DETECTION OF PHYTOESTROGENS BY CAPILLARY ELECTROPHORESIS IN COMBINATION WITH ISOLATION BY MAGNETIC PARTICLES MODIFIED BY MOLECULARLY IMPRINTED POLYMER

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Abstract

Phytoestrogens are assumed to play an important role in the prevention of tumor or heart disease, and osteoporosis. Soy food is the most significant source of these compounds present as acetylglucosides, malonylglucosides and/or methyl ethers. The most commonly used methods for phytoestrogen determination include chromatographic or electrophoretic separation techniques.

In this work, the benefits of coupling of capillary electrophoresis with molecularly imprinted polymers for analysis of phytoestrogens are shown. Polydopamin imprinted layer formed on the surface of magnetic particles enables efficient extraction/isolation of model compounds (genistein and biochanin A) from milk sample and subsequent microcolumn separation with absorbance detection enables to distinguish nonspecifically bound interferents.

Keywords: Molecularly imprinted polymers, genistein, biochanin A, micellar electrokinetic chromatography

1. INTRODUCTION

Molecularly imprinted polymers (MIPs) are stable polymers with molecular recognition abilities, provided by the presence of a template during their synthesis and are excellent materials with high selectivity for sample preparation in bioanalytical methods [1]. The use of MIPs has received much attention, not only due to high selectivity, but also for other advantages, such as the low cost of synthesis (compared to antibodies), high mechanical and chemical stability. Thus, MIPs have promising applications in recognition of amino acids and protein [2], important molecules in food [3], pesticides [4], drugs, folic acid and others substances. In more recent applications, magnetic molecularly imprinted polymers (Mag-MIPs) have been used for the recognition of antibiotics in food and environmental samples [2].

Dopamine (DA), (3, 4-dihydroxyphenyl) ethylamine, is considered an important neurotransmitter and significant member ofcatecholamine family. It plays many valuable roles in the functioning of the central nervous system (CNS), hormonal, and renal systems [5]. In addition to biologically significant properties, dopamine also has properties suitable for use in MIPs such as easy polymerization, ability of sorption on almost all inorganic and organic surfaces including superhydrophobic, etc. [6].

Phytoestrogens (PE) and their derivatives have received special attention due to their high concentrations in food, especially in vegetables and soy products. Some of the leading phytoestrogens for cancer treatment or prevention are genistein, coumestrol and glyceollin. Studies imply that countries with high consumption of soy products have a lower incidence of breast cancer in women, this suggests that phytoestrogen intake plays a substantial role in the susceptibility to this cancer type [7]. Soy PE intake may reduce the risk of cardiovascular disease and others chronic diseases such as osteoporosis [8]. Major classes of phytoestrogens include flavonoids, isoflavonoids, lignans and stilbenoids [9].

Selected phytoestrogens, genistein and biochanin A, belong to the group isoflavonoids. In plants, isoflavones are present in a biologically inactive form as glucoside conjugates. In the intestine, by the action of intestinal

bacteria, they are hydrolyzed to active forms named aglycones. Taking into account their structural similarity to the natural endogenous hormone 17-estradiol, isoflavones demonstrate the ability of binding to estrogen receptors and consequently the low estrogenic activity. Genistein and biochanin A are considered to be important constituents of animal and human food, as they have a serious influence on human health [10]. Biochanin A and genistein possess anticancer, antioxidant and antiosteoporosis effect [11].

2. MATERIALS AND METHODS

2.1. Chemicals

Dopamine hydrochloride, Trizma® base, phytoestrogen standards (genistein and biochanin A), Sodium dodecyl sulfate and acetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity. Sodium tetraborate decahydrate and magnetic particles Dynabeads™ MyOne™ Silane were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ethanol and methyl alcohol were obtained from Penta (Prague, CZ).

2.2. Capillary Electrophoresis (CE)

Quantification of phytoestrogens, genistein and biochanin A, was performed by CE instrument 7100 (Agilent Technologies, Germany) with absorbance detection at wavelength of 254 nm. Fused silica capillary with an internal diameter of 75 μm, with the total length of 64.5 cm and an effective length of 56 cm was used. The sample was introduced hydrodynamically by 35 mbar for 3 s and a separation voltage of 12 kV was applied. A background electrolyte (BGE) was composed of 30 mM sodium borate buffer, 20 mM sodium dodecyl sulfate (SDS) containing 5 % (v/v) ethanol at pH 9.6. Prior to the analysis, the capillary was washed for 60 seconds using BGE.

2.3. Preparation of Mag-MIPs

Mag-MIPs (**Figure 1**) were prepared from magnetic particles (50 μL), which ware first washed by water (200 μL) for three times. For preparation of MIPs, 400 μL of genistein (1 mg/ml) and 200 μL of 20 Mm TRIS (pH 10) were added. For preparation of non-imprinted polymers (NIPs), 400 μL of 80 % ethyl alcohol and 200 μL of 20 Mm TRIS (pH 10) were added. The mixture was stirred for 1 hour at temperature 45 °C. 100 μL of dopamine (17.5 mg/mL) was added. The mixture was stirred overnight. Then supernatant was removed, MIP/NIP were washed by methanol (200 μL) for three times.

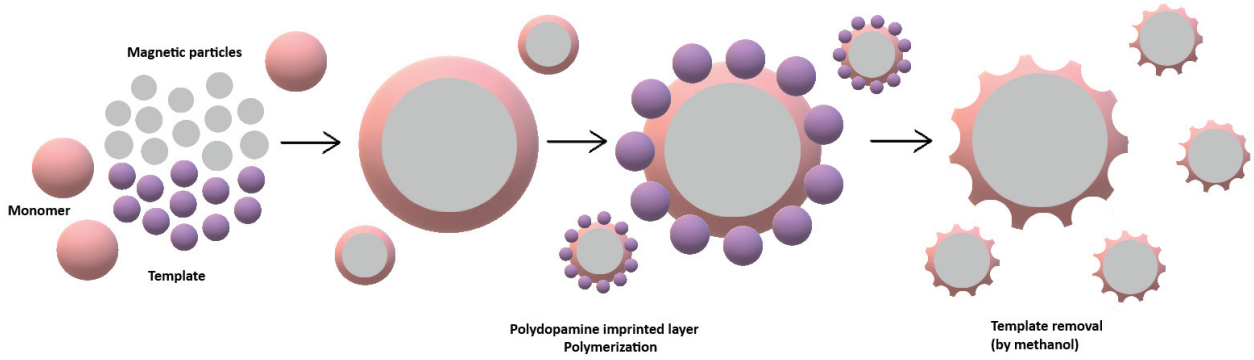

Figure 1 Scheme of preparation of Mag-MIPs

2.4. Sample Preparation

After mag-MIPs/NIPs washing by methanol, genistein (200 μL) was added to mag-MIPs/NIPs at concentrations of 0.125, 0.6, 0.3, 0.015 and 0.0075 mg/ml. Next, biochanin A (0.125 mg/ml), cow milk, milk spiked with

genistein (0.125 mg/mL) and soya milk were used as samples. The mixture was stirred for 2 hours at temperature 45 °C. After supernatant removal, mag-MIPs/NIPs were washed with methanol (200 µL) and samples were analyzed by CE.

3. RESULTS AND DISSCUSION

3.1. Detection of phytoestrogens by CE

Genistein extracted from the sample by mag-MIPs was released from the surface by methanol and analyzed by CE with absorbance detection at 254 nm. Standard solution of genistein was added to mag-MIP/NIP at different concentration and a calibration curve was constructed for quantification (**Figure 2**). The difference in signal obtained by mag-MIP and mag-NIP demonstrates that MIP surface is able to recognize the target (template) [12]. Linear dependence in genistein concentration was observed exhibiting the coefficient of determination $R^2 = 0.9329$.

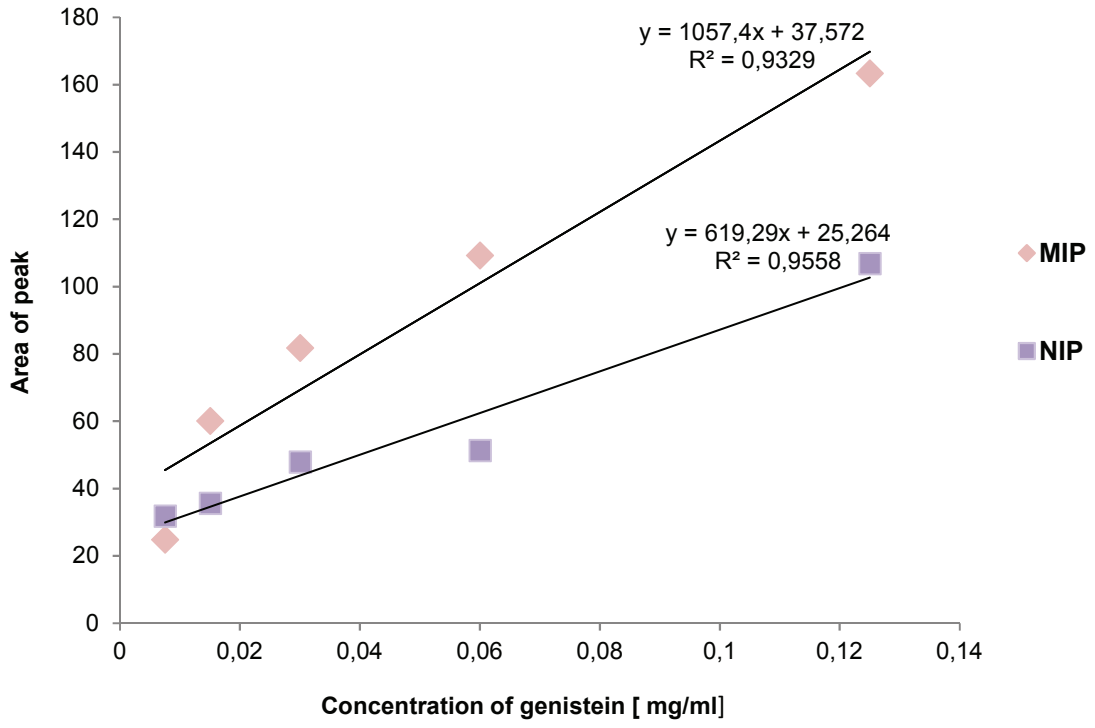

Figure 2 Calibration curve of genistein. CE analysis: BGE - 30 mM sodium borate buffer, 20 mM SDS, 5 % (v/v) ethanol, pH 9.6; detection - 254 nm; hydrodynamic injection - 35 mbar for 3 s; separation voltage - 12 kV

3.2. Real sample analysis

Determination of PEs in real samples is an important goal and therefore, cow milk and soya milk were used as model real samples to test the suggested method. At first, the cow milk was analyzed by CE (pink trace) to demonstrate that not only a number of proteins are present but, also the signal of genistein is very low (marked by asterix). Therefore, the sample preparation using mag-MIPs lead to significant improvement (orange trace) and to identify the genistein signal, the milk sample was spiked by genistein standard solution (blue trace). For comparison, crude soya milk (green trace) was analyzed as well as the soya milk sample purified by mag-MIPs (purple trace). The concentration of PEs (daidzein, glycitein, daidzin etc.) in soya milk is significantly higher compared to cow milk [13] and therefore no spiking was necessary.

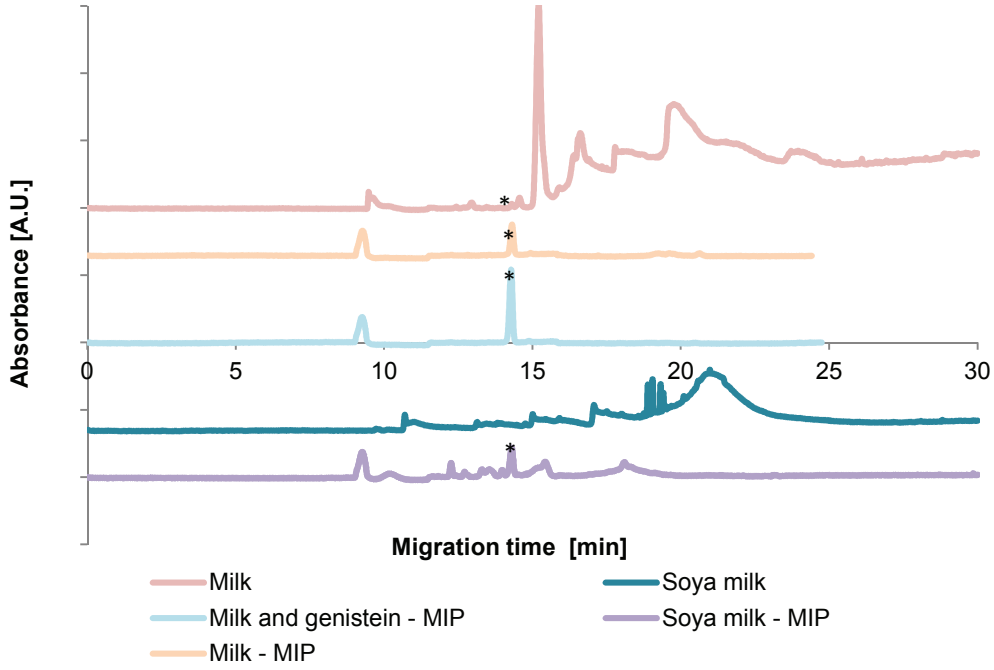

Figure 3 CE real samples (cow and soya milk) purified by mag-MIP. BGE - 30 mM sodium borate buffer, 20 mM SDS, 5 % (v/v) ethanol, pH 9.6; detection - 254 nm; hydrodynamic injection - 35 mbar for 3 s; separation voltage - 12 kV

4. CONCLUSION

In this work, sample purification method using a combination of MIP technology and magnetic particles was developed followed by capillary electrophoretic analysis. Mainly, benefits of simple preparation of polydopamine MIPs combined with effectivity of magnetic particle-based analyte extraction are demonstrated as well as powerful CE-UV/Vis detection. Successful determination of PEs in real samples as cow and soya milk was demonstrated. For comparison, soya milk contains the higher concentration of PEs than cow milk [13]. At first, CE analysis of milk samples was carried out and presence of numerous proteins is evident in electropherograms, however the signal of genistein is very low. The treatment of sample using mag-MIPs caused an increase in the genistein signal.

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