6 Appendices

6.1 Publication I

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Non-Enzymatic Electrochemistry in Characterization and Analysis of Steroid Compounds

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ABSTRACT
This review summarizes achievements in electrochemistry-related research of steroid-based compounds in clinical, pharmaceutical, and environmental analysis. Special attention is paid to compounds possessing none or only isolated double bonds at the steroid core. Their direct redox activity is limited to far positive/negative potentials under variety of conditions and electrode materials and relies on the functional groups attached to the steroid skeleton, or as the case may be its double bond or moieties present at the side chain. The possibilities of electroanalytical methods in sterol characterization and analysis are demonstrated in a table with 31 references devoted to direct voltammetric and amperometric methods of oxidation of cholesterol, phytosterols, oxysterols, and related compounds at carbon-based and metal-based nanoparticles modified electrodes, and the reduction of bile acids and their conjugates at mercury-based electrodes, and modified glassy carbon electrodes. Furthermore, methods based on the indirect oxidation of cholesterol using bromine species as a mediator at platinum electrode and non-enzymatic cholesterol biosensors are reviewed. Their drawbacks and benefits are discussed with respect to the challenging task of identification and quantitation of these compounds in biological matrices, otherwise mostly performed using expensive mass spectrometric techniques preceded by a chromatographic separation step.

KEYWORDS
Cholesterol; electroanalysis; HPLC; sterols; steroid hormones; voltammetry

Introduction

Steroids are organic compounds possessing parent four fused rings: three six-member cyclohexane rings and one five member cyclopentane ring (for numbering see the cholesterol structure in Figure 1). Different functional groups (and their configuration) attached to this four-ring (cyclopentanophenanthrene) core, its oxidation state, and the presence of the prominent side chain at C_{17} determine their physiological function in fungi, plants, and animals. Frequently, they are components of cell membrane, influencing its fluidity (e.g., cholesterol), they act as signaling molecules (e.g., steroid hormones) or facilitate physiological functions such as digestion (e.g., bile acids) (Yeagle, 1985; Beato, 1989; Hofmann and Hagey, 2008). Besides naturally occurring steroids, synthetic products succeeded in medicinal applications as progestin in fertility regulation (Erkkola, 2007). On the other side, they are also misused for doping, e.g., anabolic androgenic steroids (Fragkaki et al., 2009).

As the steroid core itself is electrochemically inactive, the redox activity of steroid-based compounds is dependent on the attached functionalities or increased bond order within the core. In this review, we have concentrated on the achievements of electrochemistry-related research of cholesterol, oxysterols, phytosterols, and bile acids and their conjugates – mostly molecules with saturated bonds or with one double bond within the steroid core. Basically, these compounds are electrochemically active under specific conditions at specific electrode materials at far positive/negative potentials. The several examples of their non-enzymatic redox activity include the electrochemical reduction of bile acids at mercury-based electrodes and modified glassy carbon electrodes (GCE) in mixed aqueous-organic (Ferri et al., 1984; Liu et al., 2006; Yilmaz et al., 2015), and electrochemical oxidation of cholesterol, phytosterols, and related compounds at glassy carbon and boron-doped diamond (BDD) electrodes in non-aqueous medium (Hosokawa et al., 2009; Hojo et al., 2011; Kotani et al., 2011). Non-enzymatic cholesterol sensors, mostly based on metal-based nanoparticle (NP) (Li et al., 2010; Ji et al., 2014) modified electrodes or employing indirect self-assembled monolayers (SAMs) (Matsumoto et al., 2008; Aghaei et al., 2010), are also described. Beside these compounds, electrochemistry of selected steroid hormones is known (Santos et al., 2010; Levent et al., 2014; Zhu et al., 2015), as they contain an increased number of conjugated double bonds within their structure leading to electrochemical activity within the potential window of common electrode materials. Moreover, the enzyme-based approach of detection of steroid-based compounds represented by a vast number of studies dealing with the development of biosensors for cholesterol detection, employing cholesterol oxidase is increasingly used.

Sterols are a subgroup of steroids, most commonly described as compounds containing a hydroxyl group on the C_3 and an

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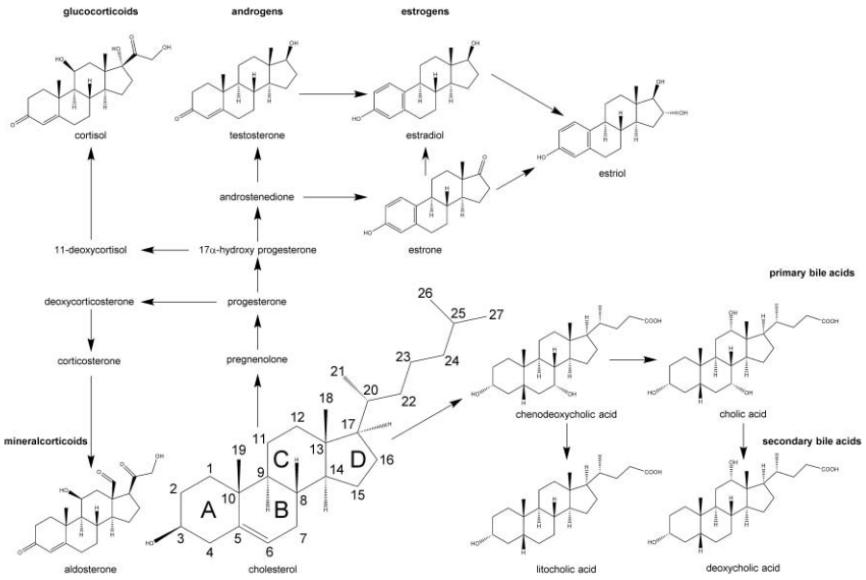

Figure 1. Simplified metabolic pathways of discussed cholesterol-derived compounds.

aliphatic side-chain of 8 to 10 carbon atoms at C_{17}. Cholesterol is one of the most important C_{27} sterols with a double bond at C_5 and an isooctane C_8 side chain (Figure 1) (Lubanda and Vecka, 2009). It is an essential component of cell membranes in eukaryotic cells and also a substrate for the biosynthesis of bile acids and important signal molecules – steroid hormones. In human body, it is either biosynthesized *de novo* by a series of enzymatic reactions using acetate as the primary precursor or it is absorbed from food. Biosynthesis of cholesterol is controlled by several regulatory proteins sensitive to cholesterol levels (Espenshade and Hughes, 2007). It is a direct regulation mechanism depending on cholesterol levels – higher intake leads to decrease in cholesterol biosynthesis and *vice versa*. Cholesterol, which is largely insoluble in aqueous media, travels through the blood circulation in form of lipoprotein complexes (Gill et al., 1985). Cholesterol bound in low-density lipoprotein (LDL) particles is at elevated levels (hypercholesterolemia) a high-risk atherosclerosis factor (Lusis, 2000). The high-density lipoprotein (HDL) particles on the other hand transport the excessive cholesterol from the peripheries to the liver and by that lower the atherosclerotic risk.

Steroid hormones control many vital physiological functions. There are two major classes of steroid hormones: adrenal steroid hormones (mineralocorticoids and glucocorticoids) and gonadal steroid hormones (androgens, estrogens, and progestogens). They are biosynthesized from cholesterol by a series of enzyme-controlled reactions, as reviewed in Miller (1988) and Payne and Hales (2004), and insinuated in Figure 1. Adrenal steroids and progestogens most commonly consist of 21 carbon atoms, androgens of 19 and estrogens of 18 (see Figure 1 for selected structures). Steroid hormones are important regulators of physiological processes. In blood, these lipophilic compounds are transported bound to specific carrier proteins. The physiological effects of steroid hormones are initiated when they enter target cells and bind to intracellular steroid receptors (Beato et al., 1996). The transcription of a large number of genes is influenced this way. However, it has been suggested that many important steroid-induced signaling events are triggered independently of transcription and are associated with the plasma membrane receptors (Hammes, 2003).

Bile acids are acidic sterols with saturated steroid nucleus and carboxylic group in the five-carbon (C_{24} bile acids) or eight-carbon (C_{27} bile acids) side chain at C_{17}. In mammals, C_{24} bile acids predominate. The formation of bile acids is considered as the most important way of cholesterol elimination (Björkhem, 1985). The side chain of cholesterol (C_{27} compound) is oxidized to a C_{24}-carboxylic acid during their synthesis. A simplified metabolic pathway for the formation of bile acids in humans is depicted in Figure 1, where primary and secondary bile acids can be distinguished. The principal primary bile acids possess hydroxyl groups at C_3, C_7, and C_{12}. Primary bile acids are synthesized from cholesterol in the hepatocytes and secreted in gut afterward. Major primary bile acids in humans are cholic and chenodeoxycholic (shown in Figure 1). After the biosynthesis from cholesterol the bile acids are conjugated by a specific linkage, in humans, predominantly with glycine, minority with taurine. For C_{24} bile acids, the conjugation with glycine converts a weak acid (pKa of ca 4.8–6.2.) to a slightly stronger acid (pKa of ca 4) (Hofmann and Hagey, 2008). The result is increased solubility and ionization at the pH conditions prevailing during digestion. Conjugated bile acids are fully ionized at the intestinal pH and are therefore impermeable to cell membranes. After the secretion in the gut, during the enterohepatic circulation, bile acids can be modified by microorganisms. These modifications involve oxidation, epimerization, or loss of the hydroxyl groups on the

steroid nucleus. Bile acids formed this way are called secondary. Disorders in bile acid synthesis and metabolism can lead to various diseases, as outlined in several reviews (Hofmann, 1999a, 1999b; Heubi et al., 2007; Van Veldhoven, 2010). Most of the physiological, pathophysiological, and metabolic properties of the bile acids can be attributed to their multifaced, amphipathic, and physical-chemical characteristics. The values of critical micellar concentration (CMC) are important for many processes (Roda et al., 1983; Hofmann and Roda, 1984; Carey, 1985). The value of CMC increases with the increasing number of hydroxyl groups in the steroid molecule, and conjugation with glycine or taurine results in a slightly lower values (Roda et al., 1983). For C_{24} acids, the CMC values range from 9 mmol L^{-1} (chenodeoxycholic acid) to 60 mmol L^{-1} (ursocholic acid) in water at 25°C. In the presence of 0.15 mol L^{-1} Na^+ ions (simulation of physiological conditions), slightly lower values are estimated (0.6 mmol L^{-1} to 39 mmol L^{-1}) (Hofmann and Roda, 1984). The arched skeleton of bile acids with about 10 chiral carbons and multiple hydrogen-bonding groups makes these compounds ideal for intermediate assemblies that relate to supramolecular properties and have a great deal of potential from the molecular and ionic recognition point of view for pharmacological and other applications (Tamminen and Kolehmainen, 2001; Miyata et al., 2007; Štěpánek et al., 2011).

Plant sterols, phytosterols, resemble cholesterol both in function (stabilization of phospholipid bilayers in cell membranes) and in structure (steroid nucleus, 3β-hydroxyl group, C_5 double bond) (Kritchevsky and Chen, 2005). Most phytosterol side chains at C_{17} contain 9 or 10 carbon atoms. The principal plant sterols are β-sitosterol, campesterol, and stigmasterol. Phytosterols have long been known to lower serum cholesterol concentrations by competing with dietary and biliary cholesterol for intestinal absorption (Pollak and Kritchevsky, 1981; Patel and Thompson, 2006). In contrast to these possibly beneficial effects of plant sterols, a rare genetic condition called sitosterolemia, an autosomal recessive disorder also known as phytosterolemia, is characterized by overabsorption of phytosterols and premature coronary artery and aortic valve disease (Patel and Thompson, 2006). Phytosterols have also recently been identified in atheromatous plaque obtained from individuals with apparently normal absorption of plant sterols raising the possibility that phytosterols are an atherosclerotic risk factor (Patel and Thompson, 2006).

Identification and quantitation of naturally occurring steroid-based compounds is a challenging task, because of their small structural differences, which occur in very complex mixtures and sometimes low target concentrations. The most common and relatively cheap separation and detection techniques have substantial drawbacks due to the structure of these compounds, e.g., only weak chromophores with absorption around 200 nm (for high-performance liquid chromatography (HPLC)-UV) or the necessity of derivatization or hydrolysis (for gas chromatography (GC)). The common techniques for the assay of these compounds in biological and physiological matrices not only include the mass spectrometric techniques, frequently preceded by chromatographic separation procedures, but also electrophoretic, enzymatic, and immunological methods. The most progressive method seems to be LC-MS; nevertheless, there is still a need for reduced cost and improvement in analytical ruggedness and reproducibility, as specified below.

The analysis of steroids using LC-MS, which is becoming the primary methodology for the analysis of steroids in biological samples, overcoming many of the limitations of immunoassays, was reviewed in Kushnir et al. (2011). Chromatographic methods (including thin-layer chromatography, solid-phase extraction, GC, HPLC, and supercritical fluid chromatography) in the analysis of cholesterol and related lipids were also reviewed in Hoving (1995). A modification of the Abell–Kendall method (Abell et al., 1952), which involves the liberation of cholesterol from the lipoprotein complex, saponification of cholesterol esters, extraction of cholesterol into petroleum ether, and measurement of cholesterol by means of the Liebermann–Burchard color reaction, is considered as a reference measurement procedure by the US Center for Disease Control and Prevention (Duncan et al., 1982). A definitive GC-IDMS (isotope dilution) method was developed by the US National Institute of Standards and Technology (Cohen et al., 1980). Accurate measurements of cholesterol, in the ranges found in total cholesterol as well as in LDL and HDL, are important. In clinical practice, cholesterol analysis is most commonly performed employing a sequence of enzymatic reactions (Warnick, 2000). Cholesterol in food can be determined by e.g., non-aqueous capillary electrophoresis (Xu et al., 2002), RP-HPLC with UV detection, preceded by an extraction step (Daneshfar et al., 2009), or GC-ion trap mass spectrometry (IT-MS) (Szterk and Pakuła, 2016). According to European Pharmacopoeia gas chromatography with flame ionization detector (GC-FID) using pregnenolone isobutyrate as the internal standard is the validated method for cholesterol assay (Council of Europe, European Directorate for the Quality of Medicines and Healthcare, 2004a). Mass spectrometry (MS) and particularly the combination GC–MS is an indispensable method for studying plant sterols (Rahier and Benveniste, 1989). The possibilities of bile acid analysis in biological matrices (e.g., bile, plasma, urine, and feces) were reviewed (Griffiths and Sjövall, 2010). GC and HPLC in combination with MS are commonly utilized for the quantification of individual bile acids. Nevertheless, high overhead costs and staff requirements limit their applications. Capillary liquid chromatography–mass spectrometry (LC-MS) with ES ionization provides the highest sensitivity, whereas the GC-MS is less sensitive but offers extensive structure-dependent fragmentation increasing the selectivity of determinations (Griffiths and Sjövall, 2010). Derivatization, e.g., with trifluoroacetate or trimethylsilyl, is required before GC analyses (Makin et al., 2010). Other methods for bile acid quantification employ HPLC with UV (Kakiyama et al., 2014) or fluorescent (Gatti et al., 1997) detection. It is also possible to employ the enzymatic reduction of NAD^+ (Hirano et al., 1987) or ELISA methods (Kobayashi et al., 1998). Titration by sodium hydroxide in ethanolic medium using phenolphthalein is the validated method for chenodeoxycholic (Council of Europe, European Directorate for the Quality of Medicines and Healthcare, 2004b) and ursodeoxycholic (Council of Europe, European Directorate for the Quality of Medicines and Healthcare, 2004c) acid assays in pharmaceutical preparations according to European Pharmacopoeia. This short overview of analytical methods adverts to

the necessity of development of alternative approaches to the detection of steroid-based compounds in various matrices opened by the drawbacks of the techniques currently used.

Electrochemistry of cholesterol, phytosterols, bile acids, and related compounds

Redox activity of steroid-based compounds without any double bond or with only isolated double bonds at steroid nucleus is rather limited. Even cholesterol had been regarded as an electrochemically inactive compound for a long time (Görög, 1983). The first studies on cholesterol electrochemical oxidation were inspired by the well-documented indirect oxidation approach (Shono et al., 1984) and have first appeared in the late eighties of the last century (Groves and Neumann, 1988; Li et al., 2003). Direct oxidation has been reported only in 2005 (Kowalski et al., 2005). In general, two approaches succeeded in steroid-based compounds electrochemical sensing. The first, very progressive one, includes the construction of enzyme-based biosensors, almost exclusively for cholesterol detection. The second one includes isolated studies on their indirect or direct oxidation and reduction at various bare and modified electrode materials and non-enzymatic biosensors.

The number of studies dealing with cholesterol biosensors development has been increasing rapidly since the turn of the millennium. A good biocompatibility of the supporting material is essential when developing enzyme-based biosensor. Today biosensors are in most cases based on electrodes modified with NPs (very often carbon nanotubes or metal-based NPs) and/or enzymes (cholesterol oxidase in most cases). A review has been published recently dealing with the use of nanomaterials in cholesterol biosensing, containing a comprehensive table with basic properties of 41 biosensors developed in the last two decades (Saxena and Das, 2016), some studies are also reviewed in Morzycki and Sobkowiak (2015). The principal mechanism of a typical cholesterol biosensor is summarized in the following text. The sensor is created by immobilizing cholesterol oxidase on gold NP-modified gold electrode (Saxena et al., 2011). It possesses a satisfactory limit of detection of 34.6 μmol L^{-1}. The immobilized cholesterol oxidase effectively catalyzes the oxidation of cholesterol, whereas the gold NPs serve as "electronic wires," which enhance the transfer of electrons generated from the enzyme catalytic redox reaction to the electrode surface. The fabricated bioelectrode was successfully used for the selective determination of cholesterol in human serum samples. In this case, sensor response stability was ~95% of the original response after 1 month.

A few enzyme-based biosensors for electrochemical determination of bile acids have been also reported, based on the detection of enzymatically produced NADH (Albery et al., 1993; Bartling et al., 2009) or H_2O_2 (Koide et al., 2007). A disposable iridium-modified carbon sensor prepared using thick-film screen-printing techniques and modified with 3α-hydroxysteroid dehydrogenase enables the detection of the enzymatically generated NADH using cyclic voltammetry and amperometry (Bartling et al., 2009). Its applicability was verified on the determination of three bile acids (cholic acid, taurocholic acid, and taurochenodeoxycholic acid) in bovine calf serum. A possibly clinically useful screen-printed disposable biosensor of taurocholic acid was successfully calibrated in human serum (Lawrance et al., 2015).

Even though the research in this area is enormous, the applicability of the majority of the enzyme-based sensors developed is often limited. Inadequate performance in terms of one or more analytical parameters including stability, sensitivity, and detection limit has been reported. Because of the reasons listed above, the non-enzymatic electrochemical detection methods are desirable. They often provide sufficient robustness, signal stability, and favorable analytical parameters mentioned above. Renovation of these properties in dependence on the condition of the electrode surface is substantially increased thanks to their simple, uncomplicated construction. The stability of bare electrode materials can be demonstrated for example on HPLC-ED method for the detection of cholesterol (Kotani et al., 2011). At least 4 month long stability of the detector is more than that for any of the sensors reported in the mentioned review on cholesterol biosensors (Saxena and Das, 2016).

Sterols redox activity on bare electrode materials is limited to far positive/negative potentials under variety of conditions, which naturally presents a substantial shortcoming. It relies on the functional groups attached to the steroid skeleton, and if present, those in close connection with the double bond, or groups present in the side chain attached on C_{17}. As these compounds represent a large group of great biological, physiological, and medicinal importance and the use of other analytical methods has substantial drawbacks as highlighted in Introduction, during the last two decades increased interest in the utilization of electrochemical methods in characterization and analysis can be traced. Naturally, this effort is driven by the development of new methods and electrode materials including their modification, and progress made in biosensing. All 31 important electroanalytical studies devoted to redox reactions of mentioned steroid-based compounds we have found since the first study published in 1981 (Kemula and Kutner, 1981) are summarized in Table 1. It is divided into four parts and summarizes methods based on the direct oxidation, direct reduction, indirect oxidation or adsorption/desorption processes, and non-enzymatic biosensors for cholesterol.

A number of studies are devoted to the possibilities of direct and indirect oxidation of cholesterol as a typical representative of sterols with an isolated C_5 double bond. This double bond is typically present also in many phytosterols (e.g., campesterol, stigmasterol, and β-sitosterol), and the studies on products of electrochemical oxidation of cholesterol confirm its importance for this process. The bile acids are considered even less electrochemically active because of the lack of a double bond in the steroid core.

Direct oxidation of cholesterol, oxysterols, phytosterols, and bile acids at bare electrodes

In general, direct electrooxidation of sterol-based structures is quite a challenging task. There are only few methods based on direct oxidation of cholesterol and related compounds using bare electrodes. For electroanalytical purposes, the most recent ones were published by Kusu et al. and employed GCE or BDD electrode and non-aqueous media with the prevalence of

Table 1. Selected electrochemical studies dealing with sterol-based compounds with none or only isolated double bonds at the steroid nucleus.

Analyte	Electrode/method (detection potential E_{det} vs. Ag/AgCl)	Conditions	Linear dynamic range	*LOD* (for *S/N* = 3 if not stated otherwise)	Comments (peak potential E_p vs. Ag/AgCl)	Reference
			Studies based on electroreduction			
Cholic a. Deoxycholic a. Ursodeoxycholic a. Chenodeoxycholic a. Lithocholic a.	DME/differential pulse polarography	$I = 0.4$ mol L^{-1} Acetate buffer pH 5.25	4–100 μmol L^{-1} 4–20 μmol L^{-1} 4–20 μmol L^{-1} 4–20 μmol L^{-1} [b] 4–20 μmol L^{-1}	—[a]	$E_p = -1.050$ V to -1.350 V Matrices: human bile, pharmaceutical products	(Ferri et al., 1984)
Cholic a.	HMDE/square wave stripping voltammetry	Britton – Robinson buffer pH 9.0 Accumulation potential: −600 mV Accumulation time 60 s	0.2–0.8 μmol L^{-1} 1–6 μmol L^{-1} 10–70 μmol L^{-1}	0.067 μmol L^{-1} 0.3 μmol L^{-1} 3.3 μmol L^{-1}	$E_p = -1.352$ V Matrices: cattle and sheep bile	(Yilmaz et al., 2015)
Deoxycholic a.	MWCNT-GCE/cyclic voltammetry	0.05 mol L^{-1} KH_2PO_4 water: methanol solution (1:1, v/v)	—[a]	—[a]	$E_p = +0.65$ V	(Liu et al., 2006)
			Studies based on direct electrooxidation			
Cholestanol	GCE/semimicro HPLC-ED $E_{det} = +2.8$ V	Mobile phase: acetonitrile + 10 mmol L^{-1} $LiClO_4$ Column: Develosil C30 UG-3 microbore column (250 mm × 1.0 mm i.d., 3 μm) Flow-rate: 100 μl min^{-1} Column temperature: 50°C	1–200 μmol L^{-1}	0.23 μmol L^{-1}	Matrices: human serum, mice serum Internal standard: 6-ketocholestanol	(Hojo et al., 2010)
Cholesterol	GCE/HPLC-ED $E_{det} = +1.9$ V	Mobile phase: acetonitrile:2-propanol (9:1, v/v) + 50 mmol l^{-1} $LiClO_4$ Column: Develosil C30 UG-3 microbore column (150 mm × 4.6 mm i.d., 3 μm) Flow-rate: 1000 μl min^{-1} Column temperature: 20°C	0.5–100 μmol L^{-1}	0.36 μmol L^{-1}	Matrix: human serum Internal standard: stigmasterol	(Hojo et al., 2007)
Cholesterol	BDD/HPLC-ED $E_{det} = +2.2$ V	Mobile phase: acetonitrile + 10 mmol l^{-1} $LiClO_4$ Column: Develosil C30 UG-120, (250 mm × 1.0 mm i.d., 5 μm) Flow-rate: 30 μl min^{-1} Column temperature: 40°C	0.02–100 μmol L^{-1}	[c] 0.008 μmol L^{-1}	Matrix: meat Internal standard: stigmasterol	(Kotani et al., 2011)
5α,6α-epoxy-chol 5β,6β-epoxy-chol 7-keto-chol 7α-OH-chol 7β-OH-chol 25-OH-chol 26-OH-chol	GCE/HPLC-ED $E_{det} = +2.8$ V	Mobile phase: acetonitrile + 50 mmol L^{-1} $LiClO_4$ Column: Develosil C30 UG-5 microbore (250 × 1.0 mm i.d., 5 μm) Flow-rate: 50 μl min^{-1} Column temperature: 18°C	2.5–50 μmol L^{-1}	0.57 μmol L^{-1} 0.73 μmol L^{-1} 0.26 μmol L^{-1} 0.27 μmol L^{-1} 0.29 μmol L^{-1} 0.21 μmol L^{-1} 0.16 μmol L^{-1}	Matrix: oxidatively modified LDL Internal standard: stigmasterol	(Matsunaga et al., 2009)
Lathosterol Cholesterol	GCE / HPLC-AD $E_{det} = +2.8$ V	Mobile phase: acetonitrile + 30 mmol L^{-1} $LiClO_4$ Column: YMC-Pack ODS-AL column (250 mm × 2.0 mm i.d., 5 μm) Flow-rate: 250 μl min^{-1} Column temperature: 50°C	0.15–300 μmol L^{-1} 15–600 μmol L^{-1}	0.3 μmol L^{-1} 0.3 μmol L^{-1}	Matrices: human serum, rat serum Internal standard: 6-ketocholestanol	(Hojo et al., 2011)
β-sitosterol Campasterol Stigmasterol	GCE / HPLC-AD $E_{det} = +2.8$ V	Mobile phase: acetonitrile + 50 mmol L^{-1} $LiClO_4$ Column: Develosil C30	10–200 μmol L^{-1}	3.4 μmol L^{-1} 2.01 μmol L^{-1} 1.06 μmol L^{-1}	Matrices: human serum, rat serum Internal standard: 6-ketocholestanol	(Ito et al., 2010)

Brassicasterol	UG-3 conventional column (150 × 4.6 mm i.d., 3 μm) Flow-rate: 1500 μl min^{-1} Column temperature 30°C	0.816 μmol L^{-1}				
Cholic a. Deoxycholic a. Chenodeoxycholic a. Glycocholic a. Glycodeoxycholic a. Glycochenodeoxycholic a. Taurocholic a. Taurodeoxycholic a. Taurochenodeoxycholic a. Ursodeoxycholic a. Lithocholic a. Glycolithocholic a. Taurolithocholic a.	Au / HPLC-ED pulse amperometric detection ($E_1 = 0.03$ V, $E_2 = 0.6$ V, $E_3 = -0.8$ V, with duration times of $t_1 = 1.6$ s, $t_2 = 0.3$, and $t_3 = 0.3$ s)	Mobile phase: water + acetonitrile + 0.5 mol L^{-1} NaOH gradient Column: reversed phase PLRP-S (150 × 4.6 mm i.d., 5 μm) Flow-rate: 1.300 mL min^{-1} Column temperature: 35°C	0.6–14 μmol L^{-1} for all	ca 0.3 μmol L^{-1} for all (if not stated otherwise) ca 0.6 μmol L^{-1} ca 0.6 μmol L^{-1} ca 0.6 μmol L^{-1} ca 0.6 μmol L^{-1}	Matrices: human duodenal bile, r at ileal fluid	(Dekker et al., 1991)
Cholic a. Deoxycholic a. Chenodeoxycholic a. Glycocholic a. Glycodeoxycholic a. Glycochenodeoxycholic a. Taurocholic a. Taurodeoxycholic a. Taurochenodeoxycholic a. Ursodeoxycholic a. Lithocholic a. Glycolithocholic a. Taurolithocholic a.	Au / HPLC-ED pulse amperometric detection ($E_1 = 0.05$ V, $E_2 = 0.6$ V, $E_3 = -0.6$ V, with duration times of $t_1 = 0.48$ s, $t_2 = 0.12$, and $t_3 = 0.06$ s)	Mobile phase: aqueous 0.9 mol L^{-1}: aqueous CH_3COONa: 0.1 mol L^{-1} NaOH + acetonitrile (15%, v/v) Column: anion exchange CarboPac PA-100 (250 mm × 4 mm i.d., 8.5 μm) Flow-rate: 0.8 mL min^{-1}	—[a]	Less than 10 μmol L^{-1} for all		(Chaplin, 1995)
Cholic a. Deoxycholic a. Ursodeoxycholic a. Chenodeoxycholic a. Lithocholic a.	Porous graphite / HPLC-ED in coulometric regime $E_{det} = +1.4$ V	Mobile phase: MeOH: acetonitrile: 0.07 mol L^{-1} aqueous CH_3COONa (55:20:25, v/v; pH 5.0) Column: Ultrasphere ODS (150 mm × 4.6 mm i.d., 5 μm) Flow-rate: 0.9 mL min^{-1}	[d] up to 20 μg	[d e] 0.20–0.25 μg	Assay: ursodeoxycholic acid and related impurities in capsule and tablet formulations	(Scalia et al., 1995)
		Studies based on indirect electrooxidation and adsorption/desorption processes				
Cholic a. Hyodeoxycholic a. Chenodeoxycholic a. Deoxycholic a. Lithocholic a.	DME / HPLC-ED (alternating voltage) polarography $E_{det} = -0.5$ V	Mobile phase: methanol:acetate buffer (7:3, pH = 4) + 0.1 mol L^{-1} $NaClO_4$ Column: LiChrosorb RP-18 (250 mm × 4 mm i.d., 10 μm) Flow-rate: 1 mL min^{-1}	250–10000 μmol L^{-1} —[a] —[a] —[a] —[a]	ca 200 μmol L^{-1} —[a] —[a] —[a] —[a]		(Kemula and Kutner, 1981)
Cholesterol	HMDE / ASV in LS mode	0.1 mol L^{-1} KCl	0.1–8 μmol L^{-1}	0.06 μmol L^{-1}	$E_p = -0.08$ V Assessment of determination in human serum	(Peng et al., 1992)
Cholesterol	Pt / AD $E_{det} = +1.8$ V	$NaClO_4$, KBr (both 10 mmol L^{-1}) in DMF	30–200 μmol L^{-1}	3.2 μmol L^{-1}	Indirect oxidation using KBr as a mediator	(Chiang et al., 2011)
Cholesterol	Pt / DPV		32–143 μmol L^{-1}	0.10 μmol L^{-1}	$E_p = +1.4$ V	

(*Continued on next page*)

		NBu_4PF_6, KBr (both 100 mmol L^{-1}) in acetonitrile			Indirect oxidation using KBr as a mediator	(Tsierkezos and Ritter, 2014)
		Non-enzymatic biosensors				
Cholesterol	Electroplated nanoporous Pt on silicon substrate / AD $E_{det} = +0.4$ V	PBS (pH 7.0) + Triton X-100, 2-propanol	10–8000 μmol L^{-1}	—[a]	Selectivity for ascorbic acid and acetaminophen	(Kim et al., 2009)
Cholesterol	PtNP on CNT/ITO electrode / AD $E_{det} = +0.7$ V	PBS (pH 7.0) + Triton X-100	5–10000 μmol L^{-1}	2.8 μmol L^{-1}	Selectivity for glucose, ascorbic acid, and Dopamine; stable for at least one months	(Yang et al., 2012)
Cholesterol	PtNP on macroporous Au / AD $E_{det} = +0.2$ V	PBS (pH 7.0) + Triton X-100, 2-propanol	Up to 5000 μmol L^{-1}	15 μmol L^{-1}	Selectivity for ascorbic acid, uric acid and acetaminophen	(Lee and Park, 2010)
Cholesterol	Porous tubular Ag NP modified GCE / AD $E_{det} = +0.35$ V (*vs.* SCE)	0.1 mol L^{-1} NaOH pH = 13	280–33000 μmol L^{-1}	180 μmol L^{-1}	Matrix: blood estimated cholesterol concentration in agreement with values obtained by HPLC	(Li et al., 2010)
Cholesterol	Cu_2S nanostructures on Cu rod / AD $E_{det} = +0.3$ V (*vs.* SCE)	Acetate buffer (pH 7.0) + Triton X-100, 2-propanol	10–6800 μmol L^{-1}	0.1 μmol L^{-1}	Selectivity for AA, L-cys, and DA,	(Ji et al., 2014)
Cholesterol	Hexadecyl mercaptan SAM on Au / indirect quantitation using $[Fe(CN)_6]^{4-}$ as a redox marker / CV	50 mmol L^{-1} $NaClO_4$	15–60 μmol L^{-1}	—[a]	Selectivity for phenol, and aromatic aminoacids. Weak response to cholic and deoxycholi acids	(Piletsky et al., 1999)
Cholesterol	Poly(2-macaptobenzimidazole) MIP on Au / indirect quantitation using $[Fe(CN)_6]^{4-}$ as a redox marker / DPV (*vs.* SCE)	Water:ethanol (1:1, v/v), 50 mmol L^{-1} $NaClO_4$	up to 20 μmol L^{-1}	0.7 μmol L^{-1}	Selectivity for ascorbic acid, phenol, and tryptophan Slight interference for vitamin D_2	(Gong et al., 2003)
Cholesterol	Hexadecyl mercaptan SAM on Au / indirect quantitation using $[Fe(CN)_6]^{4-}$ as a redox marker / CV	Water:ethanol (1:2, v/v), 33.3 mmol L^{-1} $NaClO_4$	66–700 μmol L^{-1}	—[a]		(Chou and Liu, 2005)
Cholesterol	Octadecyl mercaptan SAM on Au / indirect quantitation using $[Fe(CN)_6]^{4-}$ as a redox marker / CV	Skin cholesterol extraction from palm into EtOH solution for 60 s	—[a]	—[a]	Non- invasive skin cholesterol measurement. Coefficient of correlation of the current method and the invasive conventional HPLC method was 0.9408	(Matsumoto et al., 2008)
Cholesterol	2- mercaptobenzimidazole MIP on Au / indirect quantitation using $[Fe(CN)_6]^{4-}$ as a redox marker / capacitive measurements	0.1 mol L^{-1} $NaClO_4$	5–30 μmol L^{-1}	0.42 μmol L^{-1}	Selectivity for ascorbic acid, uric acid glucose Slight interference tryptophan, vitamin D_2, and phenol Matrix: human serum	(Aghaei et al., 2010)
Cholesterol	Octadecyl mercaptan SAM on Au / indirect quantitation using $[Fe(CN)_6]^{4-}$ as a redox marker/ CV	Ethyl acetate:ethanol (1:19, v/v)	—[a]	—[a]	Matrix: egg yolk Sensor: 1.4 mmol L^{-1} GC: 1.1 mmol L^{-1}	(Nagaoka et al., 2012)
Cholesterol	MIP-MWCNT on CCE / indirect quantitation using $[Fe(CN)_6]^{4-}$ as a redox marker / LSV	Water:ethanol (1:2, v/v), 0.1 mol L^{-1} KCl	10–300 nmol L^{-1}	1 nmol L^{-1}	Selectivity for cholic acid and deoxycholic acid	(Tong et al., 2013)
Cholesterol	p-Aminothiophenol MIP on AuNP-MWNT modified GCE / indirect quantitation using $[Fe(CN)_6]^{4-}$ as a redox marker / CV, DPV	0.1 mol L^{-1} KCl	0.1–1000 pmol L^{-1}	33 fmol L^{-1}	Selectivity for cholic acid, deoxycholic acid, ascorbic acid, uric acid	(Ji et al., 2015)

Cholesterol	β-CD modified graphene / indirect quantitation using methylene blue as a redox marker / DPV	PBS (pH 7.4) Cholesterol dissolved in EtOH	1–100 μmol L^{-1}	1 μmol L^{-1}	Negligible interference of common blood components, tryptophan being an exception Very little interference of common drugs	(Agnihotri et al., 2015)
Ursodeoxycholic a.	Self-assembled L-β-CD modified gold / indirect quantitation using ferrocene carboxylic acid as a redox marker / CE-ED	0.1 mol L^{-1} HAc-NaAc (pH 2.9) + 5%	—[a]	0.1 μmol L^{-1} for ursodeoxycholic acid		(He et al., 1997)
Deoxycholic a.	$E_{det} = +0.45$ V	CH_3OH Fused-silica capillary: (80 cm × 25 mm i.d.) Separation voltage: 20 kV Injection: electromigration 16 kV, 10 s				

[a]Not given. [b]Reducible only at pH $\geq$ 10. [c]*LOD* based on FUMI theory. [d]On column values. [e]*LOQ* values.

AA, ascorbic acid; ASV, anodic stripping voltammetry; BDD, boron doped diamond; CE, capillary electrophoresis; CNT, carbon nanotubes; CV, cyclic volammtery; DA, dopamine; DME, dropping mercury electrode; DPV, differential pulse voltammetry; ED, electrochemical detection; EtOH, ethanol; GCE, glassy carbon electrode; HMDE, hanging mercury drop electrode; HPLC, high performance liquid chromatography; ITO, indium tin oxide; L-cys, L-cysteine; LSV, linear sweep voltammetry; L-β-CD, lipoyl-β-cyclodextrin; MIP, molecularly imprinted polymer; MWCNT, multiwalled carbon nanotubes; NP, nanoparticle; PBS, phosphate-buffered saline; SAM, self-assembly monolayer; β-CD, β-cyclodextrin.

acetonitrile containing lithium perchlorate as a supporting electrolyte, as it offers sufficient solubility of sterols, and wider potential window compared to alcoholic media, especially at BDD electrode (Suryanarayanan and Noel, 2010; Benesova et al., 2014). Two studies employing chromatographic separation with pulsed amperometric detection (PAD) on gold electrode were published (Dekker et al., 1991; Chaplin, 1995). Also, an HPLC-ED method for assay of free bile acids in pharmaceutical preparations, using porous graphite as working electrode, was developed (Scalia et al., 1995). These studies, however, are mostly focused on the chromatographic aspect of the method, rather than the electrochemical process itself.

Both glassy carbon and BDD exhibit wide potential window in the anodic region in non-aqueous media and their surface can be easily mechanically renewed by mechanical polishing, although for BDD this type of surface renovation has been scaled up only in a few last years in connection with studies on the interaction of surface-active molecules with organic analytes (Yardim et al., 2011; Brycht et al., 2016; Zavazalova et al., 2016). Otherwise, anodic or cathodic activation at far positive/negative potentials in the region of water decomposition reactions is being used. Beside the variety in electrode surface activation, BDD is especially suitable material thanks to its excellent mechanical and electrochemical properties, such as a wide working potential window and enhanced signal-to-background ratios due to the low background current. A couple of reviews on properties, characterization, and utilization of BDD electrodes were published in last few years (Peckova et al., 2009; Einaga et al., 2014), including the possibility of miniaturization and biocompatibility for *in vitro* and *in vivo* applications (Peckova and Barek, 2011), and a practical guide to their applications in electrochemical research (Macpherson, 2015).

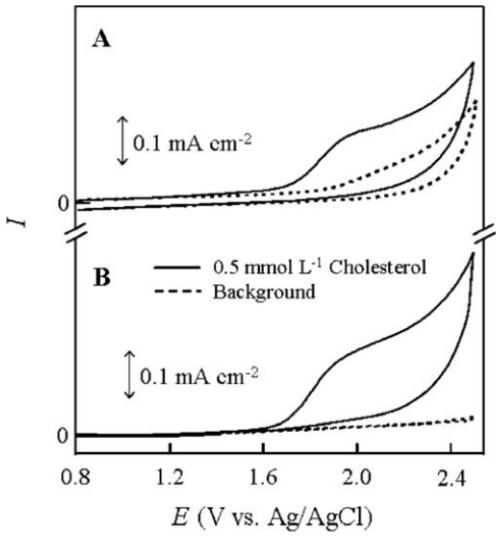

Figure 2. Cyclic voltammograms (0.02 V s^{-1}) of cholesterol (0.5 mmol L^{-1}) in acetonitrile containing 50 mmol L^{-1} $LiClO_4$ at the (A) glassy carbon and (B) BDD electrodes. Reprinted with permission from Kotani et al, 2011.

Comparison of cyclic voltammograms of cholesterol at GCE and BDD electrode based on the results of Kotani et al. (Figure 2) shows that the direct oxidation of cholesterol takes place at highly positive potential around +1.8 V vs. Ag/AgCl, it is irreversible and BDD outperforms glassy carbon (Kotani et al., 2011). The potential window is wider and the signal is slightly higher at the BDD, but the difference is not too big. The main difference lies in the background current values. The relatively low background current of the BDD is attributed to the predominant hydrogen termination of the surface as well as the compact non-porous surface. On the other hand, the GCE has relatively large background current. It was postulated that the intercalation of the solvent molecules of acetonitrile to the gap between the thin plates of the glassy carbon crystallized graphite may cause a large charging current at this electrode (Kotani et al., 2011).

Based on the studies mentioned above, determinations of cholesterol (Hojo et al., 2007; Hojo et al., 2011; Kotani et al., 2011), cholestanol (Hojo et al., 2010), oxysterols (Matsunaga et al., 2009), and phytosterols (Ito et al., 2010) were developed using HPLC-ED with detection potentials from +1.9 V to +2.8 V (vs. Ag/AgCl). GCE was used as the working electrode in a commercial radial flow cell (Hojo et al., 2007; Matsunaga et al., 2009; Hojo et al., 2010; Ito et al., 2010; Hojo et al., 2011), only in one case BDD electrode in a thin-layer flow cell was employed (Kotani et al., 2011). In all papers, C30 columns were used, with exception of the paper (Hojo et al., 2011) where C18 column was employed. The C18 column was used because it was the only one among the columns examined that could separate lathosterol from cholesterol. Most of the studies aim at cholesterol detection, as the ideal model steroid with a large physiological importance. The choice of the separation column is the crucial factor, because the necessity to use the non-aqueous mobile phase containing acetonitrile or a mixture of acetonitrile and 2-propanol (9:1, v/v) (Hojo et al., 2007) with lithium perchlorate in concentrations varying between 10 mmol L^{-1} and 50 mmol L^{-1} as the supporting electrolyte limits the

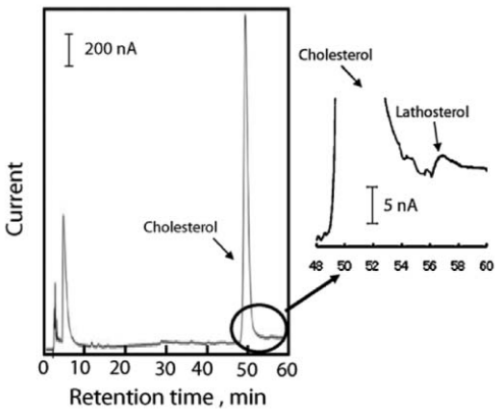

Figure 3. HPLC-ED of lathosterol and cholesterol in human serum. Mobile phase, acetonitrile containing 30 mmol L^{-1} $LiClO_4$; flow rate 0.25 mL min^{-1}; YMC-Pack ODS-AL column (250 mm × 2.0 mm i.d., 5 μm); column temperature 50°C; GCE; applied potential +2.8 V vs. Ag/AgCl; injection volume 5 μL. Reprinted with permission from Hojo et al, 2011.

variability of elution strength. For example, in an extended study on cholesterol and lathosterol separation at 25 columns including fifteen different C18 columns, two C8 columns, a C4 column, a silica column, two phenyl columns, two cyano columns, a diol column, and a C30 column, only one C18 column was successfully employed, with almost 60 min of retention time (Figure 3) (Hojo et al., 2011). In all other studies, where C30 columns of various length, width, and particle size were used, retention times of cholesterol varied from ca 20 min to 55 min. The detection potential has to be highly positive (greater than +1.9 V vs. Ag/AgCl), in concordance with the course of cyclic voltammograms in Figure 2. The *LOD*s for cholesterol ranged from 0.36 μmol L^{-1} (Hojo et al., 2007) to 0.008 μmol L^{-1} (Kotani et al., 2011). The latter *limit of detection (LOD)* is lower than for commonly employed HPLC-UV, LC/MS, and LC/MS/MS methods and was achieved at BDD electrode at +2.2 V vs. Ag/AgCl.

An example of analysis of real sample is depicted in Figure 3. It shows a representative chromatogram of lathosterol and cholesterol in human serum; analyses of rat and mice serum were also conducted (Hojo et al., 2011). Using an internal standard method (6-ketocholestanol), total lathosterol in control samples of all serum samples was determined with a recovery of more than 95.8% and an RSD ($n = 5$) lower than 7.3%. The determination of the serum level of lathosterol or the lathosterol/cholesterol ratio is a well-known marker of endogenous cholesterol synthesis. Similar HPLC-ED methods aimed at serum cholesterol (Hojo et al., 2007; Kotani et al., 2011), cholestenol (Hojo et al., 2010), and phytosterols, namely β-sitosterol, campesterol, stigmasterol, and brassicasterol (Ito et al., 2010) are summarized in Table 1. The detection limit of these phytosterols was below 3.4 μmol L^{-1} and their determination has been successfully applied to the monitoring of experimental phytosterolemia in rats (an autosomal recessive disease causing, e.g., primary coronare atherosclerosis in rats induced by a high-phytosterol diet). The other matrix analyzed beside serum was oxidatively modified low-density lipoprotein, where seven oxysterols were determined by the similar HPLC-ED method as the other sterols investigated (Matsunaga et al., 2009).

Obviously, the above-mentioned HPLC-ED methods represent an interesting alternative to the MS-based methods with high financial and staff requirements, whereas omitting the necessity of sterol derivatization for gas chromatographic applications. Nevertheless, the use of a non-aqueous medium may lead to long retention times on the reversed phase columns (Figure 3), or insufficient separation of structurally similar sterols. A compromise between their retention times and possibility of electrochemical detection needs to be made by selection of the separation column. Development of a method that would employ mixed medium of acetonitrile and a more polar solvent (e.g., water) could speed up the analysis and increase the selectivity while preserving all the advantages of the electrochemical detection that are low price, high selectivity, and high sensitivity (especially for those sterols that are hardly detectable by UV or fluorescent detectors).

These benefits are partially fulfilled employing PAD at Au electrode. Using a resin-based reversed-phase column and an optimized gradient of acetonitrile in dilute sodium hydroxide solution (Dekker et al., 1991) or isocratic elution in mixed alkaline:acetonitrile media at an anion-exchange column (Chaplin, 1995), separation of 13 bile acids and their conjugates with glycine and taurine was accomplished in 60 minutes, respectively, 35 minutes with lithocholic acid and their conjugates possessing only the 3α hydroxyl group being the last eluted compounds. The PAD detection conditions are similar to those generally chosen for carbohydrate and aliphatic alcohol analysis and rely on the oxidation of the hydroxyl groups (Johnson and LaCourse, 1990; LaCourse et al., 1991). Using a set of three potential pulses, the bile acids are detected anodically at the oxide-free Au surface at the potential of ca 0 V to +0.1 V due to the interaction and stabilization of the reaction intermediates with the partially vacant *d*-orbital of the noble metal. The other positive/negative pulses applied lead to the formation of Au surface oxides and consequently their dissolution to obtain clean Au surface. This direct anodic electrocatalytic detection leads to sufficient *LOD*s lower than 10 μmol L^{-1}. The sensitivity is dependent on the number of hydroxyl groups in the molecule. Applicability of the method was verified on determination of bile acids in human T-tube bile, human duodenal bile, and ileum fluid with high recovery and concordance with enzymatic assay of bile acid in these samples (Dekker et al., 1991). The main disadvantage of this approach is that the detection requires basic medium and thus the usage of reversed phase silica-based columns is restricted.

Mixed aqueous-organic solvent (acetonitrile and methanol) mobile phase was also used for the assay of ursodeoxycholic acid and related impurities in pharmaceutical formulations (Scalia et al., 1995). A coulometric detector containing two in-line porous graphite coulometric electrodes operating at anodic voltages of +0.60 V and +1.40 V provided accuracy and sufficient reproducibility for this purpose and also for the detection of other free bile acids. The working electrode can be activated by simply flushing it with concentrated solutions of nitric acid and/or sodium hydroxide.

All mentioned HPLC-ED studies confirm that the possible passivation of the electrode surfaces due to the adsorption of the analyzed compounds or their reaction products is easily prevented by the liquid flow or *in-situ* by electrochemical activation and thus are perspective for practical implementation.

Indirect oxidation of cholesterol, oxysterols, phytosterols, and bile acids at bare electrode materials

Several reports on indirect oxidation of steroid-based compounds exist. They are mostly devoted to cholesterol and cholic acid. The main interest, however, lies in the explanation of the oxidation mechanism and the identification of the reaction products rather than their analytical utilization. When the indirect oxidation is performed, a mediator that is present or generated in the electrolyzed solution acts as an electrochemical catalyst by lowering the activation energy needed for the reaction to take place. Indirect oxidation can be performed both in non-aqueous and aqueous media. In the aqueous medium, it remains the only possibility of oxidation, because water considerably narows the potential window in the anodic region and the potentials needed for the direct oxidation of sterols are not accessible.

Several reports have shown that cholesterol can be indirectly electrochemically oxidized by various mediators as reviewed in

Morzycki and Sobkowiak (2015): Hydroxylation of cholesterol at C_{25} has been achieved with Mn(III)-porphyrin-O_2 adduct (Groves and Neumann, 1988), giving 25-hydroxycholesterol. The terminal isopropyl group of cholesterol at the C_{25} position was selectively oxidized in acetonitrile containing $LiClO_4$ to give the same compound, 25-hydroxycholesterol, using Tl(II)-hematoporphyrin-O_2 adduct, which has been generated galvanostatically in the presence of O_2 at the cathode from the corresponding Tl(III)-hematoporphyrin complex (Maki et al., 1997). In both cases, an adduct of the reduced form of a metal, porphyrin, and O_2 served as the mediator. Cholesterol was also oxidized in dimethylformamide containing 6% water with NaBr. In this case, BrO^- species electrogenerated under galvanostatic conditions at a platinum electrode from Br^- ions served as a mediator, and among oxidation products 3,5,6-trihydroxycholesterol, 7-oxocholesterol, 5,6-epoxycholesterol, and 7-ketocholesterol were identified (Wu et al., 1992). Three studies used an indirect oxidation of cholesterol for electroanalytical purposes on a conventional platinum electrode, using bromine species in a mixed medium of dimethylformamide: water (94:6, v/v) (Wu et al., 1992), and in non-aqueous media of dimethylformamide (Chiang et al., 2011) and acetonitrile (Tsierkezos and Ritter, 2014). There are two anodic peaks present in cyclic voltammogram between +1.0 V and +1.6 V vs. Ag/AgCl that are attributed to redox pairs Br^-/Br and Br/Br^+, respectively. Their current response is significantly increased in the presence of cholesterol, especially in the case of the second peak corresponding to Br/Br^+ system (Figure 4). It is believed that cholesterol upon oxidation reduces the Br^+ cation to form neutral bromine species and thus enhances the oxidation current of the second peak (Chiang et al., 2011). Steady-state current amperometry (Chiang et al., 2011) and differential pulse voltammetry (DPV) (Tsierkezos and Ritter, 2014) were used for the quantitation of cholesterol; the obtained *LOD* for DPV (0.10 μmol L^{-1}) (Tsierkezos and Ritter, 2014) appears to be significantly lower than the ones obtained on other, enzyme modified, materials.

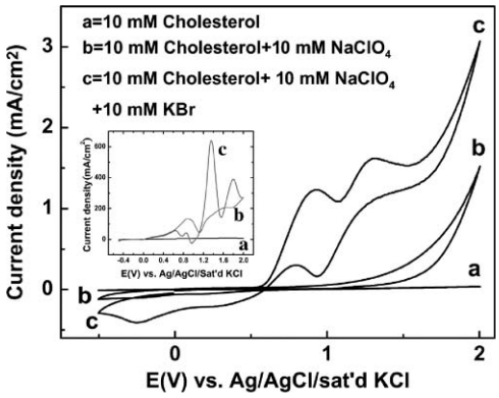

Figure 4. Cyclic voltammograms at bare platinum electrode in dimethylformamide of (a) 10 mmol L^{-1} cholesterol, (b) 10 mmol L^{-1} cholesterol plus 10 mmol L^{-1} $NaClO_4$, and (c) 10 mmol L^{-1} cholesterol plus 10 mmol L^{-1} $NaClO_4$ and 10 mmol L^{-1} KBr. Scan rate 10 mV s^{-1}, electrode area 1 cm^2. Inset: DP voltammograms of the above samples. Reprinted with permission from Chiang et al., 2011.

While cholesterol possesses only one hydroxyl group at C_3 that can be oxidized by the indirect process, other sterols may contain more of these functionalities and thus the order of their oxidizability is of interest. PbO_2 and graphite plate anodes were used for the indirect galvanostatic preparative oxidation of cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; see Figure 1) in dimethoxyethane:water (2:1, v/v, pH = 5) and a platinum foil anode in water (pH = 12; adjusted with 10% NaOH) containing NaCl as the mediator and $NaClO_4$ as the supporting electrolyte (Medici et al., 2001). Products of the anodic oxidation varied depending on the anode used, and also on the time of the reaction. It was ascertained that the alcoholic functions of cholic acid can be converted to oxo functions. The rate of oxidation was in the order $C_7 > C_{12} > C_3$ on the PbO_2 and graphite plate with dehydrocholic acid in quantitative yields as the final product, which respects the order of reactivity in the regioselective oxidation of the cholic acid by chemical oxidizing agents (Kritchevsky, 1971). Reactions carried out with platinum foil electrode are much slower and stop selectively at the 7-oxo derivative (i.e., 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic). The absence of organic chloro-derivatives among products was noticed indicating that the mediated electrochemical oxidation of cholic acid to the dehydro-derivatives is essentially a surface reaction and that the role played by volume reactions involving ClO_2 and/or HClO is secondary.

When nickel hydroxide electrode (Schäffer, 1987) is used for the indirect oxidation of cholic acid and several steroidal alcohols, the electrode material itself serves as a mediator, the electrocatalyst is presumably a nickel oxide hydroxide (NiOOH) that is continuously reformed at the electrode surface. In general, the reactivity of the different hydroxyl groups in steroids in 0.01 mol L^{-1} KOH in *tert*-butyl alcohol:water (1:1, v/v) follows the order: 3β-OH $\approx$ 3α-OH > 17β-OH >> 20β-OH > 11β-OH. For example, in steroidal alcohols containing only the 3α-OH or 3β-OH group and 17β-OH group, the former are selectively oxidized. Additional activation of the 3-OH group by an allylic C_4 double bond increases the selectivity and leads to 50% testosterone. Cholic acid is oxidized exclusively at the 3α-position (without any attack at the 7α-OH or 12α-OH groups) to 3-oxo,7α,12α-dihydroxy-5β-cholan-24-oic acid. Its yield is lowered by a competing reaction to a lactone; this is formed by oxidative cleavage of the C_3–C_4 bond, followed by lactonization. Comparable selectivity has been reported for chemical oxidations by silver carbonate on Celite® (Mckillop and Young, 1979) and by molecular oxygen with a platinum catalyst (Heyns and Blazejewicz, 1960).

Although only two examples (Chiang et al., 2011; Tsierkezos and Ritter, 2014) on an electroanalytical application of the indirect oxidation were found within the published studies, this approach is attractive because of the mild reaction conditions. On the other hand, substantial drawback in low target selectivity of electrogenerated oxidizing agents envisages the necessity of pre-separation steps and sophisticated sensing arrangements for successful applications in clinical analysis. Obviously, the types of mediator, electrode material, and conditions are the crucial factors for the successful development of detection strategies. The studies of indirect oxidation open the way to coupling of effective oxidation agents to ion-selective membranes, modified

electrodes, or molecularly imprinted polymers (MIPs) to improve selectivity of modified-electrode based systems.

Products of direct oxidation of cholesterol

Only few studies were conducted to identify products of direct electrooxidation of cholesterol (Kowalski et al., 2005; Kowalski et al., 2008; Hosokawa et al., 2009) and a review was published dealing with some aspects of the issue (Morzycki and Sobkowiak, 2015). No reports on other sterols exist according to the best of our knowledge. In 2005, Kowalski et al. used a platinum electrode in glacial acetic acid containing sodium perchlorate and sodium acetate as supporting electrolytes (Kowalski et al., 2005) and in dichlormethane with tetrabutylammonium tetrafluoroborate as a supporting electrolyte (Kowalski et al., 2008). It was found that the solvent used affected totally the reaction mechanism and structure of products. In the former case, 7α- and 7β-acetoxycholesterols (10:3 ratio) were identified as major reaction products, with minority of 7-oxocholesterol. The reaction mechanism involves two electrons – one proton oxidation at the C_7 allylic position leading to formation of C_7 carbocation followed by an attachment of a nucleophile to this intermediate. The approach of the nucleophile from a sterically less hindered α side of a steroid molecule dominates (Kowalski et al., 2005).

Preparative electrolysis in dichloromethane with separated cathodic and anodic compartments produced dicholesteryl ether in a relatively high yield of about 37% (Kowalski et al., 2008). Dichloromethane, which does not exhibit nucleophilic properties, enables the formation of cation-radical centered at the oxygen atom as the result of one-electron oxidation of cholesterol. Through the heterolysis of the C_3—O bond assisted by the π electrons of the C_5–C_6 double bond leading to the homoallylic carbocation and subsequent reaction of the oxygen atom of the second molecule of cholesterol, dicholesteryl ether is formed. Nevertheless, when the leakage of unwanted species to anodic compartment was not prevented, depending on electrolysis conditions (composition of supporting electrolyte and electrolytic cell construction) various by-products were obtained as the result of the nucleophilic attack of the present species: 3β-chloro (cathodic reduction of dichloromethane on the auxiliary electrode to chloride ions, and their diffusion to the anodic compartment), 3β-acetoxy (as the consequence of the presence of glacial acetic acid in the cathodic compartment to prevent the electrochemical reduction of dichloromethane), or 3β-acetylamino (leakage of acetonitrile from the electrolytic bridge). The formation of dicholesteryl ether is credited to the presence of the double bond in cholesterol, making its formation unlikely in sterols typically not possessing a double bond in its structure, for example in bile acids.

Hosokawa et al. oxidized cholesterol at carbon fiber column electrode in an acetonitrile-2-propanol mixture using $LiClO_4$ as a supporting electrolyte (Hosokawa et al., 2009). The reaction products were identified by IR spectroscopy, NMR, and MALDI-TOF-MS. The formation of cholesta-4,6-dien-3-one in a four-electron, four-proton electrochemical process was confirmed (Figure 5). This reaction of cholesterol as well as analogous reactions of oxysterols and phytosterols was successfully applied to the development of HPLC-ED methods for the determination of these compounds in human and rat serum (Hojo et al., 2007; Matsunaga et al., 2009; Hojo et al., 2010; Ito et al., 2010; Hojo et al., 2011; Kotani et al., 2011) as summarized in Table 1.

Reduction of bile acids

Unlike oxidation, electrochemical reduction of sterols (cholesterol and phytosterols) at reasonable negative potentials is impossible, because they possess a maximum of one double bond at the steroid nucleus and lack other electrochemically reducible moieties. Therefore, the studies aimed at reduction involve only bile acids with the carboxyl group at C_{24} or C_{27}. It is known that aliphatic and aromatic carboxylic acids that are not strongly activated by electron-withdrawing groups and do not contain more easily reducible groups give a well-developed polarographic wave in aqueous (Korshunov and Kuznetsova, 1949) or nonaqueous (acetonitrile) (Coetzee and Kolthoff, 1957) medium, but this is usually due to the reduction of protons to hydrogen or reduction to the aldehydic form (Tiribilli et al., 2015).

Using differential pulse polarography (DPP) (Ferri et al., 1984), it was found that the similar behavior of the peak height versus the concentration of the unconjugated (cholic, deoxycholic, ursodeoxycholic, chenodeoxycholic, and lithocholic) and conjugated (glycine and taurine conjugates) acids allows the evaluation of the total bile acids content by summing the independent forms in which they are present in the sample. The activity of free bile acids is characterized by the presence of a well-developed peak independent of the pH at the potential of −1.15 to −1.35 V (vs. Ag/AgCl), ursodeoxycholic acid is reduced at about 0.1 V more positive potential. Despite the proximity of reduction potentials with the hydrogen evolution reaction, total bile acids contents in human bile can be determined by these methods in weakly acidic to basic media.

The other method used for reductive determination of a representative of bile acids is square wave voltammetry (SWV) at a

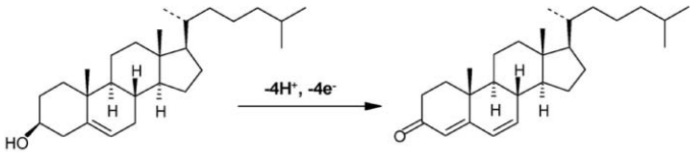

Figure 5. Cholesterol oxidation leading to the formation of cholesta-4,6-dien-3-one in the medium of acetonitrile containing lithium perchlorate (50 mmol L^{-1}) as supporting electrolyte. Reprinted with permission from Hosokawa at al., 2009.

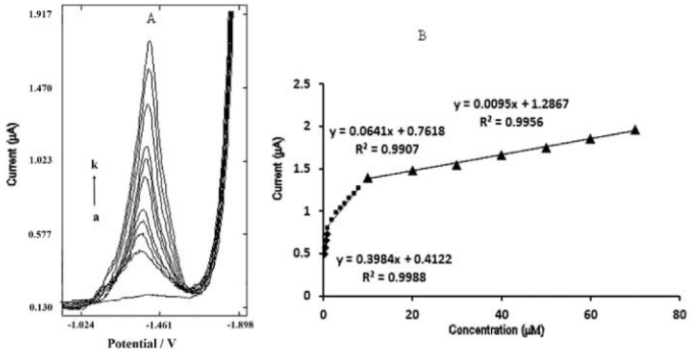

Figure 6. The square wave stripping voltammograms (A) and the calibration curve (B) of cholic acid at the HMDE in Britton-Robinson buffer (pH = 9.0). Concentration of cholic acid from curve *a* to curve *k*: 0, 0.2, 0.4, 0.6, 0.8, 2, 4, 6, 20, 40, and 60 μmol L^{-1}. Accumulation time 60 s at the potential of −0.6 V (*vs.* Ag/AgCl). Reprinted with permission from Yilmaz et al., 2015.

hanging mercury drop electrode (HMDE) (Yilmaz et al., 2015). As the bile acids adsorb strongly at the electrode surface, the adsorptive mode leads to their accumulation and enhancement of voltammetric response. A reductive peak at −1.35 V (vs. Ag/AgCl) was utilized for the determination of cholic acid in gallbladder of various animals (i.e., sheep and cattle) without the need for any time-consuming and possibly contaminating preprocessing steps such as extraction, cleanup, derivatization, or pre-concentration. A substantial drawback of the method is the non-linear course of the calibration dependence and the low sensitivity for higher concentrations of the acids obvious from Figure 6.

Presumably, quantitations of selected bile acids described in above-mentioned studies (Ferri et al., 1984; Yilmaz et al., 2015) are based on the catalytic reduction of the proton of the carboxyl group, proceeding independently of the pKa value of the carboxylic group (for unconjugated bile acids pKa is ca 4.5). The exact reaction mechanism remains relatively unclear and is not discussed in those studies. Obviously, the adsorption ability of bile acids complicates the utilization of the reductive process for analysis and the electrode material is limited to mercury-based due to the very negative potential of reduction.

The other electrode used was GCE and multiwalled carbon nanotubes modified GCE (MWCNT-GCE) employed to study reduction of deoxycholic acid (Liu et al., 2006). Cyclic voltammograms were measured in methanol:water (1:1, v/v) mixture and irreversible cathodic wave near the potential of +0.65 V (vs. SCE) was obtained only at the modified electrode. Its presence was attributed to the catalytic effect of multi-wall carbon nanotubes (MWCNT) exhibiting large specific area, particular electronic structure, and high electric conductivity, especially connected with some oxygen containing groups (Luo et al., 2001; Musameh et al., 2002). The position of the peak is independent of pH, but its current increases with increasing pH, and also increases with the upper switching potential in cyclic voltammetry. The wave was attributed to the overall four-electron reduction of the carboxyl group to the corresponding hydroxymethyl compound. Such reduction is otherwise hardly achievable and requires activation of the carboxyl group by a strong electron withdrawing group, which is less easily reducible than the carboxyl group (this excludes the nitro group and a number of nitrogen-containing heteroaromatic groups as activating groups).

The use of modified electrodes and non-enzymatic biosensors in cholesterol and bile acid sensing

Non-enzymatic biosensors working in amperometric mode and modified electrodes for voltammetric determinations represent a promising approach developed for sensing of cholesterol. Their optimized operating parameters and obtained figures of merit are summarized in Table 1. These sensors do not suffer to that big extent from the drawbacks of enzyme-based sensors as time dependence of the activity of the enzyme, pH, temperature, and humidity dependence of the signal, and possibly sensitivity of the enzyme to toxic chemicals. The main issue substantiating their sometimes complicated and time-demanding fabrication and problems with signal repeatability due to enhanced surface activity of the detected steroids and their products is the increase of selectivity necessary for cholesterol detection in real matrices (blood, food matrices).

Three main strategies for cholesterol sensing include (i) its direct oxidation at modified supporting electrode materials by metal-based NPs, (ii) indirect detection of ferrocyanide at SAM structures, or (iii) electrochemical sensing of redox-active dyes released from host-guest complexes in the presence of cholesterol.

The first approach developed relatively recently is represented by studies on cholesterol determination employing electrodes modified with NPs of Pt (Kim et al., 2009; Yang et al., 2012), Au/Pt (Lee and Park, 2010), Ag (Li et al., 2010), and Cu_2S (Ji et al., 2014). The fabrication procedure can be rather complicated including templating methods and electroplating techniques (Kim et al., 2009; Lee and Park, 2010), but also relatively simple galvanic processes were reported, e.g., for rose-like Cu_2S nanostructures at Cu rod (Ji et al., 2014) or simple evaporation of solution containing respective NPs at the supporting electrode material (Li et al., 2010). Triton-X is usually

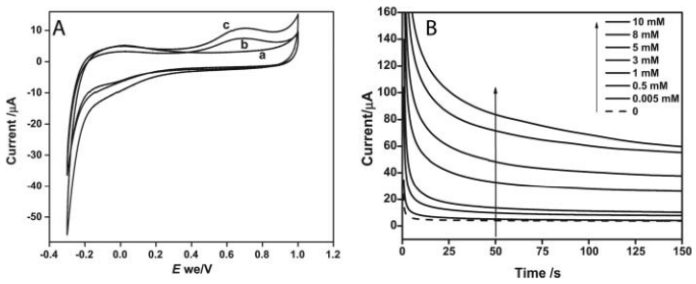

Figure 7. (A) Cyclic voltammograms at a Pt NP/$(CNT)_{24}$ bilayer electrode in phosphate buffer (pH 7) in the absence (a) and in the presence of cholesterol (2 mmol L^{-1}) (c) and at a Pt NP/(CNT)12 bilayer electrode in the presence of cholesterol (2 mmol L^{-1}) (b). (B) Amperometric response of Pt NP/(CNT)24 bilayer electrode in 50 mmol L^{-1} phosphate-buffered saline (pH 7) solution containing different concentrations of cholesterol. Applied potential +0.7 V (*vs.* Ag/AgCl). Reprinted with permission from Yang et al., 2012.

added to the tested solutions to assure sufficient solubility of cholesterol. The detection potentials reported are rather low (up to +0.7 V vs. Ag/AgCl) compared to those employed on conventional electrode materials (over +1.6 V vs. Ag/AgCl) (Hojo et al., 2007; Kotani et al., 2011). The mechanism of the oxidation was not established in the above-mentioned studies. Obviously, the enhanced signal towards cholesterol at lower potentials is connected with electrocatalytic properties of the electrode surfaces as the consequence of their enlarged area by nanostructures leading to higher electrical conductivity and faster electron transfer. An illustrative example of the effect of these properties on the analytical performance is the sensor prepared by the deposition of Pt NPs via the spontaneous reduction of H_2PtCl_6 on the functional defect sites of the layer-by-layer assembled carbon nanotube thin film at indium-tin oxide (ITO) electrode (Yang et al., 2012). Cholesterol provides a well-developed voltammetric peak at this type of sensor at +0.7 V vs. Ag/AgCl that can be utilized for its chronoamperometric determination (Figure 7). This sensor outperformed the others in terms of wide linear range over three orders of magnitude and very low *LOD* of 2.8 μmol L^{-1}. It exhibits also at least 1-month long signal stability and selectivity towards common interfering species in blood, e.g., ascorbic acid, glucose, and dopamine. Nevertheless, comparing *LOD*s in the Table 1 for this type of sensors, all of them fulfil requirements on cholesterol detection, as the normal physiological (human serum) level of cholesterol concentration is under 5.17 mmol L^{-1}, whereas most of the possible interferents have a substantial lower physiological level of ca 0.1 mmol L^{-1} (e.g., ascorbic acid, uric acid, and acetaminophen). The lowest reported *LOD* of 0.1 μmol L^{-1} at Cu_2S/Cu rod electrode (Ji et al., 2014), should be taken cautiously with respect to the lowest concentration of the estimated linear dynamic range (10 μmol L^{-1}) and the figures of concentration dependencies published in that paper.

Future research in this area is essential to understand the process of electrochemical oxidation of cholesterol at NPs modified electrodes.

Studies reported the possibility of indirect cholesterol sensing using SAMs and MIP (Piletsky et al., 1999; Gong et al., 2003; Chou and Liu, 2005; Matsumoto et al., 2008; Aghaei et al., 2010; Nagaoka et al., 2012; Tong et al., 2013; Ji et al., 2015). The general procedure for SAM preparation is as follows: A gold electrode is immersed in a solution containing a suitable substrate for the formation of SAM (capable of forming S–Au bond) and a template for the future complementary binding sites (cholesterol), which is washed away after the SAM formation. One exception includes doping of the MIP to ceramic carbon electrode (CCE), making it easy to renew the surface by smoothing (Tong et al., 2013). The decrease in ferrocyanide signal in the presence of cholesterol, which is adsorbed in the empty spots, is then directly proportional to cholesterol concentration. Sensors utilizing SAMs have a very wide potential for practical application as demonstrated in the above-mentioned studies. A detector employing non-invasive cholesterol monitoring was based on skin cholesterol concentration (Matsumoto et al., 2008), and a commercially available handheld instrument and a sensor chip for food analysis (Nagaoka et al., 2012) were developed.

β-Cyclodextrin modified graphene was used as a non-enzymatic biosensor of cholesterol using methylene blue as a redox indicator (Agnihotri et al., 2015). Methylene blue forms an inclusion complex with β-cyclodextrin, but if cholesterol is present methylene blue is replaced by it. The free methylene blue can be then determined by DPV. This elegant method provided a sufficient *LOD* lower than 1 μmol L^{-1} and a good selectivity against common blood components. Similar approach was tested when preparing self-assembled β-cyclodextrin derivative monolayers on a gold electrode, hosting ferrocene carboxylic acid. Inclusion complex with ursodeoxycholic and dehydrocholic acid enables their indirect detection after their electrophoretic separation (He et al., 1997). Preliminary experiments also proposed that lipoylamino-β- and γ-cyclodextrins can act as effective hosts for binding bile acids when they were immobilized at the surface of a gold electrode (Egawa et al., 2005), but no further work was yet published in this area.

Redox activity of steroid hormones

At this point, it should be emphasized that beside above-mentioned steroid-based compounds with limited electrochemical activity a number of other steroids are reducible or oxidizable at reasonable potentials within the potential window of common electrode materials due to the presence of conjugated double bonds, and possibly extended by oxo group (testosterone,

reducible corticoids) and the presence of phenolic moiety (ring A) contained in the structure of estrogens (estrone, estradiol, and estriol; for respective structures see Figure 1).

Estrogenes can be electrochemically oxidized at bare (Santos et al., 2010; Cesarino and Hümmelgen, 2015) and modified electrodes (Luo et al., 2013; Zhu et al., 2015, Cesarino and Hümmelgen, 2015). BDD electrode was utilized for the SWV determination of estriol hormone in a pharmaceutical product and in a urine sample taken during pregnancy (Santos et al., 2010). A poly(L-proline)-ordered mesoporous carbon film with controllable thickness on the GCE surface was fabricated by a one-step electrochemical technique and used for the construction of electrochemical sensing platform for natural estrogens (Luo et al., 2013). Furthermore, three sensors based on carbon nanostructures (graphene oxide, reduced graphene oxide, and reduced graphene oxide modified with antimony NPs) exhibited strong electrocatalytic activity toward the oxidation of estrone, estriol, and especially of estradiol (Cesarino and Hümmelgen, 2015). These sensors were applied to estriol and estradiol detection in water. It was shown that DPV enables to distinguish and quantify these two substances, which in water constitute an environmental problem (Cesarino and Hümmelgen, 2015). An electrochemical biosensor with sufficient electrocatalytic activity based on an electrodeposited graphene/ordered mesoporous carbon composite modified carbon paste electrode was prepared for the SWV determination of natural estrogens (estrone, estradiol, and estriol) (Zhu et al., 2015).

The other frequently detected compounds in pharmaceutical, physiological, and environmental matrices are corticosteroids. Their polarography was extensively studied by de Boer et al. (1978), (1979a), (1979b), (1980a), (1980b), and (1981). It was shown that both the C_3 oxo group at the steroid nucleus and the C_{20} oxo group at the side chain of corticosteroids can be reduced and both of these reduction steps can be used for analytical purposes (de Boer et al., 1978). DPP was employed for analysis of corticoids in single-component pharmaceutical preparations and it proved to be a fast and reliable method with low standard deviation (de Boer et al., 1979a, 1979b). Multi-component pharmaceutical preparations analysis is also described using DPP (de Boer et al., 1980a). Furthermore, the reduction of halogen-containing corticosteroids (de Boer et al., 1980b) and the mechanism of 3-keto corticosteroids electrochemical reduction were studied (de Boer et al., 1981).

Later, modern electrochemical materials and their modified varieties were utilized, beside the detection of corticosteroids in common matrices even *in vivo* determination was reported (Cook, 1997). The developed biosensor measured competitive binding of corticosteroids and corticosteroid-peroxidase with antibodies, immobilized on platinum electrode, by monitoring the peroxidase activity.

Electrochemistry of corticosteroids at solid electrodes was studied including both oxidation (Goyal et al., 2007) and reduction (Goyal et al., 2010; Goyal and Agrawal, 2012; Goyal et al., 2013). The electrochemical oxidation of a corticosteroid methylprednisolone, used for doping, has been studied at gold NPs modified ITO electrode (Goyal et al., 2007). The proposed DPV method was effectively applied to detect the concentration of methylprednisolone in pharmaceutical formulations, human blood plasma, and urine samples with the results corresponding to those obtained by GC/MS.

Testosterone was successfully determined in oil-based pharmaceutical preparations and urine samples without any separation using square-wave adsorptive stripping voltammetry (SWAdSV) at a GCE (Levent et al., 2014). The analysis was performed in aqueous media (pH = 5.0) containing cetyltrimethylammonium bromide enhancing substantially the reduction current signal of testosterone. Presumably, lipoaffinity created between the non-polar moiety of the adsorbed surfactant micelles and lipophilic testosterone via its surface solubilization into the surfactant film, allows the preconcentration of analyte. AdSV was further employed to testosterone determination using a lead film electrode (Tyszczuk, 2008). Possibility of employing the method to determination in a pharmaceutical preparation and human urine samples directly without any separation step was confirmed in this study. Furthermore, voltammetric investigation of testosterone, and epitestosterone reduction was carried out at bare and single-wall carbon nanotubes modified edge plane pyrolytic graphite electrode (Goyal et al., 2010). The developed protocol was implemented for the determination of both the compounds in urine samples of healthy people and patients undergoing treatment with testosterone and the results were found to be similar to those obtained by HPLC. A study based on AdSV of testosterone propionate on HMDE (Hu et al., 1993) provided a low detection limit and was applied to its assay in pharmaceutical preparations.

Electrocatalytic reduction of halobetasol propionate at a single-walled carbon nanotube modified edge plane pyrolytic graphite electrode (Goyal and Agrawal, 2012) was successfully applied to the detection of halobetasol propionate in pharmaceutical preparations. The electrochemical behavior of mometasone furoate has been studied at single-walled carbon nanotubes modified pyrolytic graphite electrode (Goyal et al., 2013). The reduction site in mometasone furoate was found to be carbonyl group at position C_3. The developed method was successfully applied for the determination of mometasone furoate in pharmaceutical preparations and in human urine.

Recently, an SWV at ordered mesoporous carbon modified GCE was developed for the determination of prednisolone, a corticosteroid abused for doping in sports, in pharmaceutical formulations and spiked human urine (Munyentwali and Zhu, 2015).

Utilization of electrochemical methods at mercury electrodes for characterization and quantitation of steroid-based compounds

A relatively high number of studies can be traced in the literature on utilization of mercury electrodes for characterization and indirect quantitation of steroid-based compounds. Also, their adsorption ability on mercury surfaces can be utilized for quantitation. Although some of these approaches are only of historical importance, there are also newer studies substantiating the use of this, nowadays somewhat problematic electrode material (Navrátil et al., 2011; European Commission Regulation of the European Parliament and of the Council 2016/0023 on Mercury, 2016).

After the WWII possibilities of polarographic techniques as the leading analytical methods for detection of steroids were investigated, methods for indirect polarographic estimation of cholesterol were published in the 1940s: Cholesterol was precipitated by digitonin and the excess of digitonin was determined by cobalt maximum suppression (Talafant, 1950). Cholesterol can be oxidized by Oppenauer oxidation (Oppenauer, 1937), a gentle method for selective oxidation of secondary alcohols to ketones, and subsequently determined at approximately −1.3 V using DC polarography (Wolfe, 1941). This method was also applicable directly to cholestenone, and after the Oppenauer oxidation to stigmasterol and other sterols.

In a series of polarographic studies (Feroci et al., 1992, 1994, 1995a, 1995b, 1996, 1997; Fini et al., 1997), interaction of bile salts with divalent metal ions such as Cd^{2+}, Pb^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}, the latter three being present in the gall bladder, were studied as reviewed in Zuman (2000). The reactions between the heavy metal ions and bile salts may be of a physiological importance. Depending on the concentration of the bile salt, they are in the monomeric form or in the form of dimeric to tetrameric aggregates or even larger aggregates formed by six or more anions of bile salt. While monomeric anions are soluble under physiological conditions and show negligible interaction with above mentioned cations, dimers and larger aggregates form slightly soluble complex compounds at pH values higher than pKa of the respective acid with stability constant depending on its structure (number and positioning of the hydroxyl group, and possible conjugation with glycine or taurine) and on the kind of the divalent cation. These data can be obtained from dependences of limiting currents of free metal ions and corresponding half-wave potentials on concentration of bile salts.

Other studies utilize the strong adsorption ability of steroid-base compounds itself on the electrode surface. The strength of adsorption of a bile salt on the mercury electrode surface can be indicated by the suppression ability of polarographic maximum (Milberg et al., 1988). The indicator is the concentration of the bile acid at the point when the maximum is suppressed to 50% of the original height. When observing the polarographic maxima of oxygen (−0.2 V) and Zn^{2+} ion (−1.2 V; both vs. Hg pool) it can be concluded that adsorption ability increases in the order monohydroxy < dihydroxy < trihydroxy sterols and the protonation of the carboxylic group has little effect. Thus, presumably the hydrophobic portions of the rings B and C are responsible for the interaction with mercury. These findings are valid for concentrations several orders of magnitude lower than CMC values of bile acids.

Furthermore, a shift in the value of half-wave potential of the first polarographic wave of the reduction of molecular oxygen was observed in the presence of bile salts (Feroci et al., 2007). This phenomenon was credited to an occurrence of a direct interaction between oxygen and bile salts, where the hydrophobic face of bile salt monomers and/or small aggregates are involved, thus enhancing dismutation of superoxide ion produced at the electrode. As a consequence, bile salts, beside the well-assessed physiological roles can behave as oxygen carrier and as antioxidant, preventing the oxidation of biological compounds by superoxide ion.

Adsorption abilities of sterols can be also used for their quantitation. Anodic stripping voltammetry (ASV) can be employed to directly measure cholesterol content in aqueous solutions (Peng et al., 1992). Cholesterol is adsorbed on an HMDE and an adsorption/desorption signal is observed at the cyclic voltammograms in 0.1 mol L^{-1} KCl. A sharp peak-counter peak at the potentials of −0.05 V and −0.075 V (vs. Ag/AgCl) corresponds to the above-mentioned processes. The peak current is linear with cholesterol concentration in the range from 0.1 μmol L^{-1} to 8 μmol L^{-1}.

An HPLC-ED method based on alternating voltage polarography measuring the changes in the double layer capacitance due to the adsorption of bile acids at the dropping mercury electrode was developed (Kemula and Kutner, 1981). The *LOD* is dependent on the value of the solution flow-rate, which increases the rate of transport of a substance to the DME surface due to convection, and especially at long drop times enables the determination of lower concentrations than in the similar system under steady-state conditions. The utilization of these processes for quantitation of other sterols can be envisaged.

Conclusion

There are a large number of studies dealing with electrochemical oxidation and reduction of steroid-based compounds with conjugated double bonds. For example, steroid hormones containing phenolic ring A or conjugation of C_3 oxo group double bond with C_4–C_5 double bond. Compounds of this type are, thanks to their structure, electrochemically active at reasonable potentials within the potential window of common electrode materials. On the other hand, the number of studies dealing with the non-enzymatic electrochemical oxidation or reduction of steroid-based compounds containing none or an isolated double bond is rather limited. Undoubtedly, the positioning of their redox activity at the edges of the potential window is a substantial shortcoming; thus, it is desirable to investigate other electrochemical strategies. It offers an open field for research with possibly large impact on the application of the new approaches to detection of these compounds in practice. This effort can be traced on a relatively high number of studies devoted to cholesterol detection as the leading compound of interest from the clinical point of view. Its oxidation on both conventional and NP modified electrodes has been studied leading to the development of non-enzymatic amperometric biosensors. Many studies are focused on indirect oxidation by inorganic mediators or utilization of SAMs or host-guest complexes immobilized at the supporting electrode materials. Nevertheless, the number of the studies is still low compared to those dealing with cholesterol enzyme-based biosensors, where an increased interest is apparent since the turn of the millennium. However, the sensors developed utilizing enzymes possess noticeable drawbacks, such as low stability, robustness, etc. The more robust, bare electrode-based sensors can offer sufficient or at least improved stability, sensitivity, and selectivity when their design and detection conditions are optimized. A good example is the concept of oxidation of cholesterol in nonaqueous media and its detection using HPLC with amperometric detection at GCE or BDD electrode, which has been successfully applied also for selected oxysterols and phytosterols. Furthermore, the reduction of bile acids was successfully

employed in several analytical studies at mercury electrodes and our preliminary results indicate that some bile acids can be oxidized at BDD electrodes in mixed organic-aqueous media (Klouda et al., 2016).

It is highly probable that electrochemistry of cholesterol, phytosterols, bile acids, and related compounds will continue to draw interest of scientists studying both direct, indirect, and enzymatic possibilities of their detection. The actual challenges can be seen in: (i) searching the ways of direct oxidation and reduction of these compounds, preferably in aqueous or mixed-aqueous media using novel electrode materials with relatively extended potential window in anodic (e.g., BDD) or cathodic (e.g., amalgam-based materials) region; (ii) development of new voltammetric and amperometric methods using the novel electrode materials and their validation so that they can be routinely used in clinical, pharmaceutical, environmental, and other laboratories; (iii) development of new non-enzymatic nanomaterials-based biosensors for a larger scale of compounds beside cholesterol, preferably with non-complicated fabrication procedure, long-term signal stability, and sufficient sensitivity and selectivity for the fit-for the purpose application; (iv) extension of the concepts described in (i)–(iii) to miniaturized scale for *in vivo/in vitro* sensing of steroid-based compounds under physiological conditions; (v) studies on adsorption-desorption processes at mercury-based and other electrode materials leading to new methods of quantitation of steroid-based compounds and clarifying their surface-activity in dependence on their concentration and conditions used.

Beside these electroanalytical areas, other scientific fields should be emphasized, where the knowledge on electrochemistry of these compounds may allow significant advances: (i) in depth studies on direct and indirect oxidation of these compounds may help to clarify their importance in physiological processes and further lead to improved electrosynthetic pathways (ii) for supramolecular systems containing steroid subunits and ligands electrochemical methods can broaden the knowledge about their structure, stability, and bonding interactions by observing how the electrochemistry of the particular steroid subunits changes in the absence/presence of convenient ligands. Furthermore, the investigation of their redox activity can clarify interactions involved in the self-assembling process. Although a limited number of studies excluding the enzymatic biosensors for detection of steroid-based compounds can be still expected in the following years, the possibilities reviewed herein show that many aspects of electrochemistry of these compounds remain unexploited or were investigated only for cholesterol. Thus, the authors believe that the employment of novel electrode materials, its modification not only by nanostructures and electrochemical-based detection strategies could open new ways in electrochemical detection of steroid-based compounds, as e.g., cholesterol, phytosterol, bile acids, and others and could enable their direct detection in physiological, pharmaceutical, and environmental matrices avoiding the time-consuming multiple extraction and separation steps.

Abbreviations

AD	amperometric detection
AdSV	adsorptive stripping voltammetry
CCE	carbon ceramic electrode
CMC	critical micellar concentration
DPP	differential pulse polarography
ELISA	enzyme-linked immunosorbent assay
FID	flame ionization detector FUMI
HDL	high density lipoprotein
ID-MS	isotopic dilution mass spectrometry
IR	infra-red
IT	ion trap
LDL	low density lipoprotein
LDR	linear dynamic range
LOD	limit of detection
LOQ	limit of quantation
LS	linear sweep
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectroscopy
MWCNT	multi-wall carbon nanotubes
NADH	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonantion
PAD	pulse amperometric detection
RP-HPLC	reversed phase HPLC
SWAdSV	square wave adsorptive stripping voltammetry
SWV	square wave voltammetry
TOF	time of flight

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6.2 Publication II

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Bile acids: Electrochemical oxidation on bare electrodes after acid-induced dehydration

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ABSTRACT

Bile acids and sterols in general have long been considered practically inactive for direct redox processes. Herein, a novel way of electrochemical oxidation of primary bile acids is reported, involving an initial acid-induced dehydration step, as confirmed by capillary electrophoresis–mass spectrometry, thereby extending the electrochemical activity of the steroid core. Oxidation potentials were found to be ca + 1.2 V *vs.* $Ag/AgNO_3$ in acetonitrile on boron doped diamond, glassy carbon, and platinum electrodes in a mixed acetonitrile–aqueous medium employing perchloric acid as a chemical reagent, and as a supporting electrolyte for the voltammetric measurements. The chemical step proved to be effective only for primary bile acids, possessing an axial 7α-hydroxyl group, which is a prerequisite for providing a well-developed voltammetric signal. Preliminary results show that other steroids, *e.g.*, cholesterol, can also be oxidized by employing a similar approach.

1. Introduction

Biosynthesis of the bile acids (BAs) is an important pathway for the metabolism and excretion of cholesterol in mammals [1]. Depending on the place of formation, literature discerns primary BAs, which originate in the liver, and secondary BAs that are formed by bacterial transformation of the primary BAs in the gut [2]. The most common primary BAs in humans are cholic (**1**; CA) and chenodeoxycholic (**2**; CDCA) acids.

The lack of double bonds or any fluorescent or electrochemically active groups in the molecules of BAs significantly limits the range of methods useful for their determination [3]. Gas chromatography after derivatization and HPLC in combination with mass spectrometry are commonly utilized for quantitation of individual BAs [4–6]. Other methods are based on detection of the products of enzymatic reactions, frequently using 3α-hydroxysteroid dehydrogenase as the key enzyme [7]. Electrochemical biosensors detecting the enzymatically generated NADH [8,9] or hydrogen peroxide [10] represent another strategy. Other reports on utilization of electrochemical methods for quantitation of BAs are scarce, as shown in our recent review [11]. BAs give electrochemical signal on mercury electrodes at far negative potentials [12,13], presumably as a result of catalytic hydrogen evolution from the carboxyl group in the side chain. Alternatively, electrooxidation has been reported in studies employing chromatographic separation with pulsed amperometric detection on gold [14,15] or porous graphite electrodes [16]. These studies, however, are mainly focused on the chromatographic aspects of the methods, rather than on the electrochemical processes themselves. Indirect oxidation using NaCl as a mediator succeeded in conversion of the hydroxyl groups of cholic acid into keto groups [17,18]. No study sufficiently characterizing the direct electrochemical oxidation of BAs has been published to date. Reports on steroids lacking any or possessing only isolated double bonds, including cholesterol, are scarce [11].

Herein, we present anodic oxidation of primary BAs on bare platinum, glassy carbon (GC), and boron doped diamond (BDD) electrodes in a mixed medium of acetonitrile–water–perchloric acid, where perchloric acid serves as a dehydrating reagent. Such an introduction of a double bond into the steroid skeleton can potentially increase the electrochemical activity. In the case of cholesterol, the double bond, together with the respective allylic positions, was identified as one of the sites of the electrochemical attack [19]. This approach, based on acid-induced dehydration, has also enabled a spectrometric determination of cholesterol (Liebermann-Burchard reaction) [20–22]. The proposed electrochemical approach could find application in the

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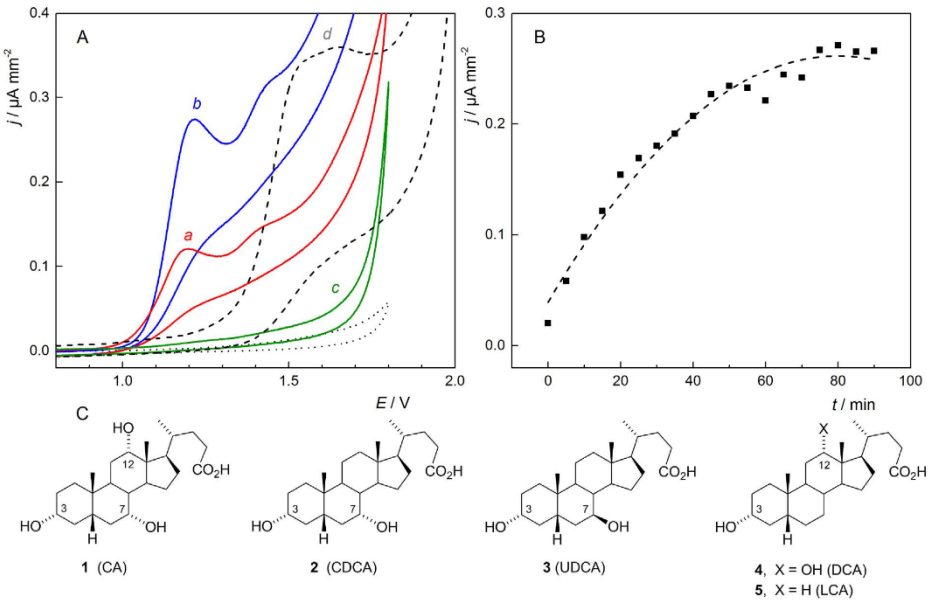

Fig. 1. (A) Cyclic voltammograms of (*a*) CA, (*b*) CDCA, (*c*) UDCA ($c = 9 \times 10^{-5}$ mol L^{-1}), and (*d*) cholesterol ($c = 5 \times 10^{-5}$ mol L^{-1}) in acetonitrile containing 0.1 mol L^{-1} $HClO_4$ and 0.43% H_2O on BDD electrode. Voltammograms recorded 90 min after the solutions were prepared from the stock solution and the supporting electrolyte. Supporting electrolyte in dotted line, scan rate 50 mV s^{-1}. (B) In-time development of the first CV peak height of CDCA ($c = 9 \times 10^{-5}$ mol L^{-1}). (C) Structural formulas of the BAs.

diagnosis of disorders of BA synthesis. A block in the biosynthesis of BAs in most cases results in a deficiency of the primary BAs [23].

2. Experimental

Cholic (**1**), chenodeoxycholic (**2**), ursodeoxycholic (**3**), deoxycholic (**4**), and lithocholic (**5**) acids, and cholesterol (all of > 99% purity, structures in Fig. 1C) were purchased from Sigma-Aldrich. All other commercially available chemicals were of analytical grade (if not stated otherwise).

Voltammetry was performed using a potentiostat PalmSens 2.0 with PSTrace 4.8 software. BDD (A = 7.07 mm^2, Windsor Scientific, UK), GC (A = 3.14 mm^2), or platinum (A = 7.07 mm^2; both Metrohm, Switzerland) working electrodes were used, routinely polished using alumina prior to each scan. Electrochemical cell with integrated reference electrode (Ag wire in 0.1 mol L^{-1} $AgNO_3$, 1 mol L^{-1} $NaClO_4$ in acetonitrile, separated from the measured solution by a salt bridge containing 0.5 mol L^{-1} $NaClO_4$ in acetonitrile) and a platinum foil counter electrode were employed. All experiments were carried out under the temperature of 21 °C. The contact time of the BA and $HClO_4$ and their concentrations are given in the caption of each voltammogram.

A modification of the previously described setup was used for capillary electrophoresis–mass spectrometry (CE-MS) [24]. The sample was hydrodynamically injected from an implemented PEEK cell: Sample volume 10 µL; injection time 2 s; separation voltage 18 kV. Parameters of fused silica capillary: Inner diameter 25 µm, outer diameter 360 µm, length 50 cm. Separation buffer: acetonitrile/1 mol L^{-1} acetic acid/10 mmol L^{-1} ammonium acetate. A Bruker microTOF (Bruker Daltonics, Germany) time-of-flight mass spectrometer equipped with a coaxial sheath liquid electrospray ionization (ESI) interface (Agilent, Waldbronn, Germany) was operated in positive ion mode; the mass range set 100–480 m/z; spectra rate 5 Hz. Source: ESI voltage: − 4000 V (grounded sprayer tip), plate offset: − 500 V; nebulizer: 1.0 bar; dry gas: 4.0 L min^{-1}; dry temperature: 190 °C. Transfer: capillary exit: 75.0 V; skimmer 1: 25.3 V; hexapole 1: 23.0 V; hexapole RF: 65.0 Vpp; skimmer 2: 23.0 V; lens 1 transfer: 38.0 µs; lens 1 pre pulse storage: 6.0 µs. Sheath liquid (2-propanol:water:formic acid, 49.9:49.9:0.2, v/v/v) was introduced by a syringe pump (KD Scientific, Holliston, MA, USA) with a flow rate of 0.48 mL h^{-1}.

3. Results and discussion

3.1. Voltammetric response of bile acids in the acetonitrile–water–perchloric acid medium

Electrochemical oxidation of two primary CA (**1**) and CDCA (**2**), and three secondary BAs, ursodeoxycholic acid (**3**, UDCA), deoxycholic acid (**4**, DCA), and lithocholic acid (**5**, LCA) in a mixed medium of acetonitrile–water containing perchloric acid was investigated.

Respective cyclic voltammograms on BDD electrode are presented in Fig. 1A (curves *a–c*). The overall process proved to be highly dependent on the structure. Only primary BAs with the axial 7α-hydroxyl group (CA, CDCA) afforded well-developed irreversible anodic signals at around + 1.2 V (curves *a, b*), *ca* 2.25 × higher for CDCA than CA, increasing in time (Fig. 1B). The difference in current densities for the different BAs can be rationalized by different, temperature-dependent rates at which each of the BAs undergoes the dehydration reaction. Such low oxidation potential has not been reported to date. That applies not only to BAs, but to any other steroid-based compounds, lacking any or possessing only isolated double bonds, including cholesterol. A proof of its oxidizability at + 1.5 V under the same conditions as those for BAs is presented by the voltammogram in Fig. 1A, curve *d*. The voltammograms of the secondary BAs, namely DCA and LCA lacking the 7α-hydroxyl group and UDCA possessing 7β-hydroxyl group, are featureless around this potential (shown for UDCA in Fig. 1A, curve *c*).

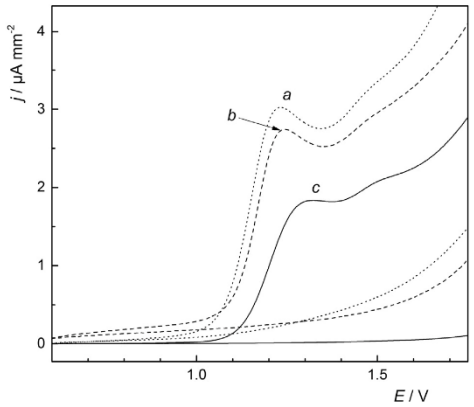

Fig. 2. Linear sweep voltammograms for CDCA ($c = 9 \times 10^{-4}$ mol L^{-1}) and supporting electrolyte on (*a*) Pt, (*b*) GC, and (*c*) BDD electrodes. Supporting electrolyte: acetonitrile containing 0.1 mol L^{-1} $HClO_4$ and 0.43% H_2O. Voltammograms were recorded 70 min after the preparation of the solutions, scan rate 50 mV s^{-1}.

Obviously, the presence of the axial 7α-hydroxyl group is the crucial factor enabling the development of the anodic signal. Note that its antiperiplanar position to the hydrogen atom at C(8) is likely to allow ready dehydration on protonation with $HClO_4$. The latter reaction was identified as the rate determining step leading to time dependency of the voltammetric signal, reaching a stabilized current value after approximately 75 min (Fig. 1B). Fig. 2 shows that the oxidation process can be achieved on various bare electrode materials including BDD, GC, and platinum with comparable positive potentials. The highest signal/background ratio is provided by BDD, which predestines this material for analytical applications. Preliminary voltammetric experiments on this electrode material suggest linear concentration dependences with detection limits in the micromolar concentration range for both BAs.

3.2. CE-MS elucidation of reaction steps

CE-MS measurements were used to confirm the dehydration reaction steps and to investigate its other products and products of electrochemical oxidation. The proposed mechanism for the CDCA **(2)** (m/z = 410.33; CDCA·NH_4^+ adduct) chemical reaction step is shown in Fig. 3B based on CE-MS measurements of the reaction mixture containing CDCA in acetonitrile/$HClO_4$ solution (water content 0.43%) (Fig. 3A). In principle, the dehydration can produce the corresponding Δ^6 or Δ^7 alkene (m/z = 392.32; (CDCA–H_2O)·NH_4^+ adduct); the latter structure **(6)** is more likely, as it should be thermodynamically preferred (Zaitsev rule) but the structure has not been confirmed at this stage. Nevertheless, dehydration of 7α-hydroxy derivatives on treatment with $POCl_3$ in pyridine at room temperature is known to afford the thermodynamically favored Δ^7-alkenes (Zaitsev rule) [25]. Therefore, formation of the Δ^7-alkene **(6)** in our case seems very likely. After a longer period (inset in Fig. 3A), a new signal was found at m/z = 416.32 (H^+ adduct), corresponding to the loss of another hydroxyl and addition of the acetamide group **(7)**. This is also supported by the migration behavior in CE that indicated a positive charge of the latter species **(7)**, presumably due to protonation of the amide group (Fig. 3A). Formation of such a product can be rationalized by Ritter reaction [26], starting with an attack of the Lewis basic nitrogen of the acetonitrile at a cationic species (presumably generated by protonation of the double bond), followed by hydrolysis of the iminium intermediate with the water that is present. Elucidation of the exact structure of this product will be the subject of future investigation.

The rise in voltammetric signal intensity correlates with the gradual rise of the peak attributed to dehydrated steroid **(6)** in the CE-MS

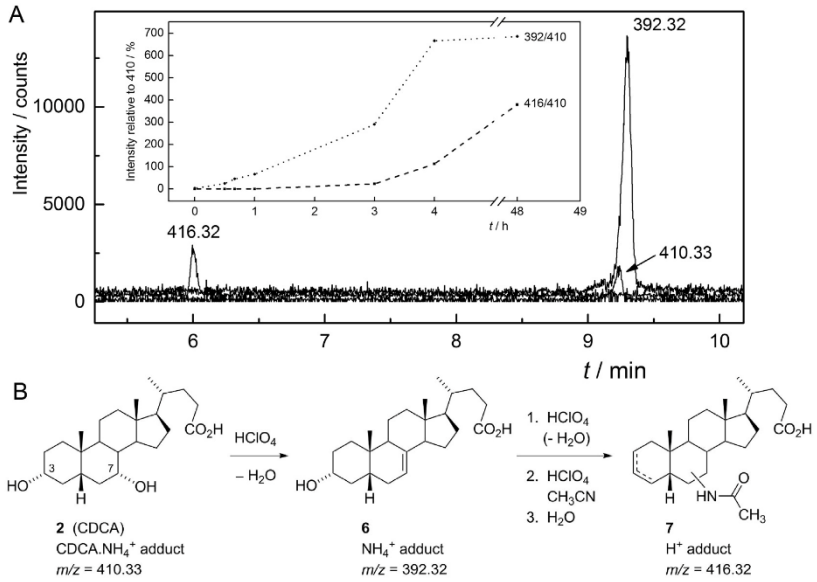

Fig. 3. (A) CE-MS of solution containing CDCA ($c = 9 \times 10^{-4}$ mol L^{-1}) in acetonitrile containing 0.1 mol L^{-1} $HClO_4$ and 0.43% H_2O obtained after 4 h. Three selected ion traces are shown (m/z 392.32, 410.33, 416.32). Inset: In-time development of ratio between respective peaks. (B) The proposed reaction mechanism for CDCA dehydration.

measurements. The introduction of double bond(s) into the steroid skeleton enables the oxidation process, as unsaturated compounds are more prone to the removal of an electron from a bonding π-orbital (HOMO) and formation of a reactive radical cation. The exact mechanism and structure of the products of both chemical and electrochemical reaction steps are under investigation. In general, formation of a number of reaction products is expected, as dehydration of steroids, promoted by aqueous acidic systems, often leads to backbone rearrangements and other transformations [20,27,28]. Further, oxidative properties of perchloric acid itself have to be considered together with the fact, that beside the final products of dehydration reaction the intermediates can undergo electrochemical oxidation.

3.3. Solvent, supporting electrolyte and water effects on voltammetric signals

The effects of the media used and the water content on the overall process were further investigated. The choice of the solvent must respect its safe miscibility with strong acids in view of the associated hazards. Furthermore, the solvent must provide sufficient potential limit that enables electrochemical oxidation within the potential window of the respective electrode material. Acetonitrile, a weak electron donor, fulfils both requirements. With other tested miscible solvents, no signal for BAs was observed, presumably as a consequence of incomplete dehydration reaction (nitromethane) or decomposition of the solvent preceding the electrochemical reaction of the primary BAs (tetrahydrofuran). The second key factor is the choice of the supporting electrolyte, which should also function as a dehydration agent. Experiments were carried out with $HClO_4$, H_2SO_4, H_3PO_4, and HCl (all 0.1 mol L^{-1}), containing respective concentrations of water in the solutions (%): 0.43, 0.04, 0.17, and 0.68. Only the presence of $HClO_4$ as the strongest among the inorganic acids used, enabled the dehydration step and consequently the electrochemical oxidation of the reaction products. Nevertheless, for other steroids with the core already activated by the presence of double bond(s), the use of other inorganic acids for further dehydration can be foreseen [20], as indicated by our preliminary experiments with cholesterol. Importantly, the voltammetric signal is not developed when using water-free medium, such as acetonitrile with $NaClO_4$ as supporting electrolyte, or the same medium with water present, confirming the necessity of the presence of a strong acid for inducing the dehydration reaction.

The initial water content proved to be an important factor influencing the chemical reaction step. The minimum amount in the solution (0.43%) is given by its presence in the concentrated solution of perchloric acid (70%). On the other hand, with water concentration higher than 2% *v/v*, only negligible voltammetric signal was obtained as the dehydration step was apparently inhibited. However, once the dehydration starts and the electrochemically active species are formed, increasing the water:acetonitrile ratio does not lead to any substantial decrease of the voltammetric signal. This finding is important for prospective applications in HPLC with electrochemical detection of steroids requiring variability in water content for their successful separation.

4. Conclusions

For the first time an approach offering anodic electrochemical oxidation of bile acids in mixed organic–aqueous medium at reasonably low potential is presented. The signal can be obtained on various electrodes (BDD, GC, Pt). The saturated steroid core, known to be electrochemically inert, is activated by introduction of double bond(s) (via dehydration). This takes place directly in the medium of acetonitrile–water containing $HClO_4$, the latter also serving as the supporting electrolyte. Only primary BAs, possessing the axial 7α-hydroxyl group, can undergo the acid-induced dehydration step, which nevertheless proceeds rather slowly. Therefore, the voltammetric signal is time dependent. Identification of the products of both chemical and electrochemical reaction steps is under investigation. Applicability to cholesterol was also confirmed. Based on the current work, development of voltammetric methods and methods based on amperometric detection in liquid flow techniques can be envisaged. The novel approach presented here, inspired by enzyme-based or acid-induced dehydration reactions, can be viewed as a promising step on the way to a long-sought after tool aiming at implementation of electrochemical methods for characterization and detection of steroids that are currently deemed to be electrochemically inert.

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6.3 Publication III

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A novel voltammetric approach to the detection of primary bile acids in serum samples

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ABSTRACT

An innovative voltammetric approach to the detection of cholic and chenodeoxycholic acids is presented. These two primary bile acids are important biomarkers of liver function in humans and are involved in many physiological processes in the human body. Herein we describe a way to reproducibly convert the hard-to-detect bile acid molecule into an easily detectable derivative *in situ* using 0.1 M $HClO_4$ in acetonitrile (water content 0.55%). Under these conditions the bile acids are dehydrated and the resulting alkenes can be subsequently oxidized electrochemically on polished boron-doped diamond electrode under unchanged conditions at approximately +1.2 V *vs.* $Ag/AgNO_3$ in acetonitrile. After optimization, differential pulse voltammetry provides competitive limits of detection of 0.5 μM and 1.0 μM for cholic and chenodeoxycholic acid, respectively, with a linear course of calibration dependency to the minimum of 80 μM. The method was applied for detection of cholic and chenodeoxycholic acids in artificial and human serum samples using single solid phase extraction on C-18 cartridge for preliminary separation of the analytes. High recoveries of 80–90% were consistently obtained by the proposed voltammetric method and reference HPLC with fluorescence detection for human serum samples, confirming good selectivity for real-life samples.

1. Introduction

In mammals, biosynthesis of amphipathic bile acids (BAs) is one of the three regulatory pathways of cholesterol homeostasis [1,2]. It is a catabolic way of converting lipophilic cholesterol into a water-soluble readily excreted molecule. In liver, cholesterol is metabolized into BAs, which are referred to as primary. Newly biosynthesized BAs are then conjugated with glycine or taurine, secreted into the bile, and delivered to the lumen of the small intestine. After the secretion, in the distal small intestine and colon, BAs are deconjugated and approximately 95% of them are recovered via portal blood circulation. As the primary BAs travel through the gastrointestinal tract, they are modified by numerous anaerobic bacteria, which results in the formation of secondary BAs [3]. In humans, the primary BAs are cholic acid (CA) and chenodeoxycholic acid (CDCA) (Fig. 3), and the most common secondary BAs are the products of bacterial dehydroxylation at C-7 of the primary BAs, namely deoxycholic acid (DCA) for CA, and lithocholic acid for CDCA [4]. In young infants, only primary BAs are present as they lack the anaerobic intestinal flora, which is developed during the first year of life [5]. In humans and other mammals, BAs play various vital roles in the organism. They emulsify cholesterol, dietary lipids, and fat-soluble vitamins [4] and possess antimicrobial functions [6]. Bile acids have also been shown to act as important regulatory molecules at the nuclear level, and in fact act as hormones [7]. Moreover, BAs were implicated as co-carcinogens, particularly in cancers of the gastrointestinal tract, due to their inherent detergent effect on biological membranes [8].

There are several disorders in BAs biosynthesis in humans, where their determination is of a clinical importance [9]. The primary BAs, *i.e.*, CA and CDCA, and partially the secondary DCA (~20%) constitute more than 90% of the biliary BAs [5]. A block in the biosynthesis of BAs results in most disorders in deficiency of the primary BAs. A mixture of CA and CDCA is then administered to the patients in order to suppress the biosynthesis of cytotoxic BA precursors/artefacts and to restore the input of primary BAs

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into the enterohepatic circulation. Accumulation of the three major BAs in the human body (CA, CDCA, DCA) in abnormally high (submillimolar) concentrations either intracellularly, or extracellularly results in cytotoxicity [5]. In the case of a complete biliary obstruction, the plasma BA levels usually rise 10- to 20-fold. Moreover, CA, CDCA, DCA and their conjugates represent 95%–99% of the BA pool in systemic circulation upon liver injury and are the only BAs that accumulate up to micromolar concentrations in serum [10]. The serum BA values vary depending on the age of the subjects in between 4.2 ± 2.0 μM (female children) and 10.5 ± 4.3 μM (elderly females) for healthy individuals, and for gallstone patients in between 67.4 ± 27.0 μM (female adults) and 376.4 ± 218.1 μM (elderly males) [11]. Furthermore, compared to healthy individuals the concentrations of CA, CDCA, DCA and their conjugates were found to be significantly higher in 331 patients with a broad range of liver impairments [10]. Total primary BA levels are 2.4 ± 0.3 μM in healthy individuals, and 48.9 ± 5.4 μM in patients with liver diseases. Several recent studies have dealt with the determination of CA as a biomarker, whose concentration sharply increases in intestinal and liver disease/damage [12–15].

Development of analytical methods for the determination of BAs in biological matrices is complicated by the absence of chromophores and fluorophores in their molecules. Consequently, methods based on enzymatic reactions or combining GC and especially LC with MS are most frequently employed [16–18]. Conventional LC-MS/MS can be used for studying metabolic pathways of BAs and their sulfate metabolites [19] and evaluating the serum CA levels as the biomarkers of drug induced liver injury [14]. Furthermore, UPLC-MS is one of the most commonly employed methods in BA profiling and determination [20–23] and is used, *e.g.*, for monitoring the BA levels during liver transplantation [20], or to monitor circulating and hepatic BAs in accordance with the FDA guidelines [21]. Supercritical fluid chromatography with MS/MS detection serves as a more exotic example of BA analysis [24]. Other approaches, such as GC-based methods, and methods utilizing fluorescence detection (FLD) require laborious and time-consuming derivatization procedures: BAs can be determined as methyl ester-trimethylsilyl ether and methyl ester-acetate derivatives using GC–MS/MS [25]. Fluorescence detection can be employed in HPLC after derivatization of the sample with 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate [26], or with 4-nitro-7-*N*-piperazino-2,1,3-benzoxadiazole in pressurized capillary electrochromatography [27]. Nanomolar quantification limits are generally reported in these studies, which could be regarded unsubstantiated in clinical analysis where the primary BA values in humans are in a micromolar range. Furthermore, the separation step significantly prolongs and complicates the instrumental part of analysis so that it becomes considerably more costly, which limits its use in clinical practice. Therefore, faster and cheaper methods of BAs determination are highly desirable and sought after.

The following two approaches illustrate the pursuit of new biosensor platforms for CA determination. In the first approach, a biosensor platform is fabricated using 4-cyano-4′-pentylbiphenyl (5CB) liquid crystals laden with surfactant molecules (such as anionic SDS) in aqueous solution. Molecules of BAs, which also exhibit surfactant properties, then competitively adsorb at the surfactant-laden 5CB/aqueous interfaces, triggering a change in 5CB liquid crystal orientation, which is subsequently utilized for BA quantitation using polarizing optical microscopy [13,28–30]. However, experiments with real-life samples were not performed in these studies, therefore, the practical applicability of this approach remains rather questionable. The detection limits are: 12 μM [13], 5 μM [28], 5 μM for CA and 1 μM for CDCA together with interference experiment with ascorbic acid [29], and 20 μM for CA together with experiments in synthetic urine [30]. Such an approach is strictly limited by the pH of the studied solution, since deprotonated BA (at high pH values relative to the BA's pK_a) generates electrostatic repulsion between the BA and anionic surfactant. These repulsive interactions prevent the BA from adsorbing on the surfactant-laden interface, resulting in sharply increased detection limits (170 μM at pH 7.5 for CA, as in ref. [13]). The second approach is based on CA's ability to competitively bind and remove CA aptamers from the surface of gold nanoparticles (AuNPs) [15]. This leads to a change in the density of the aptamer adsorbed on the surface of the AuNPs. Consequently, such a change leads to a different growth morphology of the AuNPs, and therefore to a change in the VIS absorption spectrum of the solution. The resulting red shift with the increasing concentration of CA can be used for CA determination employing UV–VIS spectrophotometric detection. The linear dynamic range of this method is 1–15 μM with the detection limit of 1 μM, which is lower than that of the previously reported colorimetric detection method based on AuNPs [31]. The method has not been applied to real-life samples and, more importantly, was not tested for cross-reactivity, which could adversely affect the selectivity of this approach.

As we reviewed recently [32], electrochemistry on bare electrodes is not commonly employed for BA determination. However, as a fresh innovation, enzymatic methods were developed recently, which employ indirect electrochemical detection of BAs [33,34]. Here, the BA molecule does not get into contact with the electrode for direct redox process; instead, it is converted into 3-ketosteroid by 3α-hydroxysteroid dehydrogenase in the presence of NAD^+, which itself is reduced to NADH. Oxidation of NADH is then exploited for the indirect detection of BAs. The linear dynamic range of this enzymatic approach is 5–400 μM [33].

Only a few studies employing the electrochemical reduction of BAs (presumably catalytic reduction of the proton of the carboxylic group) can be traced in the literature [35–38]. The use of electrochemical oxidation in analytical application is similarly uncommon, being represented by only three chromatographic studies from the 1990s [39–41]. Two of these employed HPLC with pulsed amperometric detection on gold electrode, where three pulses (E_1 = +0.03 V, +0.05 V resp.; E_2 = +0.60 V; E_3 = −0.80 V, −0.60 V resp.) were applied. Aliphatic alcohols, such as non-activated BAs, are known to be virtually electrochemically inactive in voltammetry. In contrast to their aromatic counterparts, aliphatic alcohols cannot stabilize free-radical products of one-electron oxidation by π-resonance [42]. This results in their electrochemical inactivity on carbonaceous electrode materials. On the contrary, electrode materials with unsaturated valence orbitals (Au, Pt) can bind and stabilize free-radical products of one-electron oxidation of aliphatic alcohols. However, this catalytic activity is accompanied by rapid fouling of the electrode by accumulation of oxidation products. Hence, in voltammetric techniques, such electrodes appear, again, practically unable to oxidize aliphatic alcohols. However, it has been shown that in pulsed amperometric detection on gold working electrode [39,40], the detection and cleaning of the electrode can be achieved by applying three pulses (triple-pulse amperometry) with altering cathodic and anodic polarizations that minimises electrode fouling. Cleaning is then attained through (i) oxidative desorption of oxidation products by applying highly positive potentials while the surface is oxidized to AuO/PtO and (ii) removal of the mostly inert oxides by cathodic dissolution to restore the original electrochemical activity of the surface [43]. This approach is, however, fundamentally different from that we have used in the present study. Herein, the initial dehydration introduces double bond(s) into the BAs structure, which enables oxidation of the resulting alkenic species on carbonaceous (BDD, GC [44]) electrode materials, as discussed below.

Other two studies [39,40] were mainly focused on the chromatographic aspects rather than on the electrochemical detection

step; the detection limits ranged from 0.3 to 10 μM. The main disadvantage of this approach is that pulsed amperometric detection of components containing hydroxyl groups requires a pH value approaching 13, which seriously limits the use of the reversed-phase silica-based columns. Moreover, the linear dynamic range is relatively narrow (0.6–14 μM) [39]. The third study [41] was focused on the assay of ursodeoxycholic acid and related impurities in pharmaceutical formulations. It employed a coulometric detector containing two in-line porous graphite electrodes operating at anodic voltages of +0.60 V and +1.40 V. The quantification limit was 0.20–0.25 μg on-column weight. The electrochemical part of that study does not provide any in-depth information about the process: neither a cyclic voltammogram to illustrate the electrochemical behavior of the BAs under the conditions used are shown, nor is the reference electrode stated. Therefore, direct electrochemistry of BAs and their voltammetric determination is in fact still an uncharted territory, as illustrated by the absence of such methods in recently published reviews on the analytical platforms for BA measurement [17,18].

A breakthrough discovery in electrochemistry of BAs has been recently published by our group [44]. It has been shown that the primary BAs can be chemically activated *in situ* by using 0.1 M $HClO_4$ in acetonitrile, which serves both as a supporting electrolyte and a dehydrating agent. In this environment, the primary BAs undergo a dehydration similar to that in the Liebermann-Burchard reaction that is used in a colorimetric assay of cholesterol [45]. The resulting molecule can then readily undergo electrochemical oxidation on bare electrodes, such as platinum, glassy carbon, and boron-doped diamond (BDD) at potentials of approximately +1.2 V *vs.* Ag/$AgNO_3$ in acetonitrile. The oxidation is facilitated by the introduction of a double bond into the molecule (by the initial dehydration). The resulting unsaturated compounds are more prone to the removal of an electron from a bonding π-orbital, generating a reactive radical cation. The major primary BAs in human blood, CA and CDCA, can be oxidized under these conditions, since they possess the axial 7α-hydroxyl group in the antiperiplanar position to the axial 8β-proton, which renders the molecule particularly prone to dehydration. Secondary BAs are electrochemically inactive under equal conditions, as they lack this structural feature [44].

In this work, a direct and fast voltammetric method for detection of CA and CDCA was developed and applied for their determination in artificial and human serum. Boron-doped diamond electrode was used in this work: it represents an advanced electrode material with a large potential window, especially in the region of positive potentials, and, as a rule, a relatively low proclivity to adsorption of organic molecules and their reaction (by)products, as well as species present in physiological matrices [46–48].

2. Experimental

2.1. Chemicals

Cholic and chenodeoxycholic acids (both of >99% purity), human serum albumin, and human serum (human male AB plasma, USA origin, sterile-filtered) were purchased from Merck (Darmstadt, Germany). Acetonitrile (Merck, Germany; HPLC grade), perchloric acid (Penta, Czech Republic; p.a.) were used. All other commercially available chemicals were of analytical grade (if not stated otherwise).

Artificial serum was prepared according to the literature [49,50]. The following constituents were dissolved in deionized water: 4.5 mM KCl; 5 mM $CaCl_2$; 145 mM NaCl, 4.7 mM D-(+)-glucose; 2.5 mM urea; 0.1% human serum albumin. The solution was used immediately after preparation.

2.2. Voltammetric measurements

Voltammetric measurements were performed using a potentiostat PalmSens 2.0 with PSTrace 5 software. Boron-doped diamond working electrode (A_{geom} = 7.07 mm^2, Windsor Scientific, UK) with a flat inlaid disk was employed, routinely polished using alumina suspension (particle size 1.1 μm; Elektrochemické Detektory, Turnov, Czech Republic) in deionized water prior to each voltammetric scan. Electrochemical cell model SVC-3, and both the non-aqueous reference (model RE-7, Ag wire in 0.01 M $AgNO_3$, 1 M $NaClO_4$ in acetonitrile) electrode and the platinum wire counter electrode were all purchased from ALS (Tokyo, Japan). The solutions for voltammetric measurements were prepared by mixing the appropriate volume of the stock solution of the BA in acetonitrile ($c = 1 \times 10^{-4}$ M; $c = 1 \times 10^{-3}$ M for solutions with $c \geq 1 \times 10^{-4}$ M) with the solution of $HClO_4$ in acetonitrile. The contact time and temperature of CA/CDCA and $HClO_4$ solutions and their respective concentrations are given in the text and in the caption of each figure. The determination of water content in the solutions subjected to voltammetric measurements were performed by automated Karl Fischer titration on 831 KF Coulometer (Metrohm, Herisau, Switzerland).

Cyclic voltammetry (CV) and linear sweep voltammetry (LSV) were performed at scan rate of 50 mV s^{-1} if not stated otherwise. The optimized differential pulse voltammetry (DPV) parameters were as follows: step potential (E_{step}) = 5 mV; scan rate (v_{sc}) = 50 mV s^{-1}; pulse time (t_{pulse}) = 5 ms; and pulse potential (E_{pulse}) = 100-mV. The height of the voltammetric peaks was evaluated using the fixed baseline function of PSTrace 5 software by connecting the local minima before and after the peak.

The limit of detection (LOD) was calculated as 3 × SD of the peak height calculated from ten repeated measurements of the lowest detectable concentration divided by the slope of the linear concentration dependency.

2.3. HPLC-FLD measurements

A slightly altered HPLC-FLD procedure [26] based on derivatization of BAs with 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate (BDETS) was employed as a reference method. The standard addition method was used for quantification of BAs in an eluate after SPE. Potassium citrate (500 mg), dimethyl sulfoxide (100 μL), and a 1×10^{-3} M solution of BDETS in acetonitrile were added successively to 40 μL of the eluate after SPE (or to a mixture of 20 μL of the eluate after SPE and 20 μL of the standard solution of an appropriate BA in acetonitrile). The mixture was shaken for 30 s and then heated at 95 °C for 30 min. Next, the mixture was centrifuged at 13,000*g* for 10 min, the supernatant was filtered and analysed by HPLC-FLD.

Fluorescently labelled BAs derivatives were separated on a column Supercosil LC-18 (250 × 4.6 mm; 5 μm particle) guarded by precolumn Superguard LC-18 (2 cm); the column temperature was maintained at 30 °C. The mobile phases were acetonitrile (mobile phase A) and a mixture of 40×10^{-3} M aqueous solution of ammonium formate (with pH = 3.56) and acetonitrile 50:50 (*v*/*v*; mobile phase B). The gradient program begun with 100% mobile phase B. The proportion of solvent A was increased from 0 to 70% over a 37.5 min period, next was increased to 100% over a 17.5 min period, and finally kept constant for an additional 1 min prior to recycling to initial conditions over a 4 min period. The flow rate of mobile phase was constant at 1.0 mL min^{-1}. The

injected volume of the sample was 25 μL. The fluorescence excitation and emission wavelengths, λ_{ex} and λ_{em}, were 333 and 390 nm, respectively.

2.4. Solid phase extraction procedure

A marginally modified extraction procedure found in the literature was employed [51]. Supelco Discovery DSC-18 (3 mL, bed wt. 500 mg) tubes with covalently bonded octadecyl group to the end-capped stationary phase were obtained from Merck (Darmstadt, Germany). A 5 mM solution of CA and CDCA in methanol was prepared and used to spike the serum samples. Sample of 20 μM BA in artificial/human serum was mixed 1:1 with 0.05% formic acid. The SPE column was preconditioned with 3 mL methanol and 3 mL 0.05% formic acid. Then 12 mL of the sample and formic acid mixture (1:1) was introduced on the SPE column which was washed with 2 mL H_2O and 2 mL 5% methanol aqueous solution. After washing, BAs were eluted with 2 mL of methanol and 4 mL of acetonitrile. A 1 mL aliquot of the eluate was analyzed using HPLC-FLD. The rest was evaporated under nitrogen at 80 °C, reconstituted in 3 mL acetonitrile, and then subjected to DPV. The solutions of artificial/human serum extracts for the DPV measurements were prepared as follows: 900 μL of the reconstituted eluate after SPE; 100 μL of 2.2 M $HClO_4$ in acetonitrile; corresponding volume of 1×10^{-4} M solution of BA in acetonitrile as a standard addition; and acetonitrile to give a total volume of 2.2 mL.

2.5. Data analysis

Results obtained by the newly developed DPV method and by the previously described reference HPLC-FLD method were compared using unpaired two-tailed Student's *t*-test. Statistical analysis was performed using MS Excel (Redmond, WA, USA). The confidence level for accepting/rejecting the null hypothesis was set to be 95%. The null hypothesis was therefore accepted when the p value obtained with the *t*-test was greater than 0.05.

3. Results and discussion

In our previous work [44] we have demonstrated that the key to obtain the voltammetric oxidation signal of CA and CDCA at *ca* + 1.2 V *vs.* $Ag/AgNO_3$ in acetonitrile on a bare electrode is their dehydration reaction in 0.1 M $HClO_4$ (simultaneously used as the supporting electrolyte) in acetonitrile. Preliminary results on identification of products of dehydration reaction of CDCA and CA using NMR spectroscopy indicate that the acid-catalyzed elimination of 7α-hydroxyl is followed by hydrogen shift stabilization of the cationic intermediate and results in the formation of a mixture of products, even with unsaturated C or D ring of the steroid skeleton. The following electrochemical oxidation gives rise to a mixture of ketonic and diketonic species, as confirmed by HPLC-MS measurements. Fig. 1 shows the proposed mechanism of the chemical activation and electrochemical oxidation reactions (for CDCA, similar process is expected for CA), on which the determination of CA and CDCA is based. Initially, the BAs are activated by dehydration reaction with $HClO_4$ in acetonitrile as proposed in ref. [44] and shown as the first step in Fig. 1. Then, one electron is electrochemically abstracted from the activated BA **1**. This reaction gives rise to a radical-cationic species **2** that subsequently reacts with a water molecule, acting as a nucleophile, and by losing one electron and two protons is oxidized to a ketonic species **3**. Overall, the proposed mechanism is thus a two electron two proton electrochemical process. Naturally, investigation of the mechanism of both chemical and electrochemical reaction steps, especially potential double bond migration in the steroid core and location of the keto group, is subject of our continuous effort and will be presented in a separate study.

In this study, BDD was selected as the electrode material for the voltammetric measurements, and acetonitrile as a solvent because it is hardly oxidizable and thus exhibits a wide potential window in the region of positive potentials. Other solvents miscible with water, such as methanol, ethanol, or isopropanol, are oxidizable by the HO• radicals generated at BDD electrode at positive potentials by water anodic decomposition, and thus their presence in the solution lowers the anodic potential limits [52,53]. To overcome fouling of the electrode surface (decline of peak heights by 26% for CA and 36% CDCA during 3 consecutive scans was observed), the BDD surface was mechanically polished after each scan. Anodic activation of the electrode surface, commonly used for the *in situ* regeneration of BDD surface [47], was rather ineffective. It is based on powerful oxidation of the species fouling the electrode surface by HO· radicals generated at highly positive potential as explained above. Nevertheless, their generation is presumably limited in the medium used, owing to the minimal water content and thus this approach was not effective. Cathodic activation was similarly ineffective, leading to poorly reproducible voltammetric response. This effect progressively worsened with increasing cathodic activation time; therefore, mechanical polishing was chosen as the most suitable surface renewal approach.

The nature of the electrochemical oxidation process was investigated using LSV with progressively increasing scan rate (5–500 mV s^{-1}) in consecutive measurements. After evaluating the log i_p (in nA) *vs.* log v (in mV s^{-1}) dependencies, the slope values were determined to be 0.53 ± 0.01 for CA and 0.46 ± 0.02 for CDCA, which points to a diffusion-controlled process with the theoretical slope value of 0.5. This is further supported by the obtained i_p *vs.* v and i_p *vs.* $v^{1/2}$ dependencies. On one hand, by evaluating the i_p *vs.* $v^{1/2}$ dependency, linear regression equation with correlation coefficient of 0.9989 for CA (Eq. (1)) and with correlation coefficient of 0.9977 for CDCA (Eq. (2)) were obtained on datasets that yielded reasonably straight lines (Supplementary Information Fig. S1).

$$i_p[nA] = 72.3 \times v^{1/2}\left[mV^{1/2}s^{-1/2}\right] + 71 \quad (1)$$

$$i_p[nA] = 86.2 \times v^{1/2}\left[mV^{1/2}s^{-1/2}\right] + 12 \quad (2)$$

On the other hand, i_p *vs.* v dependency yielded concave functions, obviously with significantly lower correlation coefficients of 0.9639 for CA and 0.9868 for CDCA, again pointing to a diffusion-controlled process in which i_p is directly proportional to the square root of v. Thus, naturally, attempts at accumulating the reactants on the electrode surface to use adsorptive voltammetric mode of measurement were not successful.

3.1. Optimization of conditions for the chemical and electrochemical reaction steps

Further extensive work was devoted to optimization of the conditions for effective voltammetric detection of CA and CDCA. Their dehydration reaction in 0.1 M $HClO_4$ (simultaneously used as the supporting electrolyte) in acetonitrile is a rather slow reaction, and it takes approximately an hour before the voltammetric signal at +1.2 V stabilizes [44]. Thus, for the development of a voltammetric procedure usable for determination of these primary BAs factors influencing the chemical reaction step (water content in the solution of dehydrating agent in acetonitrile, type of dehydrating agent, heating temperature/time) and electrochemical oxidation step (effect of water content and pH of the measured solution on the signal height and stability, and the parameters of DPV used for quantitation) were investigated first.

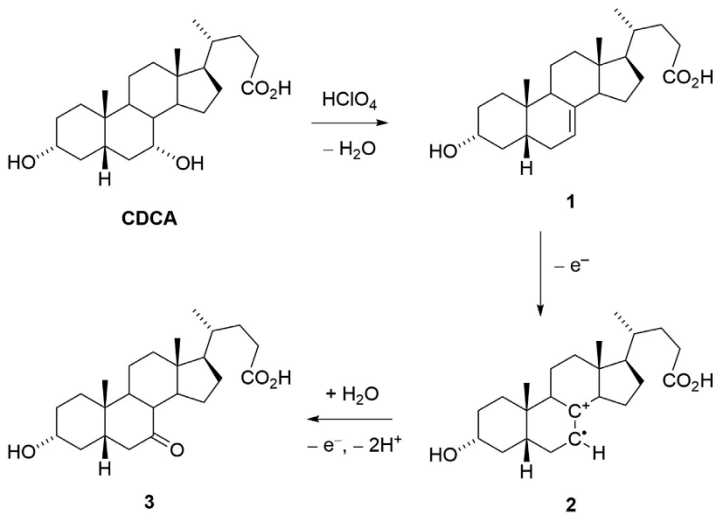

Fig. 1. Proposed mechanism of the chemical activation (dehydration) and subsequent electrochemical oxidation of CDCA.

3.1.1. Chemical reaction step

3.1.1.1. Water content in dehydrating solutions. Naturally, the water content in the mixture of acetonitrile and dehydrating agent is an important factor when considering efficiency of the dehydration reaction. For 0.1 M $HClO_4$ in acetonitrile, used in the voltammetric measurements, the calculated minimal amount in the solution (0.43%) is given by water present in the 70% concentrated solution of perchloric acid. The actual water content increased by air humidity is 0.550 ± 0.002% (240 mM), as determined by Karl Fischer titration.

Any further increase in the water content in dehydrating solutions leads to diminishing anodic voltammetric signal of the BA at *ca* + 1.20 V as illustrated in Fig. 2 for CDCA. Therefore, it is desirable to keep the water content to the minimum when dehydration reaction proceeds. Nevertheless, once dehydration is completed and the electrochemically active species are formed, increasing the water:acetonitrile ratio does not lead to any substantial decrease of the voltammetric signal as shown in Fig. 5 and discussed below.

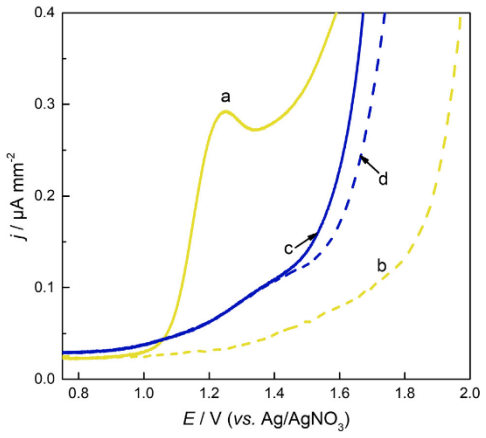

Fig. 2. Linear sweep voltammograms of CDCA ($c = 1 \times 10^{-4}$ M; curves a, c) in 0.1 M $HClO_4$ in acetonitrile. Supporting electrolyte in dashed lines (b, d). Water content (a, b) 0.550 ± 0.002%, (c, d) 10.0%; ambient temperature 21 °C. Scan rate 50 mV s^{-1}.

3.1.1.2. Heating temperature and time. At ambient temperature the dehydration step proceeds with a different rate for CA and CDCA, resulting in a CV signal *ca* 2.25 × higher for CDCA than that for CA [44]. To increase the dehydration reaction rate and to unify the signal response of both BAs a heating step was introduced. When the temperature is increased from 10 °C to 50 °C, the voltammetric signal rises in an exponential-like manner (Supplementary Information Fig. S2A), and it rises linearly with the increasing heating time (Supplementary Information Fig. S2C). After being heated up to 50 °C for 5 min, relatively well-developed, stable and under these conditions irreversible voltammetric signals of comparable intensity were obtained with the peak potentials of approximately +1.2 V in CV (Fig. 3). Corresponding CV reduction peak was not observed even at high scan rates of 5 V s^{-1}. These parameters were chosen with respect to the time consumption and signal stability – after heating the solution at 50 °C for 5 min, the signal remains stable with the relative standard deviation (RSD) of 2.9% over the course of 95 min. The same heating time applied at lower temperature proved not to be sufficient for completion of the dehydration reaction, as the voltammetric signal further slightly increases during the 95 min period (Supplementary Information Fig. S2B). Importantly, long periods of heating paradoxically lead to loss of the CA signal at +1.2 V, as demonstrated by cyclic voltammograms in Fig. 3A, where a decrease of this signal by *ca* 55% can be observed when extending the heating period to 30 min. Simultaneously, longer heating resulted in the formation of an additional peak appearing at approximately +0.67 V in the recorded cyclic voltammogram of CA (Fig. 3A). Thus, by significantly prolonging the heating period to 30 min, it is possible to distinguish between CA and CDCA. Obviously, the newly emerging, under used conditions irreversible,

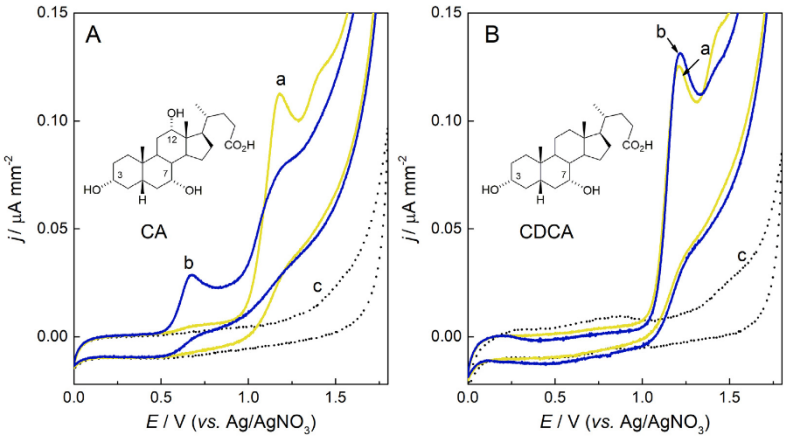

Fig. 3. Cyclic voltammograms of (A) CA, and (B) CDCA ($c = 5 \times 10^{-5}$ M) in 0.1 M $HClO_4$ in acetonitrile (water content 0.550 ± 0.002%). Yellow lines (a): heating 5 min / 50 °C; blue lines (b): heating 30 min/50 °C; dotted lines (c) correspond to supporting electrolyte. Scan rate 50 mV s^{-1}.

anodic signal of CA can be attributed to the oxidation of a further dehydrated product, whose formation is connected to C-12α-hydroxyl group (axial), which is absent in CDCA. The signal of CDCA only slightly rose at +1.2 V during the 30 min heating period (Fig. 3B), because the molecule cannot be dehydrated any further.

3.1.1.3. Supporting electrolyte/dehydration agent effect. The efficiency of the dehydration reaction is likely to be dependent on the dehydration agent [44]. Therefore, feasibility of other inorganic acids as dehydrating agents was investigated. Under ambient temperature (21 °C) only 0.1 M $HClO_4$ succeeded, and well-developed anodic signals of CA and CDCA were observed. Other acids, namely 0.1 M H_2SO_4 or H_3PO_4, were ineffective. After implementing the heating step (50 °C for 5 min), a weaker signal (approximately one third compared to that seen for $HClO_4$) was observed with H_2SO_4 (Fig. 4). No signal was detected when H_3PO_4 was tested. Thus, 0.1 M $HClO_4$ was used as an optimal dehydrating agent throughout this work.

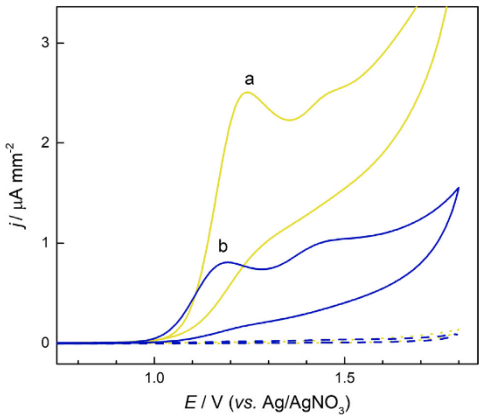

Fig. 4. Cyclic voltammograms of CDCA ($c = 9 \times 10^{-4}$ M) in (a) 0.1 M $HClO_4$ (water content 0.550 ± 0.002%), and (b) 0.1 M H_2SO_4 (water content 0.04%) in acetonitrile. Solutions were heated up to 50 °C for 5 min before the measurements. Supporting electrolyte in dotted ($HClO_4$) and dashed (H_2SO_4) lines of corresponding colour. Scan rate 50 mV s^{-1}.

3.1.2. Electrochemical step

Water content in dehydrating solution greatly affects formation of the electrochemically active species, as shown in Fig. 2 and discussed above. The dehydration reaction is most efficient when minimal water content is present. Nevertheless, after the acid-induced dehydration is completed, water content can be increased. It can be beneficial to modify (i) the ratio aqueous phase:acetonitrile and (ii) pH of the solution for selected analytical applications, *e.g.*, for HPLC, where variability in organic:aqueous phase ratio in mobile phase is often required to achieve effective separation elution of the analytes. Therefore, the effect of water content and pH of the measured solutions on the intensity and stability of anodic CV signal of CA and CDCA was investigated.

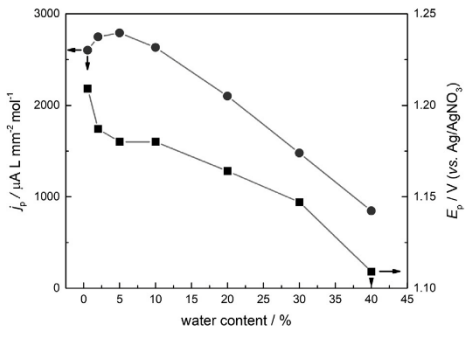

Fig. 5. Effect of water content (0.55, 2, 5, 10, 20, 30, and 40%) on (●) CV peak current densities divided by molar concentration (j_p) and on (■) CV peak potential (E_p) of CDCA ($c = 9 \times 10^{-4}$ M). Solution of CDCA was heated up to 50 °C for 5 min and left standing at ambient temperature (21 °C) for additional 90 min before the measurements. Supporting electrolyte: 0.1 M $HClO_4$ in acetonitrile. Evaluated from CV, scan rate 50 mV s^{-1}.

3.1.2.1. Water content in voltammetry. The water content was increased after completion of the dehydration reaction, *i.e.*, 5 min heating at 50 °C of the solution of the BA in 0.1 M $HClO_4$ in acetonitrile. As shown in Fig. 5 for CDCA, the anodic signal of the BA is present for water content from 0.55% (naturally present) to 40%, and its oxidation potential gradually shifts to less positive values with the total shift being approximately 100 mV between 0.55% and 40% of water. Simultaneously, the signal intensity rises slightly when the water content is increased from 0.55% (intrinsically present) to 5%, and then sharply decreases to a minimum observed at 40% (current density per molar concentration is an adjusted signal response unit to compensate for the dilution by water addition). Despite the progressive decline of the signal to approximately one third (at 40% of water) of the original value at intrinsic 0.55% of water, it is still well developed which is promising for electrochemical detection in separation techniques as mentioned above. However, for the voltammetric experiments there was no need to adjust the water content as the signal rise is minor and the inessential step brings redundant uncertainty to the procedure. Therefore, in the solutions used in this work, the intrinsic water content of 0.550 ± 0.002% was kept.

3.1.2.2. pH stability of the voltammetric signal. The effect of pH on the intensity and stability of the anodic voltammetric signal of the BAs was investigated by addition of a concentrated aqueous solution of NaOH to the reaction mixture after completion of the acid-induced dehydration step. The dependency of the signal on pH was monitored to assess the feasibility of transferring the voltammetric procedure to liquid flow systems, such as HPLC with amperometric detection. The pH requirements of such techniques are strict because of the lower stability of standard columns under extreme pH values. It is obvious from Fig. 6 that the course of the voltammograms of CDCA is stable in acidic to basic pH values (1–12.5). Only at higher pH values (pH ≥ 12.6) the signal was found to be shifted towards the less positive potentials, while the course of the cyclic voltammogram flattened. This seems to indicate a change in the mechanism of the redox reaction including salt formation in the basic media, which results in a more sluggish electron transfer. At the same time, only marginal decrease in CA signal intensity was observed up to pH 13 (Supplementary Information Fig. S3). The change in the course of cyclic voltammograms with the rising pH was found not to be as pronounced as for CDCA, indicating different structure of dehydration products of CA compared to those of CDCA. Nonetheless, in order to keep the voltammetric procedure simple, there was no need to adjust the pH, and pH 1 given by the concentration of $HClO_4$ was used.

3.2. Determination of the primary BAs using DPV

With respect to the irreversible course of the redox reaction, DPV was chosen for quantitation of CA and CDCA. It is based on evaluating the peak occurring at *ca* + 1.2 V in CV (*ca* + 1.1 V in DPV), which is obtained after 5 min of heating to 50 °C in 0.1 M $HClO_4$ in acetonitrile (water content 0.550 ± 0.002%). Differential pulse voltammetry parameters for the BAs determination were optimized (Supplementary Information Fig. S4) by varying the four DPV parameters (step potential, scan rate, pulse time, and pulse potential) – one at the time, while maintaining the remaining three parameters constant. The parameter providing the highest and well-developed signal response (*i.e.*, step potential 5 mV; pulse time 5 ms; and pulse potential 100 mV) was used for the DPV measurements. The selected scan rate of 50 mV s^{-1} is the maximal possible value of the instrument used in this study.

The signal response decreases with consecutive scans due to passivation of the working electrode. To eliminate this problem, the working electrode was polished using alumina suspension between individual scans.

To illustrate the possibility of determination of the sum of both BAs as total primary BA pool, DP voltammograms of CA, CDCA and a mixture of the two are presented in Supplementary Information

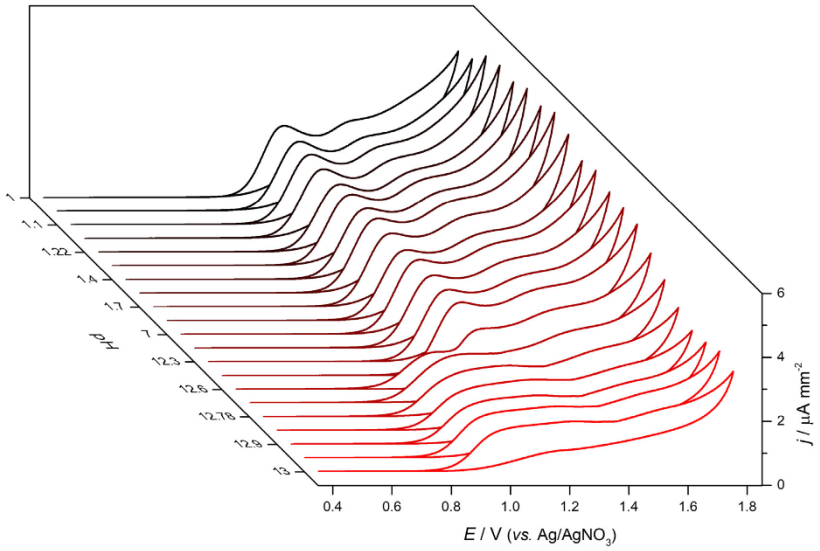

Fig. 6. Cyclic voltammograms of CDCA ($c = 9 \times 10^{-4}$ M) at various pH (1–13) after completion of the acid-induced dehydration. The solution of CDCA was heated up to 50 °C for 5 min in 0.1 M $HClO_4$ in acetonitrile (water content 0.550 ± 0.002%). Alkalization of the solution was achieved by gradually adding 10 M NaOH aqueous solution. Scan rate 50 mV s^{-1}.

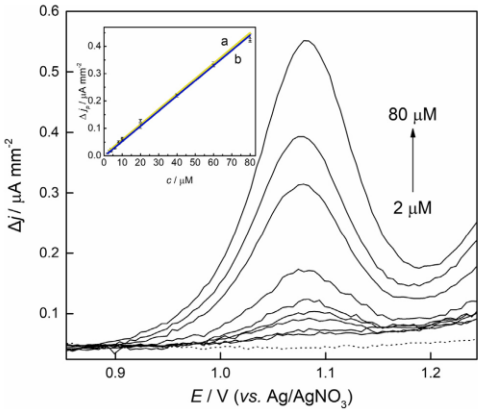

Fig. 7. Differential pulse voltammograms of CA (2, 4, 6, 8, 10, 20, 40, 60, and 80 μM) obtained under optimized conditions. Inset: Calibration dependencies of (a) CDCA, and (b) CA obtained using DPV (error bars represent SD). Each solution of the BA was heated up to 50 °C for 5 min before the measurement. Supporting electrolyte: 0.1 M $HClO_4$ in acetonitrile in dotted line (water content 0.550 ± 0.002%).

Fig. S5. While maintaining constant equimolar concentration of the primary BAs – CA, CDCA (90 μM) and CA + CDCA (each 45 μM, total BAs 90 μM), the peak height at + 1.1 V in DPV remained stable with RSD of 0.6% for the three solutions. The height of the second peak (not used for determination) at *ca* + 1.35 V varies considerably but this is not an issue for the analytical process based on the evaluation of the first peak occurring at + 1.1 V.

Differential pulse voltammograms corresponding to the concentration dependency of CA are shown in Fig. 7. The current response for CA and CDCA is comparable, as demonstrated by the inset in Fig. 7 and further by Fig. S5 in Supplementary Information. The parameters of the calibration dependencies are summarized in Table 1, where similar slope for CA and CDCA is reported. Such similarity allows determination of the sum of both BAs as the total primary BA pool. The median current density of the signal and its RSD for 5 measurements of the 6 μM calibration point is 26.2 nA mm^{-2} (RSD 8.1%) for CA and 30.3 nA mm^{-2} (RSD 9.4%) for CDCA. For the 60 μM calibration point it is 330 nA mm^{-2} (RSD 1.5%) for CA and 334 nA mm^{-2} (RSD 2.7%) for CDCA. Importantly, the interday variability (reproducibility) of the peak heights is 4.3% when evaluated from 4 consecutive days (CV, for CDCA).

The LODs were estimated from the lowest measurable concentration, *i.e.* 2 μM for CA and 3 μM for CDCA. These values are comparable or notably lower than those reported in the literature aiming at low-cost analytical methods for CA [13,15,28–31] and CDCA determination [29].

3.3. Determination of CA and CDCA in artificial and human serum

The applicability of our new voltammetric procedure was verified by determination of CA and CDCA in artificial and human serum samples, spiked with CA and CDCA to provide 20 μM concentration of the BAs. An extraction step had to be implemented into the procedure, as the excessive water content in serum would not allow the chemical activation of these BAs and thus the direct voltammetric determination. The extraction was performed employing marginally modified literature procedure using C-18 SPE cartridges [51]. Bile acids are retained in non-dissociated form on these cartridges when the acidified serum sample is loaded, as their p*Ka* values are ~4.8–5.0 [54]. They were determined using standard addition method and compared with the results obtained by a reference HPLC-FLD method, modified from ref. [26].

3.3.1. Artificial serum

The extraction procedure was tested and slightly adjusted during experiments with artificial serum. The extracts treated with 0.1 M $HClO_4$ in acetonitrile provided well-developed DPV peaks at the potential of + 1.1 V that rose linearly with the standard addition of CA/CDCA (Fig. 8A for CA, Supplementary Information Fig. S6A for CDCA). Artificial serum with no BA spike was used as a negative control. It provided no voltammetric signal in the studied potential range, which further indicates that no constituent of the artificial serum interferes with the determination at the given concentration levels. The figure of merit for the determinations of CA and CDCA is shown in Table 2. The values obtained by DPV and HPLC-FLD were compared using Student's *t*-test to determine whether there is a significant difference in the results. The null hypothesis was set to state that any discrepancies in the obtained results are purely due to random and not systematic errors. For artificial serum, recovery of CA using DPV is 95 ± 8.7%, which correlates very well with the HPLC-FLD determination (95 ± 5.5%; p = 0.90). Recovery of CDCA using DPV was found to be 73 ± 4.7%, and 79 ± 6.0% using HPLC-FLD, which is a non-significant difference according to the *t*-test result of p = 0.29. Lower recoveries are most likely due to lower extraction efficiency of the SPE cartridge towards CDCA in artificial serum. To address this issue the extraction procedure should be further optimized.

3.3.2. Human serum

Human serum was spiked, and the samples were treated using the same procedure as that for the artificial serum samples. Neither of the two BAs was detected in non-spiked control human serum using the proposed DPV procedure (dotted line in Fig. 8B for CA and Supplementary Information Fig. S6B for CDCA) and HPLC-FLD. The resulting DPV peaks for spiked human serum samples are positioned at slightly more positive potential compared to the artificial serum samples, and their development is poorer, which is most likely due to the more complex nature of human serum. Again, there is no significant difference (CA p = 0.22, CDCA p = 0.28) between the quantitation of CA and CDCA in human serum obtained with the DPV and HPLC-FLD methods. Slightly lower recoveries obtained with DPV (82% and 80% for CA and CDCA, respectively) compared to HPLC-FLD (89% and 88%, respectively) are presumably caused by passivation of the electrode prior to the oxidation reaction of the BAs in DPV. This is supported by the occurrence of peak "X" at approximately + 0.88 V, which was present in the analysed samples of human serum (illustrated in Fig. 8B), and which is not attributed to the BAs. It obviously belongs to an electrochemically active constituent of human serum that was co-extracted together with the BAs.

Table 1
Figure of merit for CA and CDCA DPV determination in 0.1 M $HClO_4$ in acetonitrile.

Bile acid	Slope (nA L mm^{-2} μmol^{-1})	Intercept (nA mm^{-2})	r	LOD (μM)
CA	5.54 ± 0.09	−2.97 ± 2.12	0.9991	0.5
CDCA	5.58 ± 0.18	+2.68 ± 2.94	0.9962	1.0

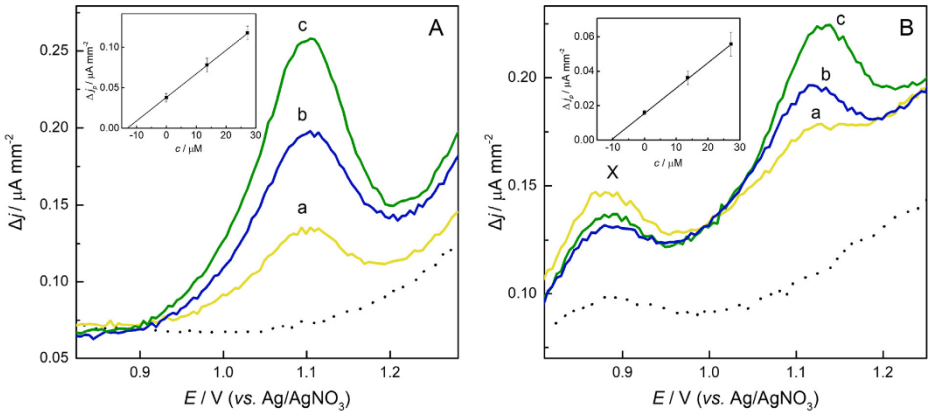

Fig. 8. Differential pulse voltammograms of CA after extraction from (A) artificial serum, and (B) human serum obtained under optimized conditions. (a) Sample, (b) standard addition of c = 13.6 μM CA, and (c) standard addition of c = 27.3 μM CA, control extract with no spike in dotted line. Solutions were heated up to 50 °C for 5 min before the measurements. Supporting electrolyte 0.1 M $HClO_4$ in acetonitrile (water content 0.550 ± 0.002%). Insets: Standard addition dependency with the respective extrapolation used for quantitation of CA, error bars represent SD. Pearson's r greater than 0.9999 for both (A) and (B).

Table 2
Figure of merit for CA and CDCA DPV determination in artificial and human serum. Uncertainty represents standard deviation (SD) for 5 repeated measurements.

BA		DPV		HPLC-FLD		t-test/p value
	c added (μM)	c found ± SD (μM)	recovery ± SD (%)	c found ± SD (μM)	recovery ± SD (%)	
Artificial serum						
CA	20.0	19.0 ± 1.7	95 ± 8.7	19.0 ± 1.1	95 ± 5.5	0.90
CDCA	20.0	14.6 ± 0.9	73 ± 4.7	15.8 ± 1.2	79 ± 6.0	0.29
Human serum						
CA	20.0	16.3 ± 0.9	82 ± 4.5	17.8 ± 1.1	89 ± 5.5	0.22
CDCA	20.0	15.9 ± 1.2	80 ± 6.0	17.5 ± 0.8	88 ± 4.1	0.28

Overall, the t-test results again suggest no significant difference between the results provided by the newly developed DPV and HPLC-FLD methods, hence, there is no statistical corroboration of a systematic error in the procedure. Recoveries of 80–90%, consistently attained by both methods for human serum samples, are most likely caused by reduced extraction efficiency of the SPE procedure. Importantly, the proposed DPV method is selective for determination of primary BAs in real-life human serum samples, representing a complex matrix, and it enables detection of concentration levels of primary BAs in serum of population with a variety of liver disease/damage. In male adult gallstone patients, the serum BA values (most of which is constituted by the primary BAs) rise more than 19-fold to 84.2 μM, and in elderly males even 42-fold to 376.4 μM, compared with healthy individuals [11]. In any case, in gallstone patients, the BA concentration rises at least twice in both males and females, and probably much more. Generally, in the case of various liver diseases, the concentration of primary BAs rises 20-fold to 48.9 μM [10]. Therefore, when only binary decision is required, extremely sensitive methods can be excessive, and simple positive or negative answer may suffice for preliminary examination.

4. Conclusion

In this work a novel proof of concept voltammetric method for CA and CDCA determination is presented. This approach enables BAs electroanalysis in a similar fashion in which the Liebermann-Burchard reaction enabled colorimetric determination of cholesterol [45]. In a very simple way, it reproducibly converts the hard-to-detect primary BA molecules into easily detectable derivatives *in situ*. Primary BAs are important biomarkers of liver function in human. This method provides competitive limits of detection of 0.5 μM for CA and 1.0 μM for CDCA. It was applied to the real-life determinations of both BAs in human blood serum. The proposed DPV method has shown satisfactory accuracy, providing results that are not significantly different from the values obtained with a previously described HPLC-FLD method. The BAs concentration usually increases several-fold to tens to hundreds of micromoles per litre levels in patients suffering from various liver and other diseases. Therefore, there is a considerable variety of possible applications in clinical diagnosis. In the future, the method could serve as an attractive alternative to the existing but instrumentally more demanding methods, such as LC-MS, especially in clinical laboratories with limited access to expensive instrumentation. The potential of applications in liquid flow systems, hyphenated with electrochemical detection, can be envisaged, based on the results presented herein, such as the possibility of pH adjustment and increasing the water content without losing the voltammetric signal. To further substantiate the feasibility and merit of this approach, pilot experiments with flow injection analysis were performed. In those experiments, no electrochemical or mechanical activation of the electrode appears to be necessary for obtaining stable response

of the primary BAs. Among the main benefits of our method are the low cost per measurement, short total analysis time, simplicity, and the associated robustness.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2020.107539.

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6.4 Confirmation of participation

1. **Klouda J.**, Barek J., Nesměrák K., Schwarzová-Pecková K.: Non-enzymatic Electrochemistry in Characterization and Analysis of Steroid Compounds, *Crit. Rev. Anal. Chem.* **47** (2017) 384–404. **80%**
2. **Klouda J.**, Barek J., Kočovský P., Herl T., Matysik F.M., Nesměrák K., Schwarzová-Pecková K., Bile acids: Electrochemical oxidation on bare electrodes after acid-induced dehydration, *Electrochem. Commun.* **86** (2018) 99–103. **70%**
3. **Klouda J.**, Nesměrák K., Kočovský P., Barek J., Schwarzová-Pecková K., A novel voltammetric approach to the detection of primary bile acids in serum samples, *Bioelectrochemistry* **134** (2020) 107539. **75%**

I confirm that the information given above is true, complete and accurate.

Prague, July 10, 2020 ……

Assoc. Prof. RNDr. Karolina Schwarzová, Ph.D.

6.5 Publications in impacted journals

1. **Klouda J.**, Barek J., Nesměrák K., Schwarzová-Pecková K.: Non-enzymatic Electrochemistry in Characterization and Analysis of Steroid Compounds, *Crit. Rev. Anal. Chem.* **47** (2017) 384–404. **IF_{2019} = 4.568** (WOS)
2. Schwarz D., Noda Y., **Klouda J.**, Schwarzová-Pecková K., Tarabek J., Rybacek J., Janousek J., Simon F., Opanasenko M.V., Cejka J., Acharjya A., Schmidt J., Selve S., Reiter-Scherer V., Severin N., Rabe J.P., Ecorchard P., He J.J., Polozij M., Nachtigall P., Bojdys M.J.: Twinned Growth of Metal-Free, Triazine-Based Photocatalyst Films as Mixed-Dimensional (2D/3D) van der Waals Heterostructures, *Adv. Mater.* **29** (2017) 1703399. **IF_{2019} = 27.398** (WOS)
3. **Klouda J.**, Barek J., Kočovský P., Herl T., Matysik F.M., Nesměrák K., Schwarzová-Pecková K., Bile acids: Electrochemical oxidation on bare electrodes after acid-induced dehydration, *Electrochem. Commun.* **86** (2018) 99–103. **IF_{2019} = 4.333** (WOS)
4. **Klouda J.**, Nesměrák K., Kočovský P., Barek J., Schwarzová-Pecková K., A novel voltammetric approach to the detection of primary bile acids in serum samples, *Bioelectrochemistry* **134** (2020) 107539. **IF_{2019} = 4.722** (WOS)

6.6 Oral presentations

6.6.1 Conference proceeding (indexed in WOS)

1. **J. Klouda**, K. Nesměrák, K. Schwarzová-Pecková Bile Acids: Possibilities of Anodic Oxidation in Non-aqueous and Mixed Media at Selected Electrode Materials, in: XXXVI. Modern Electrochemical Methods, Jetřichovice, Czech Republic, 23 – 27 May 2016. Oral Presentation, Book of Abstracts, p. 100.
2. **J. Klouda**, D. Bavol, A. Economou, J. Zima, J. Barek, H. Dejmkova, Simultaneous Determination of Antioxidants by Flow-Injection Analysis with Multiple-Pulse Amperometric Detection, in: 14th ISC 'Modern Analytical Chemistry', Prague, Czech Republic, 21 – 22 September 2017. Oral presentation. Book of Abstracts, p. 154.
3. **J. Klouda**, J. Barek, and K. Schwarzová-Pecková, Voltammetric Determination of Cholic and Chenodeoxycholic Acid on Boron Doped Diamond Electrode, in: XXXVIII. Modern Electrochemical Methods, Jetřichovice, Czech Republic, 21 – 25 May 2018. Oral Presentation, Book of Abstracts, p. 123.
4. **J. Klouda**, J. Barek, K. Nesměrák, and K. Schwarzová-Pecková, Voltammetric Determination of Cholic and Chenodeoxycholic Acids in Artificial Serum, in: XXXIX. Modern Electrochemical Methods, Jetřichovice, Czech Republic, 20 – 24 May 2019. Oral Presentation, Book of Abstracts, p. 135.

6.6.2 Conference abstracts

5. **J. Klouda**, J. Barek, K. Schwarzová-Pecková, Electrochemical Oxidation of Primary Bile Acids, in: 22nd Meeting of the Portuguese Electrochemical Society, Ponta Delgada, Portugal, 19 – 22 June 2017. Oral Presentation, Book of Abstracts, p. 25.
6. **J. Klouda**, J. Barek, and K. Schwarzová-Pecková, Rediscovering Electrochemistry for Sterol Determinations, in: 1st Cross-Border Seminar on Electroanalytical Chemistry, Furth im Wald, Germany, 4 – 6 April 2018. Oral Presentation. Book of Abstracts, p. 10.
7. **J. Klouda**, J. Barek, Frank-Michael Matysik, and K. Schwarzová-Pecková, Elektrochemická oxidace chemicky aktivovaných žlučových kyselin, in: 70. Sjezd českých a slovenských chemických společností, Zlín, Czech Republic, 9 – 12 September 2018. Oral Presentation. Book of Abstracts, p. 387. ***The presentation was part of "The Metrohm Young Chemist Award" competition national finals.***

8. **J. Klouda**, A. Zarybnická, J. Barek, and K. Schwarzová-Pecková, Voltammetric Determination of Smith-Lemli-Opitz Syndrome Biomarker 7-Dehydrocholesterol, in: 2nd Cross-Border Seminar on Electroanalytical Chemistry, Budweis, Czech Republic, 10 – 12 April 2019. Oral Presentation. Book of Abstracts. ***Best presentation award: 1st place.***

9. **J. Klouda**, A. Zarybnická, J. Barek, and K. Schwarzová-Pecková, A Novel Approach to 7-Dehydrocholesterol Determination in Smith-Lemli-Opitz syndrome Diagnosis, in: XXV International Symposium on Bioelectrochemistry and Bioenergetics, Limerick, Ireland, 26 – 30 May 2019. Oral Presentation. Book of Abstracts, p. 113.

10. **J. Klouda**, R. Roshni, K. Schwarzová, J. Barek, D.K.Y. Wong, Minimising Fouling During Dopamine Detection at Carbon Electrodes Hydrogenated by Aromatic Organosilanes, in: R&D Topics 2019, Adelaide, Australia, 1 – 4 December 2019. Oral Presentation. Book of Abstracts.

6.7 Poster presentations

1. **J. Klouda**, K. Nesměrák, K. Schwarzová-Pecková, Bile Acids: Possibilities of Anodic Oxidation in Non-aqueous and Mixed Media, in: 16th Inaternational Conference on Electroanalysis (ESEAC), Bath, United Kingdom, 12 – 16 June 2016. Poster. Book of abstracts.
2. **J. Klouda**, K. Schwarzová-Pecková, J. Barek, F.-M. Matysik, Bile Acids: Possibilities of Electrochemical Oxidation in Mixed Media of Acetonitrile and Water, in: ANAKON 2017, Tübingen, Germany, 3 – 6 April 2017. Poster. Book of Abstracts.
3. **J. Klouda**, J. Barek, and K. Schwarzová-Pecková, Voltammetric behaviour of primary bile acids after acid induced dehydration, in: 17th International Conference on Electroanalysis (ESEAC), Rhodes, Greece, 3 – 7 June 2018. Poster. Book of Abstracts, p. 129.
4. **J. Klouda**, J. Barek, and K. Schwarzová-Pecková, Voltammetric Behaviour of Primary Bile Acids Cholic and Chenodeoxycholic Acid, in: Bioelectrochemistry and Bioelectronics of Macromolecules (JW70), Brno, Czech Republic, 12 – 15 June 2018. Poster. Book of Abstracts, p. 91.

6.8 Internships

1. University of Regensburg, Regensburg, Germany

Research group of Prof. Frank-Michael Matysik; 9 January – 31 May 2017 (5 months); funding: Erasmus+ programme.

2. University of Regensburg, Regensburg, Germany

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3. Macquarie University, Sydney, Australia

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2. Czech Science Foundation

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