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Evaluation of a molecularly imprinted polymer for determination of steroids in goat milk by matrix solid phase dispersion

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ABSTRACT

A molecularly imprinted polymer–matrix solid-phase dispersion methodology for simultaneous determination of five steroids in goat milk samples was proposed. Factors affecting the extraction recovery such as sample/dispersant ratio and washing and elution solvents were investigated. The molecularly imprinted polymer used as dispersant in the matrix solid-phase dispersion procedure showed high affinity to steroids, and the obtained extracts were sufficiently cleaned to be directly analyzed. Analytical separation was performed by micellar electrokinetic chromatography using a capillary electrophoresis system equipped with a diode array detector. A background electrolyte composed of borate buffer (25 mM, pH 9.3), sodium dodecyl sulfate (10 mM) and acetonitrile (20%) was used. The developed MIP–MSPD methodology was applied for direct determination of testosterone (T), estrone (E1), 17β-estradiol (17β-E2), 17α-ethinylestradiol (EE2) and progesterone (P) in different goat milk samples. Mean recoveries obtained ranged from 81% to 110%, with relative standard deviations (RSD) ≤ 12%. The molecularly imprinted polymer–matrix solid-phase dispersion method is fast, selective, cost-effective and environment-friendly compared with other pretreatment methods used for extraction of steroids in milk.

1. Introduction

Nowadays, to ensure the safety of milk and other alimentary products before entering the food chain, the development of methodologies to determine the presence or absence of dangerous and/or forbidden substances in these products is of crucial importance. Steroid hormones have become one of the groups of analytes of concern in milk because it could constitute a risk for consumer's health, since some of these compounds are endocrine disruptors associated with many endocrine disorders and even cancer [1,2].

Testosterone (T), progesterone (P), estrone (E1) and 17β-estradiol (17β-E2) are organism-synthesized steroid compounds, naturally present in animal tissues and fluids (see Supplemental material SM1). Milk contains considerable quantities of these hormones. Milk and dairy products constitute a 60–70% of total E1 intake with the diet [3]. The growing demand of fresh milk and dairy products leads farmers to milk animals even during their gestation period, when the level of natural steroid hormones is extremely high [4]. Therefore, these modern dairy practices result in a considerable increase of hormone levels. The presence of 17β-E2 in milk is of particular concern, due to its carcinogenic risk even at low levels and it is listed within Group A in Council Directive 1996/22/EC (Group A, substances having anabolic effect and unauthorized substances).

The illegal use of some steroid hormones in dairy practices as growth promoters is also an important problem [5]. In that respect, 17α-ethinylestradiol (EE2) is one of the most important synthetic estrogens, commonly used to increase the weight gain of animals (see Supplemental material SM1). Regarding legislation, the use of these hormone active substances for animal fatting has been forbidden by the European Union (Council Directive 2003/74/EC). However, Directive 2003/74/EC allows the use of medicinal products containing 17β-E2 for some treatments in cattle.

Determination of steroids in milk samples, due to complexity of the matrix, usually requires a suitable pre-treatment step for the removal of interferences. Deproteinization, hydrolysis, cleaning or preconcentration steps (liquid–liquid and/or solid-phase extraction) have been extensively used for this purpose in milk samples in [5–12]. These pretreatments are usually relatively expensive

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in terms of time and organic solvent consumption. Current trends are focus on the development of faster, selective, cost-effective and environment friendly procedures. In this sense, matrix solid-phase dispersion (MSPD) offers an interesting alternative for the extraction of steroids from milk samples [5].

Matrix solid-phase dispersion is a relatively recent extraction and clean up technique used for the simultaneous determination of different analytes, from liquid, viscous, semi-solid and solid samples [13]. MSPD combines the use of mechanical forces generated from the gridding of samples with irregularly shaped particles of a sorbent which acts as solid support, to produce a sample/column material from which the dispersed matrix components can be selectively isolated. The method comprises sample homogenization, cellular disruption, fractionation, and purification in a single process [14]. MSPD is a simple and cheap preparation procedure that allows the reduction of extraction time and the consumption of organic solvents and sorbents, while still providing similar or higher extraction efficiency and selectivity than other procedures [13,14]. Many analytical methods based on MSPD have been developed for the extraction of a wide range of organic compounds for milk samples, using different sorbents, such as C_{18}, C_8, silica gel or florisil, but they usually lack of selectivity for target analytes [9,15–18]. In that respect, molecularly imprinted polymers (MIPs) have been successfully used as a specific sorbent for selective extraction of different compounds. In the literature not much works deal with the use of MIPs as selective sorbents in MSPD. Only a previous work of our research group has demonstrated the successful application of a MIP as a sorbent in MSPD for determination of 17β-E2 in goat milk by HPLC–DAD [19].

In this context, the aim of this work is focused on the development of a new methodology for the simultaneous extraction of T, E1, 17β-E2, EE2 and P by MSPD using a MIP as a selective sorbent (MIP–MSPD). The extraction method proposed was applied for the multiresidue determination of these hormones in goat milk samples. Separation and quantification of the target analytes were carried out by micellar electrokinetic chromatography with a diode-array detector (MECK–DAD). In comparison with HPLC–DAD, the use of MECK with the same type of detector offers some advantages such as higher peak efficiency, faster and easier method development by using a great variety of additives in the background electrolyte and lower consumption of organic solvents. To the best of our knowledge, this is the first time that the mentioned analytes are simultaneously extracted by a MIP–MSPD procedure in this matrix and also the first application of MECK–DAD for the analysis of steroids in milk.

2. Experimental

2.1. Reagents and chemicals

All reagents were of analytical grade and deionized water (18.2 MΩ/cm) was obtained from a Milli-Q water system (Millipore Iberica, Madrid, Spain). Methacrylic acid (MAA), ethylenglycol dimethacrylate (EGDMA), sodium tetraborate decahydrate (borate), 17β-estradiol (17β-E2), estrone (E1), ethynilestradiol (EE2) and progesterone (P) were supplied from Sigma-Aldrich (St. Louis, MO, USA). Azobisisobutyronitrile (AIBN), sodium dodecyl sulfate (SDS) and testosterone (T) were purchased from Fluka Analytical (St. Louis, MO, USA). Acetonitrile (ACN), dichlrometane (DCM) and methanol (MeOH) were from Panreac Química (Barcelona, Spain). Sodium sulfate (Na_2SO_4), acetic acid (HAc) and sodium hydroxide (NaOH) were supplied by Scharlab (Barcelona, Spain). Sea sand was supplied by Quality Chemicals S.L. (Esparraguera, Spain). 3 mL empty solid phase extraction (SPE) cartridges and polyethylene frits were purchased from Symta (Madrid, Spain). All solutions were filtered prior to use through 0.45 μm pore size disposable nylon filters from Análisis Vínicos (Tomelloso, Spain).

2.2. Milk samples

Fresh goat milk and pregnancy goat milk samples free of steroids (fat content 4.1% and 4.2%, respectively) were kindly provided by a farm located in Losar de la Vera (Cáceres, Spain), and belong to autochthon goat breed "Caprina Verata". Milk samples were collected in sterile bottles by direct manual milking and immediately stored in the freezer at −20 °C until use for analysis. Pasteurized goat milk samples (fat content 3.5%) were bought from a local supermarket and stored in refrigerator until use.

2.3. Standard solutions

The appropriate amounts of T, E1, 17β-E2, EE2 and P were dissolved into MeOH to get individual stock solutions with a final concentration of 2000 μg mL^{-1}. These solutions were stored in dark glass bottles and kept at −20 °C when they were not in use. Mixed working solutions of steroids were prepared daily by diluting each individual stock solution with MeOH/H_2O (50/50, v/v) to achieve concentrations ranging from 5 to 100 μg mL^{-1}.

2.4. Instrumentation

Analyses were carried out using a Beckman P/ACE MDQ Capillary electrophoresis system equipped with a diode array detector (DAD) from Beckman Coulter, Inc. (California, USA) and provided with a 32 KARAT software for data handling. The DAD wavelength range was set to 190–300 nm. Separations were performed in an untreated fused-silica capillary of 75 μm ID and 375 μm OD, purchased from Polymicro Technologies (Phoenix, AZ, USA). The employed capillary had a total length of 60.2 cm and as effective length of 50.0 cm to the detector. A Basic 20 pH-meter from Crison Instruments S.A. (Alella, Spain) was employed to adjust the pH of the separation buffers.

2.5. MEKC–DAD analysis

Separation of the steroids was carried out by MECK–DAD according to a previous work of our research group [20]. The running BGE was composed of borate buffer (pH 9.3; 25 mM), SDS (10 mM) and 20% ACN as an organic modifier and was freshly prepared prior to use. Other instrumental conditions were as follows: capillary temperature, 15 °C; injections by pressure, 0.5 psi × 3 s of sample followed by a plug of 0.1 psi × 1 s of BGE, and applied voltage, 27 kV. Before its first use, a new capillary was rinsed with 1 M NaOH for 30 min, followed by a 3 min rinsing with water. Between injections of samples, the capillary was conditioned with 0.1 M NaOH for 1 min followed by a 3 min rinsing with water and 5 min with the BGE used in the separation. The detection was performed at 249 nm for T and P, and 200 nm for E1, 17β-E2 and EE2, with a band width of 10 nm. Instrumental linearity of the MECK–DAD method was demonstrated in the range of 4.25–100 μg mL^{-1} for T, 2–100 μg mL^{-1} for E1, 3.6–100 μg mL^{-1} for 17β-E2, 5.7–100 μg mL^{-1} for EE2 and 7.3–100 μg mL^{-1} for P (R^2=0.995–0.999). For instrumental repeatability (n=6), relative standard deviations (RSD, %) for migration times (t_m) and corrected peak areas, A_c (A_c= peak area/t_m), were < 11% and 17%, respectively. For intermediate precision (n=9, k=3), RSD were between 1% and 7% for t_m and between 3% and 12% for A_c.

2.6. MIP–MSPD procedure

The molecularly imprinted polymer was synthesized by the bulk polymerization method using 17β-E2 as template molecule