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

Judith Gañán [a], Sonia Morante-Zarcero [a], Alejandrina Gallego-Picó [b], Rosa María Garcinuño [b], Pilar Fernández-Hernando [b], Isabel Sierra [a,*]

[a] Departamento de Química Inorgánica y Analítica, E.S.C.E.T, Universidad Rey Juan Carlos, C/Tulipán s/n, Móstoles, 28933 Madrid, Spain
[b] Departamento de Ciencias Analíticas, Facultad de Ciencias, Universidad Nacional de Educación a Distancia (UNED), Senda del Rey, n° 9, 28040 Madrid, Spain

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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

* Corresponding author. Tel.: +34 914887018; fax: +34 914888143.
E-mail address: isabel.sierra@urjc.es (I. Sierra).

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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

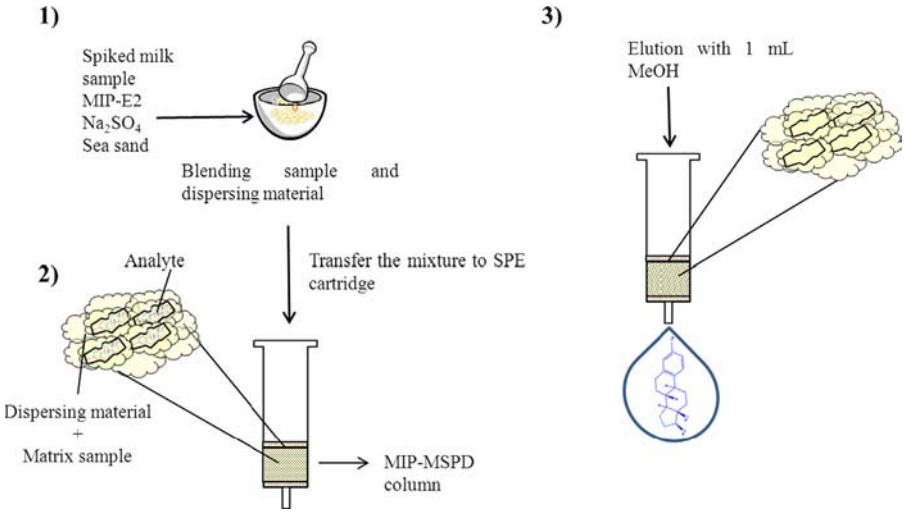

Fig. 1. Graphical scheme for the MIP–MSPD extraction procedure.

(MIP-E2), following a procedure summarized in a previous study of the authors [19] where the template: functional monomer: crosslinker (17β-E2: methacrylic acid: ethyleneglycol dimethacrylate) ratio chosen was 1:30:150. Non-imprinted polymer (NIP) was also prepared following the same procedure without adding the template molecule. An aliquot of 200 μL of fortified milk sample was placed into a glass mortar and gently blended with 0.048 g MIP-E2, 0.126 g Na_2SO_4 and 0.126 g washed sea sand using a glass nail until dry and homogeneous mixture was obtained. After the MSPD blending process, the mixture was packed into a SPE cartridge with a plugged, with porous PTFE disks at both ends that retain the entire mixture. Target analytes were directly eluted from the cartridge using 1 mL ACN, at a constant flow rate of 1 mL min^{-1}. Fig. 1 shows a graphical scheme of the MIP–MSPD procedure. Finally the eluent was dried using a vacuum line, and the residue was reconstituted with 500 μL of MeOH/H_2O (50:50, v/v) and filtered through nylon filters (0.45 μm) prior MEKC–DAD analysis.

3. Results and discussion

3.1. Optimization of the MIP–MSPD procedure

MSPD efficiency depends on careful optimization of the experimental conditions affecting competition within the matrix, the dispersant sorbent or solid support, and the extraction solvent for analytes and potential matrix interferences. Consequently, several factors such as type of sorbent, sample/sorbent ratio, and washing and elution conditions were carefully selected to achieve extracts with the highest recovery and the lowest amount of interferences from milk samples.

The nature of the solid support/dispersant sorbent will affect the retention and elution of the target analytes and the corresponding sample components. MSPD applications use conventional bounded phases (C18, alumina, florisil, etc.) as dispersant for analytes. However, these sorbents are usually non-selective. In this research, with the aim of increasing the selectivity of the MSPD process for the steroids extraction, a MIP for 17β-E2 (MIP-E2) was employed as sorbent. MIP-E2 was synthesized and characterized in a previous research of the authors and successfully applied for the selective determination of 17β-E2 in milk samples [19].

The first step in the MSPD method setup was the evaluation of a suitable sample:MIP ratio to allow complete adsorption of matrix components and to facilitate the transfer into the MSPD cartridge. In this study, different ratios of sample:MIP were evaluated (2.5/1, 1/1, 1/2.5), using 200 μL of sample spiked with the mixture standard of steroids in order to obtain a concentration of 50 μg mL^{-1}. The resulting MSPD mixtures were too wet to be transferred into the cartridge and undergo the chromatographic process, even when ratio 1/2.5 was used. Sodium sulfate and sea sand (in the same ratio) were then added to the mixture, in order to reduce the moisture content and to improve the sample disruption. Finally, using MIP:sea sand:Na_2SO_4 in a ratio 1:3:3, a homogeneous and dry mixture was obtained, which allowed the easy packing into the cartridge and also the flow of solvents through it.

An appropriate washing solvent should leave the target compounds adsorbed on the cartridge and remove matrix interferences from the sample as much as possible. For this purpose, different washing solvents (hexane, chloroform, 0.5M NaOH and water) and different volumes were investigated. In all cases, 1 mL of MeOH was employed as a elution solvent. However, due to the low recoveries observed (between 17% and 66%) and the broad range of matrix compounds extracted under the tested washing conditions, the washing step was avoided for further experiments, eluting the analytes directly from the cartridge. Subsequently, solvents of different polarities such as MeOH, ACN, MeOH/ACN (50:50, v/v), ACN/HAc (99.9:0.1, v/v) and DCM were tested to optimize the direct elution procedure. In these experiments, the cleanest extracts with the best recoveries were obtained using ACN as an elution solvent. As shown in Fig. 2, recoveries between 80% and 94% were achieved for the target compounds using this solvent, whereas using MeOH recoveries were between 60% and 75%. The mixture ACN/HAc (99.9:0.1, v/v) gave the worst results for all compounds. On the other hand, the amount of ACN loaded on the cartridge for the extraction had a great effect on recovery efficiency. Different ACN volumes were tested (0.5–2 mL) and it was found that 1 mL of ACN was the optimum value. An insufficient volume meant uncompleted elution and volumes higher than 1 mL did not improve the recovery percentage of the hormones and increase the next dryness step. Fig. 3 shows the electropherograms obtained for goat milk sample spiked with the steroids after the MIP–MSPD procedure with and without the washing step.

The selectivity and extraction efficiency of the developed MIP–MSPD procedure were also investigated by comparison with NIP–MSPD. For this purpose, the optimum conditions for the MIP–MSPD method were applied for the extraction of T, E1, 17β-E2, EE2 and P in goat milk sample preparing the dispersed sample using the NIP instead of MIP. In this case, recoveries were

between 48% and 52% for T, E1, 17β-E2 and EE2 and 80% for P. These results confirmed that MIP-E2 was a suitable solid support for MSPD that favors the selective extraction of the most of the studied steroids in milk with high efficiency.

For the optimization of all parameters affecting the MIP–MSPD procedure, recoveries were calculated by comparison of the A_c of each analyte in milk samples spiked with the steroids with the A_c of each analyte in simulated milk samples (samples prepared in the same way but spiked with the hormones at the end of the MIP–MSPD procedure).

3.2. Validation of the MIP–MSPD procedure

To validate the developed MIP–MSPD method, a series of experiments under the optimal experimental conditions were performed to obtain linear ranges, precision, accuracy, detection (LOD) and quantification (LOQ) limits. To evaluate the linearity of the method, calibration curves for all analytes were constructed using spiking samples containing increasing concentrations of the target steroids (matrix-matched standard curves). The slope and intercept values of the calibration curves were determined using regression analyses. Linear relationship was found between corrected peak areas and the concentration of the analyte in all cases, with correlation coefficients (R^2) higher than 0.99 (Table 1).

Precision and accuracy of the method were carried out spiking aliquots of 200 μL of milk sample with the five steroids at two concentration levels on the same day and different days ($n=3$). Table 2 summarizes the average recoveries obtained for each steroid, ranging from 81% to 110%. Intra-day and inter-day precision expressed as relative standard deviation (RSD) ranging from 1% to 13% for all analytes. These results demonstrated the good repeatability and accuracy of the method.

LOD and LOQ were calculated at signal-to-noise ratios of 3 and 10, respectively, following IUPAC recommendations. The LODs obtained were 1.27 μg mL^{-1} for T, 0.6 μg mL^{-1} for E1, 1.13 μg mL^{-1} for 17β-E2, 1.7 μg mL^{-1} for EE2 and 2.17 μg mL^{-1} for P. The LOQs obtained were 4.25 μg mL^{-1} for T, 2 μg mL^{-1} for E1, 3.6 μg mL^{-1} for 17β-E2, 5.7 μg mL^{-1} for EE2 and 7.3 μg mL^{-1} for P. Non-spiked samples (blanks) were also processed in order to demonstrate that

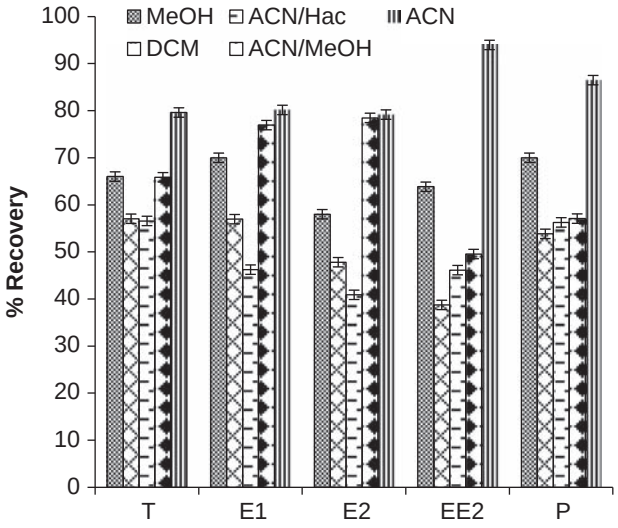

Fig. 2. Effect of different elution solvents after using MIP–MSPD procedure on the recovery of testosterone (T), estrone (E1), 17β-estradiol (17β-E2), ethynilestradiol (EE2) and progesterone (P), using 1 mL of elution volume.

Table 1
Linearity of the method.

Steroid	Linear range (μg mL^{-1})	Calibration curves[a]	R^2	Matrix effect (%)
T	4.25–100	24.511 x+115.74	0.996	79
E1	2.0–100	72.669 x+77.163	0.995	87
E2	3.6–100	68.453 x+40.467	0.987	88
EE2	5.7–100	28.440 x+410.94	0.990	43
P	7.3–100	11.473 x+66.394	0.995	81

[a] Matrix-matched standard calibration curves.

Table 2
Accuracy[a] of the MIP–MSPD–MEKC method.

Steroid	Recovery (%) low level[b]		Recovery (%) high level[c]	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
T	85 ± 1	2	93 ± 9	10
E1	89 ± 2	2	110.1 ± 0.2	0.2
E2	81 ± 2	3	83 ± 10	12
EE2	85 ± 1	1	93 ± 11	12
P	88 ± 2	3	110 ± 8	8

[a] Spiked samples analyzed by the MIP–MSPD–MEKC method ($n=3$ assays).
[b] Low level=2 μg/mL for E1, 3.6 μg/mL for 17β-E2, 5.7 μg/mL for EE2, 4.25 μg/mL for T and 7.3 μg/mL for P.
[c] High level=100 μg/mL.

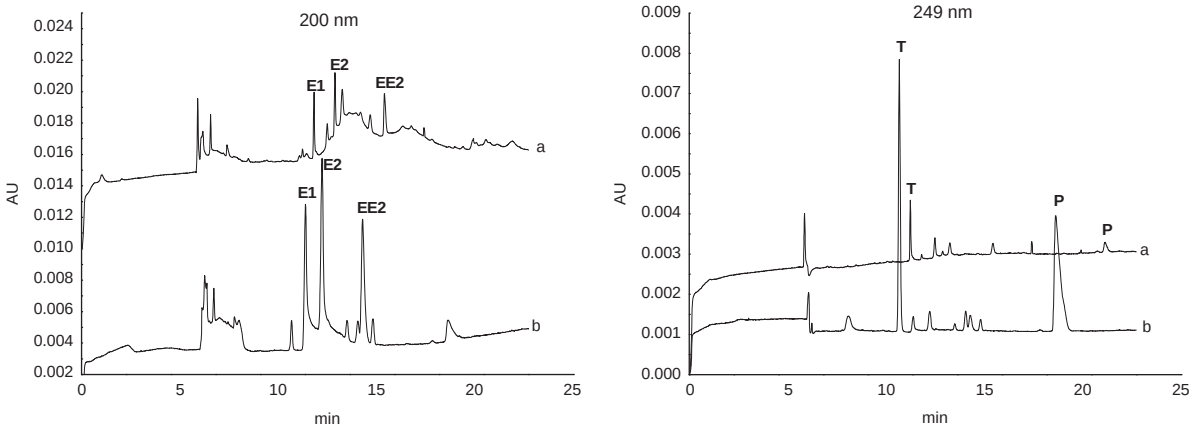

Fig. 3. Effect of the washing step and elution solvent on analyte recoveries and matrix interferences. (a) 1 mL of NaOH 0.5 M as a washing solvent and 1 mL of MeOH as an elution solvent, (b) without washing step and 1 mL of ACN as an elution solvent. Electrophoretic conditions: capillary fused silica, 50 cm effective length and 75 μm ID; running BGE, 25 mM borate buffer (pH=9.3), 10 mM SDS and 20% ACN v/v; capillary temperature, 15 °C; injections by pressure, 0.5 psi × 3 s and applied voltage, 27 kV.

the concentration of T, E1, 17β-E2, EE2 and were below the LOD of the method.

Since many macromolecular compounds present in the milk samples can influence the signal of the target analytes, the matrix effect was investigated in this study. The matrix effect (%) for each steroid was calculated as the quotient of the slope of the matrix-matched standard curve and the slope of the standard curve prepared with working solutions in $MeOH/H_2O$. As it can be seen in Table 1, matrix effects ranging from 43% (EE2) to 88% (E2) were found. These results indicated that the matrix effect exists. Then, in order to obtain accurate results, the quantitative determination of these hormones in milk samples must be carried out with matrix-matched standard calibration curves.

Finally, to evaluate the applicability of the developed MIP–MSPD procedure, three goat milk samples (fresh, fresh pregnancy and commercially pasteurized) were analyzed under the optimum conditions. Typical electropherograms of the blank and a spiked fresh goat milk sample extracted under optimized conditions are shown in Fig. 4. It can be seen that the studied steroids were not detectable in the samples, and no significant interference peaks were found at the migration times of these analytes.

3.3. Comparison of MIP–MSPD procedure with other methods

The main difficulty in determining dangerous and/or forbidden substances in complex samples such as milk lies in their extraction from the matrix. In fact, this step is the bottleneck of routine analytical methods, because several sample pretreatment steps are required in most cases. In the present work, a rapid and simple sample treatment based on MSPD for the determination of steroids in goat milk has been proposed. The greatest innovation of the developed MSPD procedure has been the use of a MIP as supporting material, that it is not usual for this purpose [13]. It is well known that in a MSPD process an appropriate solvent (or a sequence of solvents) can be used to clean the column or to directly isolate the compounds of choice [14]. In this sense, in our work the steroids were appropriately eluted from the cartridge without any previous washing step, which is an important advantage since less time consuming and organic solvents were required for the sample pretreatment. The obtained eluate was adequately "clean" for direct introduction in the CE equipment, and additional steps to remove co-eluting matrix components (usually SPE) were not needed. Thus, the MIP-E2 used as solid support in this study affords specific molecular-recognition sites for the target steroids which clearly influences the achieved selective extraction. In this manner, the milk sample is dispersed over the surface of the MIP-E2 support material, producing, through different interactions of the various sample components, a unique mixed-character phase for conducting steroids isolation. The use of an appropriate elution solvent, which elution ability to desorb the target analytes from the tailor-made recognition sites in the MIP without eluting the matrix interferences, allowed good purification efficiency and high analyte recoveries (Fig. 1).

It is well known that some matrix components such as saline constituents, macromolecules and other major compounds can disturb CE separations. For these reasons, food samples often require especially complex treatments prior to analysis by CE, especially when the analytes are present at very low concentrations [21]. As it has been already mentioned in the introduction, this is the first attempt to use a MIP–MSPD procedure for simultaneous extractions of T, E1, 17β-E2, EE2 and P in goat milk and also the first application of MECK–DAD for their analysis in this matrix. Table 3 collects some recent methods found for the determinations of the target steroids in milks by HPLC–DAD. As it can be seen, compared with other methods (liquid–liquid extraction plus molecularly imprinted solid-phase extraction; dispersive solid-phase extraction with a molecularly imprinted polymer; dynamic liquid–liquid–solid microextraction and hollow-fiber liquid-phase microextraction), the operation of the MIP–MSPD procedure is simpler and the time for the sample preparation is shorter [22–25]. In addition, in this work the steroids were appropriately eluted from the MSPD cartridge without any previous washing step, so very low organic solvents are needed for the sample pretreatment [22–25].

Recoveries obtained in the present work are in general more satisfactory, in comparison with previously reported methods. The MIP–MSPD method improves selective extraction efficiency significantly and reduces the laborious pretreatment process while reduces the assay time and shortens the organic solvent consumption. Finally, the use of CE for steroids analysis is a very interesting and environment friendly alternative to HPLC, due to its minimal sample and organic solvents consumption, and is increasingly being used not only for research purposes but also for routine analyses [21,26].

In conclusion, in this paper a new effective, selective and rapid MIP–MSPD extraction included in the MEKC–DAD method has been demonstrated. The complete method allows the separation of steroid hormones in less than 30 min avoiding, almost completely, the use of organic solvents (around 1 mL in each run). Therefore this technique is highly appropriate for the analysis of hormones and it is comparable to established separation techniques such as HPLC or GC with common detectors. The use of MIP–MSPD of milk samples was shown to be an easy, fast and efficient system for the extraction of all analytes tested.

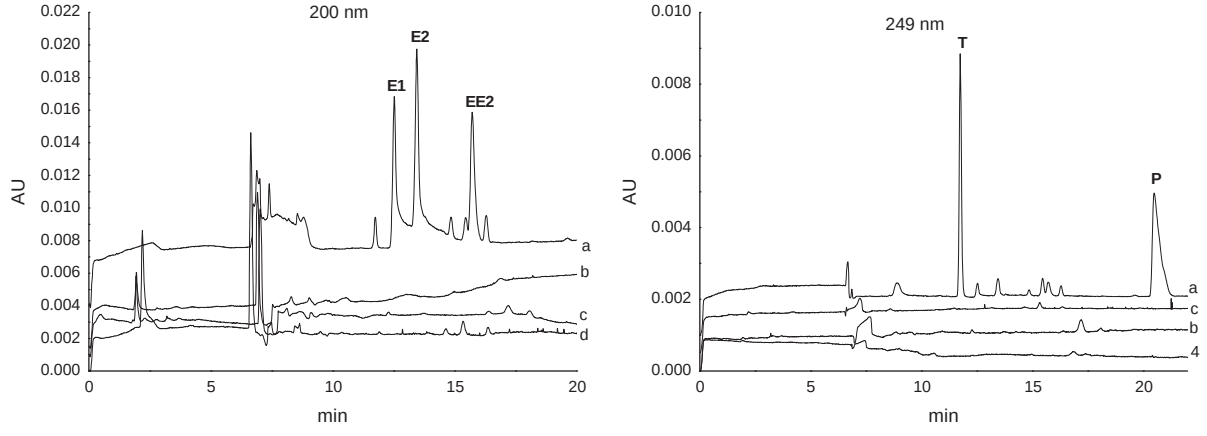

Fig. 4. Typical electropherograms of different goat milk samples treated under optimized MIP–MSPD conditions: (a) fresh milk spiked with 100 μg/mL of T, E1, 17β-E2, EE2 and P; (b) blank (not spiked fresh milk); (c) fresh pregnancy milk; (d) pasteurized milk. Electrophoretic conditions as in Fig. 3.

Table 3
Comparison of MIP–MSPD procedure with other sample preparation methods for extraction of steroids in milk.

Sample treatment procedure[a]		Recovery % (analyte)	Ref.
LLE, MISPE	500 g milk+150 mL MeOH:acetone (2 min vortex, 5 min ultrasonic, 5 min centrifug.) × 3 times. Dry and re-dissolve: 3 mL MeOH+15 mL H_2O. Conditioning cartridge 9 mL MeOH+3 mL H_2O, washing 1 mL MeOH, elution 3 mL MeOH	74% (17β-E2)	[21]
MIP–dSPE	2 g milk tablets+20 mL MeOH (30 min ultrasonic). Dried and re-dissolve: 2 mL toluene:MeOH+20 mg of MIP (30 min incubation, 10 min centrifugation). Particles dispersed in 3 mL MeOH:acetic acid (10 min ultrasonic, 10 min centrifug.). Filtrate dried and re-dissolved in 1 mL MeOH	73% (17β-E2)	[22]
DLLSME	10 mL milk+10 mL ACN (30 min ultrasonic+centrifug.). Precipitate dissolved in 2 mL acetone (10 min ultrasonic) × 3 times. Concentrate under vacuum and dilute to 10 mL with water.	94% (E1, 17β-E2 and EE2)	[23]
LLE, HP–LPME	3 mL milk+6 mL MeCN (1 min vortex, 15 min darkness, 15 min centrifug.). Dry and re-dissolve with H_2O. Adjust pH. Fiber adsorption: 60 min. Desorption: 7 min. Dry and re-dissolve in 0.1 mL mobile phase	94–118% (E1, β-E2, EE2)	[24]
MIP–MSPD	0.2 mL milk+0.085 g MIP+0.210 g Na_2SO_4+0.210 g washed sea sand. Transferred into a cartridge. Elution with 1 mL MeOH. Dried and re-dissolved in 0.5 mL MeOH	81–110% (T, E1, 17β-E2, EE2 and P)	This work

[a] LLE: Liquid–liquid extraction; MISPE: Molecularly imprinted solid-phase extraction; MIP–dSPE: Dispersive solid-phase extraction with a molecularly imprinted polymer; DLLSME: Dynamic liquid–liquid–solid microextraction; HP-LPME: Hollow-fiber liquid-phase microextraction; MIP–MSPD: Molecularly-imprinted polymer–matrix solid-phase dispersion.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.03.041.

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