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Simultaneous Determination of Daidzein, Genistein and Formononetin in Coffee by Capillary Zone Electrophoresis

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Abstract: Coffee is a favorite and beverage in Western countries that is consumed daily. In the present study, capillary zone electrophoresis (CE) was applied for the separation and quantification of three isoflavones including daidzein, genistein and formononetin in coffee. Extraction of isoflavones from the coffee sample was carried out by extraction and purification process using ether after the acid hydrolysis with the antioxidant butylated hydroxy-toluene (BHT). The experimental conditions of the CE separation method were: 20 mmol/L Na_2HPO_4 buffer solution, 25 kV applied voltage, 3 s hydrodynamic injection at 30 mbar, and UV detection at 254 nm. The results show that the three compounds can be tested within 10 min with a linearity of 0.5–50 μg/mL for all three compounds. The limits of detection were 0.0642, 0.134, and 0.0825 μg/mL for daidzein, formononetin and genistein, respectively. The corresponding average recovery was 99.39% (Relative Standard Detection (RSD) = 1.76%), 98.71% (RSD = 2.11%) and 97.37% (RSD = 3.74%).

Keywords: capillary zone electrophoresis (CE); daidzein; genistein; formononetin; acid hydrolysis

1. Introduction

Phytoestrogens (PEs), also called "dietary estrogens", are a group of natural, non-steroidal, estrogen-like compounds found in a wide variety of plants consumed by humans [1]. Currently, four different groups of plant phenolics are considered PEs, including isoflavones and lignans, coumestanes, flavonoids and stilbenes. Other less-investigated compounds include prenylflavonoids, flavones, flavans and phytosterol esters [2]. These compounds are similar to 17β-estradiol in structure and function [3]. They have a dual-directional regulation function in the human body, meaning both anti-estrogen activity and estrogenic activity [4]. Such compounds have good curative effects for the treatment of cardiovascular disease, breast cancer, Alzheimer's disease, neurodegenerative disorders, and osteoporosis, etc. [5–8]. They can also be used for the treatment of the symptoms of menopause [9].

PEs are common in nature and have many pharmacological activities [9,10]. It has been reported that the daily take of PEs for menopausal women in the Caucasus region of the United States is no more than one milligram [11]. There are some foods that contain PEs including: soybeans and soy products, tempeh, linseeds, sesame seeds, wheat berries, fenugreek, oats, barley, beans, lentils, yams, rice, alfalfa, mung beans, apples, carrots, pomegranates [12], wheat germ, rice bran, lupin, kudzu, coffee, licorice root, mint, ginseng, hops [13], bourbon, beer [14], fennel and anise [15]. As we know, the content of PEs is different between various foods. In addition, it may also occur in the same kinds of foods, such as soy beverages and tofu, since they are made of different kinds of raw materials or using different processing methods [16].

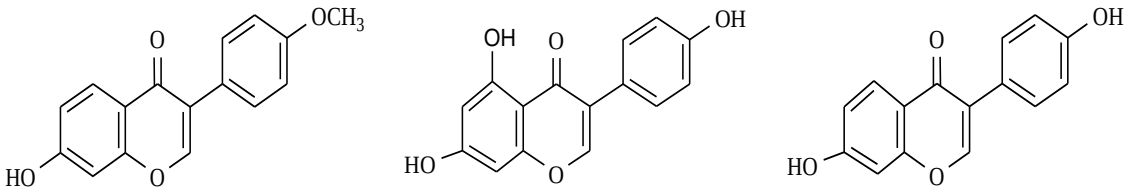
It has been proved that phytoestrogen-rich diets include soybeans and soybean products, followed by legumes, nuts, oilseeds, fruits and vegetables [17]. As is well known, there is a phenomenon

where people consume less soy and its derivatives in Western countries than in Eastern ones [18]. However, coffee is a favorite beverage in Western countries that is consumed daily; for example, in the United States, 29 September is celebrated as "National Coffee Day" [19]. Thus, it is necessary to study the content of isoflavones in coffee.

Some researchers have been studied the phytoestrogens in plants [20]. The methods used for the determination of phytoestrogens in plants are GC-MS [21,22], HPLC [17,23–31], and LC-MS [32,33]. It should be noted that HPLC separation is generally carried out on reversed-phase columns using the methanol of acetonitrile and water containing small amounts of acid as a modifier [29]. As far as we know, few studies have been reported using capillary zone electrophoresis (CE) in the analysis of isoflavones. Aramendia et al. [34] separated and identified several isoflavones using CE-ESI/MS in negative ion mode from standard samples. García-Villalba et al. [35] used capillary electrophoresis–time-of-flight-mass spectrometry to compare the metabolic profiles of conventional and genetically modified soybeans, including isoflavones, amino acids, carboxylic acids and peptides. In the above references, separation was carried out in positive mode at pH 9–9.5 and detection was achieved in negative ionization mode, but quantification was not reported. Also, the process is somewhat time-consuming, though it is useful and effective.

From the works reported so far, one can see that the quantitative determination of isoflavones in plants is mostly done by HPLC combined with acid hydrolysis or alcohol extraction. In addition, the separation system is acidic. The HPLC method uses a large amount of organic solvent in mobile phase and requires a long separation time. The acid hydrolysis and ethanol extraction are simple; however, the process cannot transform glycosides into aglycone. Also, the acid hydrolysis process may lead to the samples containing a large number of complexes and it is difficult to separate substances after they are treated by acid hydrolysis.

The aim of this work was to develop a simple, efficient and sensitive CE method, as an alternative to other methods, for the quantitative analysis of three isoflavones, including daidzein, genistein and formononetin (their chemical structures are shown in Figure 1) in coffee. For this, we improved the extraction method of isoflavones from the coffee sample with an extraction and purification process using ether instead of ethanol after the process of acid hydrolysis with the antioxidant butylated hydroxy-toluene (BHT). The proposed method was successfully applied to the determination of three isoflavones in commercially available coffee samples. To our knowledge, there are no methods that describe the quantification of isoflavones in coffee samples using the single CE. Also, the sample preparation of this method is simple, the analytical time is shorter, and the comparative cost is relatively lower. It can be used as an alternative method for the determination of isoflavones in coffee.



(**a**) Formononetin (**b**) Genistein (**c**) Daidzein

Figure 1. The chemical structures of (**a**) formononetin, (**b**) genistein, and (**c**) daidzein.

2. Materials and Methods

2.1. Chemicals and Reagents

Isoflavone standards daidzein (≥98%), genistein (≥98%) and formononetin (≥98%) were purchased from Shanghai Juyuan Biological Technology Co., Ltd. (Shanghai, China). Disodium hydrogen phosphate (Na_2HPO_4) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Methanol, acetonitrile were obtained from Kemel Chemical Reagent Co., Ltd. (Tianjin, China).

Butylated hydroxy-toluene (BHT) was used as antioxidant and was obtained from Shanghai Feige Chemical Co. Ltd. (Shanghai, China). The analytical grade regents were used to prepare the samples and the buffer solutions. The water used in all studies was ultrapure water (18.25 MΩ·cm) obtained from an ultrapure purification system (Yongjieda, Hangzhou, China). The nitrogen used for evaporation of solvents was of 99.9% purity and supplied by Feiyuan Comprehensive Service Technology (Yantai, China).

2.2. Standard and Sample Preparation

Each type of isoflavone stock solution (1 g/L) was prepared in methanol and stored at −18 °C in volumetric flask. A series of calibration solutions (0.5, 1.0, 5.0, 10.0 and 50.0 μg/mL) was prepared by diluting the stock standard solution with methanol and stored at 4 °C.

Ground coffee, purchased from the local supermarket of United States of America, was passed through a 0.45 mm sieve. A 0.2 g aliquot of ground coffee was spiked with the antioxidant (1%, 50 μL), ethanol (2 mL) and hydrochloric acid 3.4 mol/L HCL (2 mL). The acid hydrolysis was performed at 75 °C for 150 min, under reflux. This process was performed according to Alves et al. [23] with minor modification. After the hydrolysis, 1 mL of the hydrolysate was neutralized with 120 μL of sodium hydroxide (10 mol/L), vortexed and ultra-centrifuged (5000 r/min, 5 min). Then 2 mL supernatant was collected, extracted and purified by ether [36]. Then the superstratum ether was dried by nitrogen. The residue was dissolved in methanol, and volume was completed till 2 mL. And then it was filtered through a 0.45 μm Millipore filters before analysis and stored under refrigeration (4 °C) until use.

2.3. CE Apparatus

In present study, the experiments were performed on a LUMEX CAPEL 105M Capillary Electrophoresis System (LUMEX Ltd., St. Peterburg, Russia). A 60 cm (50.5 cm effective length), 75 μm i.d. fused silica capillary (Yongnian Ruifeng Chromatographic Devices Limited Company, Baoding, China) was used. A PH5500 dual-channel pH/ion meter (Crison, Singapore) was used for regulating the pH of the buffers.

2.4. Electrophoretic Conditions

Before using, each new capillary column was rinsed sequentially with ultrapure water for 10 min, 0.5 mol/L NaOH for 40 min, and then ultrapure water for 10 min. At the beginning of each day the capillary was rinsed with ultrapure water for 2 min, 0.2 mol/L NaOH for 10 min, ultrapure water for 5 min and running buffer for 10min. Between different buffers, the capillary was conditioned with ultrapure water for 2 min, 0.2 mol/L NaOH for 10 min, ultrapure water for 5 min, and buffer for 5 min. The capillary was conditioned only with running buffer for 3 min between consecutive injections. All buffers and solutions were filtered through a 0.45 μm Millipore filter before injection. Pressure injection was set at 30 mbar for 3 s. The conditions of electrophoresis was kept the temperature at 25 °C, applied voltage at 25 kV, and the UV detection at 254 nm.

3. Results and Discussion

3.1. Optimization of the CE Method

It was necessary to determine the parameters of CE before analyzing the daidzein, genistein and formononetin in the coffee sample. The UV detection wavelength of isoflavones is generally 250–265 nm. In order to determine the optimal wavelength for simultaneous determination of the three compounds, the UV absorption spectrum of the isoflavone solution from 230 to 350 nm was scanned (see Figure 2). As can be seen from the figure, the best absorption wavelengths of the three compounds were 250–260 nm, 250–260 nm, and 260–270 nm, respectively. However, all three isoflavones have a strong absorption at 254 nm, so the detection wavelength was set to 254 nm.

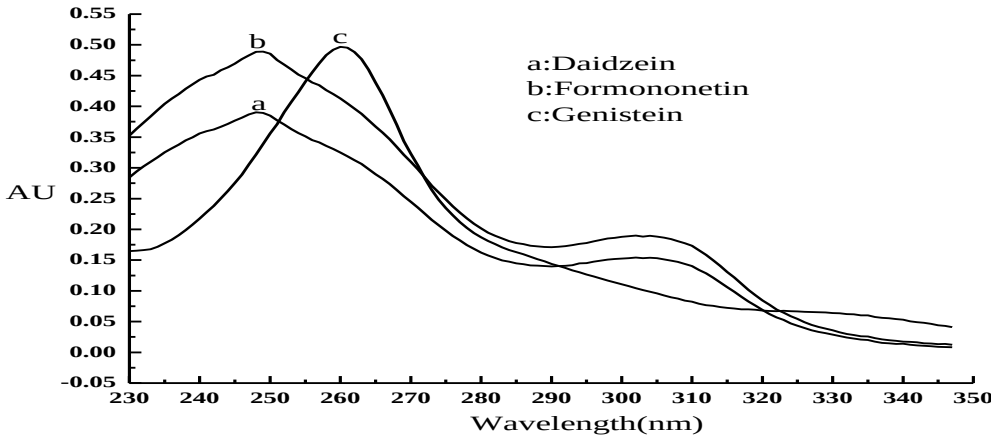


Figure 2. UV spectra of formononetin, daidzein and genistein.

Five different concentrations of Na_2HPO_4 solution at 10, 20, 30, 40 and 50 mmol/L were investigated in the present study. With the buffer solution concentration increased, the degree of separation improved, and at the same time the analysis time increased as well. However, the analysis time of 30 mmol/L was a little shorter than that of 20 mmol/L. So, a buffer concentration of 30 mmol/L buffer concentration was selected.

The buffer pH may have some effect on the migration. Using the above optimized buffer concentration (30 mmol/L Na_2HPO_4), the pH was regulated at 7.0, 8.0, 9.0, 10.0, 11.0. As the pH increased, the analytical time increased; however, the separation was still not ideal. It was almost the same as that of the basic 30 mmol/L Na_2HPO_4. So the basic system without regulating the pH value was used in the analysis.

In the following, three organic modifiers (methanol, acetonitrile and acetone) were added to 30 mmol/L Na_2HPO_4 at pH = 9.9 to investigate the influence of the separations, respectively. From the results, we can see that their presence leads to no obvious improvement in the peak shape. Therefore, none of the organic modifiers was added to the separation system.

The effects of an injection time of 1, 3, 5, 8 and 10 s at 30 mbar were also investigated in order to obtain a better sensitivity and peak shape; finally, 3 s proved to be ideal for the subsequent analysis. Similarly, effects of temperature (in the range of 15–25 °C) and applied voltage (in the range of 15–25 kV) on the separation efficiencies were also verified. Finally, a temperature of 25 °C and an applied voltage of 25 kV were selected.

From the above results, the optimal CE separation conditions were selected: 30 mM Na_2HPO_4, 3 s pressure injection at 30 mbar, 25 kV applied voltage at 25 °C and detection at 254 nm. Under these conditions, the electropherogram of daidzein, genistein and formononetin was obtained and is shown in Figure 3.

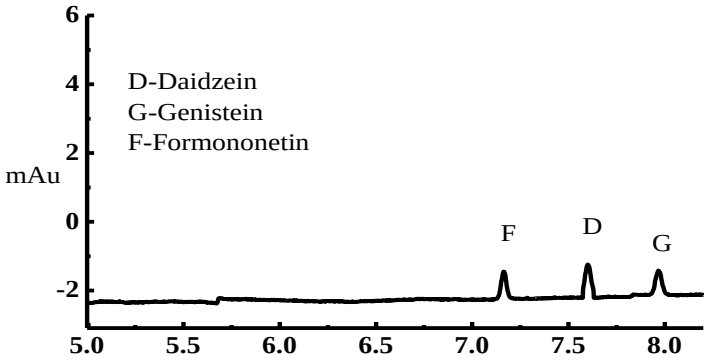


Figure 3. Electropherogram of the standard mixture of formononetin, daidzein and genistein.

3.2. Validation of the CE Method

3.2.1. Linearity, Limit of Detection and Limit of Quantification

The linear ranges were tested at 0.5–50.0 μg/mL for the three isoflavones at five concentration levels. Calibration curves were obtained by plotting the peak area of each standard as a function of the standard concentration (C). The correlations were: y = 4.0245C + 2.3062 (R^2 = 0.9995) for formononetin; y = 8.4133C − 2.8224 (R^2 = 0.9998) for daidzein; y = 6.5477C − 1.3905 (R^2 = 0.9994) for genistein. The limit of detection (LOD) and limit of quantification (LOQ) were determined as 3 σ/s and 10 σ/s, respectively, where σ is the standard deviation of the blank responses and s is the slope of the calibration curve. The LODs of formononetin, daidzein and genistein were 0.134, 0.0642 and 0.0825 μg/mL, respectively. The LOQs were 0.446 μg/mL for formononetin, 0.214 μg/mL for daidzein, and 0.275 μg/mL for genistein.

3.2.2. Study of Precision and Accuracy

The precision of the proposed method was validated by the intra-day and inter-day values of the migration time and peak area under the optimal conditions. The intra-day repeatability was determined by replicating injections of a 10 μg/mL solution of isoflavones six times in one day, while the inter-day repeatability was determined by performing injections for six consecutive days. The results are listed in Table 1. As can be seen in the table, the relative standard deviation (RSD) was less than 1.6% for the migration time and less than 5.4% for the peak area in all cases.

Table 1. Intra-day and inter-day precision of migration time and peak area for isoflavones.

Compound	Intra-Day (%)		Inter-Day (%)	
	Migration Time	Peak Area	Migration Time	Peak Area
Formononetin	0.34	4.60	0.55	5.31
Daidzein	0.62	3.45	1.23	4.51
Genistein	1.03	4.29	1.52	5.01

The method's accuracy was evaluated by the standard addition procedure. Firstly, blank samples were analyzed in order to determine the actual content of the three isoflavones in the sample. Then, three different amounts (0.5 μg/mL, 1.0 μg/mL and 2.0 μg/mL) of isoflavones were added into three coffee samples before the extraction. The results are presented in Table 2. From the table, we can see that satisfactory average recoveries (97.37%–99.39%) were obtained with an RSD lower than 3.74%. These data show that the pretreatment method has good extraction efficiency.

Table 2. Recoveries for the determination of the three isoflavones in coffee.

Compound	Concentration Added (μg/mL)	Recovery (%, n = 3)	RSD (%)
Formononetin	0.5	105.20	1.76
	1.0	95.53	
	2.0	97.44	
Daidzein	0.5	92.31	2.11
	1.0	103.34	
	2.0	100.48	
Genistein	0.5	103.58	3.74
	1.0	90.87	
	2.0	97.66	

3.3. Optimization of the Extraction Conditions and Sample Analysis

Pretreatment process optimization has to be developed since the isoflavones occur mainly as glycosides present in the coffee [37,38]. For the purpose of detecting the total amount of aglycones, the acid procedure is usually performed. According to Alves et al. [23], acid hydrolysis was performed first in the present study. However, it cannot give an ideal detection using the same procedure as in the reference. So we extracted and purified the sample with ether [36] after the process of acid hydrolysis with the antioxidant butylated hydroxy-toluene (BHT). The electropherograms of the coffee samples are shown in Figure 4. From the figure, one can see that the daidzein can be detected and at a value of 0.012 mg/g. Compared with the results of the work of Alves et al. [23], we can see that the amount of daidzein found in this study is in accordance with the value reported by the HPLC method. The work of Kuhnle et al. [31] reported total isoflavones of 1 mg per 100 g of instant coffee which is also similar to our results. The other two isoflavones could not be detected in our study. The reason may be that different kinds of coffee may contain different quantities or kinds of isoflavones [23], or the contents of the two could not be detected for this particular sample. There are other works [21,39] that have detected the coffee beverage (1 ìg of isoflavones per cup of coffee beverage (287 mL)) and coffee brew (10–66 ìg of daidzein, 15–29 ìg of genistein, and 72–78 ìg of formononetin for a 100 g cup of coffee brew) values, which indicated our results are acceptable.

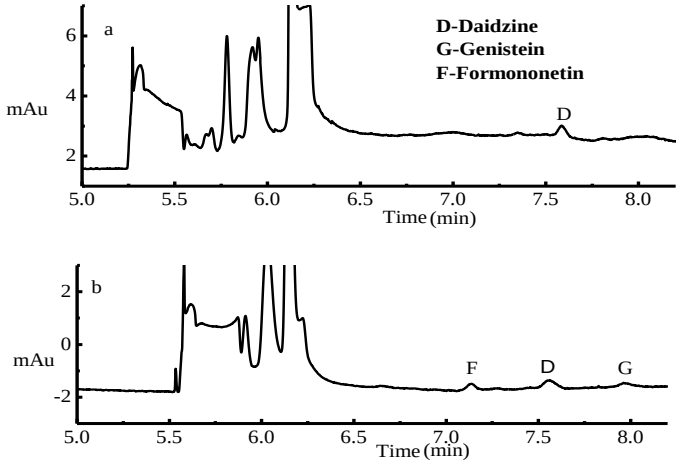


Figure 4. Electropherograms of (**a**) coffee sample; (**b**) coffee sample spiked with 1.0 μg/mL three isoflavones.

4. Conclusions

This work presents the application of CE for the determination of daidzein, genistein and formononetin in coffee. This method has the advantages of high sensitivity, shorter analytical time, minor amounts of samples and simple operation. It is concluded that the CE method is a good alternative to other methods.

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Author Contributions: Feng Luan and Huitao Liu conceived and designed the experiments; Xuanxuan Chen and Lili Tang performed the experiments; Feng Luan and Xuanxuan Chen wrote the paper.

Conflicts of Interest: The authors confirm that this article content has no conflict of interest.

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