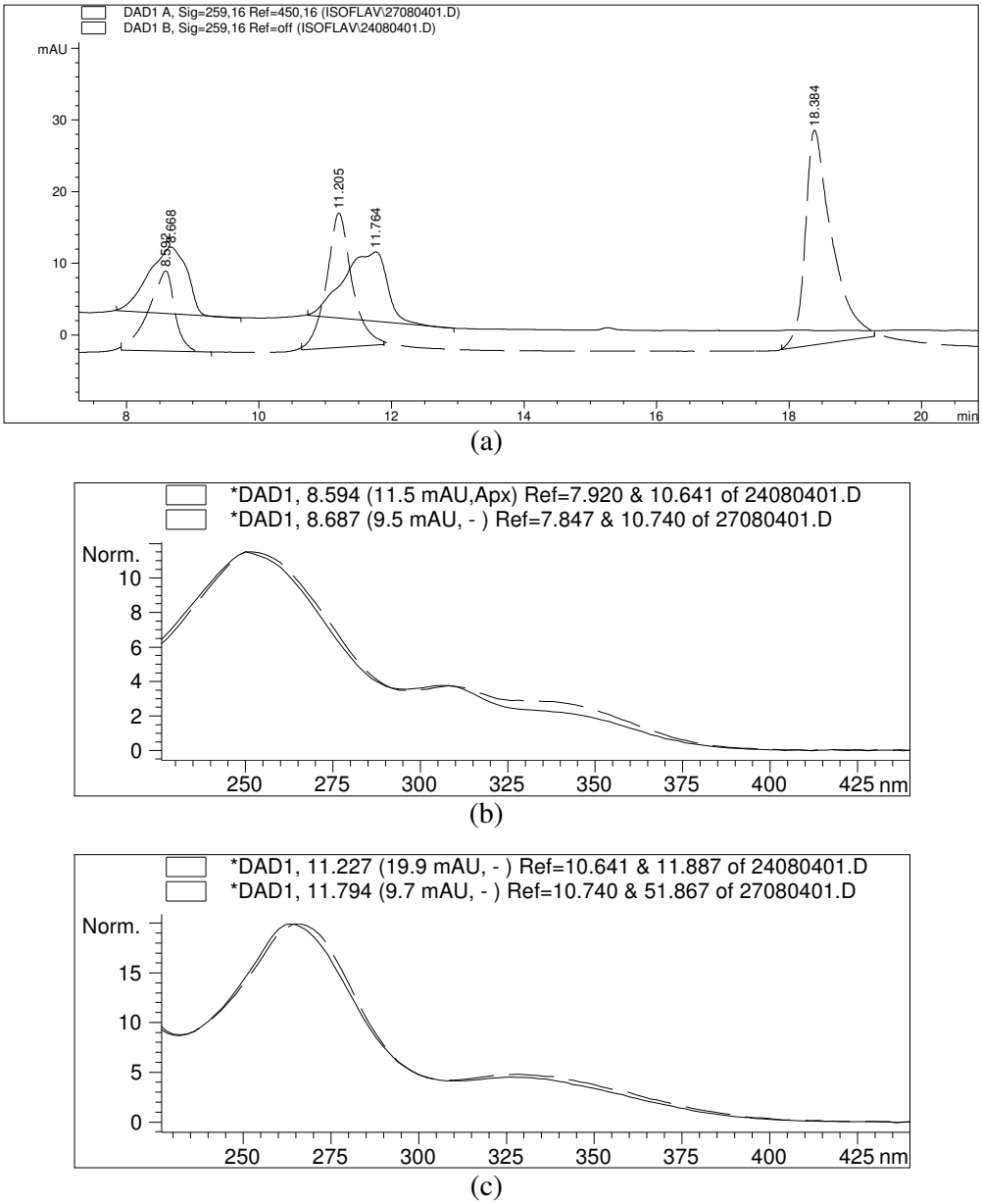


Figure 3.4. Overlaid (a) chromatograms of soybean and standard mixture (b) spectra of daidzein and peak at 8.668 min (c) spectra of genistein and peak at 11.764 min. (---) illustrates chromatograms and spectra of the standards.

3.5. Qualitative Determination of Genistein and Biochanin A in Chickpeas

Chromatograms of standard mixture and chickpeas (overlaid), spectra of genistein and peak at 12.404 min (overlaid) and spectra of biochanin A and peak at 20.327 min (overlaid) are given in Figure 3.5

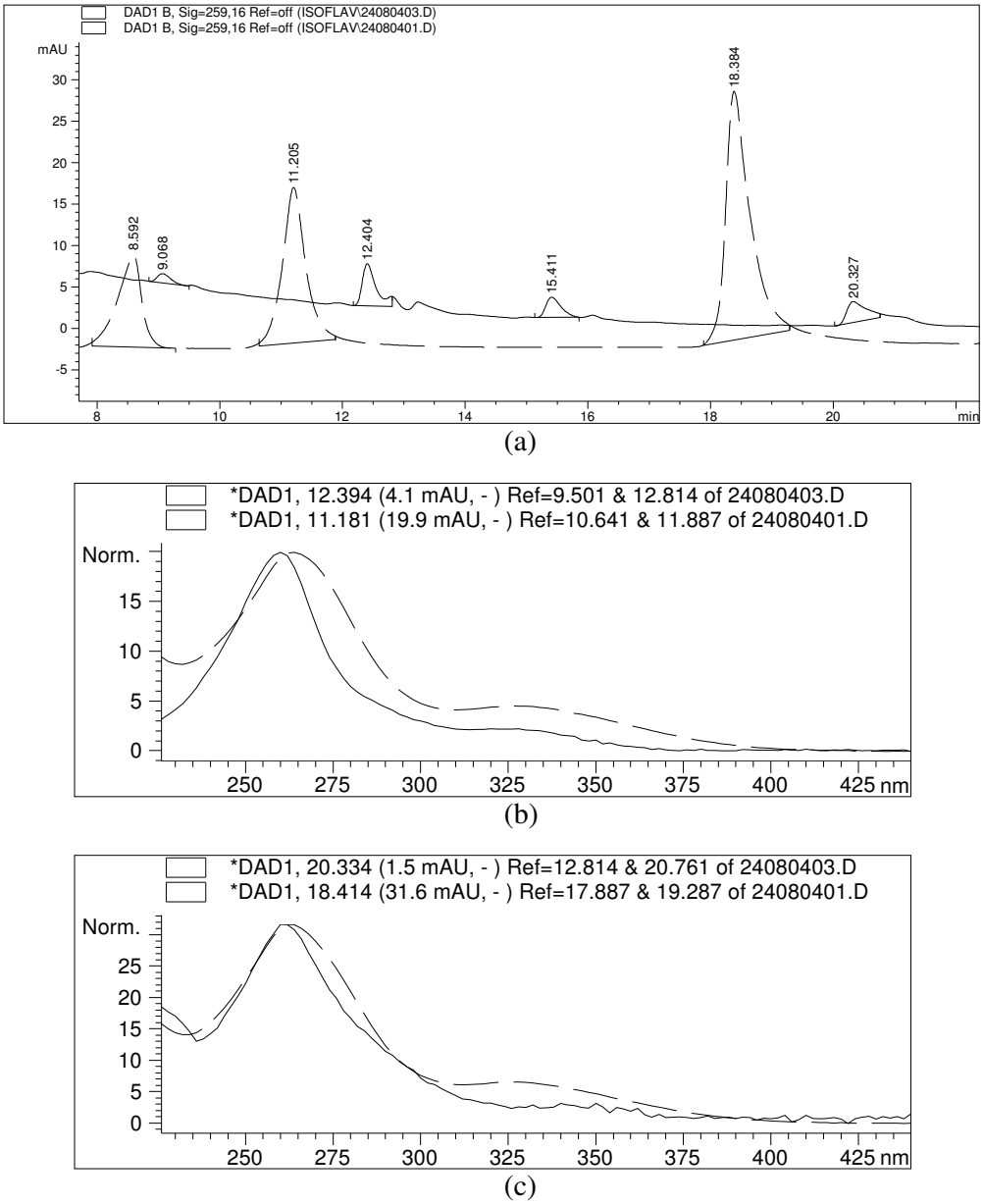
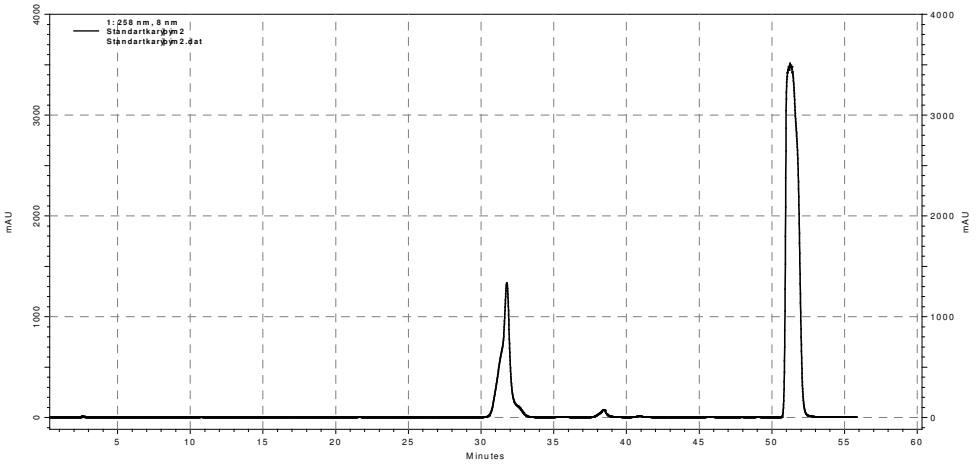
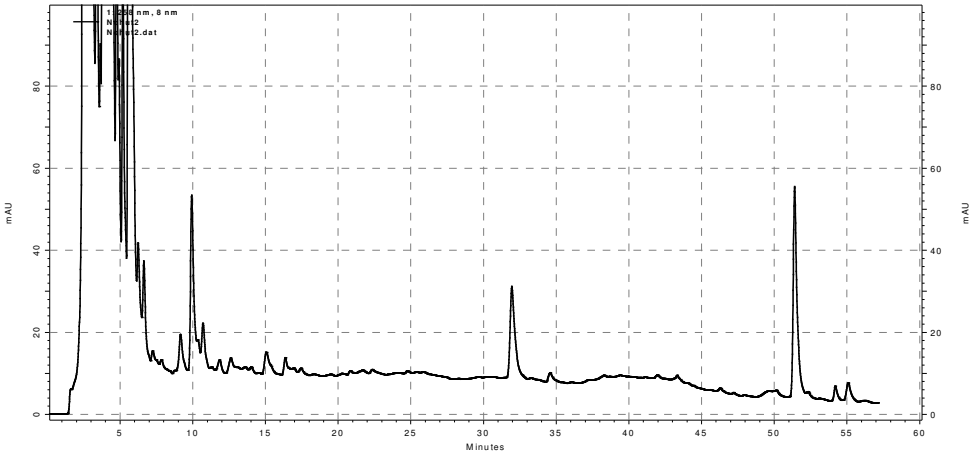


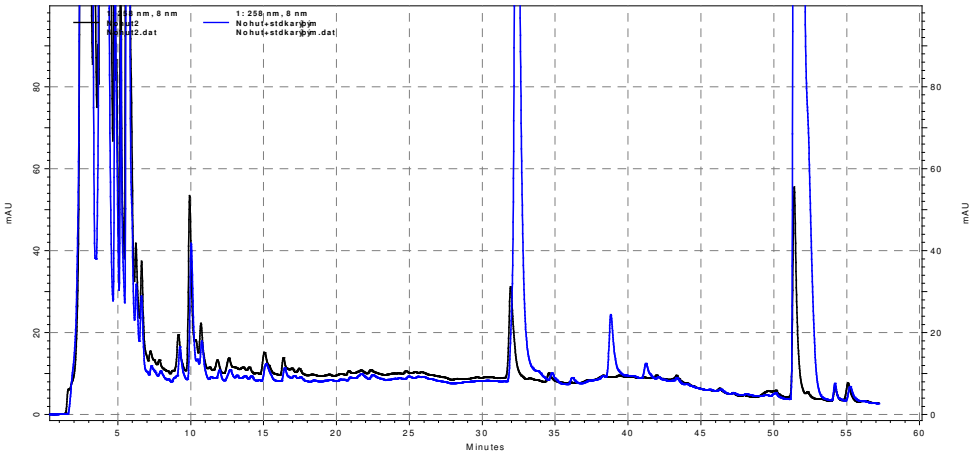
Figure 3.5. Overlaid (a) chromatograms of chickpea and standard mixture (b) spectra of genistein and peak at 12.404min (c) spectra of biochanin A and peak at 20.327min (---) illustrates chromatograms and spectra of the standards.



(a)



(b)



(c)

Figure 3.6. Chromatogram of (a) genistein and biochanin A standard mixture, (b) chickpea, (c) chickpea (black) laid over chickpea spiked with genistein and biochanin A standards (blue)

3.6. Quantitative Determination of Daidzein and Genistein in Soybeans

Having qualitatively determined daidzein and genistein in soybeans; by using the concentration versus peak area data of daidzein and genistein standards in the standard mixture (Table A.1) and by applying direct proportionation between area data of standard and area data of analyte peak in soybean chromatograms, concentrations of daidzein and genistein in soybeans were calculated and given in Table 3.3.

Table 3.3. Daidzein and genistein contents of soybeans (mg/100 g soybeans)

	Daidzein	**Genistein**
Soybeans	91.36 ± 0.245	85.57 ± 0.181

3.7. Quantitative Determination of Genistein and Biochanin A in Chickpeas

Having qualitatively determined genistein and biochanin A in chickpeas; by using the concentration versus peak area data of genistein and biochanin A standards in the standard mixture (Table A.1) and by applying direct proportionation between area data of standard and area data of analyte peak in chickpea chromatograms, concentrations of genistein and biochanin A in chickpeas were calculated and given in Table 3.4.

Table 3.4. Genistein and biochanin A contents of chickpeas (mg/100 g chickpeas)

	Genistein	**Biochanin A**
Chickpeas	0.89 ± 0.053	0.95 ± 0.061

3.8. Phytoestrogen Contents of Twenty Different Food Materials

Phytoestrogen contents of analysed all food materials, including those that do not contain any of daidzein, genistein and biochanin A, are given in Table 3.5.

Table 3.5. Daidzein, genistein and biochanin A contents of food materials (mg/100g)

Food Material	Daidzein	Genistein	Biochanin A
Haricot beans	nd[a]	nd	nd
Chickpeas	nd	0.89 ± 0.053[b]	0.95 ± 0.061
Green lentils	nd	nd	nd
Red lentils	nd	nd	nd
Soybeans	91.36 ± 0.245	85.57 ± 0.181	nd
Licorice root	nd	nd	nd
Yarrow	nd	nd	nd
Dried chestnuts	nd	nd	nd
Prunes	nd	nd	nd
Raisins	nd	nd	nd
Currants	nd	nd	nd
Black cumin	nd	nd	nd
Dried apricots	nd	nd	nd
Dried parsley	nd	nd	nd
Dried dates	nd	nd	nd
Dried figs	nd	nd	nd
Sage (Aegean region)	nd	nd	nd
Sage (Mediterranean region)	nd	nd	nd
Grapevine leaves	nd	nd	nd
Gilaburu	nd	nd	nd

[a] nd : not detected
[b] Values are expressed as “mean ± standard error”

3.9. Evaluation of the Results

The precise phytoestrogen content of many individual foods is not known and differ according to variety, location and season. (Oomah, 2002)

Soybeans, having proved to be the richest dietary source of daidzein and genistein, have been the most studied food item in the area of phytoestrogens. Therefore, there are a great number of published articles on isoflavone contents of soybeans, reporting daidzein contents in the range of 0.5-127.7 mg/100 g soybeans and genistein contents in the range of 1.1-150 mg/100 g soybeans (Table 1.2). In this study, 100 g dry soybeans were found to contain 91.36 mg daidzein and 85.57 mg genistein. These values fall within the daidzein and genistein concentration ranges determined by the previous studies.

As for biochanin A content of soybeans; USDA-Iowa State University (1999) detected 0.01 mg biochanin A in 100 g soybeans. Mazur *et. al.* (1998) reported four different varieties of soybeans, two of which contained around 0.015 mg/100 g, one contained biochanin A in trace amounts and the other contained no soybeans. (Table 1.2) In this study, no biochanin A was found in soybeans.

Since soybeans belong to the family Leguminosae, other members of this family including haricot beans, chickpeas and lentils were selected for analysis of isoflavones in this study. However, there are limited reported data on isoflavone contents of non-soy foods, including chickpeas. Genistein contents of different chickpeas in a single study (Mazur *et. al.*, 1998) vary from 0.0693 to 0.214 mg/100 g and biochanin A contents of different chickpeas in the single study vary from 0.838 to 3.08 mg/100 g (Table 1.3). In this study, 100 g chickpeas were found to contain 0.89 mg genistein and 0.95 mg biochanin A.

Limited data on daidzein contents of chickpeas in previous studies vary between 0.01 and 0.192 mg/100 g (Table 1.3). However, no daidzein was detected in the analysed chickpeas.

Similarly, there are limited number of studies reporting isoflavone contents of other beans and lentils; and the reported values are in the range of 0.0-0.4 mg/100g for beans and in the range of 0.00-0.18 mg/100 g for lentils (Table 1.4). In this study, none of the analytes were detected in either beans or lentils.

There is one study (Liggins *et. al.*, 2000) reporting daidzein and genistein contents of selected fruits and nuts, data varying between 0.0 and 0.2 mg/100 g (Table 1.5). In this study, none of the analytes was determined in selected fruits and nuts.

The reason for detecting none of daidzein, genistein and biochanin A in selected beans, lentils, fruits and nuts might be the changes in variety and location, and also the relatively high limit of detection of the method used. Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (2000) reported that the limits of detection for HPLC-UV methods are generally in the range of >0.001-0.002 mg/L. On the other hand, MS-based methods are sufficiently sensitive to measure levels of phytoestrogens in the <0.001 mg/L (Setchell *et. al.*, 1987). Therefore, HPLC-DAD system could have not detected and quantified the relatively low levels of isoflavones in some of analysed food items.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

Estrogen deficiency in post-menopausal women can lead to unpleasant symptoms such as hot flushes, sleep deprivation, forgetfulness and vaginal dryness, with a long-term increased risk of bone loss in addition to cardiovascular disease. Doctors have recommended hormone replacement therapy (HRT) for relief of these symptoms; however, by the realization that HRT is not as safe or effective as previously thought, interest in phytoestrogens has significantly increased.

Applications of phytoestrogens in industry are becoming prevalent. Phytoestrogen therapy is applied as a natural alternative to the use of post-menopausal HRT. There is a global movement towards increased consumption of foods rich in phytoestrogens (phytoestrogen-rich diets), and tablet formulations of concentrated isoflavone extracts. Phytoestrogen creams and phytoestrogen capsules are being heavily promoted.

In this study, raw materials for phytoestrogen industry were investigated. Food items belonging to different groups, including fruits, nuts, herbs and especially legumes, were selected and analysed for their daidzein, genistein and biochanin A contents.

Extraction with acetonitrile and HCl is highly recommended. Acid hydrolysis should be included if aglucones are intended to be detected. Molarity of HCl may be 1-2 M, 2 M being more effective. Heating may be done at 80 or 100 ºC, 80 ºC eliminating the possibility of degradation of aglucones.

As for chromatographic analysis; RP-HPLC-UV/DAD is the method of choice if food materials with high phytoestrogen contents are investigated. But; if phytoestrogen contents below 1 mg/100 g are to be detected, MS-based methods will provide better results.

As a result of screening of twenty different food materials, it was concluded that soybeans contain high amounts of daidzein (91.36 mg/100 g) and genistein (85.57 mg/100 g), and chickpeas contain relatively small amounts of genistein (0.89 mg/100 g) and biochanin A (0.95 mg/100 g). The remaining eighteen food materials (haricot beans, green lentils, red lentils, licorice root, yarrow, dried chestnuts, prunes, raisins, currants, black cumin, dried apricots, dried parsley, dried dates, dried figs, sage (from Aegean region of Turkey), sage (from Mediterranean region of Turkey), grapevine leaves and gilaburu) were found to contain none of daidzein, genistein and biochanin A.

Soybeans are still an important source of daidzein and genistein. Only chickpeas were found to contain genistein among all other analysed food materials. Since the genistein content of soybeans is nearly 100 times that of chickpeas, it is difficult for chickpeas to be an alternative to soybeans. In the case of biochanin A; only chickpeas were found to contain this compound, not ignoring the fact that the concentration of the compound is too low.

Being a very popular subject of interest for the research field of bioactive foods, phytoestrogens seems to remain on the agenda of many countries as a priority for the coming years. Therefore, it is highly recommended that different components of Mediterranean and Anatolian diet, particularly different varieties of legumes, should be analysed for their phytoestrogen contents and estrogenic activities which will contribute to food, medicine and cosmetics industries.

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APPENDICES

Table A.1. Peak areas of the analytes in standard mixture

	Standard Concentration (ppm)	**Peak Area[a] (mAU*s)**
Daidzein	11.1	747.86
Genistein	6	560.69
Biochanin A	7.5	876.31

[a] Values are means of three measurements