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Separation and online preconcentration by multistep stacking with large-volume injection of anabolic steroids by capillary electrokinetic chromatography using charged cyclodextrins and UV-absorption detection

The separation of three common anabolic steroids (methyltestosterone, methandrostenolone and testosterone) was performed for the first time by capillary EKC. Different charged CD derivatives and bile salts were tested as dispersed phases in order to achieve the separation. A mixture of 10 mmol/L succinylated-β-CD with 1 mmol/L β-CD in a 50 mmol/L borate buffer (pH 9) enabled the separation of the three anabolic steroids in less than 9 min. Concentration LODs, obtained for these compounds with low absorption of UV light, were $\sim 5 \times 10^{-5}$ mol/L. The use of online reverse migrating sample stacking with large-volume injection (the effective length of the capillary) enabled to improve the detection sensitivity. Sensitivity enhancement factors (SEFs) ranging from 95 (for testosterone) to 149 (for methyltestosterone) were achieved by single stacking preconcentration. Then, the possibilities of multistep stacking to improve the sensitivity for these analytes were investigated. SEFs obtained by double stacking preconcentration ranged from 138 to 185, enabling concentration LODs of 2.79×10^{-7} mol/L (for methyltestosterone), 3.47×10^{-7} mol/L (for testosterone) and 3.56×10^{-7} mol/L (for methandrostenolone). Although online triple stacking preconcentration was achieved, its repeatability was very poor and SEFs for the studied analytes were not calculated.

Key Words: Anabolic steroids; Capillary electrokinetic chromatography; Online multistep stacking

Received: March 10, 2005; revised: April 13, 2005; accepted: May 11, 2005

DOI 10.1002/jssc.200500113

1 Introduction

Although CE shows important advantages (small quantity of injected sample, high speed and resolution and low expenditure of chemicals) for the analysis of many compounds in a great variety of samples [1], the concentration LODs obtained with UV-absorption detection are still, in many cases, unsatisfactory. They can be improved by hyphenation of CE with more sensitive detectors, such as LIF [2] or electrochemical detection [3]. However, the cheapest and the most popular one is the UV-absorption detector. Although the short diameter of the capillary (25–100 μm) does not allow to measure absorbance of some compounds which do not possess good chromophores, the use of special detection windows (*e.g.* bubble cells, zeta cells) [4, 5] may slightly improve sensitivity with optical detection. In addition, CE offers a great possibility for online sample preconcentration and enables the automatisation of the process, which always is desirable in analytical chemistry, as well as in other fields.

Online sample concentration represents an effective and versatile way to enhance concentration sensitivity in CE [6]. The high electric field and tunable electrophoretic mobility of the analytes can be used to induce electrokinetic focussing within large-injection volumes of sample directly on-capillary prior to detection. Online focussing is normally performed by selecting different buffer properties to modify analyte velocity in two or more sections in the capillary, such as sample and BGE.

There are four major modes for online sample concentration in CE [6]: sample stacking, transient ITP, sweeping and dynamic pH junction. Each method relies on a distinct

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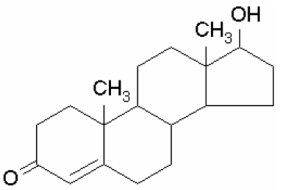
Abbreviations: CE-β-CD (DS ~ 3), carboxyethylated-β-CD (degree of substitution ~ 3); **CM-β-CD**, carboxymethylated-β-CD; **MTD**, 17β-hydroxy-17α-methyl-androsta-1,4-dien-3-one (methandrostenolone); **MTS**, 17β-hydroxy-17α-methyl-4-androsten-3-one (methyltestosterone); **SEFs**, sensitivity enhancement factors; **Suc-β-CD**, succinylated-β-CD; **TST**, 17β-hydroxy-4-androsten-3-one (testosterone)

focussing mechanism based on different electrolyte properties between sample and BGE zones, such as conductivity (ionic strength), electrolyte coion mobility, additive concentration (analyte–additive interactions) and buffer pH, respectively. Application of one of these methods frequently offers good sensitivity enhancement, at times, within several orders of magnitude.

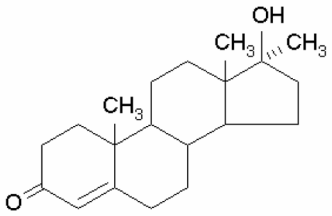
Sample stacking (in normal and reversed polarity modes, with reverse migrating micelles without and with water plug, and head column field-enhanced sample injection without and with reverse migrating micelles) occurs as ions across a boundary that separates regions of the high electric field sample zone and the low electric field background solution zone [7].

Sometimes sensitivity enhancement obtained with single stacking procedure is limited and there is a need to obtain lower concentration LODs. There are some studies describing successful application of double stacking procedure [8–11], showing that multistep stacking can be an interesting alternative to be used as online preconcentration method. Hence, the possibilities of multistep stacking and the flexibility of this online preconcentration method to adjust the desired concentration LODs, depending on the number of stacking steps and making a compromise between recovery and performance (resolution and analysis time), should be investigated.

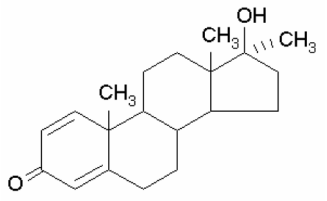
Methyltestosterone, methandrostenolone and testosterone are popular steroids (see Fig. 1), fraudly used in sports. These anabolic steroids are also used as medicines in the treatment of anaemia, renal insufficiency, endometriosis, hereditary angioedema and inoperable breast cancer [12], which reveals a huge necessity to develop new analytical methods for their quantification. Nowadays, these anabolic steroids are mainly analysed by HPLC [13, 14] or GC [14, 15]. With GC-MS, very low LODs can be achieved. However, since some anabolic steroids and their metabolites possess hydroxyl and carbonyl groups in their structure, derivatisation is necessary, as in general sample pretreatment is labourious and time-consuming [16]. HPLC and CE are highly promising methods for fast steroid screening, CE being much less employed than HPLC [17, 18]. CE has been employed for the analysis of different steroids generally with MS, fluorescence or UV detection [14, 19–21]. However, the interest in using the most widely employed detection system in CE (UV absorbance detection) for the analysis of steroids has promoted the use of preconcentration techniques previous to their analysis due to the relatively low expected sensitivity (steroids show low absorption in the UV-Vis region). In fact, offline preconcentration techniques as SPE [22, 23] and online preconcentration techniques [6, 24–27] have been employed. In spite of the results obtained for the analysis of steroids by CE, separation of



17β-Hydroxy-4-androsten-3-one (Testosterone, TST)



17β-Hydroxy-17α-methyl-4-androsten-3-one (Methyltestosterone, MTS)



7β-Hydroxy-17-methyl-androsta-1,4-dien-3-one (Methandrostenolone, MTD)

Figure 1. Structures and names of the three anabolic steroids studied in this work.

any three anabolic steroids studied in this work has previously been reported. Only the separation of testosterone and/or methyltestosterone from other steroids has been reported [6, 16, 24, 28–30]. Due to the neutral nature of these analytes they need to be separated by capillary EKC, which is based on the different distribution of analytes between a dispersed phase (CDs, micelles, *etc.*) and the mobile phase (buffer solution).

The aim of this work was to optimize the experimental conditions enabling the separation of the three above-mentioned anabolic steroids by EKC, and to develop a method for their online preconcentration in EKC in order to achieve a sensitive UV-absorption detection of these low absorbent analytes, this latter purpose involving the study of the possibilities of multistep stacking.

2 Materials and methods

2.1 Chemicals and samples

The standards of 17β-hydroxy-17α-methyl-4-androsten-3-one (methyltestosterone, MTS), 17β-hydroxy-17α-methyl-androsta-1,4-dien-3-one (methandrostenolone,

Original Paper

MTD) and 17β-hydroxy-4-androsten-3-one (testosterone, TST) were purchased from Fluka (Buchs, Switzerland) and stored at 4°C. β-CD and succinylated-β-CD (Suc-β-CD (degree of substitution (DS) ~3)) were also supplied by Fluka. Carboxymethylated-β-CD (CM-β-CD (DS ~3)) and carboxyethylated-β-CD (CE-β-CD (DS ~3)) were from Cyclolab (Budapest, Hungary). Sodium bile salts of cholic acid, taurocholic acid and taurodeoxycholic acid were purchased from Sigma (St. Louis, MO, USA) and that of deoxycholic acid from Fluka. Boric acid (Fluka), sodium dihydrogen phosphate (Merck, Darmstadt, Germany) and sodium hydroxide (Panreac, Barcelona, Spain) were used to prepare the buffers. HPLC-gradient grade methanol was from Panreac. Distilled water used throughout this work was also purified with the Milli-Q system (Millipore, Bedford, USA).

The 50 mmol/L borate buffer (pH 9) was prepared by dissolving the appropriate amount of boric acid in water and by adding 1 mol/L sodium hydroxide to the resulting solution in order to obtain the desired pH value. Then, the suitable amounts of CDs were dissolved in the buffer solution. The standards of the studied compounds were dissolved in methanol, and then diluted in water to obtain the desired concentration.

Dissolution of solids was facilitated by the use of an ultrasonic bath (Raypa, Barcelona, Spain). The pH of each buffer was adjusted using a 654 pH meter (Metrohm, Herisau, Switzerland). Both, samples and buffers, were filtered prior to the analysis using syringe filters (Titan Filtration Systems; nylon, pore size 0.45 μm, filter size 13 mm).

2.2 Instrumentation

A CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with an oncolumn diode array detector was used. The control of the instrument and data acquisition were achieved by a PC with the 3D-CE ChemStation software (A.09.03, Agilent).

Uncoated fused-silica capillary was employed: effective capillary length, 50 cm; total capillary length, 58.5 cm; ID, 75 μm; OD, 375 μm (Sugelabor S.A., Madrid, Spain). The sample and operating vials were served from a 48-vial carousel, designed for typical 1 mL snap polypropylene vials.

2.3 Analysis procedure

Experiments without stacking were performed by injecting the sample (50 mbar for 3 s) followed by the separation buffer (50 mbar for 3 s). The applied operation scheme used to achieve single, double and triple online stacking is shown in Table 1. The first stage of each run is capillary conditioning with 0.1 mol/L NaOH (1 bar for 5 min), Milli-Q water (1 bar for 2 min) and running buffer (1 bar for 5 min). Then, the sample was injected to fill 50 cm of the capillary length (from the inlet to the detection window). The injection time was calculated from the rearranged Poiseuille's equation:

$$t = 3200 \frac{Ll\eta}{d^2 P} \quad (1)$$

where P is the injection pressure (mbar), t the time duration of the pressure (s), d the ID of the capillary (μm), l the

Table 1. Analysis steps in the online multistep stacking and separation of MTS, MTD and TST. Running buffer: 10 mmol/L Suc-β-CD, 1 mmol/L β-CD, 50 mmol/L borate buffer at pH 9. During sample concentration, –20 kV indicates negative polarity and t_{s1}, t_{s2} and t_{s3} are times of the successive concentration steps (176, 209 and 211 s). Used vials: S, sample; B1–B5, running buffer; N, 0.1 mol/L NaOH; Q, Milli-Q water and W1, W2, waste

Steps	Inlet	Outlet	Single stacking	Double stacking	Triple stacking
Preconditioning	N Q B1	W1 W1 W2	5 min 0.1 mol/L NaOH 2 min water 5 min running buffer (1 bar)	5 min 0.1 mol/L NaOH 2 min water 5 min running buffer (1 bar)	5 min 0.1 mol/L NaOH 2 min water 5 min running buffer (1 bar)
Sample injection	S	B3	50 mbar, 332 s (50 cm of capillary)	50 mbar, 332 s (50 cm of capillary)	50 mbar, 332 s (50 cm of capillary)
Buffer injection	B2	B3	50 mbar, 5 s	50 mbar, 5 s	50 mbar, 5 s
Zone concentration	B2	B3	−20 kV, t_{s1}	−20 kV, t_{s1}	−20 kV, t_{s1}
Sample injection	S	B3	–	50 mbar, 332 s	50 mbar, 332 s
Buffer injection	B2	B3	–	50 mbar, 5 s	50 mbar, 5 s
Zone concentration	B2	B3	–	−20 kV, t_{s2}	−20 kV, t_{s2}
Sample injection	S	B3	–	–	50 mbar, 332 s
Buffer injection	B2	B3	–	–	50 mbar, 5 s
Zone concentration	B2	B3	–	–	−20 kV, t_{s3}
Separation	B4	B5	20 kV	20 kV	20 kV

length of the sample injection plug (mm), η the viscosity of the buffer (cP) and L the capillary length (cm). The injection time necessary to fill the effective length of the capillary (50 cm) calculated by means of Eq. (1) was 332 s. This calculated time was very similar to the experimental time estimated by filling the capillary with a solution of a mixture of the steroids (~3×10^{-4} mol/L), after filling the capillary with buffer solution, and registering the signal at 245 nm which increased when the steroids achieved the detection window. After sample injection, a short zone of the running buffer was injected to prevent loss of the analyte. Then, reverse polarity voltage (20 kV) was applied. These three steps were applied once, twice or thrice, depending on the number of stacking steps. Separation of the mixture was achieved in the last stacking step, when normal (positive) polarity voltage (20 kV) was applied.

Before the first use, the capillary was conditioned by washing it with 1 mol/L NaOH (1 bar for about 1 h) and then with Milli-Q water (1 bar for 10 min). During the whole analysis, various vials were used (see Table 1). Different buffer vials were employed for stacking and different for separation (see Table 1) so as to avoid contamination of the separation buffer which could increase the background noise. The buffer vial B1 (used for capillary preconditioning) was of 2 mL volume since this vial does not require frequent replacement. Two-millilitre vials were also employed for 0.1 mol/L NaOH (N) and water store (Q) and for the waste deposition (W1, W2). One-millilitre standard plastic vials were used for the samples/standards and buffer solutions. A temperature of 15°C was used in this work. Most of the operation steps were programmed using the preconditioning table in the ChemStation software. However, the "CE TimeTable" was used for the acquisition of the current intensity curves during the stacking preconcentration.

Since the studied compounds slightly differ in their UV-absorption maxima, a single wavelength was chosen to detect all of them (245 nm ± 5 nm), and the reference value was switched off since it did not give any improvement.

2.4 Data treatment

The curves corresponding to the variation of the current intensity as a function of the time of application of reverse polarity were obtained for mixtures of the three steroids in which each component was at concentrations ranging from 5×10^{-7} to 1×10^{-5} mol/L. Then, the 4th order polynomial curves ($ax^4 + bx^3 + cx^2 + dx + e$) were adjusted and the inflexion point x-value was calculated making the 2nd order derivative from the 4th order fitted polynomial curve ($12ax^2 + 6bx + 2c$) equal to 0. One of the two solutions of the 2nd order derivative was considered and the following equation was used:

$$x = \frac{-6b + \sqrt{36b^2 - 96ac}}{24a} \quad (2)$$

where a, b and c are the coefficients at x^4, x^3 and x^2 in the 4th order fitted polynomial curve.

LODs and LOQs in concentration (mol/L) of the steroids in a 1% v/v methanol/water solution were experimentally calculated according to the following equations [31]:

$$\mathrm{LOD} = 3S_{a'}/b' \quad (3)$$

$$\mathrm{LOQ} = 10S_{a'}/b' \quad (4)$$

where $S_{a'}$ is the standard error of the intercept and b', the slope of the straight line, both obtained by ANOVA. Experimental data were treated using Origin 7.0 (OriginLab Corporation) and Excel 7.0 (Microsoft) software.

Theoretical estimation of LOD with multistep stacking was achieved using Eq. (5):

$$\mathrm{LOD}_n = \frac{\mathrm{LOD}_0}{fn} \quad (5)$$

where LOD_0 is the LOD obtained without stacking, n the number of stacking steps (1, 2, 3…) and f the stacking amplification factor expressed as:

$$f = \frac{t_s}{t_0} \quad (6)$$

where t_s is the sample injection time in the single stacking step and t_0 the injection time in the analysis without stacking.

3 Results and discussion

3.1 Separation conditions

The separation of a mixture of neutral MTS, MTD and TST was achieved after a set of trials with various dispersed phases (CDs or bile salts) added to borate buffer at pH 9, which enabled the reverse migrating selectors to pass through the detection window when normal polarity is applied. Three anionic CDs (CM-β-CD, Suc-β-CD and CE-β-CD) and four bile salts (sodium cholate, sodium deoxycholate, sodium taurocholate and sodium taurodeoxycholate) were investigated. Figure 2 shows the electropherograms obtained for a mixture of MTS, MTD and TST at a concentration of ~3×10^{-4} mol/L in each component when adding CM-β-CD, Suc-β-CD or CE-β-CD to the electrolytic solution. It can be observed that CM-β-CD and Suc-β-CD at a 10 mmol/L concentration enabled the separation of the three neutral compounds (with CM-β-CD, the resolution between the first and the second migrating peaks (Rs_{12}) was 1.2 and the resolution between the sec-

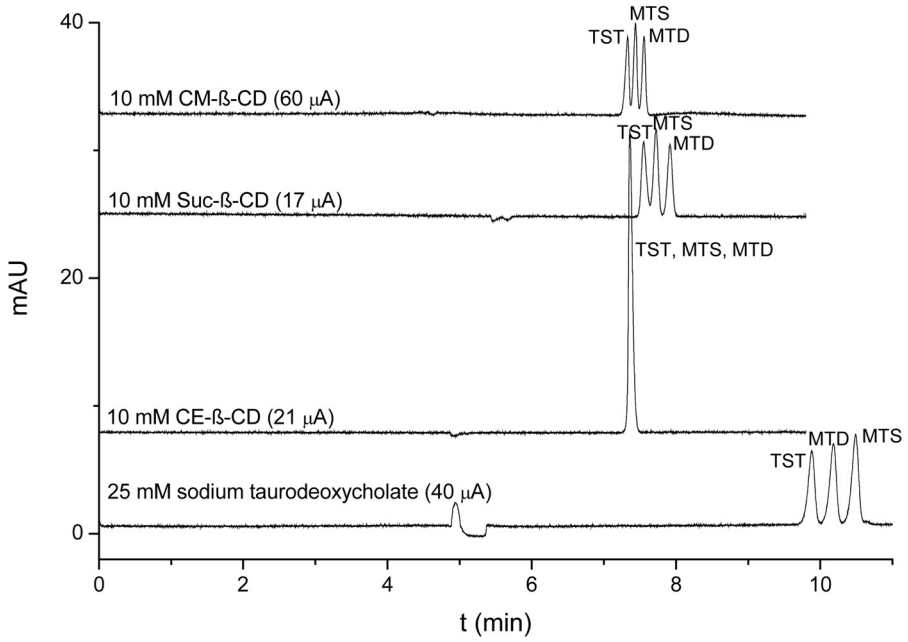


Figure 2. Separation of a mixture of MTS, MTD and TST (~3×10^{-4} mol/L in each component in 5% v/v methanol/water) with a 50 mmol/L borate buffer at pH 9 containing an anionic substituted β-CD or the bile salt taurodeoxycholic acid. Fused-silica capillary, 58.5 cm (50 cm to the detection window), 75 μm ID (375 μm OD); voltage: 20 kV (current intensity for each separation buffer is specified in the figure in parenthesis); injection: 50 mbar, 3 s and buffer injection during 3 s; temperature: 15°C; UV-absorption detection at 245 nm (±5 nm).

ond and the third migrating peaks (Rs_{23}) was 1.3, whereas with Suc-β-CD, Rs_{12} = 1.2 and Rs_{23} = 1.5). The anabolic steroids passed through the detector in the following order: TST, MTS and MTD. However, under the experimental conditions tried, CE-β-CD did not enable the separation of the mixture ($Rs_{12} = Rs_{23} = 0$). On the other hand, from the four bile salts tested, only sodium taurodeoxycholate enabled the separation of the studied compounds (Rs_{12} = 1.9 and Rs_{23} = 2.0) giving rise to a different migration order with respect to the use of CDs: TST, MTD and MTS, as it is also shown in Fig. 2.

The effect of using mixtures of dispersed phases was investigated in order to improve the separation selectivity obtained under the above conditions. For this reason, a second CD, the neutral β-CD, was added to the electrolytic solution. Figure 3 shows that the presence of β-CD at a 1 mmol/L concentration significantly improved the separation of the three steroids. The mechanism through which the separation of the anabolic steroids was improved by addition of a neutral CD is based on the simultaneous interaction of these compounds with the anionic and the neutral dispersed phases. Thus, these neutral compounds acquire mobility due to their interaction with the anionic dispersed phase (anionic CD or bile salt) which, under the normal polarity mode, migrates toward the anode. On the other hand, their interaction with the neutral CD, which migrates with the EOF due to its electroneutral nature, enables their migration towards the cathode giving rise to peaks located in the electropherogram between the signals corresponding to the EOF and the anionic CDs. Since the interaction of each steroid with the anionic and/or neutral dispersed phase is different, the discrimination increases with the addition of the neutral dispersed phase improving their separation. Thus, the addition of β-CD to the CE-β-CD system enabled to observe the separation of the analytes (Rs_{12} = 1.3 and Rs_{23} = 1.0) while the addition of β-CD to CM-β-CD, Suc-β-CD or the bile salt gave rise to an increase in the resolution. In fact, the use of 1 mmol/L β-CD with 25 mmol/L taurodeoxycholate enabled to obtain resolutions of 2.5 and 2.0 for the first–second and second–third peaks, respectively, although the broadest peaks for the analytes were obtained under these conditions (see Fig. 3). Although the mixture of 10 mmol/L CM-β-CD with 1 mmol/L β-CD offered an excellent separation of the analytes (Rs_{12} = 17.0 and Rs_{23} = 4.1), the mixture of 10 mmol/L Suc-β-CD and 1 mmol/L β-CD (Rs_{12} = 7.8 and Rs_{23} = 4.8) was chosen for further work because it provided similar shape and resolution for the peaks of the three analytes. Under these experimental conditions, concentration LODs of 4.79×10^{-5} mol/L, 5.17×10^{-5} mol/L and 6.17×10^{-5} mol/L were obtained for TST, MTS and MTD respectively. In order to improve the UV-absorption detection sensitivity, obtained for these three steroids, online sample preconcentration by stacking with large-volume injection was applied.

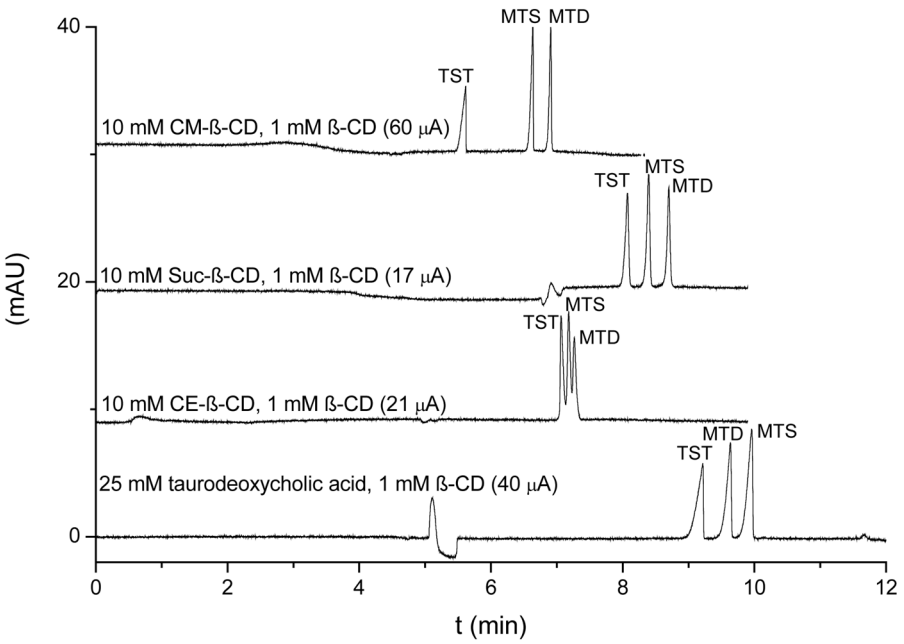


Figure 3. Separation of a mixture of MTS, MTD and TST (~3×10^{-4} mol/L in each component in 5% v/v methanol/water) with a 50 mmol/L borate buffer at pH 9 adding the native β-CD to the anionic CDs or the sodium taurodeoxycholate. Experimental conditions as in Fig. 1.

3.2 Online preconcentration by single stacking

Since the selected separation buffer contained anionic CDs with reverse migration in the normal polarity mode, the following steps were considered to perform a reverse polarity stacking mode (see Table 1): (i) sample injection to fill the effective length of the capillary, (ii) injection of the running buffer to avoid diffusion of the analytes in the early stacking stage, (iii) zone concentration applying a time of reverse polarity voltage (−20 kV) and (iv) analyte separation with normal polarity voltage (20 kV).

The key parameter for the preconcentration process was observed to be the time of application of the reverse polarity voltage (stacking time). It could be found in the literature that the stacking time should be that which allows reaching from 70 to 99% of the current intensity of the buffer solution [32–36]. The initial criterion to choose the stacking time value was to select the time corresponding to the inflexion point of the current intensity *versus* time of application of reverse polarity curve. This point was calculated after fitting the experimental current intensity data to a 4th order polynomial curve and making the second derivative of the fitted function equal to 0 (see Section 2.4). Although fitting the experimental current intensity data to a 3rd order polynomial was also achieved, the fitting was much better in the case of the 4th order functions (r >0.999) being additionally less dependent on the range of the experimental current intensity values selected to be adjusted. Therefore, the 4th order polynomial was used for further optimisation of the stacking time. In addition, due to the strong dependence of the inflexion point of the fitted curve with the sample solvent, the use of water as sample solvent was desirable during the stacking preconcentration, as it is generally known. However, due to the insolubility of the studied steroids in water, the use of 1% methanol was necessary to solubilise them before the preconcentration stage. Under all these conditions, a stacking time of 150 s was obtained for a mixture of MTS, MTD and TST, 10^{-6} mol/L of each component. However, different times (calculated as mentioned above) were obtained for different concentrations of the analytes (155 s for 5×10^{-7} mol/L, 150 s for 1×10^{-6} mol/L and 136 s for 5×10^{-6} mol/L). Then, in order to apply the same stacking time for mixtures with different concentrations of analytes, the average of the inflexion point times obtained for mixtures of concentrations ranging from 5×10^{-7} to 5×10^{-6} mol/L, was taken (147 s). This time corresponded to approximately 75% of the current intensity of the buffer solution. The electropherogram corresponding to the injection of a mixture of the three anabolic steroids using this value as stacking time enabled to observe that when this selected stacking time was applied in a single stacking run, the analytes were not separated and partially overlapped with the EOF peak (results not shown). Subsequently, the application of higher stacking times corresponding to percentages of current intensity higher than 75% was attempted. In order to achieve that the previously selected time was multiplied by the factors of 1.1 (~80% of the current intensity of the buffer solution), 1.2 (~90%), 1.3 (~95%) and 1.4 (~100%). In all these cases, the separation of the three peaks corresponding to TST, MTS and MTD could be observed enabling to obtain the calibration plots for the studied analytes. Since a similar behaviour was observed for the three analytes, as an example, Fig. 4 shows the calibration plots for TST (the 1st peak in the electropherogram) when mixtures of the

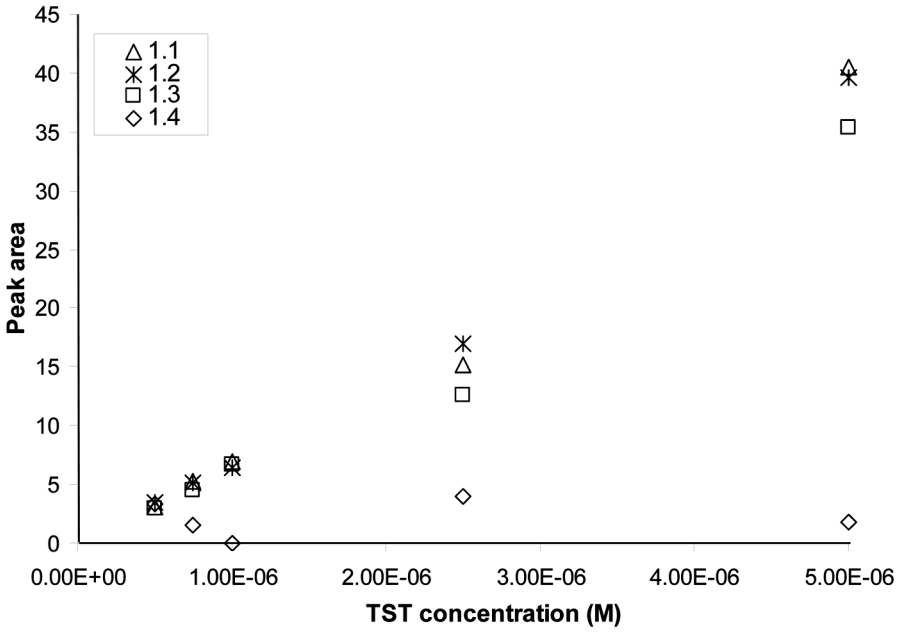


Figure 4. Calibration curves obtained plotting the peak area as a function of TST concentration (from 5×10^{-7} to 5×10^{-6} mol/L) for various factors (from 1.1 to 1.4) employed to multiply the average inflexion point of the current intensity curves. Voltage: 20 kV; injection: 50 mbar, 332 s and buffer injection during 5 s; temperature: 15°C; UV-absorption detection at 245 nm (±5 nm).

Table 2. LODs and LOQs expressed in mol/L, and SEF for LODs when the separation of the three anabolic steroids was performed without stacking and with single and double stacking

	TST			MTS			MTD		
	LOD	LOQ	SEF	LOD	LOQ	SEF	LOD	LOQ	SEF
Without stacking $n = 4$	4.79×10^{-5} ($\pm 1.12 \times 10^{-5}$)	1.60×10^{-4} ($\pm 0.37 \times 10^{-4}$)	–	5.17×10^{-5} ($\pm 1.51 \times 10^{-5}$)	1.72×10^{-4} ($\pm 0.50 \times 10^{-4}$)	–	6.17×10^{-5} ($\pm 1.94 \times 10^{-5}$)	2.06×10^{-4} ($\pm 0.65 \times 10^{-4}$)	–
Single stacking $n = 5$	5.02×10^{-7} ($\pm 4.84 \times 10^{-7}$)	1.67×10^{-6} ($\pm 1.62 \times 10^{-6}$)	95	3.47×10^{-7} ($\pm 0.81 \times 10^{-7}$)	1.16×10^{-6} ($\pm 0.27 \times 10^{-6}$)	149	4.42×10^{-7} ($\pm 2.60 \times 10^{-7}$)	1.47×10^{-6} ($\pm 0.87 \times 10^{-6}$)	139
Double stacking $n = 5$	3.47×10^{-7} ($\pm 1.53 \times 10^{-7}$)	1.16×10^{-6} ($\pm 0.51 \times 10^{-6}$)	138	2.79×10^{-7} ($\pm 1.64 \times 10^{-7}$)	9.30×10^{-7} ($\pm 5.46 \times 10^{-7}$)	185	3.56×10^{-7} ($\pm 1.56 \times 10^{-7}$)	1.19×10^{-6} ($\pm 0.52 \times 10^{-6}$)	173

three steroids were injected using, as stacking time, the above value (147 s) multiplied by the factors from 1.1 to 1.4 (stacking times ranged from 162 to 206 s). It can be clearly seen that the factors 1.1 ($r^2 = 0.984$), 1.2 ($r^2 = 0.995$) and 1.3 ($r^2 = 0.975$) showed good linearity and similar sensitivity (slope of the calibration lines), which means that there is no loss of the analytes when reverse polarity is applied. However, the use of the longest stacking time (206 s) caused losses of the analytes by the inlet position. Since the use of the shortest stacking time (162 s) produced broad peaks, caused by a too long stacked zone, a factor of 1.2 ($r = 0.997$) was chosen for further experiments because the resultant stacking time, 176 s, produced the highest sensitivity and we ensured that any part of the analyte zone is not escaping from the capillary.

Using the selected stacking time (176 s, *i.e.* the time to reach ~90% current intensity of the buffer solution) for online single stacking preconcentration, the LOD and LOQ were calculated from the calibration lines obtained plotting corrected peak areas (area/migration time) *versus* concentration (five values ranging from 5×10^{-7} to 5×10^{-6} mol/L). The correction of the peak areas was made to compensate the variations of migration time for different concentrations of the analytes [37]. Table 2 shows the sensitivity enhancement factors (SEFs) obtained for the three analytes studied under these conditions. The lowest SEF was obtained for the first peak (TST, SEF 95) whereas the second and the third peaks exhibited higher SEF values (149 for MTS and 139 for MTD), which were higher than the theoretical signal amplification value calculated from Eq. (6), which is 111. However, the obtained SEF values are close to 170-fold analyte concentration achieved by Chun and Chung [38] when filling the whole capillary with the sample (only the effective length of the capillary was filled with the sample in this work).

3.3 Online preconcentration by multistep stacking

The theoretical LODs estimated for TST, MTS and MTD, when using multistep stacking preconcentration, are shown in Fig. 5. These values were obtained from the LODs of these compounds without preconcentration (see Table 2) and the expected amplification factor (from Eq. (6), 332/3 = 111). It can be observed that there is a signifi-

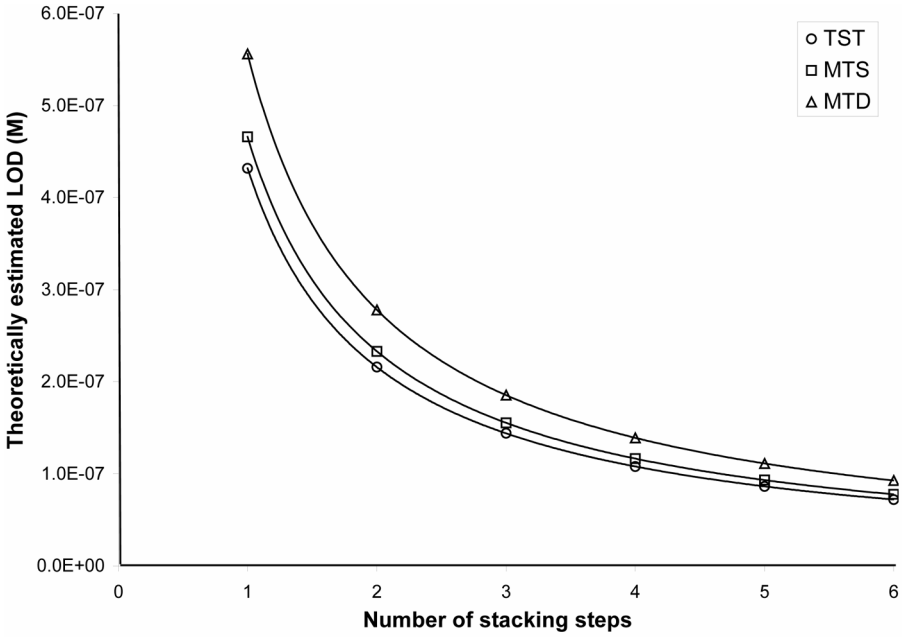


Figure 5. Theoretically estimated LOD with multistep stacking. LOD for the analysis without stacking corresponds to the value: 4.79×10^{-5} mol/L. Amplification of the signal: 111 times (332/3 s).

cant decrease in the theoretical LODs for single and double stacking, while triple and more exhaustive stacking steps seem to be less effective for improving the sensitivity.

Table 1 also shows the steps followed to achieve double and triple stacking preconcentrations. Again, the stacking time for each stacking step was the crucial parameter to be calculated for online multistep stacking preconcentration. The stacking times for the second and third stacking steps were calculated by multiplying the respective inflexion point *x*-values, obtained from current intensity curves, by the factor of 1.2 (optimised for the first stacking step). Thus, successive stacking times of 176, 209 and 211 s were calculated for single, double and triple stacking, respectively. Although the stacking time increased with the number of stacking steps, it seemed to stabilize with the number of stacking steps.

LOD and LOQ for the three anabolic steroids when using online double stacking preconcentration (see Table 2) were also calculated using corrected peak areas. In this case, SEF values obtained (138, 185 and 173 for TST, MTS and MTD, respectively) were lower than the expected theoretical amplification for the double stacking, which is 222. This result could be due to the errors associated with the integration of tailing peaks and the loss of resolution which became considerable when using double stacking preconcentration (see Fig. 6). The comparison of the theoretically estimated LOD for TST (Fig. 5) with the corresponding experimental value (Table 2), confirmed this discrepancy which can be justified considering that Eq. (5) does not take into account the number of theoretical plates (*N*) that usually decreases with the number of stacking steps. In fact, *N* for the last migrating compound (MTD), which corresponds to the peak more affected by broadening effects, was 11 000, 11 500 and 7000 for the single, double and triple stacking respectively.

Quantification of the triple stacking was not possible because of its poor repeatability, especially for the lowest concentrations. Peak symmetry and resolution decreased in this case, as it can be observed in Fig. 6, causing huge errors during integration of peaks. In addition, online preconcentration by triple stacking seems to be very susceptible to changes in the experimental conditions. Temperature variations, especially in the unthermostatised part of the capillary, influence the viscosity of buffer and sample affecting the analyte recovery. This makes the triple stacking preconcentration a doubtful way to improve sensitivity of the analysis. In addition, the analysis with multistep preconcentration is much longer, almost 1 h in the case of the triple stacking.

4 Concluding remarks

The separation of the three structurally related steroids, methyltestosterone, methandrostenolone and testosterone, has been performed for the first time by EKC using a dual CD system based on an anionic CD and a neutral one (*i.e.*, Suc-β-CD + β-CD) as dispersed phases. Due to the low absorption of UV light of these anabolic steroids, they were used as model compounds to study the possibilities of online multistep stacking preconcentration with large-volume injection in order to improve their sensitivity using UV-absorption detection. It has been demonstrated

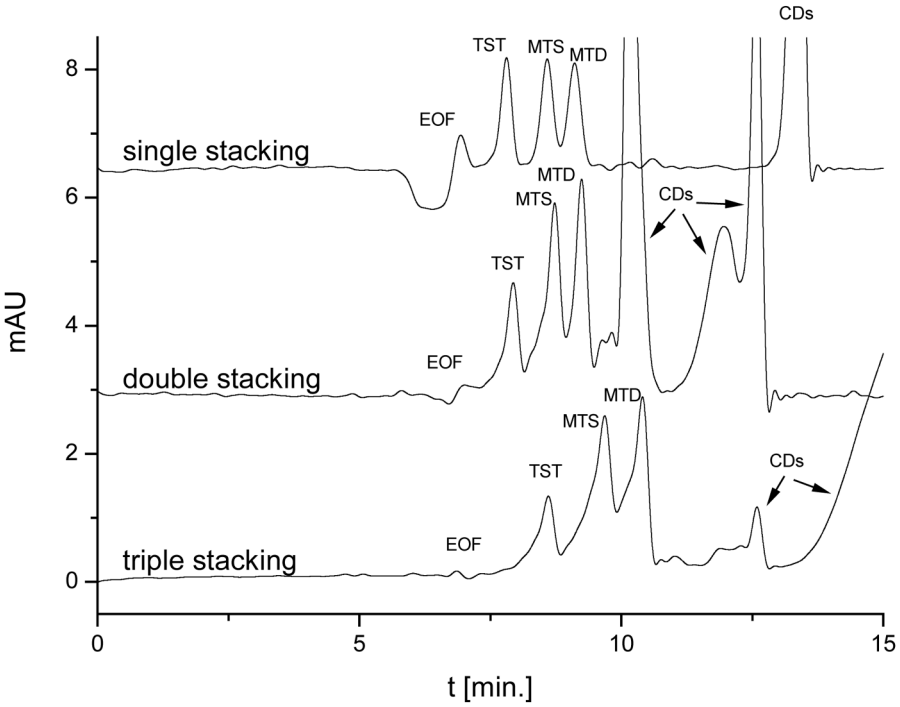


Figure 6. Comparison of the electropherograms obtained by single, double and triple stacking for a mixture of MTS, MTD and TST (2.5×10^{-6} mol/L of each one in 1% v/v in methanol:water). Experimental conditions as in Fig. 4.

that single and double stacking allow SEFs from 100 to 150 for single stacking and from 140 to 190 for double stacking. In spite of this, triple stacking was not repeatable due to multiplication of errors associated with little changes of experimental conditions.

The authors thank the Ministerio de Ciencia y Tecnología (Spain) for project BQU2003-03638. Dr. C. García-Ruiz thanks to the same institution for her contract from the Ramón y Cajal Program (RYC-2003-001). P. L. Urban thanks the European Union for his Erasmus fellowship.

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