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Short communication

Use of hollow fiber liquid phase microextraction and HPLC for extraction and determination of apigenin in human urine after consumption of *Satureja sahendica Bornm.*

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ABSTRACT

The applicability of hollow fiber liquid phase microextraction (HF-LPME) was evaluated for extraction and preconcentration of apigenin prior to its determination by HPLC. Different parameters affecting the HF-LPME recovery such as nature of organic solvent, pH of donor and acceptor phases, extraction time, stirring speed, salt addition were optimized. Under optimum conditions (1-octanol as organic solvent, pH of the donor phase = 3 and pH of acceptor phase = 11.5, extraction time of 75 min, stirring speed of 1000 rpm) limit of detection (LOD) of 0.1 ng/mL, linear range of 0.5–300 ng/mL and correlation of determination (R^2) of 0.9956 were obtained. The relative intra and inter-day standard deviations (RSD%) based on five replicate measurement were 3.5% and 10.7% respectively. Enrichment factor of 315 and recovery 85% were achieved. Finally, the applicability of the proposed method was evaluated by extraction and determination of apigenin in urine sample after consumption of *Satureja sahendica Bornm.* which is a native medicinal plant from Iran. Concentration of apigenin in urine sample was found to be 6.20 ng/mL.

1. Introduction

Apigenin belongs to a subclass of flavones with anticarcinogenic, anti-inflammatory, and antimutagenic properties [1–3] and is a commonly used dietary component of some vegetables, fruits and traditional medical herbs [4,5]. *Satureja sahendica Bornm.* (*SSB*), a member of the Labiateae family, is an endemic species of Iran, distributed in west and northwest of Iran. It is a late flowering species (late summer and fall), growing on rock walls, and rocky slopes [6] and in traditional medicine is used as a tonic, antiseptic, stomachic, antiexpectorant, relief of rheumatoid and nervous ailments [7,8]. Our preliminary experiments revealed that apigenin is one of the main flavonoids of (*SSB*) and therefore this species could be considered as a source of apigenin. Because of the importance of flavonoids to human health, the analysis, identification and structural determination of these compounds in biological fluids are of the uttermost importance in various areas of science. Several liquid chromatography methods have been reported for quantitative determination of apigenin in biological fluids. In these methods the sample preparation techniques are based on solid-phase extraction (SPE) [9] or liquid–liquid extraction (LLE) [10]. Although LLE offers high reproducibility and high sample capacity, it is a time-consuming procedure and requires large amounts of high-purity solvents which are expensive and produce toxic laboratory waste. SPE is also a time-consuming method since requires solvent evaporation in order to preconcentrate the analytes before final analysis [11]. Moreover, in these methods determination was performed after administration of drugs which usually have high concentrations in biological samples. In this work a hollow fiber liquid phase microextraction method, as a solventless method, was used to preconcentrate the ultra trace amount of apigenin in human urine sample after consumption of *S. sahendica Bornm.* which is a medicinal plant of Iran. Liquid phase microextraction (LPME) is a miniaturized liquid–liquid extraction technique. It has successfully overcomes the mentioned drawbacks of conventional LLE or SPE. Also it is sensitive, simple and inexpensive. Further it consumes low volume of organic solvent [12–15].

2. Experimental

2.1. Materials

Apigenin (Fig. 1) was purchased from Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile, benzyl alcohol, 5-nonanol and 1-undecanol were obtained from Fluka (Buchs, Switzerland). Phosphoric acid, sodium carbonate, HCl, THF (tetrahydrofuran), 1-octanol and toluene were bought from Merk (Darmstadt, Germany). The aerial parts of *S. sahendica* were collected from

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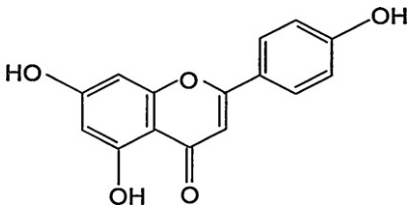

Fig. 1. Chemical structure of apigenin.

west Iran (Kurdistan province). A voucher specimen (No. 9715) was deposited at the herbarium of research center of agriculture and natural resources of Kurdistan. All other reagents were of analytical grade. Accurel Q3/2 polypropylene hollow fiber (200 μm wall thickness, 600 μm internal diameter and 0.2 μm average pore size), purchased from Membrana Company (Wuppertal, Germany), was used for all HF-LPME experiments.

2.2. Chromatographic conditions

All HPLC analysis was carried out using a HPLC system, consisting of 10 LC pump, a UV–Vis detector model LC-95 set at 370 nm and model 7125i manual injector with a 20 μL sample loop (Perkin-Elmer, Norwalk, CT, USA). Chromatographic separation was performed using C_{18}Column (250 mm × 4.6 mm, 5 μm particle size) from Waters (Milford, MA, USA). The mobile phase used for determination of apigenin was acetonitrile:0.1% phosphoric acid:THF (44.5:55:0.5, v/v) with a flow-rate of 1.0 mL/min at the ambient temperature.

2.3. Preparation of standard solutions and biological samples

Stock standard solution of apigenin (500 μg/mL) was prepared by dissolving it in methanol and stored at 4 °C and reprepared every 1 month. All the working standard solutions were freshly prepared by diluting stock solution with deionized double distilled water (pH 3.0 by additions of 1 mol/L HCl) to the required concentration.

The urine sample was obtained from a healthy volunteer that was fulfilled under following criterion: no smoking, without consuming fruits and vegetables 2 days before the study. A healthy volunteer consumed 5 g of SSB and samples were collected at 0, 1, 2, 4, 6, 8, 10 and 12 h after consumption. For preparation of biological sample 2.0 mL of 32% HCl was added into 10.0 mL of human urine and mixed well. After the urine sample was hydrolyzed for 30 min at 80 °C in water bath, hydrolyzed sample was centrifuged for 15 min at 3500 rpm. The supernatant was diluted two times with deionized double distilled water. pH of this solution is very low because of presence of concentrate HCl. Finally pH of the mixture was adjusted to 3.0 by addition of 4 mol/L NaOH. The HF-LPME–HPLC procedure was performed under optimum conditions.

2.4. Extraction procedure

A sample vial (12 mL) containing 11 mL of aqueous sample (100 ng/mL of apigenin with pH 3) with stir bar (14 mm × 4 mm) was placed on a magnetic stirrer. Each hollow fiber was cut manually into 8.8 cm length pieces, in which their internal volume is approximately 24 μL. Before use, the hollow fiber was ultrasonically cleaned in acetone for 5 min to remove any contaminations and dried in air. For extraction 24 μL of acceptor phase (carbonate buffer with pH 3) was withdrawn into a 25 μL Hamiltone syringe and its needle was inserted into the lumen of the hollow fiber. The hollow fiber was immersed in the organic solvent (1-octanol) for 10 s. Next, hollow fiber was placed into water for 5 s in order to wash the extra organic solvent from its surface. Then acceptor phase was injected into hollow fiber and one end of it was sealed with a piece of aluminum foil. The hollow fiber bent to U shape and was placed into the aqueous sample. The extraction was performed at room temperature and sample was stirred at 1000 rpm during the extraction (75 min). After extraction the hollow fiber was opened and the acceptor phase (24 μL) was withdrawn into the micro-syringe and directly injected into the HPLC system

3. Results and discussion

3.1. Optimization of HF-LPME conditions

3.1.1. Selection of organic extraction solvent

In order to select a suitable solvent for extraction, different organic solvents including: 1-undecanol, benzyl alcohol, toluene, 1-octanol and 5-nonanol were used. Solvent efficiency was evaluated for the extraction of a 11 mL aqueous sample containing 0.1 μg/mL of apigenin with pH 3.0. The hollow fiber impregnated with the organic solvent and filled with carbonate buffer (pH 10), was inserted as U shape into the vial and the extraction was performed for 30 min and the recovery was obtained. Results showed that 1-octanol with the recovery of 40% is a proper solvent for apigenin extraction.

3.1.2. Effect of pH of the donor phase

Apigenin has slightly acidic properties [16], therefore their existing forms (neutral or ionic form) could be changed by changes in pH. In order to reduce the solubility of analyte in water and increase the extraction yield of apigenin into the organic solvent, apigenin in donor phase should be in neutral form, therefore, the pH of donor phase were adjusted in the range of 1.0–6.0 by addition of 1 mol/L HCl. Maximum extraction recovery (44%) was obtained at pH 3.0. Therefore, the pH of donor phase was adjusted at 3.0 and next experiments were carried out at this pH value.

3.1.3. Effect of pH of the acceptor phase

Four buffers including: borate, ammonia, carbonate and Tris with the concentration of 50 mmol/L were tested (pH of buffer solutions was adjusted to 10). The highest analyte recovery was obtained using 50 mmol/L of carbonate. In the next step in order to study the effect of the pH on the extraction recovery, pH of carbonate buffer was changed in the range of 9.0–12.0. According to the results, pH 11.5 provided the highest recovery (60%) and it was selected as the appropriate pH for the acceptor phase.

3.1.4. Effect of stirring speed

Stirring of solution increases mass transfer in the donor phase and also reduces the time required to reach the thermodynamic equilibrium. Unfortunately, high stirring speed generates some problems such as promotion of solvent evaporation and production of air bubbles on the surface of the hollow fiber. To attain optimal stirring speeds, different stirring speed ranging from 250 to 1250 rpm was examined. Results showed that the stirring speed of 1000 rpm yielded the highest recovery (72%) and it was selected for subsequent experiments.

3.1.5. Effect of extraction time

In order to investigate the effect of extraction time on the extraction recovery, various extraction times in the range of 15–120 min was investigated. Results indicated that the recovery of apigenin was increased by increasing the exposure time up to 75 min (recovery 85%) and longer extraction times caused reduction of recovery. The decrease of recovery may be due to potential solvent loss with extending extraction time. Based on these observations, 75 min was selected as the optimum extraction time for the next experiments.

3.1.6. Effect of salt addition

The effect of salt addition on the recovery of apigenin extraction was evaluated by addition of NaCl from 0.0 to 2.0 mol/L in sample solution. Results showed that addition of NaCl had an adverse affect on recovery, so no salt was added into the sample solution. It may be thought that in the presence of salt, interaction may take place between the analyte and salt that would tend to restrict the movement of analyte from donor phase to the membrane solvent [17].

Table 1
Figures of merit of the proposed HF-LPME of apigenin.

LOD (ng/mL)	0.1
Regression equation	$y = 16{,}316x - 352.26$
R^2	>0.9956
Linear range (ng/mL)	0.5–300
RSD% (intra-day, $n = 5$)	3.5
RSD% (inter-day, $n = 5$)	10.7
EF	315
R%	85

3.2. Method validation

3.2.1. Analytical performance

The figures of merit including; linearity, limit of detection (LOD), enrichment factor (EF), repeatability, reproducibility and recovery were obtained to evaluate the practical applicability of the proposed HF-LPME technique for extraction of apigenin from the aqueous solutions under the optimum conditions (Table 1). The linearity in the samples was verified at concentrations ranging from 0.5 to 300 ng/mL by analyzing each concentration in

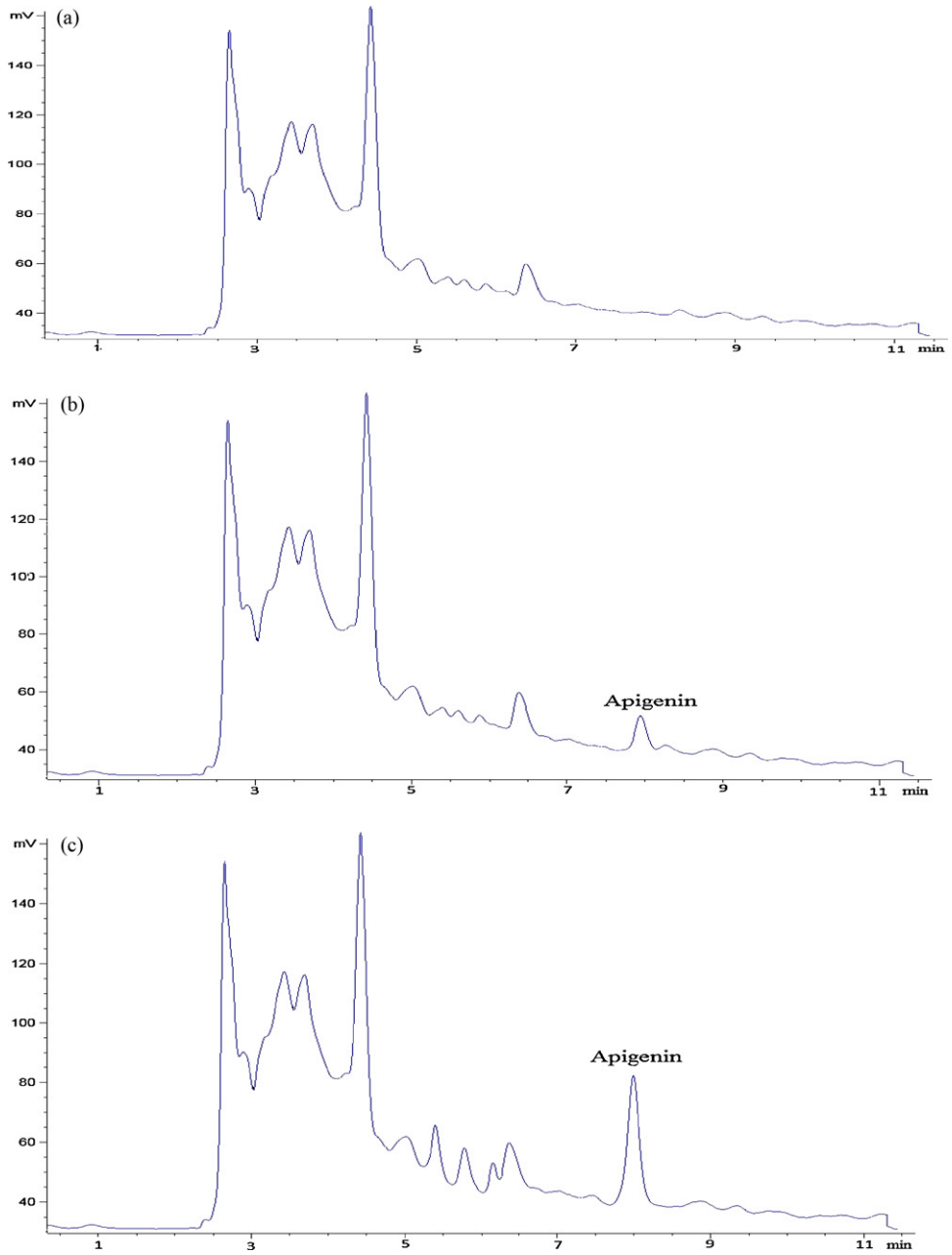

Fig. 2. Chromatograms of apigenin using HF-LPME–HPLC method under optimized conditions. (a) Blank urine sample, (b) non-spiked urine sample after consumption of SSB by a volunteer and (c) urine sample of a volunteer after consumption of SSB spiked with 15 ng/mL apigenin. Optimum conditions: organic solvent = 1-octanol; donor phase = urine sample with pH 3; acceptor phase = 24 μL of the aqueous solution of carbonate buffer with pH 11.5; stirring speed = 1000 rpm and extraction time = 75 min. HPLC condition: mobile phase = 0.1% phosphoric acid:acetonitrile:THF (55:44.5:0.5, v/v) with a flow-rate of 1.0 mL/min; λ = 340 nm.

Table 2
Comparison of the figures of merit of the proposed method with those of the other methods applied for the extraction and determination of apigenin.

Analytical technique	LR (ng/mL)[a]	LOD (ng/mL)	RSD%	Reference
HF-LPME–HPLC-UV	0.5–300	0.1	3.5	Proposed method
SPE–HPLC-UV	100–20,000	5	<5	[9]
SPE–GC–MS	20–15,000	3.3	<4.96	[19]
μSPE–UPLC–MS–MS	54–3000	18.91	2.9	[20]

[a] Linear range.

triplicate. The analyte exhibited good linearity with the correlation of determination of $R^2 > 0.9956$, in the studied range. Based on the signal-to-noise ratio of 3 (S/N = 3), LOD of 0.1 ng/mL was obtained. Under the optimum conditions, the enrichment factor and the extraction recovery (*R*%) were 315 and 85% respectively. The repeatability (intra-day) and reproducibility (inter-day) were obtained as RSD = 3.5% and 10.7% respectively. Enrichment factor (EF) and recovery (*R*%) were calculated based on the following equation:

$$EF = \frac{C_A}{C_D} \quad (1)$$

$$R(\%) = EF \times \left(\frac{V_A}{V_D}\right) \times 100 \quad (2)$$

where C_A, C_D, V_A and V_D are the concentration of analyte in the acceptor phase, the initial concentration of analyte in the donor phase, the volumes of the acceptor and donor phases, respectively.

Table 2 compares the figures of merit of the analytical performance obtained by the present method and the other methods for the extraction of apigenin from biological samples. The comparison results showed that the proposed method is more sensitive (lower LOD) and also, the table clearly shows that RSD of HF-LPME method is similar to those obtained from the other methods. Indeed despite of taking long time, this method surpasses previous methods because of these advantages.

3.2.2. Determination of apigenin in urine sample

In order to assess the applicability of the proposed extraction method for quantification of apigenin in urine sample, the urine samples were extracted and analyzed using this method under the optimum conditions. In order to reduce the matrix effect, the urine samples were diluted at the ratio of 1:1 with deionized double distilled water. Since flavonoids exist as glucuronide and glycolysed conjugates in biological fluids and usually, only the total aglycone content is determined, hydrolysis step is necessary before extraction [7] as was done in Section 2.3. The analytical results are listed in Table 3. Chromatograms of blank urine sample, non-spiked urine sample, urine sample spiked at 15 ng/mL are shown in Fig. 2. These chromatograms show that the proposed method has a high clean-up power and extraction of apigenin was not affected significantly by the matrix of urine samples. In order to study the extracted apigenin in the human urine is as glucuronides or aglycon form, the urine samples hydrolyzed by acid were compared with unhydrolyzed urine samples. Results showed that no apigenin was existed in unhydrolyzed sample. The level of apigenin was maximum in urine at 2 h after consumption of SSB. It might be attributed to its fast absorption from the gastrointestinal tract, due to rapid hydrolysis of apigenin glycosides by the enzymes of intestinal bacteria [18].

Table 3
Results of determination of apigenin in urine sample.

EF	220
RSD% (intra-day, $n = 3$)	5.2
Concentration of apigenin (ng/mL)	6.20

4. Conclusion

The three-phase hollow fiber liquid phase microextraction method reported in this work proved to be a simple, inexpensive and sensitive analytical procedure for the determination of apigenin. This method presented a high enrichment and enabled efficient sample clean-up, while yielding very good selectivity. HF-LPME–HPLC offers a large linear range, good analytical precision and low limit of detection (0.1 ng/mL). Overall, the advantages of the method allow its potential application for apigenin analysis at low levels from urine sample.

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