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Review Article

Recent Developments in Capillary Electrophoresis of Steroids and Sterols

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Abstract

Steroid hormones are intensively studied from environmental water systems. In environmental samples their concentrations are very low, but their quantities are not meaningless since steroids belong to the group of endocrine disruptors, causing cancer and developmental disorders in humans. On the contrary, only a few recent articles deal with the research of plant steroid alcohols (sterols) in biological and environmental water systems. The reason may be that sterols are not categorized as high-risk contaminants, although their chemical structures are similar with the human steroids. Mostly, steroids are analyzed with chromatography and mass spectrometry. In addition, capillary electrophoresis has shown to be a promising separation technique in metabolic studies because of the possibility to obtain simultaneous differentiation of glucosides and the corresponding native steroids. That is not easily processed in chromatography.

This review highlights the determination of human and plant steroids detected in biological samples and environmental water systems with capillary electrophoresis.

Keywords: capillary electrophoresis, steroid, sterol, sample concentration, biological fluids, water

Introduction

Human steroids and plant steroids (sterols) are observed to be global pollutants in the aquatic environment [1,2]. Steroids belong to endocrine disruption compounds (ECDs) and their existence in the aquatic organisms has been reported from many communal regions all over the world. They have often been linked to different types of effluents. Lately, microbial water quality and concentrations of fecal sterols have been used to estimate the degree of sewage contamination in marine environment [3].

Many biological compounds and synthetic chemicals may have influence on hormonal receptors and hormone activity by changing remarkably the performance of receptors in multicellular organisms. Because EDCs are not classified by their chemical features but by their biological effects, different kinds of analysis methods have been developed to determine the compounds with wide chemical differences [4]. They have concentration limits that should not be exceeded in the purified water, and especially not in the final product of the process in manufacturing drinking water [2].

It has also been justified that steroids exist in both influent and effluent water of purification plants, but also in clean tap water samples of households, since the clean-up processes of drinking water are not specifically established for steroid removal. Because water is used for drinking and food manufacturing, detectability of steroids needs to be extremely sensitive and reliable. Determination of human based steroids or plant sterols need effective isolation of the analytes from biological and environmental samples but especially their extraction from wastewater. Unfortunately, the released biological steroid contaminants to environmental water systems issue also from wastewater treatment plants (WWTPs) [5,6].

Steroids

Structures: One major class of lipids is the steroids, which have structures very different from the other classes of lipids. They are polycyclic compounds that can be either naturally occurring or chemically processed. All steroidal compounds have the primary structure. They are arranged into groups that are gonane (C17), estrane (C18), androstane (C_{19}), pregnane (C_{21}), cholane (C_{24}), and cholestane (C_{27}) (Figure 1) [7]. The human steroids exist as free and conjugated compounds due to metabolism. Their determination has importance in industrial processes using water, but especially also in diagnostics, doping control, forensics, and especially, in environmental monitoring [8], since water is used for food industry and for drinking water production. Steroids are bioactive at low concentrations, which increase challenges to measure their individual quantities.

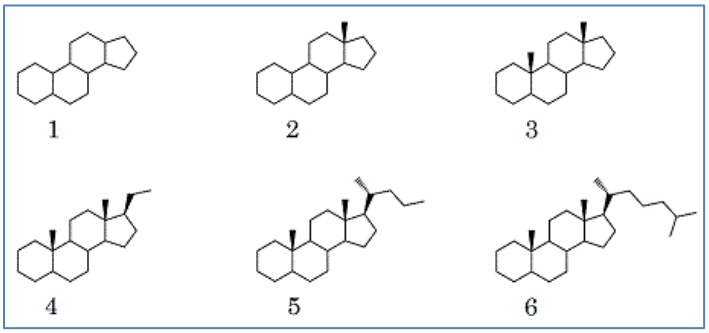

Figure 1: Steroid structures: **(1)** gonane, **(2)** estrane, **(3)** anrostane, **(4)** pregnane, **(5)** cholane and **(6)** cholestane

Synthesis: Steroids resemble terpenoids from the biosynthetical point of view. The process starts from triterpene lanosterol that originates from cationic cyclization of the acyclic hydrocarbon squalene [9]. In animal and fungi cells, steroids are formed from lanosterol, and in plants from cycloartenol [10]. They can also be synthetized in the natural process of steroidogenesis from the source compound cholesterol. In synthetic way, the microbial catabolism process produces steroids from phytosterols. The chemical products contain C19 or C22 carbon structures, which are modified to the steroids (Figure 2).

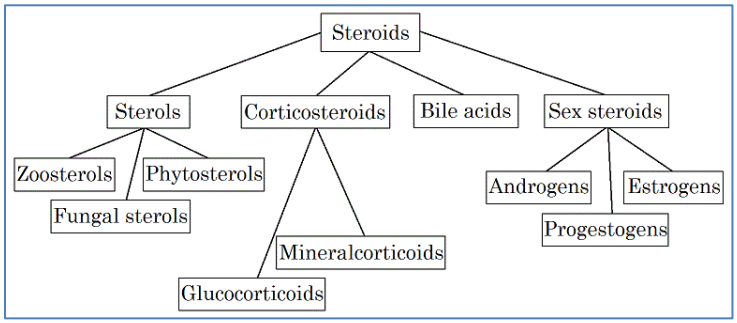

Figure 2: Sub-groups of steroids

All steroid hormones, adrenocortical hormones and sex hormones are synthetized from cholesterol. Adrenocortical hormones regulate many metabolic processes, whereas sex hormones are responsible for maturation, reproduction, and tissue growth [11]. Sex steroids are androgens, estrogens, progestogens, and glucocorticoids that effect glucose metabolism. Glucocorticoids aid the catabolic reactions of fats and proteins, when the body is in stress for synthesis of glucose [11]. Hydrocortisone is one of the most essential glucocorticoid and responsible for nutrient catabolism by preventing allergic reactions [12].

Sterols

Structures: Over 200 naturally occurring sterols have already been identified [13]. From the research point of view, there are already 13 sterol-related Nobel prizes in the years 1910-1985 [14]. Many phytosterols have important health and nutrition effects, making them industrially significant [15]. Sterols or "steroid alcohols" are divided into zoosterols (animals), phytosterols (plants), and mycosterols (yeast and fungi). Phytosterols and mycosterols are both plant sterols (Figure 3) [12]. They have similar structures as sterane.

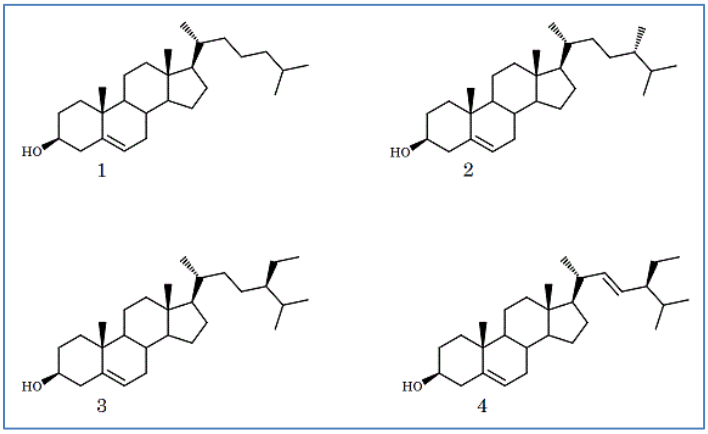

Figure 3: Representatives of sterols stuctures: (1) cholesterol (zoosterol), (2) campesterol (phytosterol), (3) sitosterol (phytosterol), and (4) stigmasterol (mycosterol)

According to literature, sterols are precursors for steroidal saponins and alkaloids [14] in insects for molting hormones and in humans for pregnane and androstane [14,15]. Although stanols are closely related to sterols, they are not as common in the nature as sterols. In hydrogenation, sterols form saturated sterols. Therefore, sterols may both exist as unsaturated and saturated forms [16].

Occurrence: Many sterols act as precursors to vitamins. They can be found in oils, vegetable fats, and plant cells. Approximately 45-95% [13] of the total amount of sterols in plants consist only of sitosterol. Other common phytosterols are campesterol and stigmasterol [16]. Ergosterol, which is the main fungal sterol, has also found in corn, peanut, cotton seed, and linseed oils [17], which are used in food industry. Besides plants, phytosterols are collected from pulp and paper industries as byproducts. Anyhow, they can be migrated to environmental with the outflow waters containing organic solvents. In addition, some degradation of sterols occurs since high process temperatures are used [18]. Sterols have also been studied from olive oil [19], walnut [20], and from several plants [10] because of their health benefits to humans.

Synthesis: In organisms, sterols are initially synthesized from acetic acid (Figure 4). In mammals and fungi, the sterol is lanosterol, whereas in plants cycloartenol and triterpenes are the products. The final compound in mammals is cholesterol and in fungi ergosterol. However, in plants different phytosterols, such as sitosterol and

campesterol are processed [21]. Interestingly, plant cells are capable of producing phytosterols not only from cycloartenol and triterpenes but also from lanosterol [22]. Since sterols can also be processed from steroids and not only from acetic acid, the bioprocess uses microbial hydroxylation of steroids that enables a large scale of different steroids to be produced from a few steroids [23]. The synthesis is possible in oxygen-depended biosynthesis by acetyl-coenzyme A via HMG-CoA reductase pathway [24,25]. The first-grade product is mevalonate before further synthesis with farnesyl diphosphate molecules to form squalene. The synthesis route goes on forming cycloartenol and triterpenes (e.g. phytosterols and brassinosteroids) via enzymatic reactions [15]. The elimation of sterols to environmental water goes through metabolism to sulphates, glucosides, esters, and alkyl ethers. That synthesis is needed essentially for plant growth, reproduction, and response to various abiotic and biotic stresses [12,25].

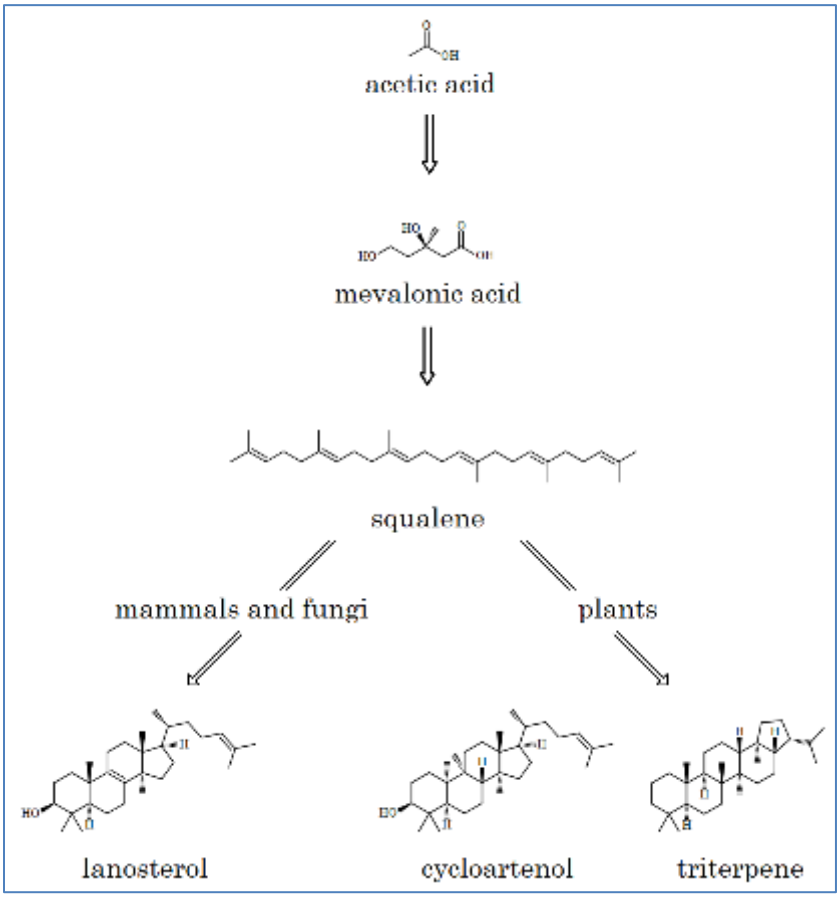

Figure 4: The sterol synthesis in organisms. The initial compound is acetic acid. In the reaction steps, first it forms mevalonic acid, then squalene. The reaction is then divided into two paths depending on the organism. In mammals and fungi, lanosterol is produced following with the formation of cholesterol (mammals) or ergosterol (fungi). In plants either cycloartenol or triterpene is formed, following with the formation of the final product, for instance sitosterol

Hydrophobicity of steroids and sterols

In environmental monitoring purposes, the solubility of steroids is not favorable since they are hydrophobic. To extract them to analysis from water samples need attention. It is discussed below in Ch. 2. Figure 5 shows the relation between octanol/water-distribution coefficient (logP) and applied double bond equivalent (aDBE) of common steroids and sterols. The aDBE is closely related to logP, since the higher the aDBE, the higher the logP. The

model does not predict the behavior of sterols in water environment, even though there is a correlation between the factors.

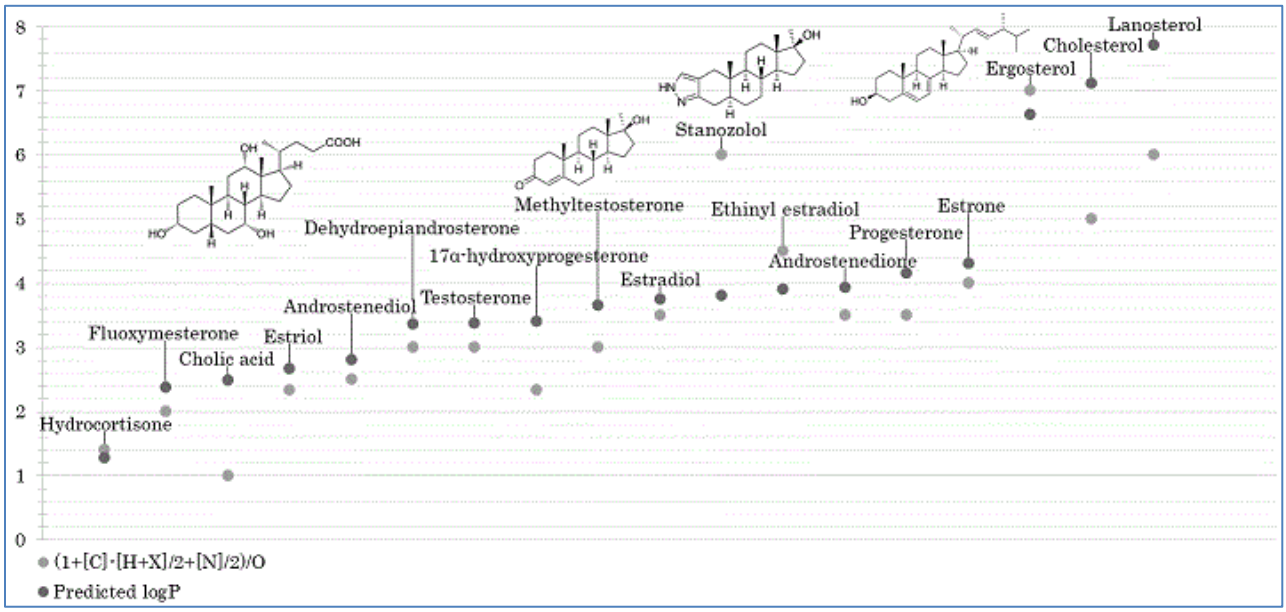

Figure 5: The characteristic behaviour of some steroids and sterols. The octanol/water-distribution coefficient (logP value) and the applied double bond equivalent (aDBE) of selected steroid and sterol compounds are compared. The steroids are hydrocortisone, fluoxymesterone, cholic acid, estriol, androstenediol, dehydroepiandrosterone, testosterone, 17α-hydroxyprogesterone, methyltestosterone, estradiol, stanozolol, ethinyl estradiol, androstenedione, progesterone, estrone, ergosterol, cholesterol, and lanosterol. The aDBE is calculated with the number of atoms in the molecule (C) being carbon, (H) hydrogen, (X) halogen, (N) nitrogen, and (O) oxygen. The equation for aDBE is (1+(C)-(H+X)/2+(N)/2)/(O). The logP values are theoretical values provided by ChemAxon [26,27]

Methods

Water pretreatment

Contamination of water systems cannot be thoroughly purified by processes at traditional communal WWTPs. In the effluent water the amount of micro contamination is ng/L - μg/L level. Extraction of contaminants from water is not necessarily comparable with decrease of biological activity, since the contaminated metabolites and side-products may further be bioactive. It has been observed that purification of wastewater decreases solid material that has been produced by suspension. In addition, purification decreases chemical and biochemical oxygen consumption and activity of the stress factors [1,2,5,28].

Generally, methods that make possible a fast, safety, and easy sample pre-treatment and which enable the use of small volumes of samples and solvents, are significantly needed. Usually, all compounds are studied with separation techniques and therefore, aqueous samples are used. Then, the studies with small sample volumes need sensitive detectors to identify the steroid compounds sufficiently.

Solid phase extraction (SPE) is the most general extraction and concentration method of steroids that exist in small concentrations in the environments water. Generally, the system sorbent is silicon-based polymer containing long hydrocarbon chains. Much favour is obtained by using N-vinyl pyrrolidonedivinylbenzene base materials, since it adsorbs intensively both polar and nonpolar compounds. Usually then, estrogens, androgens, and progesterones are

extracted simultaneously. Determination limits for estrogens containing phenolic functional groups like in estrone, 17α-estradiol, and 17β-estradiol and in17α-ethinylestradiol, were 20-200 pg/L. In gas chromatography, determination of steroids need pentafluorobenzoyl esterification reaction for enhancement of their evaporation. [29] One comparison study showed that steroids in German drinking water were 0.33 ng/L for endogenic steroids that was a little higher result than the previously mentioned. The quantities of steroids E2 and E1 were quite high, being 0.11 and 1.5 ng/L in river water.

Endeavour to decrease the consumption of organic solvents has led to development of new SPE methods. One of these new techniques for steroid isolation is stir bar sorptive extraction (SBSE) and solid phase microextraction (SPME). In both techniques along with the sample solution, no other solvents are needed, unlike in SPE. Isolation in both cases is based on the features of steroids and extraction material interactions. In SBSE technique steroid hormones estriol, estradiol, ethynylestradiol, estrone, progesterone, medroxyprogesterone, levonorgestrel, and norethindrone can be extracted onto polydimethyl coated siloxane-phenyltrimethylsiloxane-β-cyclodextrin sol gel; even using 50 times the same stir bar [30].

It is important in water analytics that the samples are analysed here and now when they are sampled. In our study, we researched the matter and observed that the water samples need to be immediately pretreated in order to be reliable. The reason is that estrogens concentrations in water decrease during storing in a freezer. Traditionally, liquid chromatography and gas chromatography with a selective detector such as a mass spectrometer (MS) has been applied. Routinely, already separation techniques with on-line coupled mass spectrometry (tandem-MS) are used for target and non-targeted analyses of steroids [6,31,32].

Capillary electrophoresis techniques in steroid analytics

Capillary electrophoresis (CE) is an electro aided miniaturized separation technique that is used for determination of both ionic and neutral compounds having molar masses from small (~18 Da) to macro size (~17 MDa) even simultaneously in one analysis. Recently, a paper on analytical techniques for steroid estrogens focused on their quantification in water samples [33]. The paper concluded that the complexity of water samples and low estrogen concentrations need highly sensitive instrumental analytical techniques (GC-MS, LC-MS) but also non-instrumental analytical techniques ((enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA)) for quantification. Surprisingly, based on the published data the non-instrumental technique ELISA appeared to be the most reliable. Usually various reference systems are needed, and at present there is a demand of sensitive and accurate separation methods for determining human based androgens, estrogens, progestogens, sterols and corticosteroids in aqueous and non-aqueous samples. Thus, in this review, capillary electrophoresis (CE) techniques are introduced with applications for steroids and sterols research. It is an interesting miniaturized censoring technique, since CE has evidenced to perform long-term on-line monitoring of industrial processes in pulp and paper industry but also a robot in detecting flotation chemicals in hydrometallurgical processes [34].

The mostly used CE techniques are capillary zone electrophoresis and electrokinetic chromatography. The methods are discussed below.

Capillary zone electrophoresis: Capillary zone electrophoresis (CZE) is suitable for determination of steroids, since electric field of aqueous and non-aqueous electrolyte solutions separates charged steroid glucosides. However, mostly CE is used to perform pharmaceutical, food and environmental analyses. To handle the water research of steroids, various sub-techniques of CE are used. The choice of the techniques is based on the studied compounds and their chemistry, like compounds structures, ionization, size, and solubility.

CE has not been used as commonly in steroid analyses as HPLC and GC. The advantage of capillary electrophoresis over the chromatographic methods is that the samples can be examined without sample preparation. Exception is the situation when the studied compounds are at very low concentrations. Then SPE concentration is needed, as in the other separation techniques, too.

The separations are made in silica capillaries (internal diameter 50 μm and total length 60-80 cm). The degree of ionization of silanol is modified by both acidity and alkalinity (pH) of the filling solution. Then, the potential difference between electrical Stern layer near the silica inner surface, and the bulk of the capillary solution, called zeta-potential determines the velocity of the electroosmotic flow (EOF) [35]. Zeta-potential is heavily affected by pH and ionic strength that in turn it has an influence on the separation of steroid group compounds. Furthermore, many instrumental parameters are available for modifying the electrical separation for selective steroid and sterol CE analytics. Liu et al. reviewed the manufacturing processes of drinking water and identified the metabolite of testosterone (testosterone-glucoside) among with androstenedione and progesterone [36]. Recently, the testosterone and estradiol glucoside metabolites were determined from WWTPs water samples in Finland [1,2,37].

Electrokinetic chromatography: Electrokinetic chromatography (EKC) is the most used technique for separation of nonionic compounds. It is based on electrophoresis and interactions between the studied compounds and the additives in the filling solution of the capillary [38]. EKC utilizes pseudo-stationary phase (PSP) as the separation medium, which is achieved by additives (micelles or microemulsion) in the filling solution called a background electrolyte solution (BGE) [39]. Micellar electrokinetic chromatography (MEKC) is based on the addition of micelle-forming surfactants into the BGE solution. Micelles are needed for dissolving purposes and separation of difficultly ionizable steroids, but they have also a wide biological compatibility [40]. The advantage of using nonionic micelles is that they have great biodegradability and low toxicity [40,41].

Lately, steroids were determined with partial-filling micellar electrokinetic chromatography (PF-MEKC) (Figure 6) that combines CZE with MEKC. The sandwich-type solution system is arranged in CE capillary by using two solutions: First, the capillary is filled with the BGE solution and then, a small plug of micellar solution is placed before the injection zone (the sample) [42]. The studied compounds will interact with the micelles and then they move into the BGE solution. In MEKC, both neutral and ionic compounds can be separated. PF-MEKC has proven to separate simultaneously neutral steroids and steroid glucoside conjugates. It is suitable for determination of androgens, estrogens, and progestogens in environmental water, and in industry processed waters, like wastewater and tap water [2,31,38,42].

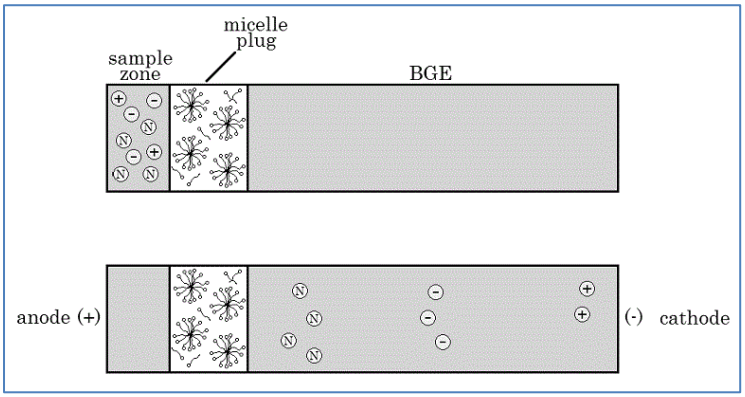

Figure 6: The working principle of PF-MEKC

Microemulsion electrokinetic chromatography (MEEKC) is another EKC separation technique that utilizes microemulsion (ME) instead of micelles in steroids separation [43]. The structure of microemulsion droplets is unique, since the nonpolar solvent droplets are coated with surfactants [44]. In addition, by attaching short-carbon chain alcohol to the droplet, the surface tension is decreased, and the system is stabilized [45]. Commonly, the medium in MEEKC separation is made of sodium dodecyl sulphate (SDS) and non-ionic surfactant or mixture of surfactants, and bile salts [45] into a BGE solution that is made of either phosphate or borate salts or their acids [44]. Thus, the analysis is not depending on the water-solubility or non-solubility of the compounds [44,45].

MEEKC method was applied for separation of corticosteroids and steroids (hydrocortisone, androstenedione, dehydroepiandrosterone, androsterone, 17-α-hydroxyprogesterone, testosterone, 17-α-methyl testosterone, pregnelone, and progesterone) using rapeseed, walnut, olive, and linseed plant oils in bio microemulsions (bioME) [46]. More recently, the approach was to couple MEEKC to mass spectrometry (MS). Intrinsically, MEEKC concentrates the samples on-line and when MS is coupled, the detectability of the steroids is considerably still improved due to the accurate and specific mass-per-charge ratios used in detection [43].

Since the steroids are hydrophobic, the separation is done in non-aqueous electrolyte solutions with addition of organic modifiers or cyclodextrins (CDs). Increase in velocities of the lipophilic steroids correlates with decrease in hydrophobic interactions between a steroid and micelles [41]. The use of β-cyclodextrin in EKC is reported to be useful in increasing selectivity between similar derivatized phytosterols [47]. Mixed-mode CDs can also enable high sensitivity in UV detection of campesterol (CAM), whose chemical structure is similar to the structure of cholesterol. The CDs assist even the separation of CAM enantiomers in aqueous EKC systems (Figure 7). Enantiomer separation was obtained in 30 mM Tris-borate electrolyte (pH 8.2) containing mixture designed solutions of γ-HS-CD and β-CD.

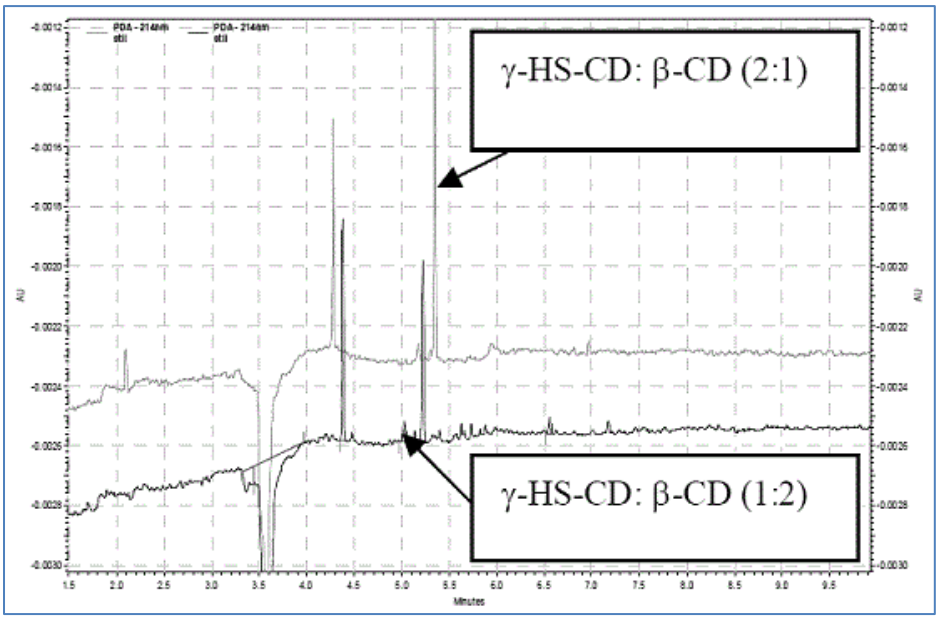

Figure 7: Enantiomer separation of campesterol (CAM) with EKC. Cyclodextrins (γ-HS-CD and β-CD additives) in 30 mM Tris-borate electrolyte solution (pH 8.2). Sample concentration: 10 mg/mL; Separation voltage +30 kV; Detection wavelength UV-214 nm; Sample introduction with pressure 0.5 p.s.i for 2 s; Analysis temperature 22 oC; Silica capillary (50 μm i.d. and 60 cm Ltot)

On-line Concentration of the Studied Compounds

Concentration in capillary

Many CE analyses are made using UV-detection, because steroids and sterols have absorptivity. Specific absorption is used for steroid carbonyl groups at 280 nm [48,49], compounds with aromatic structure at 184 nm and at 203 nm, androgens at 247 nm [2,38], and estrogens at 200 nm [2,38].

When the native absorption gives not sensitive enough for analyses, in CE analysis there are numerous on-line concentration techniques available to enhance the detectability of the studied compounds and to speed up their movement in capillary. On-line concentration procedures are very important, when the CE instrument is coupled with an industrial process for automatic sampling [34]. The most used techniques in steroid and sterol on-line concentration are sweeping [50], micelle to solvent stacking (MSS) [51], solvent and pH stacking [52], and field-amplified stacking [53] in MEKC (Figure 8). Staking affects compound velocity can be optimized by modifying composition or ionic strength of both sample and electrolyte solution. In sample stacking, the studied compounds have higher velocity in the sample solution than in the BGE, and thus they concentrate spontaneously at the boundary of the two solutions. In sweeping, the situation is contrary. The sample compounds have slower velocity in the sample than in the BGE, which contains the surfactants. When a sample is introduced into the capillary, the BGE behind and front of the sample concentrates the sample, only when the conductivities of sample and BGE are equal (and pH $\ll$ 7) and only when the system does not contain any compelling forces [51]. Sweeping is the most effective technique for hydrophobic and cationic compounds [52] like steroids, sterols, and corticosteroids. The advantage of sweeping is its effectiveness in concentrating anionic compounds (enhancement up to 5000) [54,55].

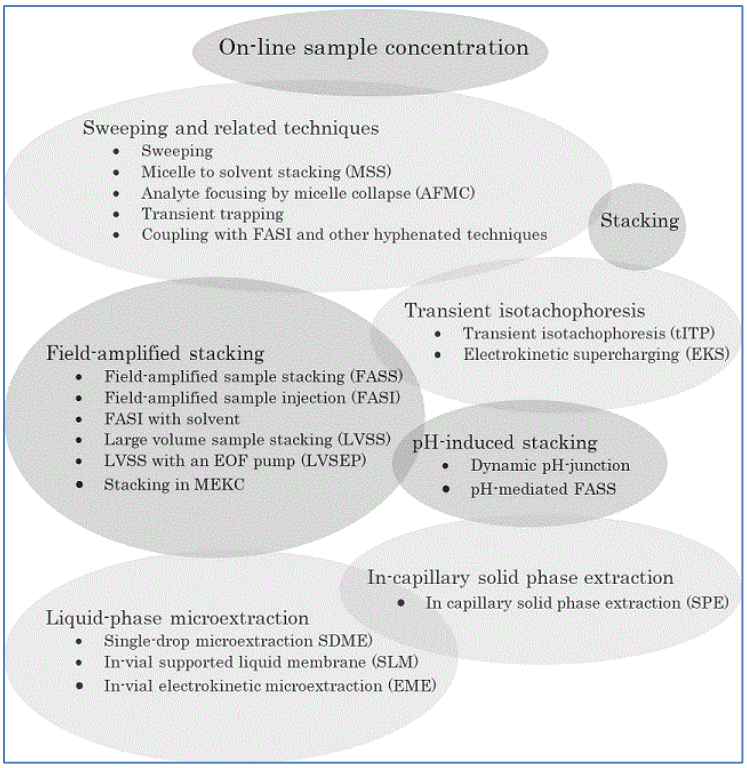

Figure 8: On-line sample concentration techniques in capillary electrophoresis

According to literature, the use of MSS-MEKC method instead of the normal MEKC can improve the detection sensitivity of the studied compounds by 113-123-fold [55,56]. Another method, namely field-amplified stacking (FAS) can also be performed using MEKC. Especially, it is useful for compounds that are not ionizable at the pH of the BGE [54]. Thus, the sample compounds are dissolved in a micellar buffer solution (c>CMC) followed by colliding to 10 times lower SDS concentration zone than it is in the BGE [54]. Therefore, in MEKC stacking, the PSP in the sample compounds have higher velocity than they have in the BGE. The PSP and the co-migrating sample compounds are then concentrated at the border of the two solutions. An example of stacking application, MEKC solution is itself the pH-mediated stacking zone for separation of α,β-dihydroxyprogesterone, ethynylestradiol, 17β-estradiol, estrone, hydroxyprogesterone, 11-ketotestosterone, progesterone, and testosterone after extracted from whole blood or plasma of fish species [57]. Furthermore, it possible to detect those steroids by using stacking at 0.2-2 ng/mL level in zebrafish, which were used as target organisms to monitor environmental water contamination. Then, they all, except progesterone and its hydroxyl metabolite were separated in five-minutes [58]. That fast method was also suitable for measuring steroids mixture in large number samples since quick knowledge on their toxicity was needed in screening. Another example of a fast analysis is the FASS-MEKC, which successfully allowed separation of cortisone, cortisol, prednisolone, corticosterone, testosterone, 17α-methyltestosterone, epitestosterone, and progesterone within 12 minutes in a MEKC electrolyte made of sodium tetraborate, boric acid, SDS, and methanol. Detection of the steroids was improved by dispersive liquid-liquid microextraction (DLLME) to decrease the limit of detection (LOD) and the limit of quantification (LOQ) to 15 ng/mL and 50 ng/mL, respectively. Those values were higher than obtained with pH-stacking, and anyhow the method fulfilled optimization criteria in biomedical applications [59].

Off-line sample pretreatment

In environmental samples, steroids and sterols occur at very low concentrations (pg/L-ng/L) [1,2,57,58]. Therefore, when on-line stacking methods cannot be used, off-line sample pretreatment is needed to achieve suitable concentration for each compound in detection. The most common way is to use solid phase extraction (SPE) prior to CE analysis. Using SPE, the studied compounds are isolated from a liquid sample matrix. In the process, they are also concentrated, since the original sample volume is usually much larger than the eluate volume that is collected and evaporated. The precipitate produced is dissolved into smaller solvent volume than the initial sample was. In steroid and sterol analyses, the most typical SPE columns contain nonpolar C18 –functionalized sorbent or other hydrophobic materials (Table 1). Usually for CZE and EKC analyses, one SPE step is requirement.

Lately, neutral and anionic glucoside conjugated steroids were studied from water samples of WWTPs [27,30,37]. Then, for determination of both neutral and anionic hormones, two-step SPE pretreatment was performed. Water samples were extracted with hydrophobic materials to retain neutral steroids and to remove most of the hydrophilic compounds. Next, the eluates, which were collected in the first step, were extracted with ion-exchanger sorbent to retain the conjugated steroids. The overall purpose was to change aqueous phase into organic phase for easy sample handling, to concentrate the steroids, and to isolate the main matrix compounds. The system concentrated the samples by 20000-fold (Figure 9). Thus, the concentrations of testosterone-glucoside, androstenedione, progesterone, and estradiol-glucoside could be determined in the WWTP process samples. The steroids in influent and effluent waters were 0-429 ng/L and 0-207 ng/L, respectively. The PF-MEKC separation was completed within 20 minutes, resulting in simultaneous identification of steroid glucosides and the parent steroids [2,37].

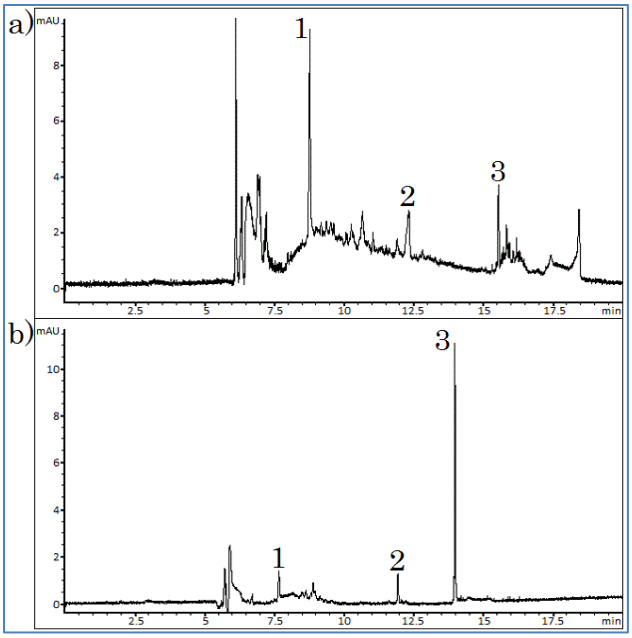

Figure 9: PF-MEKC separation of wastewater samples from a) an influent sample without SPE treatment and b) influent water pretreated with SPE (C18 sorbent). Compounds 1 testosterone-glucoside, 2 androstenedione, and 3 progesterone (unpublished results). Experimental conditions: The total length of the capillary 80 cm and the effective length 71.5 cm. Electric field: voltage (+25 kV) and current (17 ‧A): Specific UV detection at 247 (± 2) nm. Electrolyte: 20 mM ammonium acetate. Micelle: 440 µL of 100 mM SDS in 20 mM AA, and 50 µL of 100 mM sodium taurocholate

Official International Organization for Standardization (ISO) methods ISO 12228-1:2014 [60] and ISO 12228-2:2014 [61] for sterol analyses contain guidelines of sample preparation procedures for saponification, extraction, TLC, and derivatization prior to analysis. The methods are laborious [62]. In addition, derivatization has a major role in the standard guidelines, but for instance, in the analysis of conjugated sterols, then the analysis is not quantitative. Moreover, since usually both the native sterol and its conjugates are analyzed as one specific sterol (which can be quantified only with its commercial chemicals), their concentrations are not accurate [12].

To quantify the total concentration of a specific steroid, enzymatic hydrolysis procedure is used to transform the conjugated to native compounds. Then the system was possible to couple on-line with CE [34]. Examples of hydrolysis methods are enzymatic hydrolysis with β-glucuronidase and sulfatase [63]. Specific enzymes are added into the samples and the hydrolysis reaction is carried out at the temperatures from 35°C to 60°C [63,64]. However, in the concurrent analysis of free and conjugated sterols, the efficiency of hydrolysis depended on the amounts of the corresponding conjugated sterols [12].

Liquid-liquid extraction (LLE) used in lipid extraction, isolate sterols also excellently. Nonpolar solvents performing as the extraction phase, work well for quantitative isolation of sterols and sterol esters from aqueous phases. For instance, hexane is commonly used solvent for plant sterols that were extracted from vegetable oils [22]. Another quantitative extraction method is supercritical fluid extraction (SFE), in which CO_2 or methanol modified CO2 extracts sterols in an environmentally friendly way [14]. SFE is used in pulp and paper industries for removing phytosterols from pulp material, which is then used for food industry [14,19]. The extracted phytosterols were obtained as byproducts from the processes in pulp and paper industry. The sources were taken from tall oil, which contains 3-7 % (w/w) esterified phytosterols and 18 % (w/w) deodorizer distillates [19].

Table 1: Selected applications for details on analytes, sample matrices, pretreatment and analysis methods of steroids

Analyte	Sample	Pretreatment procedure	Analysis method	Reference
androstenedione, testosterone, epitestosterone, boldenone, clostebol	urine	LLE with *n*-hexane	MEKC-UV	[65]
20β-hydroxyprogesterone, estrone, testosterone, estradiol, ethinyl estradiol, progesterone, 20α-hydroxyprogesterone	fish plasma	SPE with C18 sorbent	MEKC-UV	[66]
testosterone, methyltestosterone, epitestosterone, nandrolone, gestrinone, dihydrogestrinone, tetrahydrogestrinone	urine	SPE with C18 sorbent	MEKC-UV	[67]
androstenedione, estriol, dehydroepiandrosterone sulfate, testosterone, dehydroepiandrosterone, estrone, progesterone, and estradiol	urine	hydrolysis, SPE with Sep-Pak C18	MEKC-UV	[64]
aldosterone, cortisone acetate, dexamethasone, hydrocortisone, hydrocortisone acetate, prednisolone, prednisolone acetate, prednisone, triamcinolone, triamcinolone acetonide	urine	dilution of the spiked sample	MEKC-UV	[68]
17α-hydroxyprogesterone, androstenedione, fluoxymesterone, progesterone, methyltestosterone, testosterone glucuronide, testosterone	wastewater	SPE with C18 (Strata-X) and quaternary amine (N+) sorbents, LLE with diethyl ether	PF-MEKC-UV	[2,37]
testosterone	male urine	enzymatic hydrolysis	PF-MEKC	[54]
progesterone	rat testicular tumor cells (R2C)	washing with phosphate buffer, silylation	CE-Laser-induced fluorescence	[54]

Case Studies

Determination of steroids

Capillary electrophoresis techniques showed to work excellently for comprehensive profiling of androgens, estrogens, and progesterone steroids after their SPE enrichment from environmental water samples. Usually, the water was 500 mL for SPE, but in case of low steroid amounts in influent and effluent water and their low UV sensitivity, extracted sample volume was 1 L or more [37]. Estrogenic hormones were detected to be 6 ng/L concentration in surface waters. They were detected in the sources of drinking water and in pre-treatment plants but not usually in tap water [32]. For analysis, the separation conditions for anabolic androgenic steroids (androstenedione, metandienone, fluoxymesterone, methyltestosterone, 17-epimetandienone, and testosterone) were introduced in EKC [64]. The micellar solution was mixed from SDS and sodium taurocholate. The electrolyte was made of ammonium acetate adjusted to alkaline pH. Separation and identification of the steroids was made with MEKC coupled with ion trap electrospray ionization - mass spectrometer (ESI-MS). Separation was achieved in less than 14 minutes (Figure 10) with MS-detection at the range of mass-to-charge ratio (m/z) 50 and 800 Da.

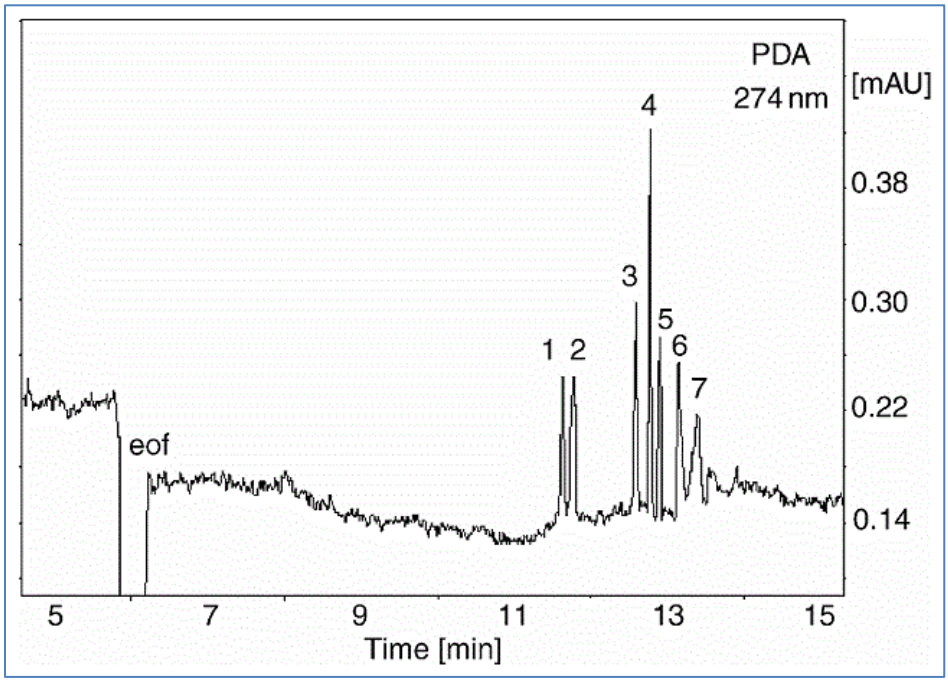

Figure 10: PF-MEKC separation of anabolic androgenic steroids at concentrations of 1-4 µg/mL. The analytes are (1) fluoxymesterone, (2) androstenedione, (3) metandienone, (4) testosterone, (5) methyltestosterone, (6) 17-epimetandienone, and (7) taurocholate. 20 mM ammonium acetate (pH 9.5) was used as BGE and the micelle consisted of 29.3 mM SDS, 1.1 mM sodium taurocholate, and 6.7 % methanol, prepared into the respective BGE. The micelle was injected at 34 mbar pressure for 99.9 s and the sample at 34 mbar for 5 s, respectively. Fused-silica capillary of 80 cm (effective length of 70 cm) was used in 22°C temperature and with separation voltage of +25 kV. Detection at 247 nm [64]

Testosterone, progesterone, estrone, 17-β-estradiol, and ethynilestradiol were also successfully separated without the need for adding organic solvents or other co-surfactants in MEKC. The method limits of concentrations in UV detection were 1.27, 2.17, 0.6, 1.13, and 1.7 µg/mL for the steroids, respectively [62]. The values show that the real samples could not be analysed without sample preparation and steroid concentration. Lately, a combination of large

volume injection and sweeping in MEKC (rLVSI-sweeping MEKC) were developed to identify androgenic steroids [66]. Then, the method was suitable for testosterone (T), epitestosterone (ET), and epitesteosterone-glucoside (ET-gluc) in biological samples at LODs of 1.0 ng/mL, 5.0 ng/mL, and 200.0 pg/mL, respectively.

However, sometimes cyclodextrins (CDs) were also needed to modify the aqueous phase, but they increased the detection limits It was recognized from the results in modifying selectivity of a steroid separation in phosphate electrolyte (pH 5.5). Because of CDs absorption, the calibration ranges were 5-200 ng/mL, 20-200 ng/mL, and 0.5-500 ng/mL for T, ET, and ET-gluc, respectively, which are more than 3 times higher than without CD [69].

In MEKC separation, cetyltrimethyl ammonium bromide (CTAB) was used instead of SDS as the cationic surfactant [63]. It was good for determination of hydrophobic steroid hormones, androgens, estrogens, progestens, and glucocorticoids. The optimized method enabled determination of the steroids cortisone, hydrocortisone, estriol, testosterone, estrone, progesterone, and estradiol. Their quantitative limits in detection with UV were 1.2-1.7 μM using a BGE made of Tris-borate solution containing both CTAB and 2-propanol. In another MEKC study, five natural and synthetic anabolic steroids, androstenedione, 19-norandrostenedione, 1,4-androstadiene-3,17-dione, methandrostenolone, and methyltestosterone were separated. The steroids were detected in an alkaline BGE solution (pH 9.0) containing 15 mM Britton-Robinson buffer, 50 mM sodium cholate, and 0.1% (v/v) Triton X-100 [64]. The detection limits were 1000-1000000 times higher than the known values in biological (μg/L) and environmental (ng/L - pg/L) samples, since they were in the range of 0.20 to 0.51 μg/mL.

Earlier, the water samples of wastewater pretreatment plants were documented to contain steroids from low 10 ng/L to nearly 1200 ng/L [69]. Recent experiments refer those, but especially the concentrations of estrogenic steroids in wastewater were very low and needed comprehensive studies [62]. Therefore, instead of PF-MEKC, CZE methods were used to reduce the UV absorption of micelles. Then, estradiol-glucoside (E2-gluc) amounts were 11.3 ng/L and 22.7 ng/L in two influent water samples [2]. Other detected steroids, testosterone-glucoside (T-gluc), androstenedione, and progesterone were at 77.5-120 ng/L, 247.7-284 ng/L, and from zero to 128.3 ng/L concentrations in the influent water. In effluent water the concentrations were little lower, but not extinct being 8.3-43.5 ng/L, 53.5-171 ng/L, and 0-4.8 ng/L, respectively. The results were in correlation with the published data in the literature [69].

Corticosteroids were used as the target compounds when the comparison of method performance between MEKC and MEEKC were studied [67]. The separated steroids were aldosterone, cortisone acetate, dexamethasone, hydrocortisone, hydrocortisone acetate, prednisolone, prednisolone acetate, prednisone, triamcinolone, and triamcinolone acetonide. It was found that microemulsion (ME) made of SDS and diethyl-L-tartrate (solution pH 7.0) was an excellent separation medium. Then MEEKC was superior to MEKC when using the respective surfactants. The conclusion was based on the result; a better differentiation was achieved with MEEKC than with MEKC [54].

The separation was excellent for androstenedione, testosterone (T), epitestosterone (ET), boldenone, and clostebol. The research was carried out with a specific sweeping technique that included full-capillary injection of the sample. Usually, the human samples are purified with liquid-liquid extraction (LLE) prior to CE analysis. On-line stacking methods have provided 108-175-fold improvement in sensitivity of steroids [70]. The calibration concentrations of the steroids were 0.05-1.00 μg/mL. However, before analysis the sample needs hydrolysis and concentration. LLE is commonly used for isolation of steroids. Especially, it has been used for nandrolone, epitestosterone (ET), testosterone (T), gestrinone, methyltestosterone, dihydrogestrinone, and tetrahydrogestrinone was successfully studied with MEKC using tetraborate complexing in taurocholate micellar mixture [71]. In the corresponding study, also nonpolar sorbents were used in SPE pretreatment for blanks. In comparison, blank urine

and LLE pretreated spiked urine were monitored at the UV-254 and UV-340 nm. Then the limit of detection was too high, since the lowest value was 1 μg/mL, which was 1000 times higher than needed with MEKC.

Steroid hormones in fish plasma have been studied using also SDS micelles in MEKC [65]. Before the analysis, ion-exchange extraction was used in SPE to remove fatty acids from the sample. Then, the eluted solution was extracted with a nonpolar sorbent and the hydrophobic steroids were eluted with ethyl acetate. The results showed that SPE was effective enough to concentrate analytes and to remove matrix before quantifying estradiol, testosterone (T), and 20β-hydroxyprogesterone. The conclusion was that the MEKC method was sensitive enough for identification of even more compounds than the mentioned, since also ethinyl estradiol, progesterone, and estrane could simultaneously be separated. The spiked samples, as also in this case, have concentrations at μg/mL or higher, which are not suitable for analyzing real urine samples with the method [66]. The concentrations need to be at least 0.005 μg/mL [72]. The problem solved similarly to environmental samples by increasing the sample volume.

The future of MEKC separation methods is continuing. Lately a new separation technique was revealed [63] and the research focuses on polymeric-mixed micelle made of cholic acid, sodium dodecyl sulphate, and polymer poloxamine Tetronic® 1107. Then, hydrocortisone, androstenedione, estriol, dehydroepiandrosterone sulphate, testosterone, dehydroepiandrosterone, estrone, progesterone, and estradiol were separated and quantified at 5-45 ng/mL level. To obtain this low concentration, time-taking multi-step sample pre-treatment was documented: hydrolysis for conjugated steroids to transform them into free steroids and SPE with nonpolar sorbents [73].

In MEKC the sensitivity is not high enough due to the absorbing micelle. Furthermore, an in-line modified concentration step is needed to enhance the detectability. An example about the success to use the methodology is in the research done with progesterone, 11-deoxycortisol, 17α-hydroxyprogesterone, deoxycorticosterone, corticosterone, 11-dehydro-corticosterone, cortisone, and hydrocortisone (Table 2) [54]. Then steroids were determined in phosphoric acid modified with SDS and urea at pH 2.5. It was found that on-line stacking improved the limit of steroid detection by 15-35-fold, whereas the sweeping technique increased it even by 100-600-fold. The limits of detection were reached 2.5-3 μg/mL with UV detection. Another example gives information on a high-sensitivity stacking-MEKC method that was invented for trace amounts of non-ionic compounds [58]. That has even higher effect on sample component sensitivities and the 1000-3000-fold enrichment in concentrations was achieved. What is more, the method does not limit the sample or its volume. Stacking has used in the quantification of steroids from biological samples [74]. It was reported in sample stacking; 5 to 50 mM in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) electrolyte solution could be used in detection of steroids at the range of 0.0002-0.002 μg/mL (0.8-6 nM) [75].

Sweeping technique in MEKC to detect the migration order of progesterone, 17α-hydroxyprogesterone, 11-deoxycortisol, corticosterone, cortisone, and hydrocortisone was used in buffers containing various kinds of surfactants [76] and also the anionic SDS and cationic tetradecyl trimethylammonium bromide (TDMAB). With SDS, the LOD was 1.0-1.9 μg/mL, but in TDMABe the values which were more than twice higher than in SDS (2.0-5.0 μg/mL). When sweeping was applied in MEKC, the LOD concentrations were lowered from μg/mL to ng/mL levels.

Mass spectrometry detection is an option for UV. It is commonly used because high-resolution 2D images are needed for steroids detection. However, the MS sensitivity is not always better than the UV detection sensitivity. Anyhow, to obtain accurate identification of unknown compounds in the sample, mass spectrometry with electrospray ionization (ESI-MS) is usually the option when coupled with CE. Since during many years, CE-MS modification has been used also been used for many years in sterol analysis. Similar nano-ESI-MS system was used for the oxime

forms of progesterone, pregnenolone, and dehydroepiandrosterone [77]. In CE-MS method, the combination was sensitive, since the LOD concentrations were 0.0025 μg/mL, 0.0050 μg/mL, and 0.0250 μg/mL, respectively.

Table 2: Examples of conditions in CE analyses of corticosteroids

CE mode	Steroid	Electrolyte	Separation voltage [kV]/ Field strength [V/cm]	Temperature [°C]	Detection	Reference
CZE	hydrocortisone, cortisone, 11-dehydrocorticosterone, corticosterone, testosterone, deoxycorticosterone, 11-deoxycortisol, prednisolone, cortisone acetate, fludrocortisone, dexamethasone	6.25 mM sodium tetraborate (pH 9.3), 1.0-6.0 mM of sulfo-β-cyclodextrin	+20	ambient temperature	UV: 254 nm	[54]
MEKC	progesterone, cortisone 11-deoxycortisol, 17α-hydroxyprogesterone, deoxycorticosterone, corticosterone, 11-dehydrocorticosterone, hydrocortisone	25 mM phosphate buffer (pH 2.5), 15 mM SDS, and 7 mM urea Stacking: 5 mM β-cyclodextrin	-25	ambient temperature	UV: 254 nm	[54]
PF-MEKC	17α-hydroxyprogesterone, androstenedione, fluoxymesterone, progesterone, testosterone, methyltestosterone, testosterone glucuronide,	20 mM ammonium acetate (pH 9.68) Pseudo-stationary phase: 1,000 μL BGE, 440 μL 100 mM SDS, and 50 μL 100 mM sodium taurocholate	+25	20	UV: 247 nm	[2,37]
MEEKC	cortisone acetate, dexamethasone, hydrocortisone, hydrocortisone acetate, prednisolone, prednisone, prednisolone acetate,	40 mM phosphate buffer (pH 7.0)–ACN–SDS–1-butanol–diethyl *L*-tartrate	+10	ambient temperature	UV: 254 nm	[68]

Sterols and cholesterols

The MEKC techniques of sterol and cholesterol analyses contained similar components for micelle phase and BGE. Phytosterols studied have been cholesteryl heptanoate, cholesteryl oleate, cholesteryl decylate, cholesteryl nonanoate, cholesteryl acetate, cholesterol, cholesteryl palmitate, cholesteryl dodecanoate, cholesteryl linoleate, cholesteryl butyrate, cholesteryl pentanoate, cholesteryl hexanoate, and cholesteryl octanoate. Since sterols are hydrophobic, they have poor aqueous solubility in their initial form. The C2-C14 sterols can be separated with traditional CZE, but larger sterols require MEKC or high organic nonaqueous solvent environment. Typical organic mobile phases include methanol, acetonitrile, dimethylformamide, and tetrahydrofuran. The use of organic or partly organic electrolytes results in quantitative determination of structurally similar compounds. However, in MEKC, organic solvents reduce micelle formation and diminish micelle size [78] and therefore the BGE cannot totally be

organic. Another disadvantage is the extreme hydrophobicity of sterols. For instance, the analysis of cholesterol and its ester derivatives is extremely challenging with MEKC, because sterols are very hydrophobic. Steroids may absorb into SDS micelles, why they may migrate inside the micelle phase. That prevents their detection. Moreover, reducing micelle size organic solvent may prevent detection of cholesterol ester [79].

Derivatization of sterols may also be a requisite option, when the sample volumes are low, and detection needs improvement. It helps with forming ionisable and sensitive derivatives of phytosterols for making them [79] cationic and detectable at 284 nm (pyridinium group).

Conclusions

The literature on free steroids and sterols show that their simultaneous analyses with corresponding conjugates is important. Sterols are not so frequently studied than steroids, although their structure resemblance with steroids inform that they need to be actively monitored for identification of EDCs. The individual steroid and sterol compounds are at very low concentrations (pg/L-ng/L) in environment matrices, although their amounts in biological matrices are even more than 1000 times higher. In addition, in their amounts in the emission sources of environmental areas are 1000-100000 times higher than the detected amounts.

Steroid enrichment with matrix isolation and compound degradation with hydrolysis are very commonly used. The differences based on sample preparation and detection makes it difficult to compare especially the steroids quantities released into environment.

There are not many recently published papers on the methods and applications with capillary electrophoresis. The common trend in steroids analytics is that the studies are made with chromatographic techniques coupled with mass spectrometers. Because the capillary electrophoresis techniques can separate free steroids from their glucoside and sulphate conjugates, it has shown potential to be more used in determination and identification of individual steroids and sterols in multicomponent mixtures.

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