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Analysis of steroid derivatives by LC-MS/MS methods: selective sample preparation procedures by using mixed-mode solid phase extraction and pH control

1. Summary

The analysis of veterinary drug residues and banned crop yield boosters in foods of animal origin is one of the largest and most important areas of food analysis. A prerequisite for effective tests is the development of accurate methods that satisfy the requirements of today's analysis for selectivity, low limits of detection and accuracy. The high sensitivity and selectivity of liquid chromatographic triple quadrupole tandem mass spectrometric (LC-MS/MS) methods allows for the detection of trace amounts of the organic target compounds even in complex samples. However, the reliability of LC-MS/MS methods depends greatly on the sample preparation preceding the analysis, the objective of which is to decrease the concentrations of matrix components co-eluting with the target compounds, thus minimizing the matrix effect in the ion source of the instrument. During sample preparation, low performance liquid chromatographic clean-up, the so-called solid phase extraction (SPE) is often used. The pH of the eluent is one of the most important parameters in liquid chromatography, and so the proper selection of pH during extraction can have a a critical influence on sample preparation and, consequently, the accuracy of the analysis. This statement holds especially true when matrix compounds with functional groups susceptible to protonation have to be removed from the analytical sample. The objective of this paper is to present mixed-mode SPE sample preparation methods that demonstrate clearly the necessity for pH control during the extraction. Examples include the determination of both neutral and basic target compounds using mixed-mode strong ion exchange SPE columns.

2. Foreword

I always listened with great interest to the lectures of Professor Dr. Jenő Fekete on separation methods at the Budapest University of Technology and Economics. Our joint research work with him began in 2008, within the framework of the Transition Facility Project of the European Union. In five years, publications that were published in 11 international journals were prepared together, and in these the importance of the sample preparation and the selection of pH in the methods developed were emphasized. It was already repeatedly pointed out by Professor Fekete during his academic lectures that proper adjustment of the pH is a key element of sample preparation and liquid chromatographic separation. With this paper, we would like to honor the memory of Professor Dr. Jenő Fekete.

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3. Introduction

3.1. Monitoring analyses

The right to safe food is considered a fundamental right, laid down in basic law, which is an inherent and inalienable human right of all people [1]. It is among the common goals of the European Union (EU), and so Hungary, to implement this fundamental right as broadly as possible, therefore, great emphasis is placed on continuous performance of food analyses and the continuous development of the necessary analytical methods.

In Hungary, where agricultural output plays a prominent role in the production of the gross national product, monitoring of agricultural products also deserves special attention because of the free competition within the EU. In Hungary, food toxicology surveillance tests and inspections (monitoring analyses), the procedures and the process of preparing the monitoring plan for the given year are prescribed and determined by FVM decree 10/2002 (I.23.) FVM [2]. The tests include checking the presence of veterinary drug residues in animals, their drinking water, and all other matrices related to the breeding and farming of animals [2]. The objective of monitoring tests is the supervision of the illegal use of prohibited substances and to detect improper use of authorized substances. The number of samples varies year by year with to the slaughter number, and thousands of samples are analyzed annually by the Food Toxicological National Reference Laboratory, to check the quantities of different residual substances. One of the major responsibilities of the food control authorities since 2008 have been the analysis of corticosteroid drug residues and stanozolol metabolites in the urine of food animals and in foods of animal origin.

It is a prerequisite for the effectiveness of monitoring activities to use rapid, accurate and precise analytical methods, which requires the application of modern analytical techniques. Confirmation tests of veterinary drug residues are performed by the 5th subject group of the Food Toxicological National Reference Laboratory are performed using high performance liquid chromatography (HPLC). Due to the complexity of the matrices and the need to determine low concentrations, sensitivities and selectivities that can be obtained using traditional UV or diode array (DAD) detection are not always satisfactory, and fluorescence detection (FLD) is not suitable for the analysis of each molecule. The required selectivity and the necessary low limits of detection (LOD) are achieved by today's state-of-the-art liquid chromatographic (LC) methods by using coupled techniques [3]. Of these coupled techniques, the liquid chromatographic triple quadrupole tandem mass spectrometric (LC-MS/MS) separation is one of the best techniques to provide excellent qualitative and quantitative results. It should be noted though that, even when using methods based on the LC-MS/MS technique, concentration values with proper performance characteristics can only be obtained after sufficiently thorough sample preparation.

3.2. Sample preparation, solid phase extraction

During LC-MS/MS measurements, the goal of sample preparation is to reduce the number and concentrations of matrix components co-eluting with target compounds, and so to minimize the matrix effect. The reason for this is that the ionization of target compounds in the ion source is influenced by co-eluting matrix components. In an ideal case, matrix components do not affect the ionization of the target component. In practice, however, the so-called ion suppression, when the ionization of the target component in the ion source is reduced by co-eluting matrix components is quite common. A phenomenon may also occur, when the ionization of the analyte is not suppressed in the ion source by the matrix compounds, but it is improved, and in this case we talk about ion strengthening [3]. Effects influencing the ionization of target components in the ion source are called the matrix effect in the case of the LC-MS/MS technique. Another objective of sample preparation can be the enrichment of target components, when target components are concentrated during the extraction steps. It is important to emphasize that the concentrations of the matrix components are also increased by the enrichment of the sample, which can result in a stronger matrix effect.

Sample preparation is comprised of two main parts: extraction of the sample and the purification of the extract (clean-up). The sample purification step can be avoided if furhter dilution of the extract is made possible by the sensitivity of the instrument or the high concentration of the target compound („dilute and shoot" methods). In the case, however, when sample enrichment is necessary, purification steps play an important role.

For the purification and enrichment of the extracts, solid phase extraction (SPE) is most commonly used. Solid phase extraction is a low performance purification used during sample preparation. SPE steps used in sample preparation serve two purposes: purification of the sample and enrichment of the components to be measured. SPE column packings are similar to the packings of columns used for analytical purposes, and so a column chromatographic purification is performed during SPE. The classification of SPE packings is identical to that of the packing of HPLC columns, and solvent strength is nearly identical. For example, in the case of hydrophilic modified copolymer SPE, methanol is a stronger solvent than acetonitrile.

SPE column packings used for sample purification:

• Polar (normal phase) e.g.: silica gel, $-NH_2$.

• Apolar (reverse phase) e.g.: alkyl modified silica, polymer based phase.

• Ion exchange (strong, weak ion exchangers, mixed-mode).

Of reverse phase packings, most commonly used are C-18 and post-silanized (end-capped) C-18 packings, but the application of polymer-based (e.g., styrene-divinylbenzene) stationary phases is also widespread, especially during LC-MS/MS analyses [4]. The advantage of polymer-based SPE columns is that they can be used in the 0-14 pH range, while the pH range of applicability of silica-based stationary phases is much narrower, it is between 2 and 9. In LC-MS/MS methods, copolymer SPE columns are often used, the packings of which contain polar parts in addition to the apolar surface (hydrophilic modified SPE). This way, their retention is suitable for both hydrophilic and lipophilic compounds [5]. When developing a copolymer stationary phase, N-vinylpyrrolidone groups are inserted into the divinylbenzene phase (**Figure 1**). This way, an easily wettable packing is generated, adsorbing more polar molecules on the N-vinylpyrrolidone groups through dipole–dipole interactions and/or hydrogen bonds, while apolar compounds are bounds to the reverse phase through π-π bonds or hydrophobic interactions.

During SPE, samples are applied to a well-conditioned column (**Figure 2**). The goal of conditioning is to wet the packing, and to remove technical contaminants left behind during production and air from the pores. During conditioning, the column is always washed using a strong organic solvent first, and the weakest one is used last (aqueous solvent). It could be important for the pH of the solvent used last during conditioning to identical to the pH of the sample solution. The solvent of the sample applied to the column also has to be as weak as possible, in order for the sorption of the components to take place on the stationary phase. However, care must be taken to ensure that the solvent of the sample dissolves the sample well, and components do not precipitate before transferring to the column. It is important that the flow of the sample through the column is slow, because the diffusion of the target components from the solvent onto the solid surface takes time. Matrix compounds are removed from beside components bound to the packing by washing the column (**Figure 2**). It is important here as well that the washing solution is a weak solvent, which does not initiate the elution of the analytes to be determined. Target components can be eluted, following the vacuum drying of the column, using a strong organic solvent (methanol, acetonitrile, ethyl acetate). The strength and pH of both the washing solution and the eluting solvent play important roles in sample purification.

During solid phase extraction, only some of the matrix components can be removed from the sample. Matrix compounds with physico-chemical properties similar to the physico-chemical properties of the target compound will adsorb, concentrate and elute together with the target components (**Figure 2**). These matrix components are separated from the target compounds during the LC-MS/MS analysis.

The application of the so-called "dilute and shoot" method is common during LC-MS/MS measurements when determining target components with higher limit values (>100 µg/kg). In practice, this means that, following the extraction of the sample, the extract is only diluted and then injected into the instrument after filtration, with no sample purification (clean-up) step used. However, for this purpose, application of high sensitivity instruments is necessary, if the limit values for the target components are low (<1-10 µg/kg). The best solution is to combine the „dilute and shoot" method with isotope dilution because, in this case, the matrix effect can be compensated for by the isotopically labeled internal standard. In the case of liquid samples (e.g., urine, milk), solid-liquid extraction cannot be used, the sample cannot be separated from the extract, making the application of the „dilute and shoot" method harder. In addition, in the case of liquid food samples and bodily fluids, limit values can be one or two orders of magnitude lower, compared to limit values given for solid food samples, therefore, enrichment of liquid samples might be necessary during sample preparation.

3.3. Polymer-based mixed-mode SPE columns

The efficiency of solid phase extraction can be enhanced further if the packing of the SPE column contains not only the reverse phase, but also (strong or weak) ion exchange groups (Figure 3). SPE columns containing packing developed this way are called mixed-mode ones. When using mixed-mode SPE columns, minimal loss of target components during extraction is ensured by the high level of retention of copolymer SPE packings modified by hydrophilic side chains [5]. In addition to target compounds, the retention of matrix components on the SPE column also increases, which in turn increases the matrix effect in the LC-MS/MS analysis. Therefore, in the case of complex samples (e.g., bodily fluids), application of SPE providing adequate selectivity for the target compounds might be necessary.

Polymer-based mixed-mode strong anion exchange SPE columns (Mixed Anion eXchange - MAX) contain quaternary ammonium groups, in addition to the reverse phase. Not too polar neutral and basic target compounds are adsorbed on the reverse phase of the SPE column, while compounds of weakly acidic character are adsorbed on the anion exchange groups or the reverse phase, depending on the pH. Mixed-mode strong cation exchange SPE columns (MCX) contain benzenesulfonic acid groups. Neutral and acidic compounds are adsorbed on the reverse phase of the MCX column, while weakly basic compounds can be absorbed on the ion exchange sulfonic acid groups or the reverse phase, depending on the pH.

3.4. Corticosteroids

Corticosteroids are antiinflammatory drugs used often and widely in veterinary medicine [6]. Active ingredients of the most frequently used synthetic corticosteroid-containing preparations are dexamethasone, prednisolone or methylprednisolone, and other derivatives of these (*Table 1*). Corticosteroids can be used legally to reduce inflammation, they have maximum residue levels (MRL) in foods of animal origin [7], [8]. However, because of their bulking effect, their excessive use is prohibited, and the urine of food animals is not allowed to contain synthetic corticosteroid residues. In food analysis, their determination is mainly performed in urine samples coming from slaughterhouses or from live animals. For urine samples, concentration values that the given analytical method must be able to detect, the so-called MRPL levels (Minimum Required Performance Limit) have been identified by the EU. Among corticosteroids, only dexamethasone (DXM) and betamethasone (BTM) have an MRPL value, which is currently 2 ng/mL [9].

Corticosteroids are neutral compounds, but at high pH (>13) dissociation of the hydroxyl group(s) on the steroid skeleton begins, resulting in a very weak acidic character for corticosteroids. From a chromatographic point of view, corticosteroids can be classified as neutral compounds of medium polarity (Log P = 0,32 – 2,31) (*Table 1*). During reverse phase liquid chromatographic analysis, on completely porous, shell structure columns, they can be separated with adequate retention and peak symmetry. During mass spectrometric (MS) detection, they give rise to precursor ions both in positive and negative ionization modes (*Figure 4*), and can also be ionized with high efficiency using electrospray (ESI) and atmospheric pressure chemical ionization (APCI) ion sources. In positive mode, they can be measured as protonated molecule ions ([M+H]$^+$), while in the case of negative polarization, they can be detected as formate ([M+HCOO]$^-$) or acetate ([M+CH$_3$COO]$^-$) adduct parent ions, depending on the composition of the mobile phase [5], [10], [11], [12], [13]. *Figure 4* shows the ion transitions of prednisolone in positive and negative ionization modes, with an eluent composition of acetonitrile – water containing 0.1% formic acid (v/v). Changing the polarization mode provides an opportunity to increase selectivity. The matrix compound eluting at 5.9 minutes only appears in the chromatogram in negative ionization mode. However, the sensitivity of the positive ionization is an order of magnitude lower, compared to negative ionization. Changing the polarization mode provides an opportunity to differentiate between screening and confirmation methods. While in the case of screening, corticosteroids are detected in positive mode, negative ionization can be used in the case of confirmation [13].

3.5. Stanozolol metabolites

Stanozolol is a synthetic steroid, belonging to the group of illegal yield enhancers. In the body, stanozolol metabolizes rapidly. Its main metabolites are 16-hydroxystanozolol (16-OH-STN), 3'-hydroxystanozolol (3'-OH-STN) and 4-hydroxystanozolol (4-OH-STN) (*Figure 5*), which can be detected in urine [14], [15]. Stanozolol metabolites are weakly basic compounds (pKa 3.05 – 5.35), they can be measured with high sensitivity after reverse phase LC separation, using positive mode ESI ionization [15].

3.6. Matrix effect

Matrix components co-eluting with target compounds influence the ionization of the latter in the ion source. Ionization of the analytes can be suppressed or, in certain cases enhanced by matrix compounds. Ion suppression can also be caused not only by matrix components, but also by co-eluting target compounds. The direction (suppression/enhancement) and size (%) of the effect of matrix components on the ionization of a given target compound can be determined by analyzing the absolute matrix effect [3]. The absolute matrix effect (ME%) can be calculated easily by comparing the slopes of the matrix matched and no matrix (pure solvent) calibrations. ME (%) = (a$_{mátrix}$/a$_{oldószer}$ -1) x 100, where 'a$_{mátrix}$' is the slope of the matrix matched calibration and 'a$_{oldószer}$' is the slope of the calibration with no matrix. While ME (%) <0 indicates ion suppression, ME (%) >0 means ion enhancement. The reproducibility of the absolute matrix effect provides the value of the relative matrix effect [3]. The relative matrix effect can be calculated from the standard deviation of the slopes of matrix matched calibrations. In this case, the slopes of the calibrations recorded from 5 identical samples of different origin have to be determined. For example, 5 calibrations have to be prepared from urine samples coming from 5 different cows, by spiking the extracts of blank urines to given concentration levels, and recording the five calibrations from these samples. The value of the relative matrix effect is given by the relative standard deviation (RSD%) of the slopes of the calibrations [3]. The relative matrix effect at a given concentration level can also be determined, for example, at the limit value of the target component. In this case, extracts of the blanks (five different cow urine samples) are spiked to the appropriate concentration after sample preparation.

Samples are analyzed, and the value of the relative matrix effect at the concentration level in question is given as the relative standard deviation (RSD%) of the chromatographic peak areas. Analyzing the reproducibility of the matrix effect is important, because the matrix effect in the test samples is compensated during the measurements by the matrix matched calibration. If the matrix effect is reproducible, then the matrix effect on the target components will be almost the same in the test sample and the calibration sample, and so it will be easy to compensate for the matrix effect well.

To compensate for the matrix effect, isotope dilution can be used. In this case, the test sample is spiked with the analogue of the target compound, labelled with stable isotopes, as an internal standard (ISTD). Because of the co-elution of the target component and the ISTD, the effect of the matrix on the unlabeled compound and the labeled analogue, so the ratio of their responses (their chromatographic peak areas), the isotope ration will be independent of the matrix effect.

4. Preparation of urine samples for LC-MS/MS analysis

During the analysis of urine samples, one should always count on the presence of weakly acidic (pKa 3-7) matrix components, which have a major effect on the outcome of the analysis [12]. Utilizing the proton function of weakly acidic matrices, their separation from neutral and basic compounds can be achieved on strong anion exchange SPE columns.

4.1. Corticosteroids

Weakly acidic matrices can be separated from neutral corticosteroids easily, using mixed-mode strong anion exchange SPE columns and alkaline pH control [12]. At alkaline pH, weakly acidic components bind to the quaternary ammonium groups of the mixed-mode strong anion exchange (MAX) SPE column through ionic interactions, while neutral target compounds are adsorbed on the reverse phase of the SPE column. Using neutral elution (e.g., acetonitrile or dichloromethane), acidic compounds will still bind to the SPE column throuh ionic interactions, while neutral corticosteroids will elute, and so a selective extraction step can be achieved. Removal of weakly acidic matrices is important, because sensitive parent ions are obtained from corticosteroids as acetate $[M+CH_3COO]^-$ or formate $[M+HCOO]^-$ adducts in negative ionization mode. Weakly acidic matrix components are also ionized well in negative ionization mode, so they can cause the ion suppression of corticosteroids in the case of co-elution.

Proper selection of the pH during solid phase extraction is very important, because if weakly acidic matrix components are not present in the completely dissociated state, then they will be adsorbed on the reverse phase of the SPE column, and then will co-elute with target compounds.

Table 2 shows the repeatability of the matrix effect during the analysis of corticosteroids by LC-MS/MS in urine samples after SPE purification at different pH values [12]. Six different blank samples from cow urine were prepared on mixed-mode strong anion exchange SPE columns. Sample purification was performed at two different pH values. In the first round, the six urine samples were prepared at pH 5.2, then new aliquots of the same six urine samples were purified at an alkaline pH (9-9.5). Following aqueous washing, samples were eluted from the SPE column using pure acetonitrile, and then dichloromethane. After evaporation to dryness, samples were redissolved in 50% methanol and spiked to a 2 ng/mL concentration level using 6 corticosteroid components. When analyzing the samples using an LC-MS/MS method, peak areas were compared component by component. The matrix effect was evaluated as the relative standard deviation of the chromatographic peak areas at the 2 ng/mL concentration level. Table 2 clearly shows that at an acidic pH the repeatability of the matrix effect is low (21% - 43.1%), while at an alkaline pH the repeatability is significantly better (2.8% - 5.7%). This can be explained by the fact that at the acidic pH of 5.2 weakly acidic components are unable to bind selectively to ion exchange groups through ionic interactions, and so they are concentrated on the reverse phase, together with the corticosteroids.

On the other hand, at an alkaline pH selective extraction worked well, because weakly acidic matrix components were bound to the packing of the SPE column in the completely dissociated state through ionic interactions. By using neutral elution, only components adsorbed on the reverse phase were eluted, so the separation of weakly acidic matrix components from neutral corticosteroids could be achieved by this step [12].

The optimized method was used in international proficiency testing for the determination of methylprednisolone (METPRED) and methylprednisone (METPREDON) in cow urine. The task was to determine methylprednisolone and its metabolite, methylprednisone in four urine samples (A, B, C and D). In samples A and B only methylprednisolone could be measured in concentrations of 0.12 to 0.67 ng/mL. Sample C was the blank (<0.05 ng/mL), while in sample D methylprednisone could be detected in a concentration of 0.84 ng/mL. Sample D also contained methyl prednisolone, but its evaluation was not requested by the organizing laboratory (EU-RL Rikilt, Wageningen, Hollandia). The detected concentrations below X,XX ng/mL were adequate for all four samples (Table 3), and so the applicability of the method was fully confirmed [12]. Results of the proficiency testing were considered adequate if the individual Z values were in the -2 – +2 range.

4.2. Stanozolol metabolites

Stanozolol metabolites (Figure 5) are weakly basic compounds (pKa 3.05 – 5.35), and so at an acidic pH (pH < pKa - 2) they bind strongly to the sulfonic acid groups of the mixed-mode cation exchange SPE column (MCX) through ionic interactions. Adjusting the pH of the sample to 1, selective extraction of the metabolites can be performed easily on a mixed-mode cation exchange SPE column. Acidic and neutral matrix components are adsorbed on the reverse face surface of the MCX SPE column, and

can be separated from basic target compounds by washing with a neutral organic solvent. Application of pH values above 1 is not possible, because in a less acidic medium selective binding of the metabolites to the cation exchange groups of the SPE column could not be achieved, since they would only be partially ionized. Metabolites can be eluted from the cation exchange SPE column using a basic organic solvent (methanol or acetonitrile containing ammonia). Relevant recoveries in the urine matrix ranged from 10% to 71% [15]. The reason for low recovery is that at pH 1 all of the basic matrix compounds in the sample are bound to the cation exchange groups, and so basic matrix components are concentrated on the cation exchange groups together with the metabolites. On the one hand, basic matrix compounds interfere with the binding of the metabolites to the sulfonic acid groups at low pH, and on the other hand, in the case of co-elution during HPLC separation, they influence the ionization of the metabolites, which can result in low recovery values.

By using a mixed-mode strong anion exchanger (MAX), at pH 10 metabolites are adsorbed on the reverse phase, while acidic matrix components again on the ion exchange groups through ionic interactions. Depending on their polarities, basic matric components can bind to the reverse phase surface, and their retention is lower than on the MCX column at acidic pH. Recoveries of the metabolites in urine samples after diethyl ether elution range from 78% to 97% [15]. Even though stanozolol metabolites are basic compounds, the purification and enrichment method using a MAX SPE column proved to be better than using an MCX column.

The applicability of the method was confirmed by the analysis of control samples. In our experiments, the main metabolite, 16-OH-STN had to be determined in the urine of cows treated with stanozolol (2 samples). Detected concentration were 0.99 ng/mL and 2.83 ng/mL. Values assigned to the samples were 0.90 ± 0.53 ng/mL and 2.20 ± 1.22 ng/mL. The accuracy of the method is also satisfactory when analyzing urine samples coming from treated animals [15].

5. Preparation of milk samples for LC-MS/MS analysis

During the analysis of milk samples, one should count on the presence of matrix components susceptible to protonation. These are mainly zwitterionic compounds: amino acids, peptides and proteins. Thus, the use of both acidic and alkaline pH control have to be examined, and the mixed-mode SPA column to be used in the method has to be chosen accordingly. When determining corticosteroids in milk, mixed-mode strong anion exchange and mixed-mode strong cation exchange SPE columns were tested at different pH values. On the MAX column, spiked (0.3 – 6 µg/kg) milk samples (n = 3) were purified at pH 11. On the MCX column, we worked at a pH ad-

justed to 2.3. When eluting the samples with acetone, recoveries (*Figure 6*) obtained on the MCX column were in the range of 94% to 113%, which are higher than the recovery data detected after sample preparation on the MAX SPE column (56% – 73%) [16]. Purification on the MCX column was also tried using other eluents. When eluting the samples with dichloromethane (DCM), recoveries for the steroids were in the range of 43% to 97%. When acetonitrile and dichloromethane (ACN + DCM) were used for elution, recoveries improved somewhat, they were between 58% and 89% (*Figure 7*, but recovery values obtained with acetone elution were not duplicated [16]. This example shows clearly that to achieve adequate performance characteristics, the selection of eluent is important in the case of mixed-mode SPE columns as well. After a pH-optimized SPE, the matrix effect was significantly reduced during the LC-MS/MS analysis, making high enrichment of milk samples possible, as well as the reduction of the limits of detection (LOD) to ng/kg levels (*Table 4*). Even lower LOD values could be obtained (1 – 6 ng/kg) when using a modified ESI ion source, compared to the LODs obtained using a multimode (MMI) ion source in APCI mode (20-70 ng/kg) [16].

6. Preparation of animal tissue samples for LC-MS/MS analysis

The retention of polymer-based SPE columns for corticosteroids were clearly demonstrated by the above methods. Therefore, in the case of tissue samples (bovine muscle, liver and kidney), during SPE optimization, the emphasis was place only on the examination of the matrix effect, because the tissue matrix is significantly different from the samples analyzed earlier (milk, urine). Tissue samples were extracted at three different pH values (acidic, neutral and alkaline), maintaining the pH control during the solid liquid extraction as well. After acidic (i) extraction, samples were prepared on an MCX SPE column at pH 2.3. The copolymer SPE column was used at a neutral (ii) pH of 7, while at the alkaline (iii) pH of 11, the MAX SPE column was tried [17]. In the case of acidic pH control, the value for the absolute matrix effect for corticosteroids in the case of the three tissue samples ranged from -30.2% to +15.0%. The value of the relative matrix effect was between 0.7% and 10.7%. When performing sample preparation at pH 7, the extent of ion suppression (-68.4% and -18.5%) was larger (especially in the case of muscle samples) than at the acidic pH. The reproducibility of the matrix effect was also lower (4.0% and 11.2%), compared to the MCX SPE sample preparation. This is due to the fact that the copolymer SPE packing does not have the required selectivity, guaranteed by the mixed-mode MCX SPE packing at the acidic pH [17]. In order to ascertain the role of the pH in sample preparation completely, the matrix effect was also evaluated with alkaline pH control in all 3 tissue samples. After using the MAX SPE column, the value of the absolute matrix effect was between -44.4%

and -4.1%. The reproducibility of the matrix effect varied between 6.5% and 12.9%. For all three tissue types, application of either the mixed-mode MCX, or the MAX SPE column was preferred, compared to the hydrophilic modified copolymer SPE column. In the case of muscle samples, the smallest matrix effect was obtained using the MCX SPE column, while in the case of kidney samples, the use of the MAX SPE column proved to be advantageous. There was no significant difference between the MCX and MAX SPE columns in terms of matrix effect in the case of liver samples [17]. Our experience also clearly shows how the matrix effect of the LC-MS/MS analysis varies in the case of different tissue samples.

Another advantage of the method is the separation from each other of the corticosteroid epimers dexamethasone (DXM) and betamethasone (BTM). The two epimers differ in terms of the spatial position of the methyl group on C-16 of the steroid skeleton (*Table 1*), and so application of an HPLC column of adequate selectivity is necessary for their baseline separation. As large a chromatographic resolution between the two epimers is important because the ion transitions of DXM and BTM are the same, and so the MS/MS detector is unable to detect the two corticosteroids on separate mass channels. A selectivity factor of 1.05 can be achieved between the two epimers using isocratic separation, with a mobile phase of methanol/(5 mM ammonium acetate) and 0.01% acetic acid in water (50/50, pH 5.4), using a shell structure phenylhexyl column (**Figure 8**) [17].

A control sample was also analyzed using the LC-MS/MS method developed according to the above description, to verify the accuracy of the method. As a natural contaminant, the sample contained dexamethasone. The detected dexamethasone concentrations were 1.63 µg/kg, 1.58 µg/kg and 2.18 µg/kg as the results of three independent analyses. The average value was 1.78 ± 0.35 µg/kg. According to the certificate of the control sample, concentrations are acceptable in the range of 0.85 to 5.97 µg/kg, so the accuracy of the method was confirmed using a sample coming from a treated animal [17].

7. Mixed-mode vs. hydrophilic modified copolymer SPE

In our previous examples, target compounds to be determined simultaneously belonged to the same group from a chromatographic point of view. All corticosteroids are very weakly acidic,rather neutral compounds. Stanozolol metabolites are of weakly basic character. When neutral components and compounds susceptible to protonation have to be determined together (e.g.: *Alternaria* toxins), it is possible that the selectivity provided by mixed-mode SPE cannot be exploited. *Alternaria* toxins are weakly acidic compounds, with the exception of tentoxin, the molecule of which is neutral. Thus, on mixed-mode anion exchange SPE columns, tentoxin is unable to bind to the anion exchange groups. On mixed-mode cation exchangers, at an acidic pH, toxins are adsorbed on the reverse phase surface, and matrices of a basic nature can be easily removed from the sample. Nevertheless, even after purification on the MCX SPE column packing, toxins are subject to high matrix effects during the LC-MS/MS measurement, which indicates that ion suppression of *Alternaria* toxins in the ion source is caused by neutral or weakly acidic matrix components [18]. In this case, when compounds with different physico-chemical properties (acidic and neutral) need to be determined simultaneously, advantages of mixed-mode SPE column packings cannot be exploited and, instead, utilization of hydrophilic modified SPE packings is most suitable.

8. Conclusions

The reliability of methods based on the LC-MS/MS technique depends significantly on the method of sample preparation. The goal of the purification steps is to minimize the concentrations of matrix components that co-elute with the target compounds, but which are invisible to the detector. During traditional reverse phase solid phase extraction, matrix components having the same polarity as the target compounds are concentrated together with the analytes. If the satisfactory resolution between matrix components and target compounds cannot be achieved by the liquid chromatographic separation, then it can be assumed that the ionization of the analyte in the ion source may be influenced by the matrix. Therefore, the application of SPE columns might be necessary, which provide selective binding sites for the target compounds and matrix components both. Through their ion exchange groups, mixed-mode SPE columns make extensive purification of even the most complex samples (e.g., urine) possible, while minimizing the loss of target compounds.

9. References

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