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A NEW APPROACH TO THE ELECTROANALYSIS OF PRIMARY BILE ACIDS AND RELATED STEROIDS

NOVÝ PŘÍSTUP K ELEKTROANALÝZE PRIMÁRNÍCH ŽLUČOVÝCH KYSELIN A PŘÍBUZNÝCH STEROIDŮ

Ph.D. Thesis

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List of symbols and abbreviations

3α-HSD	3α-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
7-DHC	7-dehydrocholesterol
AD	amperometric detection
A_{geom}	geometrical area [mm^2]
BAs	bile acids
BDD	boron-doped diamond
C	molar concentration [$\mu mol\ L^{-1}$]
CA	cholic acid
CDCA	chenodeoxycholic acid
CMC	critical micellar concentration
CV	cyclic voltammetry
DCA	deoxycholic acid
DPV	differential pulse voltammetry
E	potential [V]
ELISA	enzyme-linked immuno sorbent assay
FIA	flow-injection analysis
FLD	fluorescence detection
GC	glassy carbon
GC-MS	gas chromatography-mass spectrometry

HDL	high-density lipoprotein
IM-MS	ion mobility-mass spectrometry
j	current density [μA mm^{-2}]
LCA	lithocholic acid
LC-ID-MS	liquid chromatography-isotope dilution-mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
LDL	low-density lipoprotein
LOD	limit of detection
NADH	reduced form of nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
SE	supporting electrolyte
t	time [min]
UDCA	ursodeoxycholic acid
UPLC-MS	ultra-performance liquid chromatography-mass spectrometry

Abstract

In this doctoral thesis, a novel method for the determination of primary bile acids cholic acid and chenodeoxycholic acid is presented. Bile acids play various vital roles in the mammalian body. Moreover, their determination is extremely helpful in liver and biliary disease diagnosis and management. These saturated organic compounds lack strong chromophores and fluorophores in their structure, and thus are usually hard to detect in spectroscopy. For this reason, either instrumentally advanced but expensive methods, such as mass spectrometry, or less reliable enzymatic methods are commonly employed in bile acids quantitation. Hence, the demand for simple and reliable methods for their determination is strong. Bile acids are also known to be virtually inert for direct electrochemical oxidation. Herein, a simple method for their chemical activation for electrochemical oxidation on bare electrode materials was developed, optimized and applied to cholic acid and chenodeoxycholic acid determination. The activation is based on a dehydration reaction of a primary bile acid with 0.1 mol L^{-1} $HClO_4$ in acetonitrile (water content 0.55%) that introduces double bond(s) into the originally fully saturated steroid core. This naturally increases the electron density in the structure, and thus allows electrochemical oxidation of the bile acids under unchanged conditions directly in the dehydrating medium at +1.2 V (*vs.* $Ag/AgNO_3$ in acetonitrile). Increased electrochemical activity of cholesterol under equivalent conditions was also observed. The mechanism for the activation reaction was proposed based on results of CE-MS experiments. This activation reaction was optimized and implemented into an electroanalytical procedure employing differential pulse voltammetry on boron-doped diamond electrode, and the bile acids were determined in artificial and real-life serum samples with good selectivity. The results of the differential pulse voltammetric method were compared to the ones obtained with a previously proposed method (HPLC with fluorescence detection) confirming good accuracy of the present method. The limits of detection were estimated to be 0.5 μmol L^{-1} for cholic acid and 1.0 μmol L^{-1} for chenodeoxycholic acid. Moreover, application in liquid-flow techniques was envisaged. Pilot experiments with flow-injection analysis with amperometric detection in wall-jet arrangement using boron-doped diamond working electrode were performed with limits of detection of *ca* 1 μmol L^{-1} for both compounds, thus confirming favourable potential of the method in the amperometric mode of operation. An innovative, simple and fast electroanalytical approach to the detection of primary bile acids with a potential of clinical application was developed in this doctoral thesis.

Abstrakt

V této dizertační práci byla vyvinuta nová metoda pro stanovení primárních žlučových kyselin: cholové kyseliny a chenodeoxycholové kyseliny. V těle savců plní žlučové kyseliny rozmanité významné funkce. Jejich stanovení je rovněž důležitým nástrojem v diagnóze aléčbě onemocnění jater a žlučníku. Tyto nasycené organické sloučeniny postrádají ve své struktuře silné chromofory a fluorofory, což zásadně znesnadňuje jejich spektrometrickou detekci. Proto jsou pro účely detekce využívány instrumentálně pokročilé, ale nákladné metody, jako je hmotnostní spektrometrie, nebo méně spolehlivé enzymatické metody. Z těchto důvodů je neustálá silná poptávka po jednoduchých a spolehlivých metodách pro stanovení žlučových kyselin. Je rovněž známo, že žlučové kyseliny je prakticky nemožné přímo elektrochemicky oxidovat. V této dizertační práci byla vyvinuta a optimalizována metoda, která umožňuje chemickou aktivaci žlučových kyselin pro jejich elektrochemickou oxidaci na nemodifikovaných elektrodových materiálech a která byla aplikována pro stanovení cholové kyseliny a chenodeoxycholové kyseliny. Tato aktivace je založena na dehydratační reakci primární žlučové kyseliny s 0,1 mol l^{-1} $HClO_4$ v acetonitrilu (obsah vody 0,55 %), která do původně plně nasyceného steroidního jádra zavede jednu nebo více dvojných vazeb. To přirozeně zvýší elektronovou hustotu, a tím umožní elektrochemickou oxidaci žlučové kyseliny při +1,2 V (*vs.* $Ag/AgNO_3$ v acetonitrilu) za nezměněných podmínek přímo v dehydratujícím roztoku. Za stejných podmínek byla také pozorována zvýšená elektrochemická aktivita cholesterolu. Na základě výsledků CE-MS experimentů byl navržen mechanismus této reakce. Aktivační reakce byla optimalizována a implementována do elektroanalytického postupu využívajícího diferenční pulsní voltametrii na borem dopované diamantové elektrodě a uvedené dvě žlučové kyseliny byly stanoveny ve vzorcích umělého a lidského séra s dobrou selektivitou. Výsledky měření za využití diferenční pulsní voltametrie byly porovnány s dříve popsanou metodou (HPLC s fluorescenční detekcí), čímž byla potvrzena přesnost navržené metody. Byly stanoveny limity detekce: 0,5 μmol l^{-1} pro cholovou kyselinu a 1,0 μmol l^{-1} pro chenodeoxycholovou kyselinu. Dále byla vyhodnocena možnost aplikace tohoto postupu v průtokových metodách. Proběhly pilotní experimenty za využití průtokové injekční analýzy s amperometrickou detekcí ve *wall-jet* uspořádání a s borem dopovanou diamantovou elektrodou s limity detekce okolo 1 μmol l^{-1} analytu, čímž byl potvrzen potenciál metody v amperometrickém uspořádání. V této dizertační práci byla tedy popsána inovativní, jednoduchá a rychlá elektroanalytická metoda stanovení primárních žlučových kyselin s potenciálem pro aplikaci v klinické praxi.

Key words

Bile acids, boron-doped diamond electrode, dehydration, human serum, solid phase extraction, voltammetry

Klíčová slova

Borem dopovaná diamantová elektroda, dehydratace, extrakce na tuhé fázi, lidské sérum, voltametrie, žlučové kyseliny

This Ph.D. thesis was composed between years 2015 and 2020 at Charles University, Faculty of Science, Department of Analytical Chemistry in the UNESCO Laboratory of Environmental Electrochemistry in the Czech Republic. The focus of this scientific workgroup is on the development of electrochemical methods for sensitive monitoring of hazardous and/or bioactive organic compounds found in the human body or in the environment.

The experimental work was partially carried out during the internship in year 2017 in the laboratory of Prof. Frank-Michael Matysik at University of Regensburg, Institute of Analytical Chemistry, Chemo- and Biosensors in Germany.

1 Aims of the work

The primary aim of this work was to develop a method for bile acids (BAs) determination based on their electrochemical oxidation. Employing a voltammetric or amperometric approach for this purpose carries some inherent obstacles that needed to be addressed and overcome. Firstly, saturated steroid molecules, such as BAs, possess low intrinsic activity towards electrochemical oxidation both in aqueous and non-aqueous media. Moreover, such analytes are usually found in complex matrices as blood serum or bile, therefore, the matrix can interfere with the determination and its effect needs to be minimized. Overcoming the mentioned issues, and the consequent development of a method for BA determination were realised in the following steps:

(i) Review of the up to date literature sources, assessment of possible approaches.
(ii) Investigation of possibilities of increasing the susceptibility of BAs towards electrochemical oxidation.
(iii) Preliminary investigation of applicability of the developed procedure to related steroid compounds such as cholesterol.
(iv) In-depth characterization and optimization of the analytical process of BAs determination.
(v) Development of a novel electroanalytical method for BAs determination based on the established protocol, and its application to biological matrices.

The results of the work were published in the following journal papers available as appendices 6.1 – 6.3:

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. ***Aim (i).***
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. ***Aims (ii) and (iii).***

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. ***Aims (iv) and (v).***

2 Introduction

The topic was comprehensively reviewed in ref. [1], which is available in appendices as Publication I and further highlighted in bold script.

2.1 Steroids of interest

In humans, sterols are vital biomolecules with various diverse functions. The main sterol biomolecule is cholesterol (Fig. 1). It serves as an essential building block in cell membranes [2], and moreover it serves as a precursor for biosynthesis of biomolecules such as bile acids [3], or steroid hormones [4]. In the human body, cholesterol is either biosynthesized *de novo* from acetyl coenzyme A by a series of enzyme-assisted reactions heavily regulated at several points [5], or it can be absorbed from the diet. A block in cholesterol biosynthesis can lead to disease. Smith-Lemli-Opitz syndrome, a congenial malformation disorder, serves as an example of such disease. It is caused by a deficient enzyme 7-dehydrocholesterol reductase, which catalyzes the last step of cholesterol biosynthesis – reduction of the $\Delta^{7,8}$ double bond in 7-dehydrocholesterol (7-DHC) [6]. Yet, excessively high levels of cholesterol in plasma can be also harmful and may cause a condition known as atherosclerosis. Atherosclerosis is the primary cause of heart disease and stroke, and the underlying cause of approximately 50% of all deaths in westernized societies [7]. This results in the ever increasing interest in cholesterol determination [8].

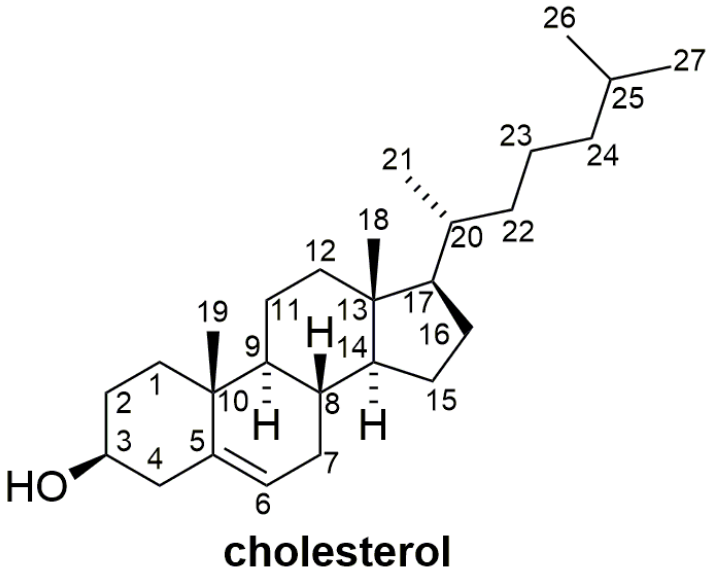

Fig. 1 Structure of cholesterol with numbered carbon atoms. Analogical numbering is used with BAs and other steroids.

The end-product of cholesterol catabolism, BAs, are amphipathic water-soluble readily excreted biomolecules. Based on the number of carbon atoms in their structure C_{24} and C_{27} BAs are discerned [3]. In mammals, C_{24} BAs are predominant. Most of the physiological, pathophysiological and metabolic properties of BAs can be attributed to their detergent-like behaviour [9]. Hydroxyl groups in common BAs are oriented in one direction, resulting in a concave hydrophilic α face and a convex hydrophobic β face of the molecule, which is devoid of the hydrophilic substituents [10]. If the concentration of a BA is sufficient, it will self-associate to form a micelle (at critical micellar concentration – CMC), presumably due to the hydrophobic effect, which aims at minimizing the hydrophobic surface, as well as hydrogen binding, which is determined by the number, location and orientation of the hydroxyl groups [11]. When the concentration of a BA is below its respective CMC, it is present in monomeric form in a solution. Basic physicochemical properties of the studied BAs are summarized in Tab. 1.

In humans, there are two main BAs that are biosynthesized in the liver, termed primary, cholic acid (CA) and chenodeoxycholic acid (CDCA) (Fig. 2). As components of bile, they are secreted into the gastrointestinal tract, where they can be modified by bacteria. This way, predominantly by removal of the hydroxyl group at C7, secondary BAs are formed. Major secondary BAs in humans are products of C7 dehydroxylation of CA – deoxycholic acid

(DCA); and of CDCA – lithocholic acid (LCA) (Fig. 2) [3,12]. Two most important functions of BAs are cholesterol level regulation, and formation of mixed micelles with dietary lipids in the small intestine to facilitate digestion of hydrophobic nutrients [13]. Ursodeoxycholic acid (UDCA) (Fig. 2) is often used as a medical drug in reduction of biliary cholesterol and treatment of diseases such as primary biliary cirrhosis or primary sclerosing cholangitis [14]. Moreover, BAs are important regulatory molecules [15], and semi-synthetic BA analogues are promising new drug candidates for treatment of chronic liver disease, hepatocellular cancer and extrahepatic inflammatory and metabolic diseases due to their intracellular nuclear course of action [16]. On one hand, BAs possess antimicrobial properties [17], but on the other, they were implicated as co-carcinogens, particularly in cancers of the gastrointestinal tract, due to their inherent detergent effect on biological membranes [18]. Importantly, BA levels sharply increase in patients suffering from gallstones [19], and a wide range of liver impairments [20–24]. For all these reasons, knowledge of BAs levels is crucial in the decision-making process in clinical practise.

Tab. 1 Critical micellar concentration, pK_a, and water solubility (pH 3, 25 °C) values of the studied BAs. Data from [25].

Bile acid	**CMC / mmol L^{-1}**		**pK_a**	**Water solubility / µmol L^{-1}**
	Water	**0.15 mol L^{-1} Na$^+$**		
CA	13	11	~5[a]	273
CDCA	9	4	~5[a]	27
DCA	10	3	~5[a]	28
LCA	n/a	n/a	~5[a]	0.05
UDCA	19	7	~5[a]	0.9

[a]The ionization properties of the side chain of bile acids is that of isopentanoic acid; the nuclear substituents do not influence ionization.

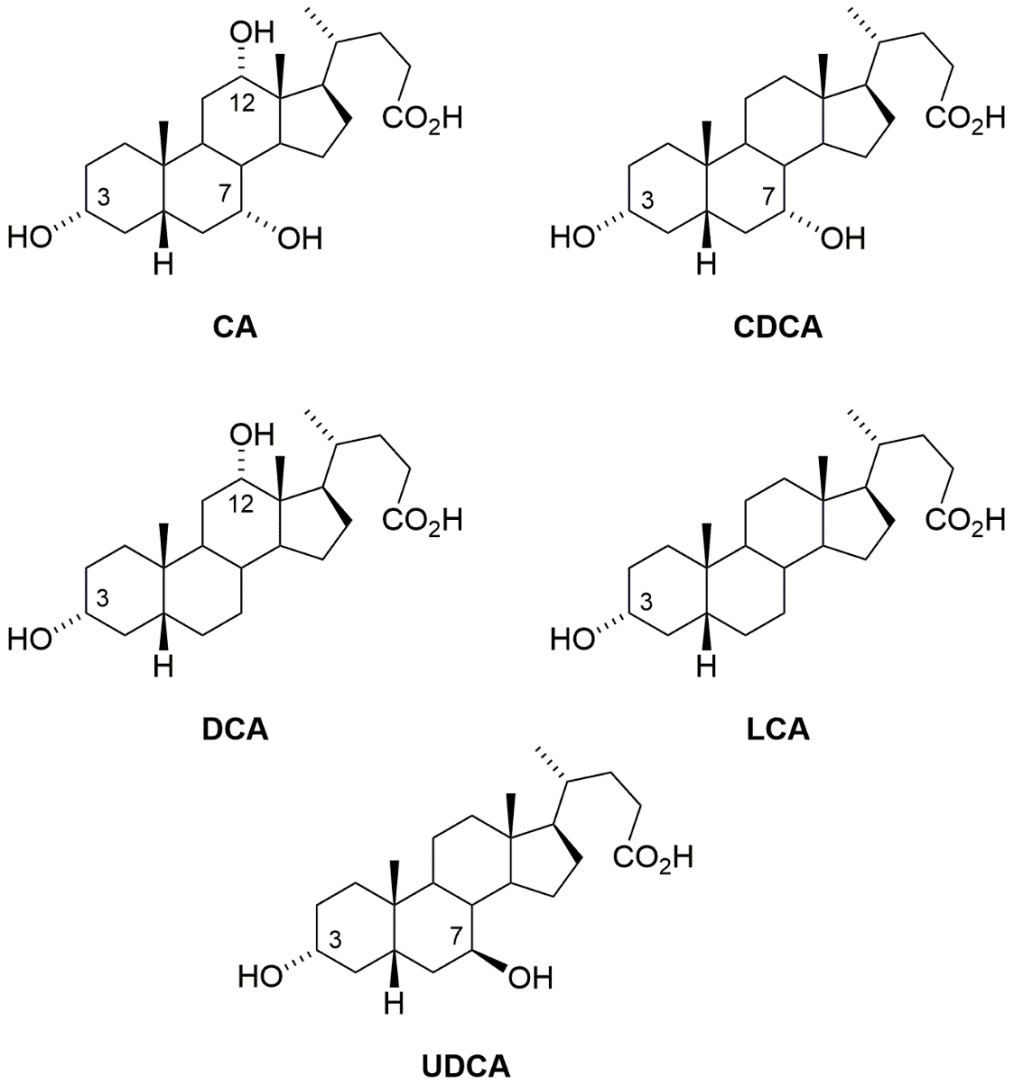

Fig. 2 Structures of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA).

2.2 Determination of cholesterol and BAs

Even though determination of both cholesterol and BAs is critical in clinical practise (mainly in blood and bile), and cholesterol alone also in food products (predominantly milk and meat products), simple methods of their detection are scarce, and/or suffer from setbacks that cannot be ignored. This is mainly because analysis and determination of saturated steroid compounds (BAs) and steroid compounds containing single double bond (cholesterol) are complicated by the absence of strong chromophores or fluorophores. Methods towards the analysis of cholesterol [1,8,26,27] and BAs [1,28–31], very often utilizing enzymatic, mass spectrometric and/or chromatographic techniques, were reviewed extensively in the past.

In food products, cholesterol determination process consists of three fundamental steps: saponification, extraction and detection [8]. In routine analyses of food, colorimetric and more often chromatographic methods are utilized. In colorimetric determination of cholesterol, termed Abell-Kendall method, a chemical reaction between cholesterol and Liebermann-Burchard reagent (acetic acid, acetic anhydride and sulphuric acid) yields a coloured solution [32,33]. More instrumentally advanced methods, such as LC-ID-MS are usually employed in quality control. In clinical practise, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol is differentiated (amongst others). Treatment decisions are based on the concentration levels of LDL cholesterol, considered to be the "bad" cholesterol, commonly associated with lesion formation in atherosclerosis [26]. At the same time, HDL, the "good" cholesterol, is strongly protective against atherosclerosis as it removes the excess cholesterol from peripheral tissues [7].

Clinical analysis of BAs is predominantly carried out using enzymatic methods, mostly because of the simplicity, high throughput, and the potential for automation. In patients suffering from gallstones, the total BA levels can rise by a 42-fold increase (up to a median of 376.4 μmol L^{-1}) compared to healthy individuals (median 8.87 μmol L^{-1}) [19]. On average, in healthy individuals, the total BA values lie in the range between 2.6 and 15.9 μmol L^{-1}. In various liver diseases, a 20-fold rise in CA and CDCA (from 2.4 μmol L^{-1} in healthy individuals to 48.9 μmol L^{-1}) has been reported [24]. Advanced analytical methods as GC-MS and LC-MS are also employed, especially for identification and quantification of individual BAs in complex biological matrices and for fundamental research purposes [28–31]. Conventional LC-MS/MS can be used for studying biochemical processes in the human body [34] or in clinical analysis [22]. For BA profiling, UPLC-MS is commonly employed [35–38]. Methods that are GC-based or use fluorescence detection (FLD) require derivatization procedures: in GC-MS, BAs can be determined as methyl ester-trimethylsilyl ether and methyl ester-acetate derivatives [39]. To allow fluorescence detection, BAs have to be derivatized with compounds such as 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-*p*-toluenesulfonate [40], or 4-nitro-7-*N*-piperazino-2,1,3-benzoxadiazole [41]. Enzymatic methods rely on a reaction between BAs and an enzyme (usually 3α-hydroxysteroid dehydrogenase, 3α-HSD). These methods are used to obtain the total serum BA values, as the enzymatic oxidation is non-specific. In principle, the enzyme catalysed oxidation of the 3α hydroxyl group of the BAs produces NADH (or a corresponding derivative), which can be detected by UV or fluorescence spectroscopy. These methods had been used historically [42],

and still remain in the spotlight of researchers [43–45]. Very sensitive indirect enzymatic electrochemical methods of BAs detection have emerged lately [46,47]. Moreover, ELISA methods can be used for the determination of total BAs. In one study, five major components of total BAs (CA, CDCA, LCA, DCA and hyodeoxycholic acid) were determined by using an indirect competitive ELISA test combining 4 different monoclonal antibodies with the LOD of 0.73 μmol L^{-1} and a relatively narrow linear range of 0.3 to 9.2 μmol L^{-1}. Also, the pursuit of simple, fit-for-purpose platforms for determination of CA and CDCA as biomarkers of liver disease/damage has been apparent in recent years [21,23,48–51]. There are two different approaches used in these studies, however, the fundamental idea is the same. In both approaches, BAs interact with a (bio)chemical entity in a defined way, which allows their determination using relatively simple and reliable optical methods such as colorimetry [23,51] or polarizing microscopy [21,48–50]. In these studies, LODs of 12 μmol L^{-1} [21], 1 μmol L^{-1} [23], 5 μmol L^{-1} [48], 5 μmol L^{-1} for CA, and 1 μmol L^{-1} for CDCA [49], and 20 μmol L^{-1} for CA [50] are reported.

All the above-mentioned methods suffer from various difficulties. In the case of methods relying on MS detection, high acquisition and overhead cost is apparent. When derivatization is required in GC and fluorescence detection, it proves to be laborious and time-consuming. While the above-mentioned, instrumentally advanced, methods possess extremely low LODs (generally nanomolar range), and favourably so for research purposes, in clinical analysis such low LODs can be regarded unsubstantiated, as the BA levels tend to lie in the tens to hundreds micromolar range in most clinical samples. Enzymatic methods based on 3α-HSD risk falsely high readings when the enzyme is contaminated with 3β-HSD (cholesterol interference), and, moreover, are unable to distinguish between individual BAs. In ELISA, risk of cross reactivity is relatively high. Simpler, fit-for-purpose methods deal with different hurdles involving selectivity, specificity, stability and applicability to real-life matrices. For all these reasons, demand for simple, cheap and reliable methods for BA analysis remains strong.

2.3 Electrochemical oxidation of cholesterol and BAs

Direct electrochemical oxidation has the potential to meet all the criteria of a reliable BA detection method. Electroanalytical methods employing bare electrodes are relatively cheap, robust and do not require complicated equipment prone to failure. However, they are not

commonly employed for BAs nor cholesterol electroanalysis, owing to the low electrochemical activity of these compounds in both positive and negative region. Relative merits of non-enzymatic methods in the analysis and characterization of sterol compounds were comprehensively reviewed in ref. **[1]**.

A series of six papers has been published by the group of prof. Kusu on electrochemical detection of cholesterol and related compounds [52–57]. They used glassy carbon (GC) and boron-doped diamond (BDD) electrodes to oxidize cholesterol and related compounds in non-aqueous media (mostly acetonitrile/$LiClO_4$) at very high potentials over +1.9 V (*vs.* Ag/AgCl). Moreover, attempts to deeper characterize electrochemical oxidation of cholesterol can be traced in the literature [58–60]. Development in cholesterol biosensors based on nanomaterials-modified electrodes was reviewed [61]. Such biosensors very often suffer from significantly decreased response over days to a couple of months period.

Indirect method for CA oxidation can be traced in the literature; Medici *et al.* used PbO_2, platinum, and graphite electrodes and NaCl as an electron transfer mediator for indirect oxidation of CA. Only three studies employing direct oxidation of BAs were found in the literature [62–64]. In the first two studies [62,63], HPLC hyphenated with triple-pulsed amperometric detection on gold electrode was employed for BA detection. Bile acids are essentially aliphatic alcohols, and these molecules are known to be practically electrochemically inactive in voltammetry. In contrast to aromatic steroids containing hydroxyl group, such as estrone, estradiol or estriol, aliphatic saturated BAs are unable to stabilize the radical product of their one-electron oxidation by π-resonance [65]. Therefore, BAs cannot be directly oxidized on carbon-based electrode materials. Electrode materials with unsaturated valence orbitals (gold, platinum) can stabilize these radicals and are able to directly oxidize BAs [62,63]. Nevertheless, this process is accompanied with rapid fouling of the electrode surface, thus, pre-treatment pulse (E_1), detection pulse (E_2) and cleaning pulse (E_3) have to be applied in a rapid sequence [66], practically ruling out the use of voltammetric techniques (E_1 = +0.03 V, +0.05 V resp.; E_2 = +0.60 V; E_3 = –0.80 V, –0.60 V resp. *vs.* Ag/AgCl). In these methods, a relatively narrow linear dynamic range is reported (0.6 to 14 μmol L^{-1} [62]). Also, a pH value approaching 13 is required, which limits the use of the reversed-phase silica-based columns. The third study employed HPLC with a detector consisting of two in-line porous graphite electrodes (+0.60 V and +1.40 V) used for UDCA detection [64]. However, the electrochemical process on which the detection is based is not

very well characterized in the study; the authors do not state what reference electrode was used, and do not provide any basic electrochemical characterization of the process, such as CVs of UDCA oxidation. Therefore, thus far, no well-established and characterized methods of BAs direct electroanalytical determination exist. As mentioned above, electrode material plays a significant role in the electrochemical process of oxidation. It affects the mechanism of oxidation, but also the extent of fouling of the electrode, charging current intensity, or the width of the potential window. For all these reasons, a great deal of care must be paid to choosing the right material for the selected application.

Boron-doped diamond represents a modern electrode material with superb electrochemical and mechanical properties [67–69]. These include the ability to prepare the material in various architectures, a wide working potential window, enhanced signal-to-background ratios due to the low background current, weak molecular adsorption, and optical transparency. Weak molecular adsorption, resulting in low proclivity to fouling, is especially favourable in electroanalysis. Although modern, the functionality of BDD is very well understood and appreciated nowadays [70]. For all the reasons listed above, BDD was selected as the main electrode material throughout this work.

The knowledge summarized in the previous paragraphs served as a stepping-stone for the development of a novel and ingenious approach that enables direct electrochemical detection of CA and CDCA.

3 Results and discussion

Major parts of this work were published in refs. [71] and [72], which are available in appendices as Publication II and Publication III and further highlighted in bold script.

3.1 Activating steroids for electroanalysis

Intrinsically, BAs do not show much electrochemical activity in the non-aqueous medium of acetonitrile containing 0.1 mol L^{-1} $NaClO_4$ as a supporting electrolyte (SE), as illustrated in Fig. 3A for CA and CDCA. There is only a hint of a peak at high potential over +1.8 V (*vs.* $Ag/AgNO_3$ in acetonitrile) close to the SE decomposition. Voltammetric response so close to the SE decomposition is hardly employable in electroanalysis because of the difficulty of peak evaluation, and because of the level of interference that is to be expected at highly positive potentials. Therefore, a way to increase the electrochemical oxidizability of BAs (*i.e.*, to "activate" them) had to be implemented into the analytical procedure.

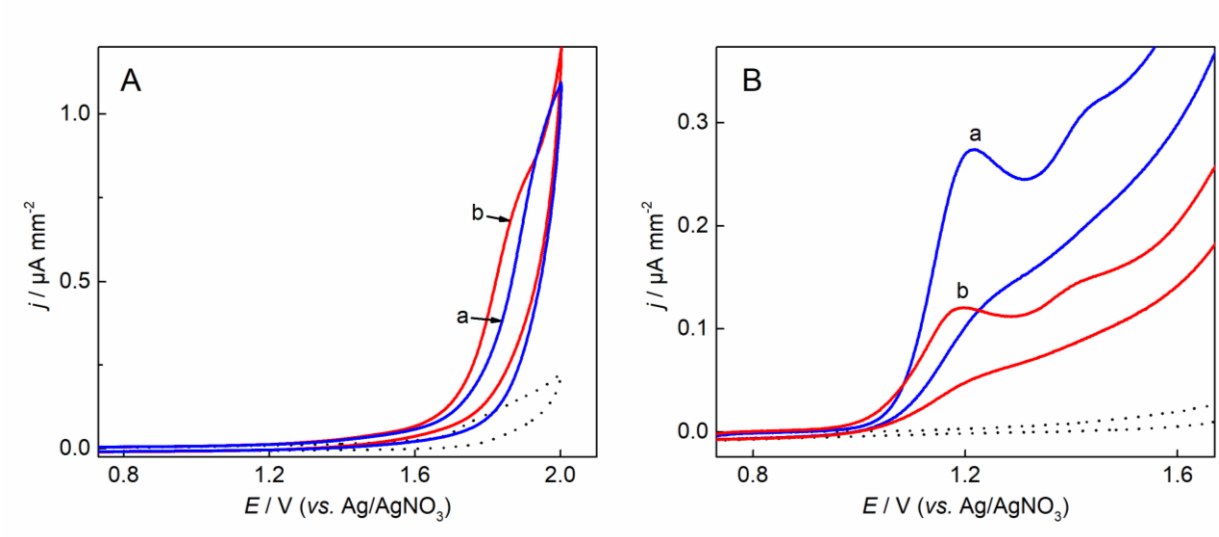

Fig. 3 Cyclic voltammograms of (a) CDCA and (b) CA ($c = 9\times10^{-5}$ mol L^{-1}) in 0.1 mol L^{-1} (A) $NaClO_4$ and (B) $HClO_4$ in acetonitrile. Supporting electrolyte in dashed line. Water content (B) 0.550 ± 0.002%. Ambient temperature 21 °C. Scan rate 50 mV s^{-1}. Polished BDD electrode (A_{geom} = 7.07 mm^2). Voltammograms recorded 90 min after the solutions were prepared from respective stock solutions.

One way to increase the proclivity of an organic compound to electrochemical oxidation is to increase the electron density in its structure. That can be achieved by introducing double bond(s) in the molecule. In the case of BAs, it can be done by removing one of the hydroxyl groups present at the steroid backbone through a dehydration reaction. A well-known dehydration reaction that is widely applied to the principal sterol, cholesterol, is the aforementioned Liebermann-Burchard reaction. It is the reaction between cholesterol and a mixture of acetic acid, acetic anhydride and sulphuric acid used in Abell-Kendall method for cholesterol determination to allow its colorimetric detection [32]. It converts the poorly absorbing cholesterol molecule into a mixture of derivatives that absorbs wavelengths in the VIS spectrum (blue-green coloured solutions) [33]. In this work, an analogical approach was utilized not to increase the UV-VIS absorbance of BAs but rather their electrochemical activity. By using 0.1 mol L^{-1} $HClO_4$ in acetonitrile, serving concurrently as a dehydrating agent and as the SE, a very well developed voltammetric signal of CA and CDCA is obtained at *ca* +1.2 V (*vs.* $Ag/AgNO_3$) (Fig. 3B) in cyclic voltammetry (CV) on BDD, GC and Pt electrodes. Other common BAs in humans, such as DCA and LCA, or UDCA, that is often used in pharmaceutics, do not show similar electrochemical activity after being in contact with 0.1 mol L^{-1} $HClO_4$ in acetonitrile (Fig. 4). Such extremely different behaviour of relatively similar molecules points to the crucial role of the axial 7α-hydroxyl group, which is present in the primary BAs, CA and CDCA. The secondary BAs, DCA and LCA, lack the hydroxyl group at C7 altogether. A stereoisomer of CDCA, UDCA, possesses the equatorial 7β-hydroxyl group (as opposed to the axial 7α-hydroxyl of CDCA), which results in a very different spatial arrangement of these two seemingly closely similar BAs [73].

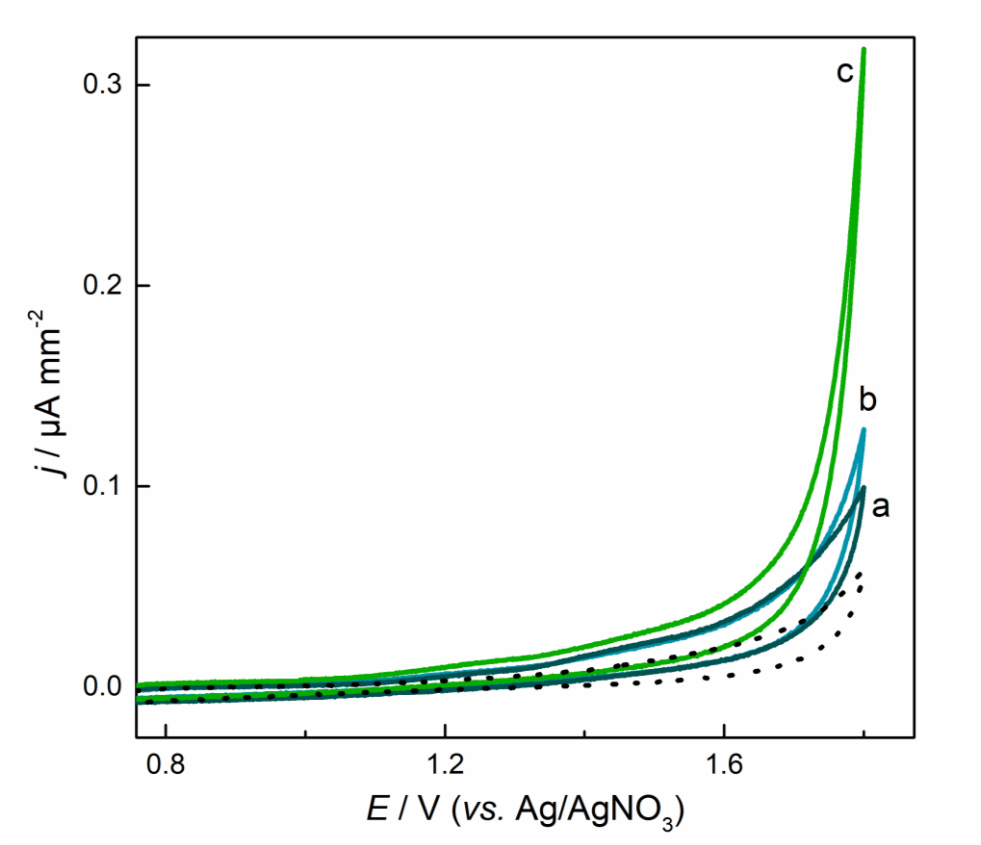

Fig. 4 Cyclic voltammogram of (a) LCA, (b) DCA and (c) UDCA (c = 9×10^{-5} mol L^{-1}) in 0.1 mol L^{-1} $HClO_4$ in acetonitrile. Supporting electrolyte in dashed line. Water content 0.550 ± 0.002%. Ambient temperature 21 °C. Scan rate 50 mV s^{-1}. Polished BDD electrode (A_{geom} = 7.07 mm^2). Voltammograms recorded 90 min after the solutions were prepared from respective stock solutions.

In CA and CDCA, the axial 7α-hydroxyl group is in the antiperiplanar position to the axial 8β-proton, which renders the molecule particularly prone to dehydration. After dehydration, double bond is introduced into the steroid core of CA and CDCA. The resulting alkene is more prone to electrochemical oxidation by removal of an electron from the bonding π-orbital. This is supported by the CE-MS results shown in Fig. 5. In principle, CDCA (**1**) (m/z = 410.33; CDCA·NH_4^+ adduct) is dehydrated by the strong acid ($HClO_4$), likely yielding Δ^7 alkene (**2**) (m/z = 392.32; CDCA–H2O·NH_4^+ adduct), which is thermodynamically preferred to Δ^6 alkene (Zaitsev rule). After a longer period (>3 h), a new signal was observed in CE-MS, which corresponds to the loss of another hydroxyl group (C3) and addition of the acetamide group (**3**). This is further supported by the migration behaviour of the compound (**3**), which indicates a positively charged species (protonation of the acetamide). This product

can be rationalized by Ritter reaction [74]. It starts 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. Obviously, the acetamide is not primarily responsible for the increased voltammetric activity of the BAs, as it was detected after >3 h, long after maximum voltammetric activity was observed (~75 min) (Fig. 6).

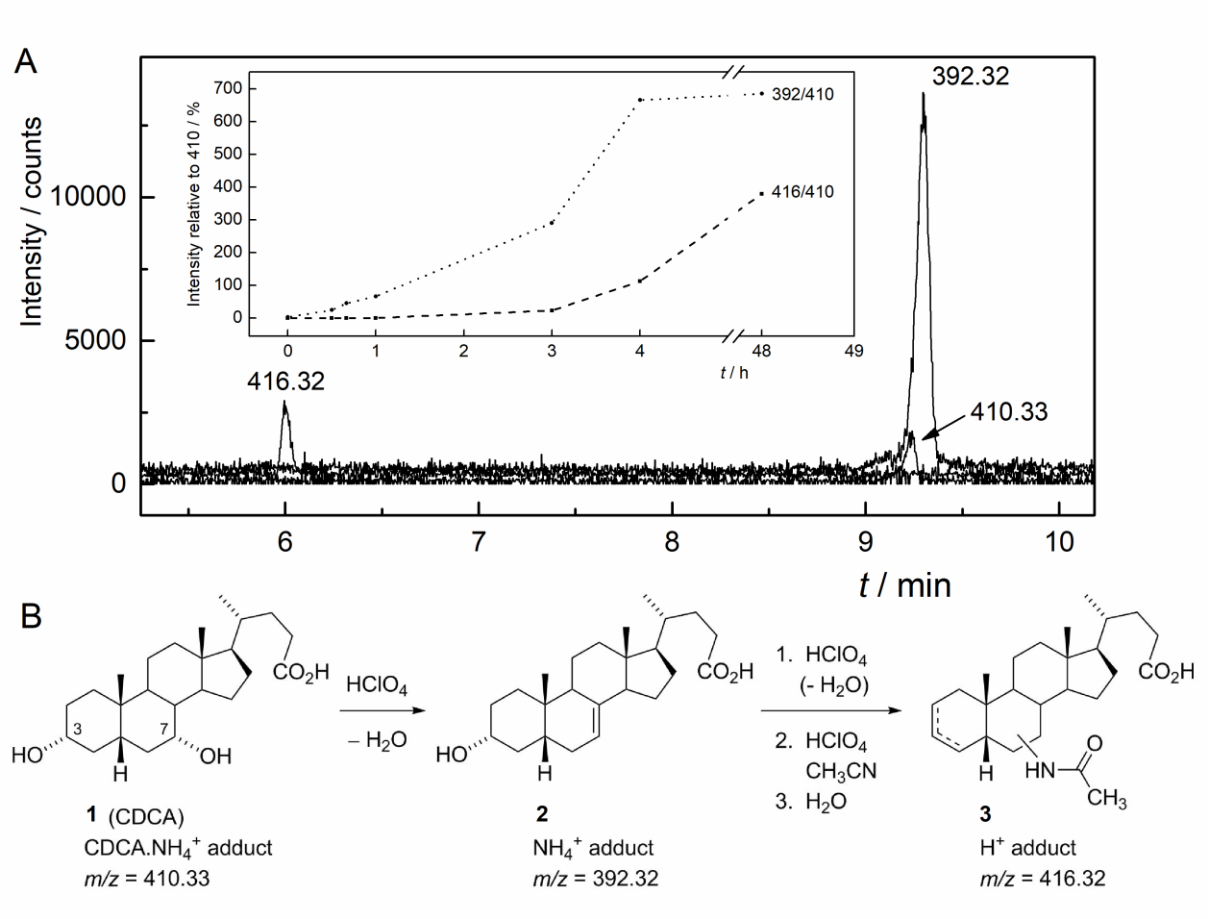

Fig. 5 (A) CE-MS electropherogram of solution containing CDCA ($c = 9\times10^{-4}$ mol L^{-1}) in acetonitrile containing 0.1 mol L^{-1} $HClO_4$ and $0.550 \pm 0.002\%$ H_2O obtained after 4 h. Three selected ion traces are shown (m/z 392.32, 410.33, 416.32). Inset: In-time development of ratio between respective peaks. (B) The proposed reaction mechanism for CDCA dehydration. Reprinted with permission from **[71]**.

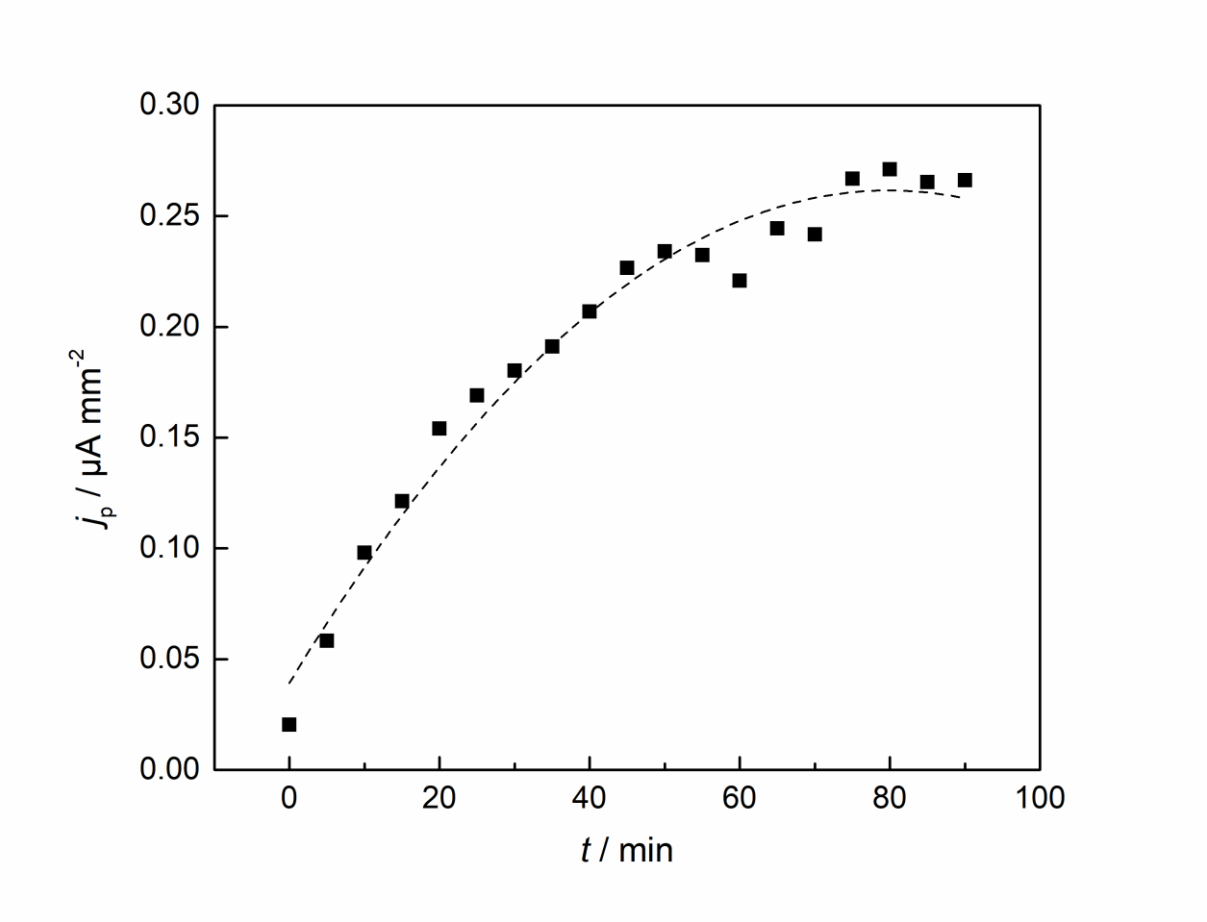

Fig. 6 In-time development of the first CV peak height of CDCA (c = 9×10^{-5} mol L^{-1}) in 0.1 mol L^{-1} $HClO_4$ in acetonitrile. Supporting electrolyte in dashed line. Water content 0.550 ± 0.002%. Ambient temperature 21 °C. Scan rate 50 mV s^{-1}. Polished BDD electrode (A_{geom} = 7.07 mm^2). Reprinted with permission from **[71]**.

The dehydration reaction, responsible for the formation of electrochemically active species, proceeds relatively slowly as apparent form Fig. 6. After placing the BA in contact with $HClO_4$, the voltammetric signal of CDCA gradually increased, until it stabilized after approximately 75 min. Moreover, a very important factor that affects the feasibility of the dehydration reaction is the water content in the dehydrating solutions. Intrinsically, the water content, coming from the concentrated $HClO_4$ stock solution and air humidity, is 0.550 ± 0.002% (240 mmol L^{-1}) as determined by Karl Fischer titration. The well-developed signal is only observed when the water content is kept to the minimum (0.550%), otherwise, no signal of the BA is obtained (Fig. 7). Obviously, excessive water content does not allow for the dehydration reaction of the BA to proceed fully.

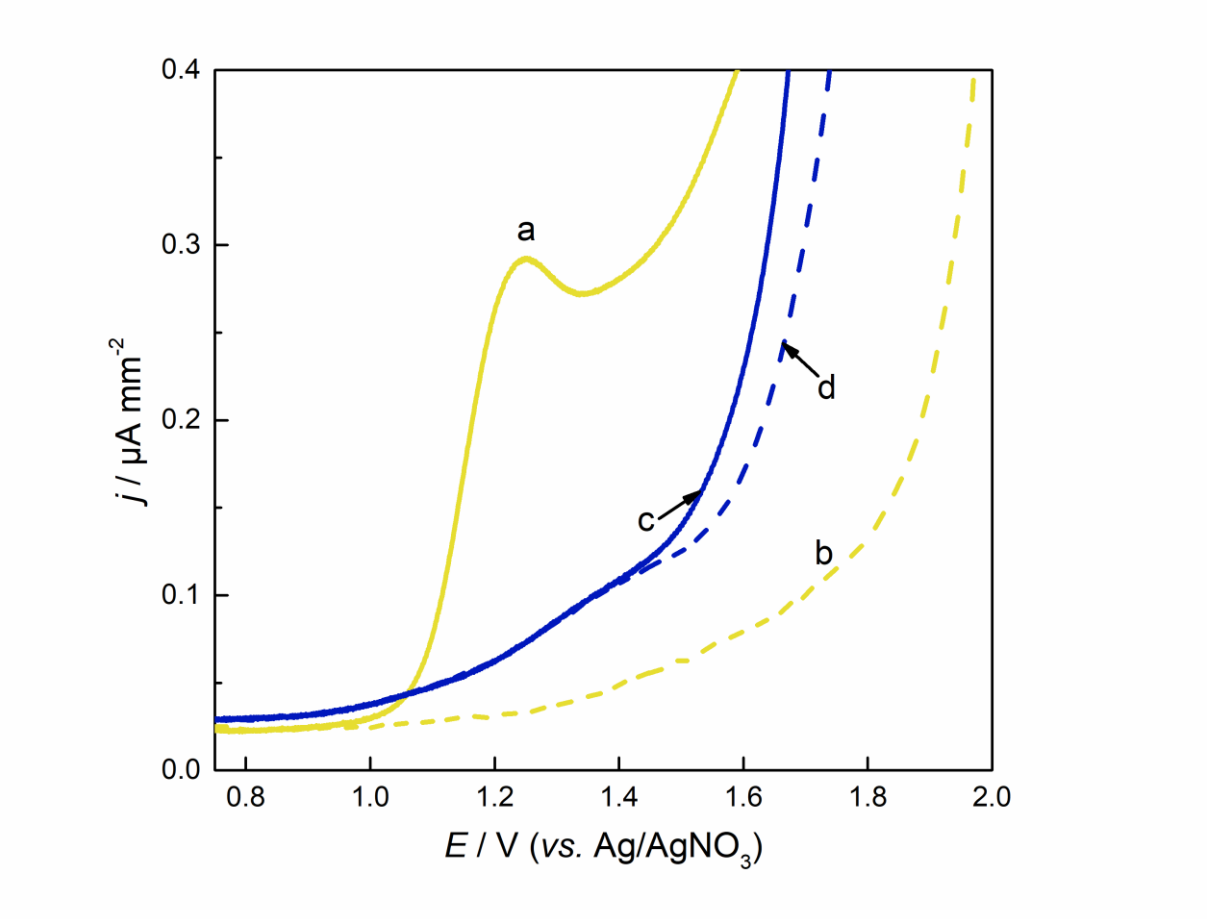

Fig. 7 Linear sweep voltammograms of CDCA (c = 1×10^{-4} mol L^{-1}); curves a, c) in 0.1 mol L^{-1} $HClO_4$ in acetonitrile. Supporting electrolyte in dashed lines (b, d). Water content (a, b) 0.550 ± 0.002%, (c, d) 10.0%; ambient temperature 21 °C. Scan rate 50 mV s^{-1}. Polished BDD electrode (A_{geom} = 7.07 mm^2). Reprinted with permission from **[72]**.

A similar process that is observed for the primary BAs is expected for cholesterol (Fig. 8). In contact with 0.1 mol L^{-1} $HClO_4$ in acetonitrile it provides a very well-developed voltammetric signal at *ca* +1.5 V (*vs.* $Ag/AgNO_3$) (Fig. 8), which is significantly less positive than previously reported (+1.8 V *vs.* Ag/AgCl [57]). This finding paved a road to novel methods of cholesterol determination [75], in which cholesterol was for the first time electrochemically determined in batch and liquid-flow systems in a mixed medium of acetonitrile-water, owing to the relatively low oxidation potential. Methods of cholesterol determination are being developed in a separate dissertation project at author's home institution (L. Benešová). Moreover, based on this finding, a method for the determination of 7-DHC, an important biomarker of Smith-Lemli-Opitz syndrome, has been developed [76].

Therefore, methods of cholesterol and 7-DHC determination were not further pursued in this work, and it was focused solely on methods for primary BAs determination.

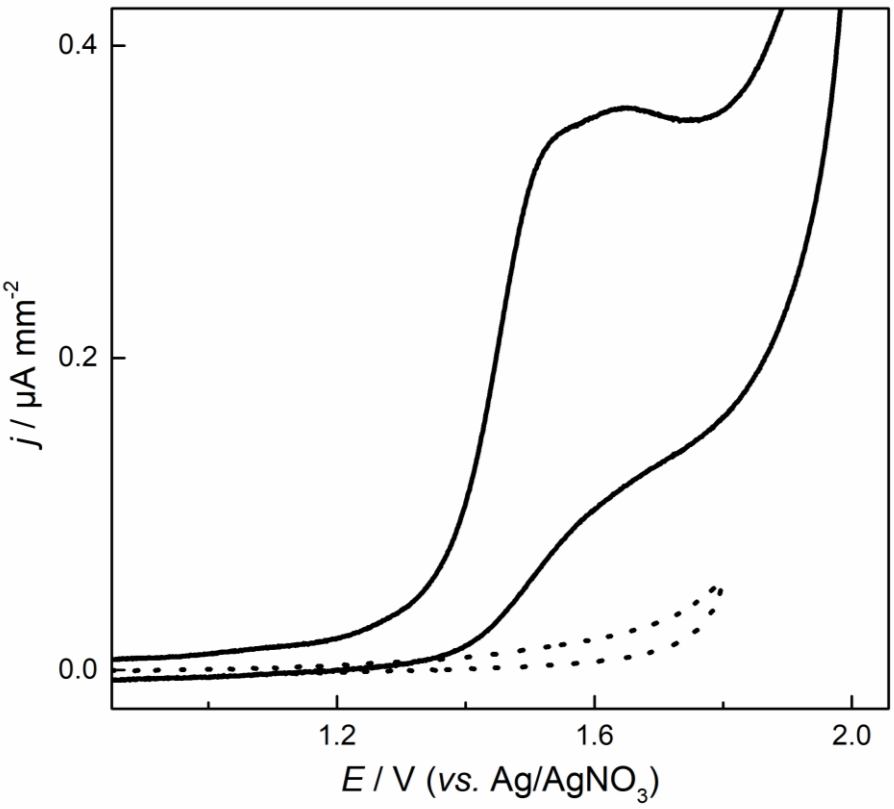

Fig. 8 Cyclic voltammogram of cholesterol (c = 5×10^{-5} mol L^{-1}) in 0.1 mol L^{-1} $HClO_4$ in acetonitrile. Supporting electrolyte in dashed line. Water content 0.550 ± 0.002%. Ambient temperature 21 °C. Scan rate 50 mV s^{-1}. Polished BDD electrode (A_{geom} = 7.07 mm^2). Voltammogram recorded 90 min after the solutions were prepared from respective stock solutions.

3.2 Optimization of the chemical activation of CA and CDCA

The current intensity of voltammetric response for activated CA and CDCA differs under ambient conditions, as apparent in Fig. 3B. This is problematic, as these two biomolecules are found together in the human body, and, under ambient conditions, it is virtually impossible to distinguish between them based on their oxidation potentials, which are very similar. Moreover, the response of activated BAs is not stable in time as apparent from Fig. 6. For

these reasons, steps were taken to ensure stable and equivalent molar response from both BAs.

Increasing the reaction temperature for the BA and 0.1 mol L^{-1} $HClO_4$ in acetonitrile results in a unified and stable voltammetric response of the BAs. This is presumably due to increased rate of the activation reaction. The current response rose exponentially with increasing temperature, and linearly with increasing time. When the reaction mixture was heated up to 50 °C for 5 minutes, the current response did not further rise and was stable with the relative standard deviation (RSD) of 2.9% over the course of 95 minutes. Longer heating time paradoxically leads to a loss of signal at +1.2 V (*vs.* $Ag/AgNO_3$) in the case of CA (30 minutes heating) while a new signal appears at *ca* +0.67 V (*vs.* $Ag/AgNO_3$) (Fig. 9A), allowing differentiation between CA and CDCA. This phenomenon is apparently caused by further dehydration of the activated CA at the axial 12α-hydroxyl group that is lacking in CDCA. Thus, in the case of CDCA, there was only a slight increment in the signal at +1.2 V (*vs.* $Ag/AgNO_3$) after 30 minutes of heating, evidently because it could not be further dehydrated (Fig. 9B). Therefore, to ensure equal and stable response of both CA and CDCA, heating temperature and time of 50 °C/5 minutes was implemented in the protocol for BAs activation.

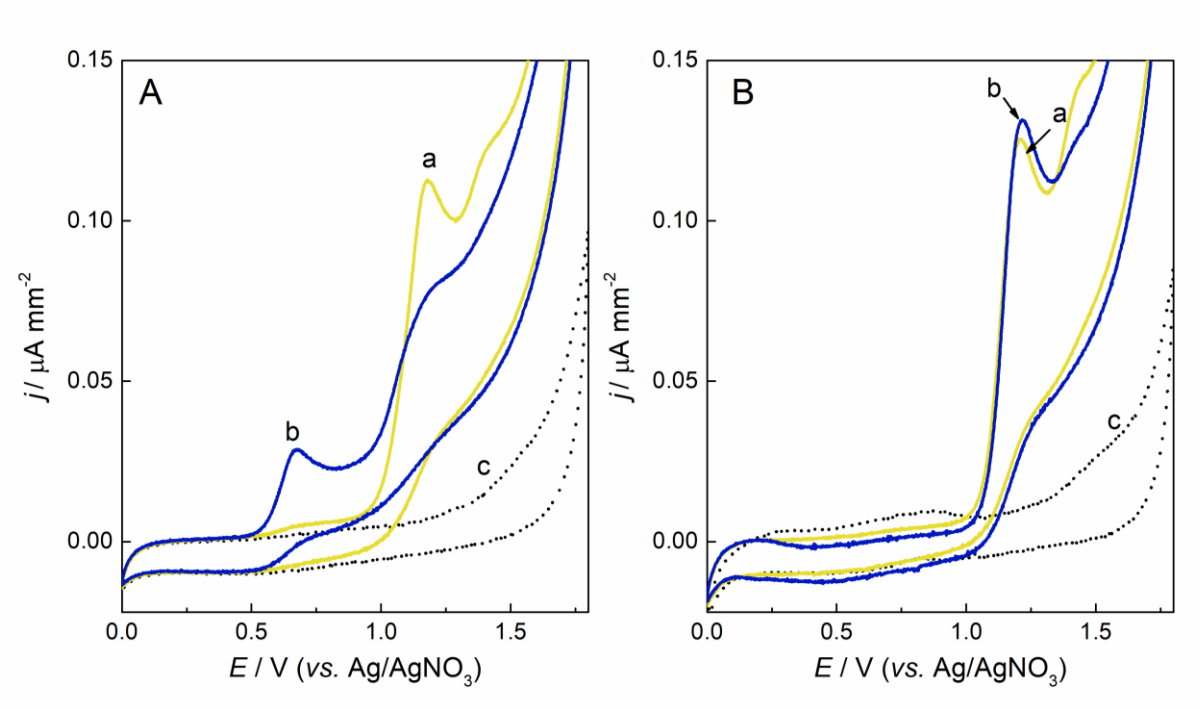

Fig. 9 Cyclic voltammograms of (A) CA, and (B) CDCA ($c = 5\times10^{-5}$ mol L^{-1}) in 0.1 mol L^{-1} $HClO_4$ in acetonitrile (water content 0.550 ± 0.002%). Yellow lines (a): heating 5 min / 50 °C; blue lines (b): heating 30 min / 50 °C; dotted lines (c) correspond to supporting electrolyte. Scan rate 50 mV s^{-1}. Polished BDD electrode (A_{geom} = 7.07 mm^2). Reprinted with permission from **[72]**.

Various dehydrating agents were investigated in this work, as strong acids different from $HClO_4$ could prove more efficient. In addition to $HClO_4$, experiments were carried out with H_2SO_4, H_3PO_4, and HCl (all 0.1 mol L^{-1}). Under ambient temperature (21 °C), only the presence of $HClO_4$ enabled the electrochemical oxidation of the BAs by their activation. However, after implementing the heating step (50 °C for 5 min), a signal was observed with H_2SO_4, approximately of one third intensity compared to that seen for $HClO_4$. Nevertheless, 0.1 mol L^{-1} $HClO_4$ in acetonitrile was used throughout this work as the dehydrating agent providing the highest current response, and as the SE at the same time.

The unified response that is attained by implementing the heating protocol and using $HClO_4$ as the dehydrating agent is illustrated in Fig. 10. Equimolar (90 µmol L^{-1}) solutions of CA and CDCA provided peaks of similar height in differential pulse voltammetry (DPV). Also, a mixture of CA and CDCA (both 45 µmol L^{-1}, 90 µmol L^{-1} total primary BAs) leads to a peak at a similar potential and with similar height (RSD of all 3 peak heights is 0.6%).

Owing to the unified signal intensity, both BAs can be simultaneously determined as the total primary BA pool.

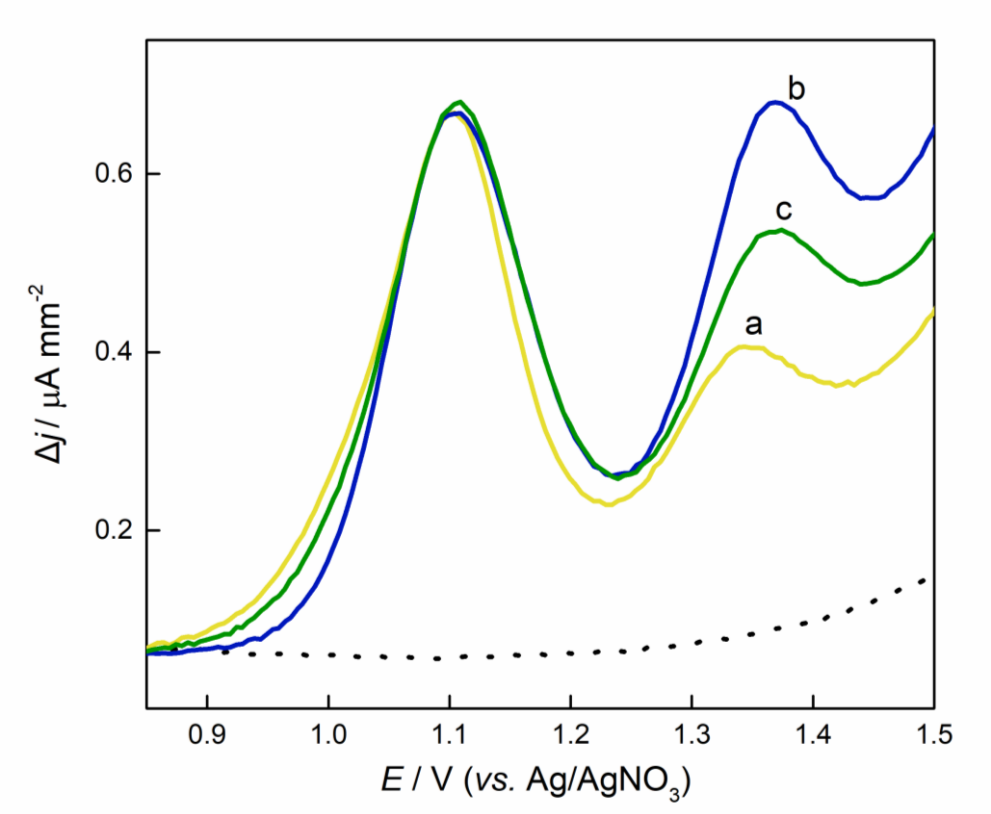

Fig. 10 Differential pulse voltammograms of (a) CA, $c = 9\times10^{-5}$ µmol L^{-1}; (b) CDCA, $c = 9\times10^{-5}$ µmol L^{-1}; and (c) mixture of both CA, $c = 4.5\times10^{-5}$ µmol L^{-1} and CDCA, $c = 4.5\times10^{-5}$ µmol L^{-1} recorded under optimized conditions. Solutions were heated up to 50 °C for 5 minutes before the measurements. Supporting electrolyte 0.1 µmol L^{-1} $HClO_4$ in acetonitrile (water content 0.550 ± 0.002%) in dotted line. Polished BDD electrode (A_{geom} = 7.07 mm^2). Reprinted with permission from **[72]**.

To assess the feasibility of transferring the procedure to liquid-flow systems (FIA or HPLC), the effect of increased water content and the pH dependency of the voltammetric signal was investigated. These parameters are highly important, especially in HPLC, because standard chromatographic columns are sensitive to extreme pH values. Adjusting the water content in HPLC allows for higher variability in polarity of the mobile phase used, thus for potentially increased separation efficiency. In FIA, the option to use mixed organic-aqueous phase results in a decrease in consumption of expensive and potentially toxic organic

solvents, especially in analyses conducted at large scale. It was shown that after the activation reaction proceeds (BA in contact with 0.1 mol L^{-1} $HClO_4$ in acetonitrile at 50 °C for 5 min) both the water content and pH can be increased without losing the voltammetric signal. This allows for a wide range of pH (confirmed 1–13) and water content (confirmed up to 40%) adjustments according to the requirements of the subsequently employed liquid-flow technique. Throughout this work, there was no need to adjust neither the pH of the studied solutions, as the techniques used are robust enough to operate under highly acidic conditions, nor the water content simply because it would not add any benefit to the selectivity or sensitivity of the method.

3.3 Basic characteristics of the electrochemical oxidation of CA and CDCA

Understanding the nature of the reaction on which an analytical procedure is based on is beneficial for selecting the optimal parameters of such procedure. Thus, in Fig. 11, the mechanism of the electrochemical oxidation (steps two and three) of activated (step one) CDCA is proposed. A similar process is expected for CA. First, an electron is abstracted from the double bond of the activated CDCA (**2**) giving rise to a radical cationic species (**4**). This species is then approached by a water molecule, which, acting as a nucleophile, is added on the steroid core. After that, one more electron and two protons are abstracted from the intermediate, and a ketonic species (**5**) is formed. Overall, it is a two electron, two proton electrochemical process. Obviously, the likelihood of double bond migration is not negligible, therefore, further experiments are being conducted to confirm the exact position of the double bond, and the ketonic function, respectively. To evaluate the nature of the electrochemical process, linear sweep voltammograms with increasing scan rate (5–500 mV s^{-1}) were recorded and evaluated. Slope of the log i_p (in nA) *vs.* log v (in mV s^{-1}) dependencies was 0.53 ± 0.01 for CA and 0.46 ± 0.02 for CDCA, respectively. This points to a diffusion-controlled process, for which the theoretical slope value is 0.5. Therefore, attempts to preconcentrate the analytes on the electrode surface were of no avail. A complementary reduction peak was not observed in CV even at high scan rates up to 5 V s^{-1} when the scan was terminated right after the first peak. Therefore, the process can be considered irreversible under the conditions used.

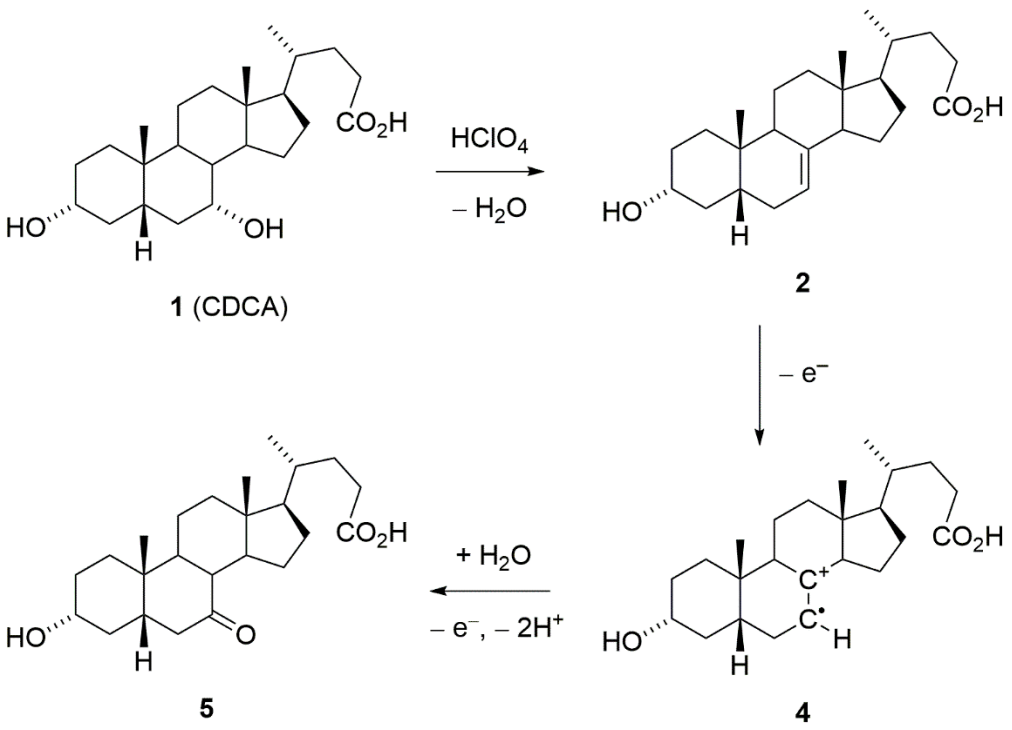

Fig. 11 Proposed mechanism of chemical activation and subsequent electrochemical oxidation of CDCA. Reprinted with permission from **[72]**.

3.4 Determination of activated CA and CDCA

3.4.1 Differential pulse voltammetry

After the BAs are activated by 0.1 mol L^{-1} $HClO_4$ in acetonitrile and their signal is stabilized and unified by implementing the heating protocol (50 °C for 5 min), they can be determined electrochemically by their oxidation. Considering the irreversibility of the BAs electrochemical oxidation process, DPV was employed as the batch quantitation technique. The following parameters were found to be optimal: step potential 5 mV; pulse time 5 ms; and pulse potential 100 mV. The selected scan rate of 50 mV s^{-1} is the maximal possible value of the instrument used in this study. To eliminate the negative effect of fouling, the electrode was polished using alumina/deionized water slurry between measurements. Neither anodic, nor cathodic activation of the BDD surface led to improved response. Calibration dependencies for CA and CDCA are summarized in Tab. 2 and shown in Fig. 12. The LODs are lower than/comparable to other studies that were aimed at low-cost platforms for BA determination [21,23,48–51]. Also, as apparent from the slope values in Tab. 2, the response

of both BAs is equivalent, which is beneficial for their simultaneous determination as the sum of the primary BAs.

Tab. 2 Figure of merit for CA and CDCA determination in 0.1 M $HClO_4$ in acetonitrile by DPV at polished BDD electrode. Reprinted with permission from **[72]**.

Bile acid	**slope (nA L mm^{-2} µmol^{-1})**	**intercept (nA mm^{-2})**	***r***	**LOD (µmol^{-1})**
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

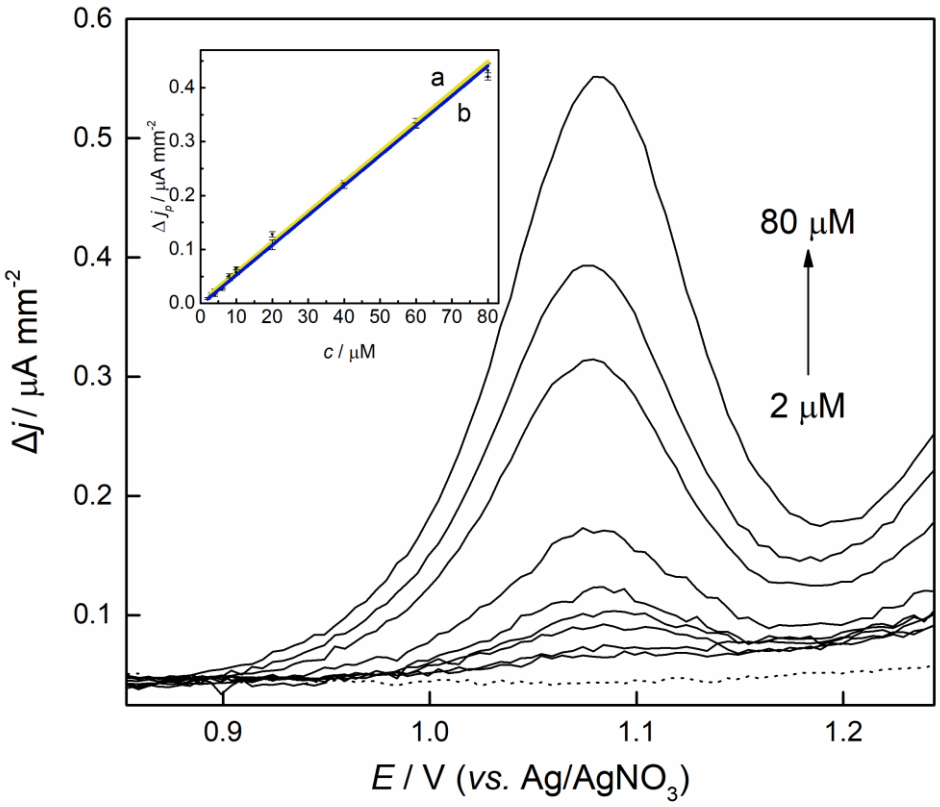

Fig. 12 Differential pulse voltammograms of CA (2, 4, 6, 8, 10, 20, 40, 60, and 80 µmol L^{-1}) obtained under optimized conditions. Inset: Calibration dependencies of (a) CDCA, and (b) CA obtained using DPV (error bars represent SD). Each solution of the BA was heated up to 50 °C for 5 minutes before the measurement. Supporting electrolyte: 0.1 mol L^{-1} $HClO_4$ in acetonitrile in dotted line (water content 0.550 ± 0.002%). Polished BDD electrode (A_{geom} = 7.07 mm^2). Reprinted with permission from **[72]**.

The most common matrix in which BAs are determined is blood serum. Therefore, experiments were conducted in artificial serum (prepared according to refs. [77,78]) to optimize the analytical procedure, and subsequently in human blood serum. Both were spiked with CA and CDCA to provide the total concentration of 20 µmol L^{-1}. As mentioned before, excessive water content does not allow the dehydration reaction to proceed. For that reason, a simple solid-phase extraction on C18 cartridge, modified from ref. [51], had to be implemented in the procedure. After extraction, the BAs were determined by standard addition method using DPV and previously proposed HPLC-FLD used as a reference method [40].

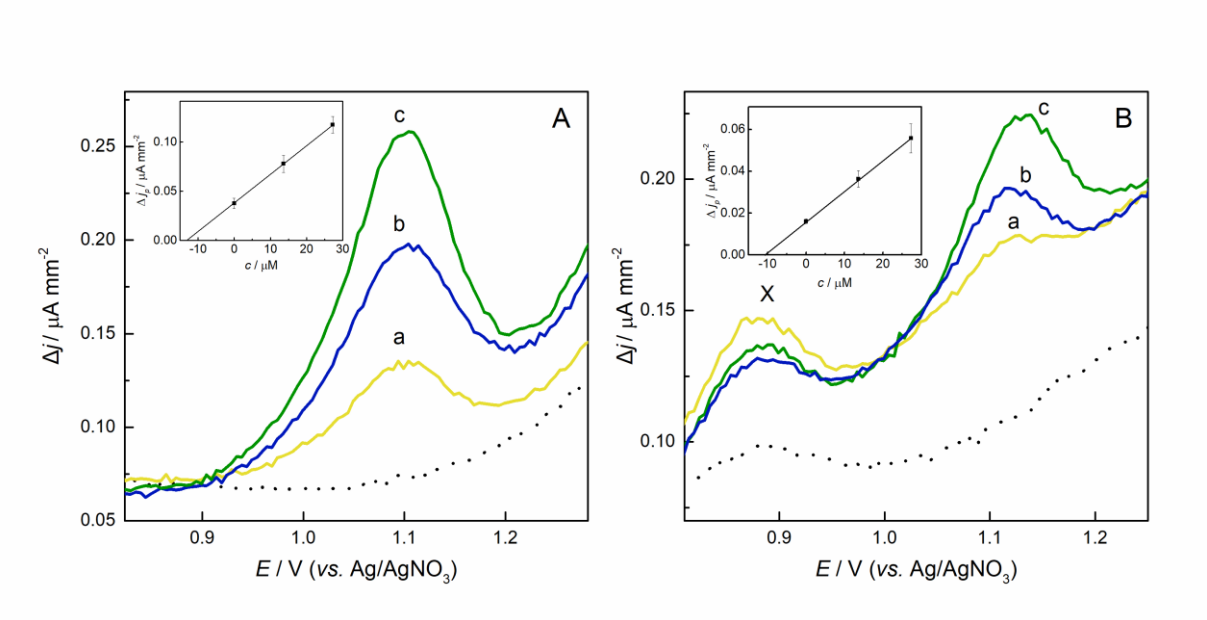

Fig. 13 Differential pulse voltammograms of CA after extraction from (A) artificial serum, and (B) human serum obtained under optimized conditions. (a) Sample, (b) standard addition of c = 13.6 µmol L^{-1} CA, and (c) standard addition of c = 27.3 µmol L^{-1} CA, control extract with no spike in dotted line. Solutions were heated up to 50 °C for 5 minutes before the measurements. Supporting electrolyte 0.1 mol L^{-1} $HClO_4$ in acetonitrile (water content 0.550 ± 0.002%). Polished BDD electrode (A_{geom} = 7.07 mm^2). Insets: Standard addition dependency with the respective extrapolation used for quantitation of CA, error bars represent SD. Pearson's r > 0.9999 for both (A) and (B). Reprinted with permission from **[72]**.

The voltammetric response of CA extracted from artificial and human serum is shown in Fig. 13. It is apparent that after the extraction from both artificial (3.13A) and human (3.13B) serum sample a well-developed peak rising linearly with increasing concentration of the BA

was observed. Slightly poorer development of the peaks and a potential shift to more positive potentials in the case of human serum is naturally caused by its more complex nature. A similar response was attained with CDCA. The results obtained by both DPV and HPLC-FLD are summarized in Tab. 3. Student's *t*-test was employed to determine whether there is a significant difference between these two methods. The null hypothesis was set to state that any discrepancies in the obtained results are purely due to random and not systematic errors. It is apparent from the p values that both in artificial serum and in human serum the results do not differ significantly ($p > 0.05$). The recoveries were slightly lower for CDCA compared to CA in artificial serum. This is most likely caused by lowered extraction efficiency of the SPE towards CDCA in artificial serum. In human serum, the recoveries for CA and CDCA were slightly lower compared to HPLC-FLD (82% and 80%, 89% and 88% respectively). This is presumably caused by passivation of the electrode by an electroactive compound, that is co-extracted from human serum with the BAs, and is apparent as peak "X" in the recorded voltammograms (Fig. 13B).

Tab. 3 Figure of merit for CA and CDCA determination in artificial and human serum by DPV. Uncertainty represents standard deviation (SD) for 5 repeated measurements. Reprinted with permission from **[72]**.

BA		**DPV**		**HPLC-FLD**		*t*-test / p value
	c added (μmol L^{-1})	*c* found ± SD (μmol L^{-1})	recovery ± SD (%)	*c* found ± SD (μmol L^{-1})	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 proposed voltammetric method can determine the BAs in both artificial and human serum providing results not significantly different from the HPLC-FLD method. Relatively high recoveries between 80% and 90% were consistently attained. The method proved to be selective for CA and CDCA even in a complex matrix such as human serum. However, determination of an individual primary BA (CA or CDCA), in the presence of the other one, is not possible under these conditions due to the similarity in their detection potentials. Only total primary BAs can be determined this way. In future works, steps ought to be taken to further optimize the extraction procedure to increase the extraction efficiency and eliminate (electrochemically active) interferents.

3.4.2 Flow injection analysis with amperometric detection

To assess the feasibility of transferring the voltammetric method to liquid-flow systems, pilot experiments in FIA-AD (amperometric detection) were performed using wall-jet detection cell with BDD electrode. Several parameters were optimized, such as detection potential (E_{det} = +1.8 V *vs.* Ag/AgCl), flow rate (3.5 ml min^{-1}) and injection volume (V_{inj} = 49 µL). After the chemical activation of CA and CDCA, their concentration dependencies were obtained (Fig. 14, Tab. 4). The LODs were estimated to be *ca* 1 µmol L^{-1}, which is comparable to the LODs obtained in DPV (0.5 µmol L^{-1} for CA, 1.0 µmol L^{-1} for CDCA). It is apparent that both BAs show linear course of the concentration dependencies, however, their slopes differ. The culprit of this discrepancy is most likely a slight difference in the positioning of the electrode within the detection cell for the individual BAs. However, there is a major benefit of this approach compared to DPV, taking advantage of BDD's low propensity to fouling. The working electrode in this experimental setup does not require mechanical polishing between individual scans, hence, when the method is fully transferred to a liquid-flow system, it will become significantly more user-friendly and cost-effective. However, the construction of the detection cell needs to be addressed so that a unified current response between measuring sessions is attained. Hopefully, these pilot results will lead to a full FIA/HPLC method applicable on real-life matrices in the future.

Tab. 4 Figure of merit for CA and CDCA determination. Measured by FIA-AD in 0.1 M $HClO_4$ in acetonitrile. The following conditions were used: E_{det} = +1.8 V (*vs.* Ag/AgCl), flow rate: 3.5 ml min^{-1}, injection volume (V_{inj} = 49 µL).

Bile acid	slope (nA L mm^{-2} $µmol^{-1}$)	intercept (nA mm^{-2})	*r*	LOD (µmol L^{-1})
CA	10.38 ± 0.13	–2.97 ± 2.12	0.9994	1
CDCA	8.34 ± 0.40	+2.68 ± 2.94	0.9908	1

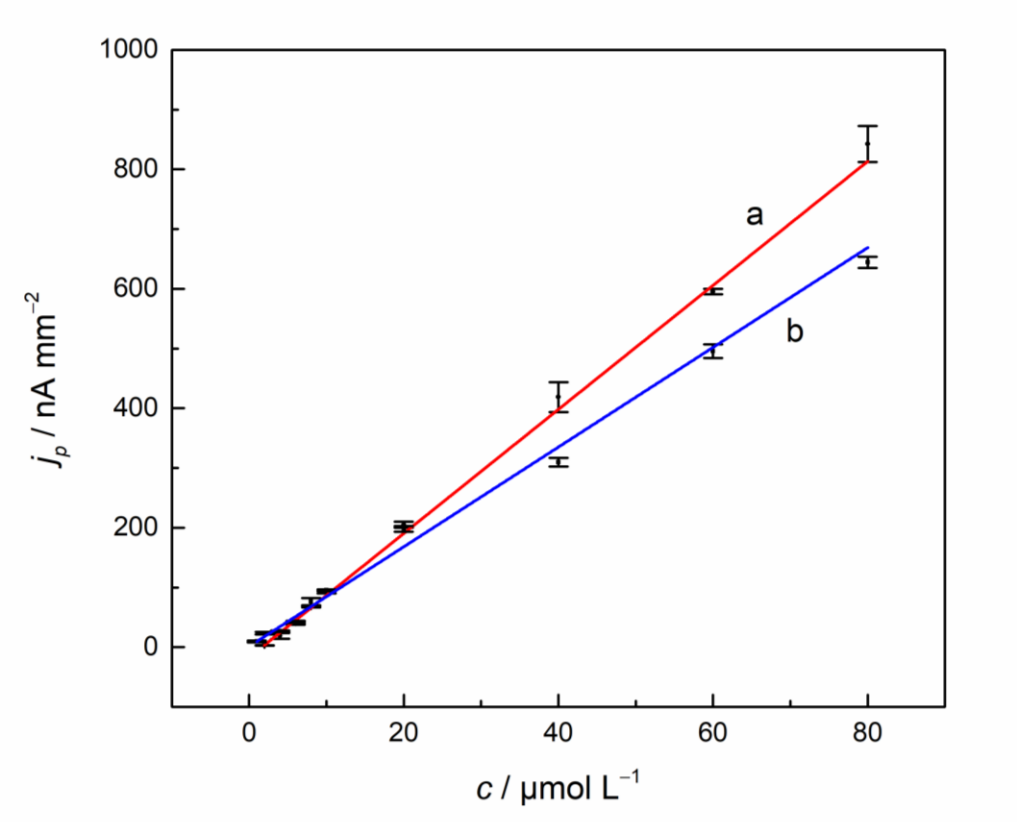

Fig. 14 Concentration dependency of (a) CA and (b) CDCA obtained by FIA-AD. Run electrolyte 0.1 mol L^{-1} $HClO_4$ in acetonitrile (water content 0.550±0.002%). Mobile phase 0.1 mol L^{-1} $HClO_4$ in acetonitrile (water content 0.550±0.002%). Boron doped diamond working electrode (A_{geom} = 7.07 mm^2), reference Ag/AgCl (3 mol L^{-1} KCl) electrode. Solutions were heated up to 50 °C for 5 minutes before the measurements. Each calibration point was measured 5 times, error bars represent SD. The following conditions were used: E_{det} = +1.8 V (*vs.* Ag/AgCl), flow rate: 3.5 ml min^{-1}, injection volume (V_{inj} = 49 µL).

4 Conclusion

In this doctoral thesis, a novel electrochemical method for the determination of two major primary BAs in human, CA and CDCA, is proposed and characterized. The method consists of two steps: (i) chemical activation of the BAs to increase their susceptibility to electrochemical oxidation and (ii) quantitation of the activated BAs by their oxidation in voltammetry or amperometry. This approach is inspired by the Liebermann-Burchard reaction of cholesterol, which, by multiple dehydrations and other modifications of the steroid core, increases the VIS absorption properties of cholesterol and enables its colorimetric determination. In this work, instead of VIS absorption, the electrochemical activity of CA and CDCA is increased by a chemical reaction. The chemical activation is attained by dehydrating the BAs with a solution of $HClO_4$ in acetonitrile so that it provides well-developed voltammetric response at *ca* +1.2 V (*vs*. $Ag/AgNO_3$ in acetonitrile) in CV.

The nature of the chemical activation process was investigated using CE-MS, and the results corroborate a loss of H_2O of the parent BA molecule upon becoming electrochemically active. The mechanism of the electrochemical oxidation has been proposed, it consists of electrochemical abstraction of an electron from the double bond newly introduced to the structure of the BA by the chemical activation. Then, this newly formed radical cationic species is approached by a water molecule, acting as a nucleophile, which is added on the steroid core. The resulting intermediate is further oxidized to a ketonic species by losing an additional electron and two protons. Overall, this is a two electron two proton electrochemical process. Obviously, there is a possibility of double bond migration within the steroid core, therefore, the exact position of the resulting ketone cannot be stated with high certainty at this point. Moreover, a mixture of products can be also expected after the chemical activation. Experiments including NMR and cyclic IM-MS characterization of the product(s) of both the chemical activation and the electrochemical oxidation are underway, and the results will be duly published in a separate study.

The chemical activation reaction is significantly affected by the water content in the dehydrating solution (should be kept as low as possible, 0.55% in this work), reaction temperature (if increased, the reaction proceeds with higher rate, heating at 50 °C for 5 minutes was used in this work), and by the choice of the dehydrating agent (signal was

observed only with $HClO_4$ and H_2SO_4; HCl and H_3PO_4 proved incapable to activate the BAs for electroanalysis). All these parameters must be strictly followed for the dehydration reaction to proceed fully. Nevertheless, once the dehydration reaction proceeds, the water content, as well as the pH of the solution, can be increased. This allows for greater variability of the solution parameters (polarity, pH) in liquid flow techniques, especially in HPLC.

The method is applicable to primary bile acids, *i.e.*, CA and CDCA. Other common BAs in humans, such as DCA or LCA, or a BA widely used in medical preparations, UDCA, cannot be activated in the same way. This highlights the importance of a structural feature present only in CA and CDCA amongst these BAs – the axial 7α hydroxyl group. Both DCA and LCA lack hydroxyl group at C7 altogether, and UDCA, a stereoisomer of CDCA, possesses the equatorial 7β hydroxyl group (for structures see Fig. 1). It is obvious that the axial 7α hydroxyl group renders the BA molecule especially prone to dehydration. For this reason, it is hypothesized that other BAs that possess the axial 7α hydroxyl group, natural or synthetic, should become electrochemically active under similar conditions. This would significantly widen the limited determination options for molecules such as α-muricholic acid, a major BA in mice, or various synthetic BAs that are candidate molecules for applications as medical drugs. All these possibilities should be further evaluated and pursued in follow-up studies conducted beyond the scope of this thesis. Moreover, similar positive effect on electrochemical activity was observed with related steroid compounds, cholesterol and 7-DHC. Based on these findings, methods of their voltammetric and amperometric determination are being developed in separate diploma/dissertation projects at author's home institution.

The LODs of the newly developed DPV method for the CA and CDCA determination are 0.5 mol L^{-1}, and 1.0 mol L^{-1} respectively. In a pilot FIA-AD experiment, LODs of *ca* 1 mol L^{-1} were obtained. These are competitive LOD values, especially when compared to biosensor platforms aimed at fast and low-cost determination of CA and CDCA. The LOD values of the newly developed method are comparable or lower than the ones of the currently existing methods [21,23,48–51]. Methods employing technically advanced equipment such as MS usually report notably lower LODs in the nanomolar range. On one hand, extremely low LODs can be viewed as unsubstantiated in clinical practice where, very often, CA and CDCA are of interest, and their levels usually range between tens to hundreds of micromoles per litre levels in patients with various diseases. On the other hand, in research applications, methods

combining a separation technique and MS detection can prove invaluable, as they allow for differentiation of several minor BAs and their derivatives present in the human body. This is something that is hardly achievable with simpler and more straightforward techniques, such as the one presented in this work.

Overall, a simple and robust method for the electroanalytical determination of CA and CDCA was developed. This method allows simultaneous determination of both these BAs as the total primary BA pool. Moreover, with prolonged heating, it allows for their differentiation. It is applicable to both batch measurements (DPV), as well as liquid-flow techniques (FIA-AD). Owing to its competitive LODs, simplicity, and low time consumption, it has the potential to serve as a point of care technique in diagnosis of the number of diseases where CA or CDCA levels are of clinical importance.

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