DEVELOPMENT OF A MOLECULARLY IMPRINTED POLYMER-MATRIX SOLID PHASE DISPERSION METHOD FOR SELECTIVE DETERMINATION OF β-ESTRADIOL AS ANABOLIC GROWTH PROMOTER IN GOAT MILK

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Abstract

A simple, fast and sensitive method for the determination of 17 β-estradiol (E2) from goat milk samples was developed by selective molecularly imprinted matrix solid-phase dispersion (MIP-MSPD) coupled with liquid chromatographic-DAD determination. The molecularly imprinted polymer was synthesized using 17β-estradiol as template molecule, methacrylic acid as functional monomer, ethyleneglycol dimethacrylate as crosslinker monomer, azobisisobutyronitrile as initiator and acetonitrile as porogen, and was applied as selective solid support for matrix solid phase dispersion. The selected dispersant showed high affinity to E2 in goat milk matrix and the obtained extract was sufficiently clean to be directly injected into HPLC for further analysis, without any interferences from the matrix. The proposed MIP-MSPD method has been validated according to European Commission Decision 2002/657/EC criteria, in terms of linearity, precision, accuracy, decision limit (CCα) and detection capability (CCβ). Linearity ranged from 0.3-10 $\mu g\ g^{-1}$ with the correlation coefficient $r^2 > 0.999$. Mean recoveries of E2 from goat milk samples at different spiked levels were between from 89.5-92.2 % with RSD values within 1.3-2 %. CCα and CCβ values were 0.36 and 0.39 $\mu g\ g^{-1}$, respectively. The developed MIP-MSPD method was successfully applied to direct determination of E2 in goat milk samples.

Keywords: β-estradiol, growth promoters, milk, MSPD, HPLC

Introduction

Anabolic steroids have been widely used as growth promoting agents in cattle to increase the weight gain of animals and reduce the feed conversion efficiency. Administration of naturally and synthetic anabolic growth promoters in food-producing animals is now prohibited by the EU [1] because of their potential risk to human beings. A lot of evidence has been documented indicating that exposure to both natural and synthetic chemicals at low levels may lead to toxic effects in humans, and is associated with many diseases such as breast and uterine cancer [2]. Some hormonal anabolic compounds, such as 17β-estradiol, are still being used illegally in livestock production to promote growth rate. This compound is a natural oestrogen which can be carcinogenic even at low levels, and it is listed within Group A in Annex I of the Council Directive 96/22/EC (Group A, substances having anabolic effect and unauthorized substances) [1]. For Group A substances, "zero tolerance" is established by EU, except for melengestrol acetate which maximum residue limit (MRL) has been set at 1 μg Kg^{-1} in cow fat.

Monitoring of E2 in milk samples is a challenging task due to low levels concentrations that should be detected and the complexity of the matrix. In general, the determination of E2 in milk has been dominated by immunological [3] and microbiological [4] techniques, which are easy to perform, but are not specific enough to ensure accurate identification. Chromatographic techniques (LC or GC), using mass spectrometry detection [5-10] can overcome these problems. However, previously sample preparation processes are necessary to achieve the optimal sensitivity, selectivity and specificity. In recent years, several sample preparation procedures for isolation of the analyte and purification of the sample have been developed for concentration and clean-up of E2 in milk samples. These include liquid-liquid extraction (LLE) [5], solid-liquid extration (SPE) [6], molecularly imprinted solid phase extraction (MISPE) [7], and multi step solid phase extraction (MSPEE) [2], which can be time-consuming and tedious.

Matrix solid phase dispersion (MSPD) technique offers an effective alternative to traditional methods for sample preparation. This methodology combines aspects of several analytical techniques performing sample disruption whilst dispersing the components of the sample on and into a solid support, thereby generating a chromatographic material with a particular characteristic for the extraction of compounds from the dispersed sample

[11]. MSPD is one of the most promising techniques to reduce matrix interferences. Most common dispersants used in MSPD, such as C18, C8, silica and florisil, lack selectivity for the target compound. Therefore, there is a need for continuous improvement of the selectivity and efficiency of the solid support [12]. Recently, the use of molecularly imprinted polymers (MIPs) as selective solid supports in MSPD has been successfully employed for the selective extraction of fluoroquinolones antibiotics in matrices serum [13, 14] and chicken tissues [15]. MIPs are synthetic polymeric materials that have specific molecular recognition properties for target compounds. The synthesis of MIPs involves the complexation in solution of a template with functional monomers, through non-covalent or covalent interactions, followed by polymerization of these monomers around the template in the presence of a cross-linker and of an initiator [16]. The synthesized polymer presents many advantages, such as high stability and resistance to harsh conditions, low cost and ease of preparation, which make MIPs very useful in practical applications such as synthetic recognition elements. The combination of MIP and MSPD techniques can be a valuable alternative for a rapid selective sample treatment and may lead to higher recoveries and lower detection limits.

The aim of the present paper is to develop a rapid, selective, sensitive and reliable method for the determination of the oestrogen 17β-estradiol from goat milk samples using MIP in combination con MSPD technique (MIP-MSPD), coupled with liquid chromatographic and DAD detection. An imprinted polymer for 17β-estradiol was prepared through bulk polymerization method using 17β-estradiol template molecule, methacrylic acid (MAA) as the functional monomer and ethylene glycol dimethacrylate (EGDMA) as the cross-linking agent and was successfully applied as selective sorbent in MSPD for extraction of the target compound from goat milk. The proposed method has been validated according to the European Commission Decision 2002/657/EC [17], and it has been successful in determining E2 in goat milk samples. ~~To the best of our knowledge, this is the first attempt of using MIP as selective solid support for the determination of E2 in goat milk samples, offering an advantage alternative in routine analysis for quality control.~~

Experimental

Chemicals and reagents

Methacrylic acid (MAA), ethylenglycol dimethacrylate (EGDMA) and β-estradiol (E2) were obtained from Sigma Aldrich (Madrid, Spain). Azobisisobutyronitrile (AIBN) was supplied from Fluka (Buchs, Switzerland). Analytical grade acetic acid was supplied by Merck (Darmstadt, Germany). HPLC-grade solvents used were acetonitrile and methanol, purchased by Sigma-Aldrich and ethyl acetate by Merck. Anhydrous sodium sulphate and washed sea sand (0.25-0.30 mm) were purchased from Sigma-Aldrich. All reagents used were of analytical grade. Deionized water (18.2 MΩ/cm quality) was obtained from a Milli-Q water system (Millipore Iberica, Madrid, Spain).
Stock solution of E2 at 500 mg/L was prepared by dissolving 5 mg of standard substance in 10 mL of methanol. This solution was stored in dark glass bottles and kept at -20 ºC when not in use. Working standard solutions were prepared daily by diluting the stock solution.

Samples

Fresh goat milk used for this study was kindly provided by a farm located in Losar de la Vera (Cáceres, Spain), and belongs 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 ºC until use for analysis. Samples were thawed at room temperature and homogenized by heating at 40 ºC for 2 minutes, before processing. All recovery experiments were performed using spiked samples and were prepared by adding the desired amount of E2 to 0.2 mL homogenized raw milk. The spiked milk samples were maintained at room temperature for 15 minutes before treatment to allow the analyte to penetrate into the milk matrix.

Apparatus

A UV lamp (model CN-6T, Vilber Lourmat, France) was used to iniciate the polymerization process. Fluorescence intensity was measured using a model L55S (Perkin-Elmer, UK). Instrumental parameters and processing data were controlled by FL Winlab software. The excitation and emission wavelenghts were set at 281 and 305 nm respectively. Chromatographic analysis for this study were performed using a VARIAN PROSTAR liquid chromatographic system (Varian Ibérica, Spain) equiped with a 230

ProStar tertiary pump, a ProStar 410 auto sampler with a six-port injection valve equipped with a 25 µL sample loop (Rheodyne), a thermostatic column compartment,an Ascentis C18 column (250mm X 4.6mm), particle size 5 µm (Supelco, USA) a DAD 335 ProStar UV–vis detector and a PC-based data acquisition system Varian Star Workstation.

Preparation of the molecularly imprinted polymer

The molecularly imprinted polymer to E2 (MIP-E2) were synthesised by bulk polymerization method which has been summarized in a previous study of the authors [18], using 17β-estradiol as template molecule (27.4 mg) methacrylic acid (MAA) as a functional monomer (0.254 mL), ethyleneglycol dimethacrylate (EGDMA) as crosslinker monomer (2.832 mL), azobisisobutyronitrile as initiator (62.5 mg) and acetonitrile (ACN) as porogen. The polymerization was induced by UV irradiation at 365 nm and 4 ºC for 2 h followed by heating at 60 ºC in an oven for 16 h. The polymer obtained was ground in a mortar and sieved to a range of particle sizes from >32 µm to > 55 µm. The template was removed by microwave-assisted extraction (MAE), following the protocol described in authors previous study mentioned above. Non imprinted polymer (NIP) was prepared following the same procedure without adding the E2.

MSPD procedure

An aliquot of spiked milk sample (200 µL) was poured into a glass mortar and 0.085 g of MIP-E2 particles, 0.210 g of Na_2SO_4 and 0.210 g of washed sea sand were added. The mixture was gently blended with the glass pestle to obtain an apparently dry and homogeneous material. After the MSPD blending process, the homogenized mixture was packed into a solid phase extraction column with a plug of silanized glass wool at the bottom that retains the entire sample. The column was lightly tapped to avoid channels and not over-compress or compact the material. Finally, the head of the column was covered with a disc of filter paper. Analyte elution from the resulting MSPD cartridge was performed dropwise by gravity using one millilitre of methanol. A Teflon valve was used to obtain a constant flow rate of approximately 1 $mL \cdot min^{-1}$. The extracts were collected into a conical tube and evaporated to dryness under a gentle stream of argon at room temperature. The residue was redissolved in 0.5 mL of methanol and an aliquot of 20 µL was directly injected into the HPLC system.

Chromatographic analyses

Chromatographic analyses of E2 were performed using as mobile phase a binary mixture consisting of acetonitrile (A) and water (B), under gradient conditions (0-6 min 10 % A, 6-10 min 100 % A). The flow rate was set up at 1 mL·min^{-1} during 6 min, decreased to 0.7 mL·min^{-1}, to be maintained to the end of the chromatogram. Identification of the compound was carried out by chromatographic comparison with a standard and by their UV spectra. Quantitative measurements of the peak area by LC-UV-DAD were made at 200 nm, which provides maximum sensitivity of the compound.

Results and discussion

Optimization of MSPD extraction procedure

MSPD efficiency depends on careful optimization of the experimental parameters affecting competition within the matrix, the dispersant sorbent, and the extraction solvent for both, analytes and potential matrix interferences [19]. Accordingly, type and amount of sorbents, cleanup and eluting solvents were carefully selected to achieve extracts with the highest recovery and the lowest amount of matrix interferences from the goat milk sample.

Taking into that the objective of this work was to carry out the selective extraction of 17β-estradiol, a MIP-E2 previously synthesized, was chosen as solid phase for sample dispersion. In this previous paper [18], recognition properties of MIP-E2 were investigated. The resulting MIP proved to be specific for 17β-estradiol, with no detecting response to others estrogens (estrone, estriol and bisphenol A). These results indicate that MIP can be used to prepare selective recognition matrices for E2, which may be of potential use for analytical purposes.

The following step in the 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 column. In this study, different ratios of sample:MIP were evaluated (1/1, 2.5/1, 1/2.5), using 200 μL of sample. The resulting MSPD mixtures were too wet to be

transferred into the cartridge and undergo a chromatographic process, even when ratio 2.5/1 was used. Sodium sulphate and sea sand in the same ratio were added to the mixture, in order to achieve total adsorption matrix components, including its relatively high moisture content. Finally, using a ratio 1:3:3 (MIP: sea sand: Na_2SO_4), a homogeneous and dry mixture was obtained that allowed the easy packing in the SPE cartridge and also the flow of solvents through it.
An appropriate washing solvent should leave the target compounds absorbed on the cartridge and remove matrix interferences from the sample as much as possible. Subsequently, chloroform and hexane were investigated as washing solvent in order to eliminate the large amount of fat contained in goat milk samples. For this purpose, 3 mL of washing solvent was tested using methanol as elution solvent (2 mL). When the column was washed using hexane, recoveries were below 5 %, improving slightly (20 %) by using chloroform as defatting solvent. Taking into account these results, it was desided to test analytes elution directly from the MSPD column, by eliminating the washing step. An enhancement of recovery (92 %) and also extract free of interferences were obtained, evidenced by the clear colour of the extract after its reconstitution.

Solvents of different polarities were tested to optimize the elution procedure, such as acetonitrile, methanol, or mixture of solvents, methanol/ethyl acetate (50/50), methanol/acetonitrile (50/50) and methanol/water (70/30). This set of assays was carried out using an elution volume of 2 mL. The best results were achieved when methanol was used as elution solvent, obtaining recoveries around 93%. Figure 1 revealed that methanol was the best solvent for the elution of E2 from the MSPD column. Volume of elution solvent was investigated. For this, different volumes of methanol as elution solvent (0.5, 1, 1.5 and 2 mL) were tested. The results (Figure 2) showed that the optimal elution volume was 1 mL, obtaining E2 recovery of 90%.

The selectivity and extraction efficiencies of the developed MIP-MSPD were also investigated by comparison with NIP-MSPD. For this purpose, the optimum conditions for the MIP-MSPD developed method were applied for the extraction of E2 in goat milk sample preparing the dispersed sample using the NIP instead of MIP. In this case, recovery was lower than 30 %, which indicate that MIP was a suitable solid support for selective extraction of E2 in milk samples.

An additional MSPD experiment was carried out using a non-spiked goat milk sample as blank assay. Figure 3 shows the obtained chromatogram which revealed that samples did not contain E2.

Validation of the method

The developed MIP/MSPD-LC/DAD method was validated in terms of linearity, precision, recovery, decision limit (CC*α*) and detection capability (CC*β*), according to procedures described in Commission Decision 2002/657/EC.

The linearity of the method was tested for 17β-estradiol. Calibration curve was constructed by preparing spiked milk samples in triplicate, containing increasing concentration of 17β-estradiol, in the range of 0.3-10 $\mu g\ g^{-1}$. The results showed good linearity for 17β-estradiol with correlation coefficient (R^2) higher than 0.999.

Precision was calculated in terms of intra-day repeatability (n=5) and inter-day reproducibility (five consecutive days) and was determined by duplicate assays at three spiking levels (1, 1.5 and 3 $\mu g\ g^{-1}$). The intra-day repeatability evaluated as relative standard desviation (RSD) was around 1.5 % and the inter-day reproducibility was lower than 2%. These results reflects the robustness of the method.

Accuracy of the method was assessed by the analysis of spiked milk samples at three concentration levels (1, 1.5 and 3 $\mu g\ g^{-1}$). The recoveries obtained varied from 89.5-92.2 % with RSD values within 1.3-2%, as shown in Table 1. These results are according to the European Communities guidelines, which recommend a recovery in the range of 80–110% of the target concentration and RSD lower than 15% (European Commission, Regulation 2002/657/EC).

According to 2002/657/EC, the decision limit (CC*α*) is defined as the limit above which it can be concluded with an error probability of α that a sample contains the analyte. An *α* value equal to 1% is applied to prohibited substances [16]. The detection capability (CC*β*) is the smallest content of analyte that may be detected, identified and quantified in a sample, with a certainty of 1-*β*, were *β*= 5%.

The value of $CC\alpha$ and $CC\beta$ were calculated using the intercept (value of the signal were the concentration is equal to zero) and the standard error of the intercept at three levels of concentration (five replicates). Milk samples were fortified with 1, 1.5 and 3 $\mu g\ g^{-1}$ of 17β-estradiol. $CC\alpha$ is the concentration corresponding to intercept + 2.33 times RSD and $CC\beta$ is the concentration corresponding to $CC\alpha$ + 1.64 times RSD. $CC\alpha$ and $CC\beta$ values 0.36 and 0.39 $\mu g\ g^{-1}$, respectively. Table 2 summarizes main parameters of the method validation.

Conclusions

A novel MIP-MSPD method followed by HPLC-DAD analysis for the selective determination of 17β-estradiol (E2) from goat milk samples has been developed and validated according to European Commission Decision 2002/657/EC criteria. This method employed E2 molecularly imprinted polymer, sea sand and Na_2SO_4 as solid support to disperse the sample and methanol to elute the analyte from MSPD column. The recoveries of the analyte from spiked goat milk samples were within the range 89.5-92.2 %, with RSD less than 2 %. A good linearity of the method was obtained ranged from 0.3-10 μgg^{-1} with the correlation coefficient $r^2 > 0.999$ and CCα and CCβ values were 0.36 and 0.39 μgg^{-1}, respectively. The proposed procedure did not require proteins precipitations or additional clean-up steps because sample extraction and clean-up were carried out in the same step. In addition, the current method requires small amount of sample and volumes of solvents, with significant reduction in sample manipulation, material costs and analysis time. It is possible to process five samples simultaneously in a total time of 15 min. This developed MIP-MSPD method was successfully applied to direct determination of E2 in goat milk samples, and to the best of our knowledge, this is the first example of using MIP as selective solid support for the determination of E2 in goat milk samples, offering an advantageous alternative in routine analysis for quality control.

Acknowledgements

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Table 1

Spiked level	**1.0** µg g^{-1}		**1.5** µg g^{-1}		**3.0** µg g^{-1}	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery %)	RSD (%)
E2	92.2	2.0	90	1.3	89.5	1.5

Table 2

Parameter	**Value (E2)**
Concentration range	0.3-10 μg g^{-1}
Precision Intraday (n=3), RSD Interday (n=3), RSD	 1 -1.5 % 1.5 -2 %
Accuracy RSD	92 % 1.3 %
CCα	0.36 μg g^{-1}
CCβ	0.39 μg g^{-1}

RSD= Relative Standard Deviation
n=number of derminations

Figure 1

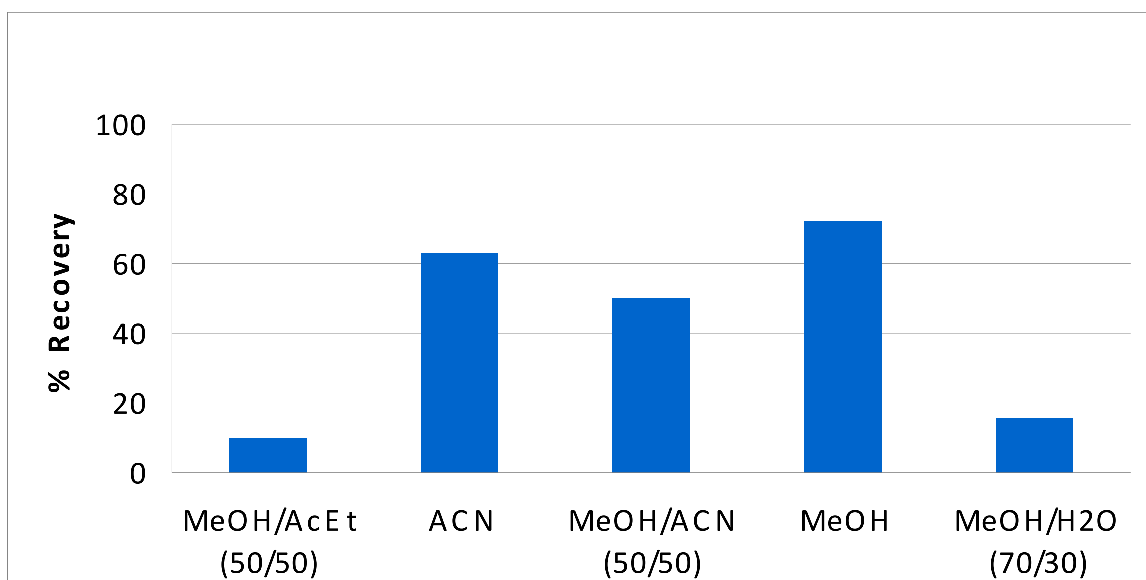


Figure 2

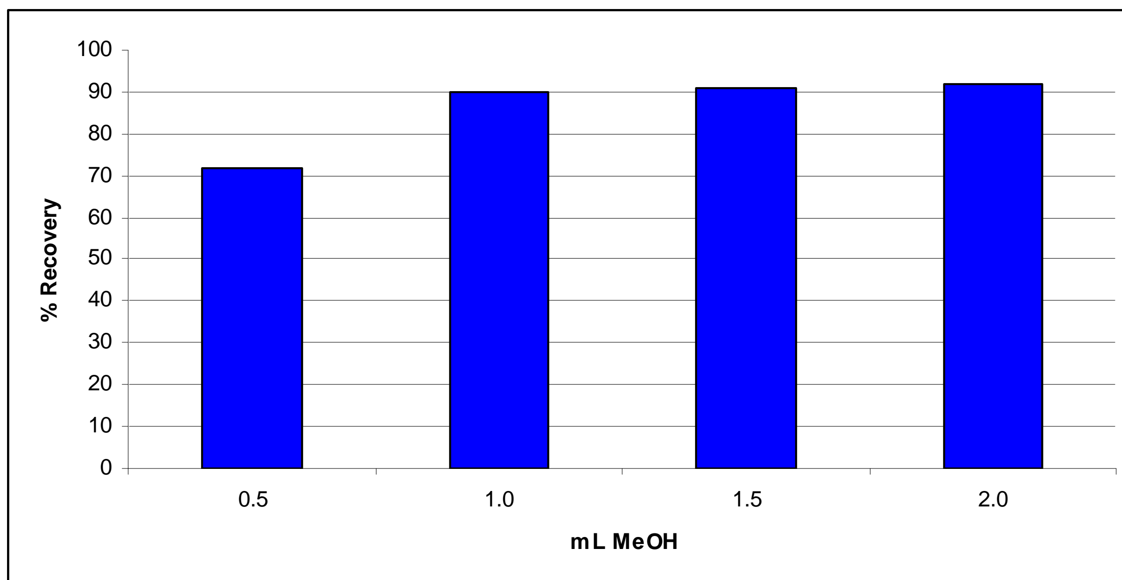


Figure 3

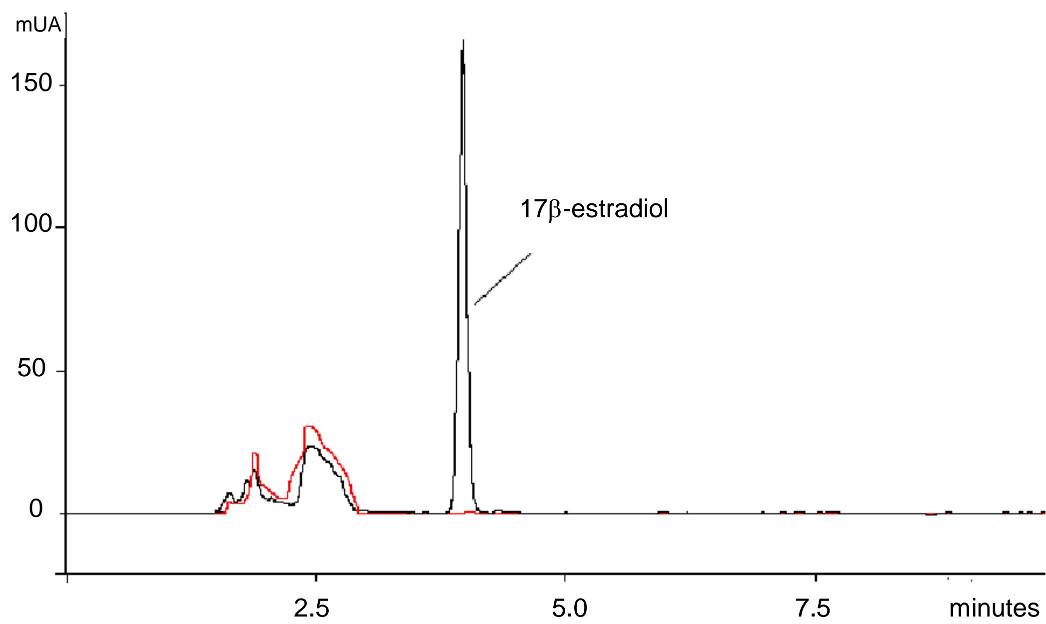


Figure captions

Fig. 1. Influence of the elution solvents on the recovery of E2. Experiments were carried out using 2 mL of each solvent. MSPD assays were carried out using 200 μL of milk sample at 1.5 μg g^{-1} spiked level.

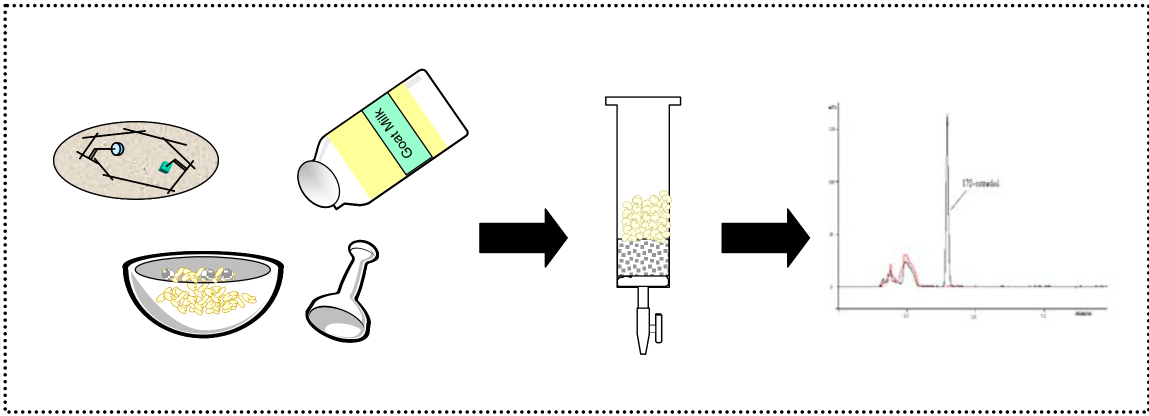
Figure 2. Optimization of the volume of methanol, used as elution solvent of E2 from MSPD column. Experiments were carried out using 200 μL of milk sample at 1.5 μg g^{-1} spiked level.

Figure 3. The chromatogram of goat milk sample and spiked goat milk sample. —— goat milk sample; —— E2 spiken goat milk sample; mobile phase: acetonitrile: water (70:30); flow rate 1.0 mL min^{-1} **;** injection volume: 20 μL

Table captions

Table 1. Mean recoveries (%) for E2 in milk samples spiked at three concentration levels (n=5), under optimum experimental conditions.

Table 2. Summary of validation parameters of the developed MSPD method in goat milk samples.



17 β-Estradiol (E2)

MIP-E2

17β-estradiol

On- line Abstract Figure

Figure Chaption

Determination of 17β-Estradiol by using a MIP-MSPD method in goat milk sample.