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Application of electroanalytical methods in food and pharmaceutical analysis

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Doctoral Thesis

Pardubice 2023

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This doctoral thesis is dedicated to my father, who has left our world on 1st April, 2013. I would like to thank my family and friends for giving me a lot of help and support during my studies. It was an honour to be granted this opportunity to study at the University of Pardubice. I am thankful for all that was given and for all that I have taken. I thank you all for the support and generosity that helped me so much.

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TITLE

Application of electroanalytical methods in food and pharmaceutical analysis

ANNOTATION

The main purpose of the research conducted within the frame of this doctoral thesis was to develop new electroanalytical approaches and methods applicable in food and pharmaceutical analysis. Due to the importance of the presence of lipophilic vitamins in food and pharmaceutical formulations, the simultaneous detection of lipophilic vitamins (A, E and K), and related compounds had been the primary goal of the dissertation. Since lipophilic analytes are usually occurring in complex matrices, the simplification of real samples treatment prior the analysis and elimination of toxic organic solvents in comparison with the previous methods have been the other benefits of this work. In addition, the development of low-cost and easy-to-use disposable sensors for the determination of selected biologically active compounds was yet another target of the whole research.

KEYWORDS

Lipophilic vitamins, foodstuffs, pharmaceuticals, voltammetry, electrodes and sensors

NÁZEV

Využití elektroanalytických metod v potravinářské a farmaceutické analýze

ANOTACE

Hlavním účelem výzkumu uskutečněného v rámci předkládané dizertační práce bylo vyvinout nové elektroanalytické přístupy a metody aplikovatelné v potravinářské a farmaceutické analýze. Vzhledem k důležitosti přítomnosti lipofilních vitaminů v potravinách a farmaceutických přípravcích byla hlavním cílem simultánní detekce těchto vitaminů (A, E a K) a příbuzných látek. Jelikož se tyto lipofilní látky obvykle vyskytují ve složitých matricích, dalším zlepšením v rámci této práce bylo zjednodušení zpracování reálných vzorků a eliminace použití toxických rozpouštědel ve srovnání s dosud publikovanými metodami. Navíc byly vyvíjeny levné a snadno použitelné jednorázové sensory pro stanovení vybraných biologicky aktivních látek.

KLÍČOVÁ SLOVA

Lipofilní vitaminy, potraviny, farmaceutika, voltametrie, elektrody a sensory

Table of Contents

List of Abbreviations

SWAdSV	square-wave adsorptive stripping voltammetry
APP	atactic polypropylene
ASV	anodic stripping voltammetry
AuE	gold electrode
BCA	*β*-carotene
CPE	carbon paste electrode
CSV	cathodic stripping voltammetry
DMF	*N*, *N*-dimethylformamide
DPV	differential pulse voltammetry
E_{ampl}	potential amplitude
ERGO	electrochemically reduced graphene oxide
E_{step}	potential step
EtOH	ethanol
ExSV	extractive stripping voltammetry
f	frequency
FIA	flow injection analysis
GC	gas chromatography
GCE	glassy carbon electrode
GCPE	glassy carbon paste electrode ~~made with glassy carbon particles~~
HPLC	high performance liquid chromatography
I_p	peak current
LC	liquid chromatography
LSV	linear sweep voltammetry
MeCN	acetonitrile
MeOH	methanol
MO	mineral oil
MWCNTs	multi-walled carbon nanotubes
PW	paraffin wax

RAc	retinyl acetate
RAN	ranitidine
RPa	retinyl palmitate
RSD	relative standard deviation
SDBS	sodium *o*-dodecylbenzenesulphonate
SDS	sodium dodecyl sulphate
SO	silicone oil
SWV	square-wave voltammetry
TCP	tricresyl phosphate
UV	ultraviolet
VA	vitamin A
VD	vitamin D
VE	vitamin E
VK	vitamin K
VK_1	vitamin K_1
α-TOAc	α-tocopheryl acetate
α-TOH	α-tocopherol
γ-TOH	gamma tocopherol
δ-TOH	delta tocopherol

Introduction

Biologically active compounds are essential for human body, neutralize free radicals that can cause damages. Thus, in the last decades there is an increased need to monitor them. [1-4]. Data from numerous scientific studies have shown the effect of these compounds on human health for the prevention of many diseases such as atherosclerosis, cardiovascular, metabolic, diabetes, rheumatism, inflammation, age-related eye diseases, and cancer [5-11]. To prevent such damage from harmful free radicals, it is recommended to maintain a healthy diet, with fruits and vegetables that are naturally rich in various antioxidants and phytochemicals such as polyphenols, flavonoids, oligosaccharides, and cyclic polyols including cyclitols [12-14]. Electroanalytical methods can provide very sensitive procedures for the determination of many compounds. They are also characterized by many other favourable characteristics, including relatively low-cost instrumentation, fast analysis times, no special sample handling required, miniaturized equipment, smaller reagents consumption, and the possibility to determine several analytes simultaneously [15-19].

The main purpose of this dissertation research was the development of new electroanalytical methods that can be used in food and pharmaceutical analysis. The focus was mainly on the development of new approaches for the simultaneous detection of lipophilic vitamins (A, E and K), vitamin D was not included in this study. Considering the physicochemical properties of lipophilic vitamins, which are not soluble in aqueous solvents, many parameters that play a key role, such as working electrode material (metal, carbonaceous, boron-doped diamond), different organic solvents, different ratios of organic solvents with water, the effect of anionic, cationic, and non-ionic surfactants, parameters of electrochemical methods, etc., have been investigated.

In previous work, the electroanalytical group in the Department of Analytical Chemistry, University of Pardubice, reported several electrochemical methods for the determination of lipophilic vitamins using square-wave adsorptive stripping voltammetry (SWAdV) on carbon-based electrodes. The methods mainly relied on two steps: the accumulation of substances in an organic solvent or in a mixture of organic solvent with water for a certain period, and then the electrochemical detection in a predominantly acidic buffer medium when using square-wave voltammetry (SWV) [16-21]. The respective methods developed shown results that were promising for the simultaneous determination of lipophilic vitamins. In the works presented in this thesis, the nonaqueous carbon paste electrode (CPE) was initially studied for the possibility of use in the analysis of lipophilic vitamins **(Publication**

1) [22]. Simultaneous determination of retinyl acetate (RAc) or palmitate (RPA), and alpha-tocopheryl acetate (α-TOAc) in cosmetic products has been achieved for the first time **(Publication 2)** [23]. Monitoring vitamin A as sum of retinoids and carotenoids (VA) in cow's milk and cream was possible without any need for sample treatment, where the extraction of analytes was done directly on glassy carbon paste electrode (GCPE) with subsequent electrochemical detection by SWV **(Publication 3)** [24]. A gold electrode (AuE) was used to detect *β*-Carotene (BCA) using nontoxic solvents in raw vegetables and pharmaceutical preparations **(Publication 4)** [25]**.** Simultaneous determination of vitamin E (VE) and vitamin K (VK) at the glassy carbon electrode (GCE) using SWAdSV in pharmaceutical preparations was also achieved **(Publication 5)** [26]. The electrochemical behaviour of alpha, gamma, and delta tocopherols (α-TOH, γ-TOH, and δ-TOH) was investigated to find out optimum working conditions for their simultaneous voltammetric detection. After thorough optimization of working conditions, their voltammetric determination has been possible using mathematical evaluation of current signals **(Publication 6)** [27].

The development of fast and low-cost methods for the determination of the selected biologically active substances was another goal of this doctoral thesis. Considering the large consumption of meat products and alcoholic beverages, two biologically active analytes, nitrites (used as additives in meat products) and ethanol (consumed by humans in alcoholic beverages), which have a major impact on human health, their monitoring is very important in the food industry. A new voltammetric method for the determination of nitrites in meat products has been developed. The cathodic reduction of 2-methyl-2H-furan-3-one at -0.210 V on GCE covered with a thin layer of electrochemically reduced graphene oxide (ERGO) and adsorbed sodium o-dodecylbenzenesulphonate (SDBS) surfactant was more preferred over the anodic oxidation of *N*-nitroso-dimethylamine at +0.8 V **(Publication7)** [28]. Immobilization of alcohol dehydrogenase into graphene-based composite material was utilized to construct a relatively simple bioanalytical device suitable for amperometric detection in the flow injection analysis (FIA) regime and applicable to the determination of ethanol in highly alcoholic drinks and some white wines **(Publication 8)** [29].

1. Lipophilic vitamins and selected biologically active compounds

1.1 Physico-chemical properties of lipophilic vitamins A, E and K

Lipophilic vitamins are found naturally in low amounts in different types of food products. The presence of these vitamins in our dietary food is essential since the human body cannot synthesize them [1-3]. Vitamins (A, D, E, and K) are four major lipid-soluble vitamins located in the phospholipid bilayer membranes that form cell walls [30]. Deficiencies of these vitamins in the daily intake may be associated with an increase in the presence of various diseases such as light blindness, xeropthalmia, keratomalacia (VA) [3,5], rickets, osteomalacia (vitamin D VD) [6], neuromuscular and neurological disorders (VE) [7-9], and reduced blood clotting leading to excessive bleeding (VK) [10,11]. Nowadays, people's awareness about the food they consume, based on the importance of vitamins in our diet, has increased significantly. For this reason, monitoring vitamins in the food, and clinical and pharmaceutical analysis are very important.

1.1.1 Vitamin A

Vitamin A consist of group of compounds, such as retinol, retinal, retinoic acid, and pro-vitamin A, such as BCA, their chemical structures are shown in Figure 1 (Fig. 1). All these organic compounds are mainly taken from animals and plant resources [31-34]. VA activity comes from the presence of the *β*-ionone ring and the isoprenoid chain. Carotenoids contribute significantly to the activity of VA, both in animals and in vegetables. Over 500 carotenoid compounds are known, of which about 50 belong to the group of provitamin A. The most important provitamin A is BCA. Retinol may be present in food in the form of structurally different analogues and metabolites. In food of animal origin, the most often found compounds are retinol and its esters, retinal, and retinoic acid. Retinoids are used to fortify (enrich) food or in pharmacy. RPA is mainly used for the fortification of dairy products [35]. VA is very easily oxidized in the presence of light and its stability is mainly affected by heat, temperature, and pH. The loss of activity occurs even in the free presence of radicals. All-*trans*-retinoids can be converted to *cis* isomers at higher temperatures, leading to lower activity of vitamins. Retinoids and carotenoids are two forms of VA that predominate in foods [35-37]. The main food sources of VA include dairy products, liver, fish, and fortified cereals, while the main sources of provitamin A are carrots, sweet potato, broccoli, spinach, melon, and squashes. Increased interest in VA is associated with performing certain functions throughout the body; for example, retinoic acid has a role in the metabolism of VA in the liver, regulating the metabolism of

carbohydrates, proteins, and lipids. As an antioxidant, VA protects cholesterol from oxidation, which has a big role in the inhibition of the growth of tumour cells. Moreover, all-*trans*-retinol has several beneficial effects on various biological processes in the skin. In addition, getting enough VA in the body reduces the risk of heart attack [3,5,38]. The deficiency of VA causes many disorders in the body, including the deposition of cholesterol within the arteries by the rapid oxidation of cholesterol, which is the main cause of heart attack, inflamed skin, infertility, delayed growth, and respiratory infections [3,39,40].

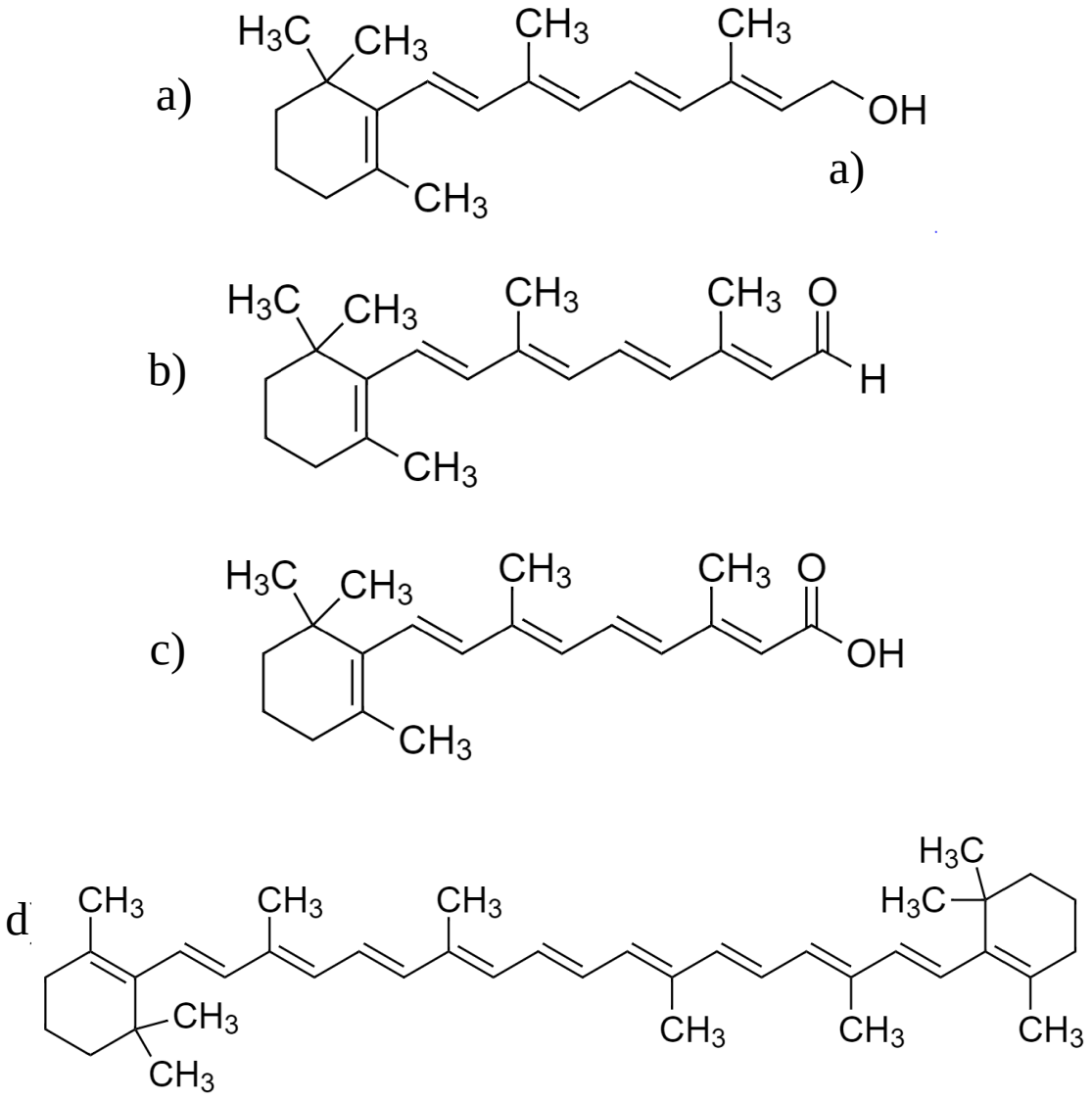

Figure 1. Chemical structures of retinol (a), retinal (b), retinoic acid (c), and β-carotene (d).

Retinoids and carotenoids can be determined spectrophotometrically in the ultraviolet (UV) and the visible-light region, respectively, because of their conjugated double bonds system. Due to their lipophilic character, the preparation of samples such as margarines and

dairy products for analysis is very complex and require a long time: sample is firstly saponified in an alkaline solution of ethanol (EtOH) and water, followed by neutralization and dilution. After the saponification, retinoids turn into retinol and the fats are washed away as fatty acids. Individual retinoids can be analysed using reverse phase high-performance liquid chromatography (HPLC). HPLC with a UV detection (VA absorbs from 310 nm to 328 nm) is the most common technique used for the determination of VA [41-44]. The measurement of carotenoids is a very complex process due to many naturally occurring chemical forms present in foods [45]. VA in the blood serum can also be determined by capillary zone electrophoresis with fluorescence detection [46]. VA can be electrochemically determined at different types of electrodes using various electrolytes. More details about the electrochemistry and electroanalysis are presented in chapters 1.2.1 and 1.3.1, respectively.

1.1.2 Vitamin E

VE is a fat-soluble antioxidant consisting of eight isomers, namely four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ). Their chemical structures are shown in Fig. 2 [3,47-49]. They differ from each other by the number and positions of the methyl groups in the chromanol ring relating to a hydroxyl group. VE isomers can donate hydrogen atom to reduce free radicals. All chromanol rings contain the hydrophobic side chain that enables the penetration of these compounds into biological membranes. Tocotrienols differ from tocopherols only in the presence of three double bonds in the hydrophobic chain. Such structure of side chain give rise to a lower biological activity of tocotrienol compared to α-tocopherol. α-TOH is the most biologically active form, although γ-TOH dominates in nature [50,51]. Synthetic α-TOAc is used to fortify foods, most edible oils, and cosmetic products. The main food sources which contain VE are edible oils, margarine, cereals, fish, nuts, and almost all dark leafy green vegetables.

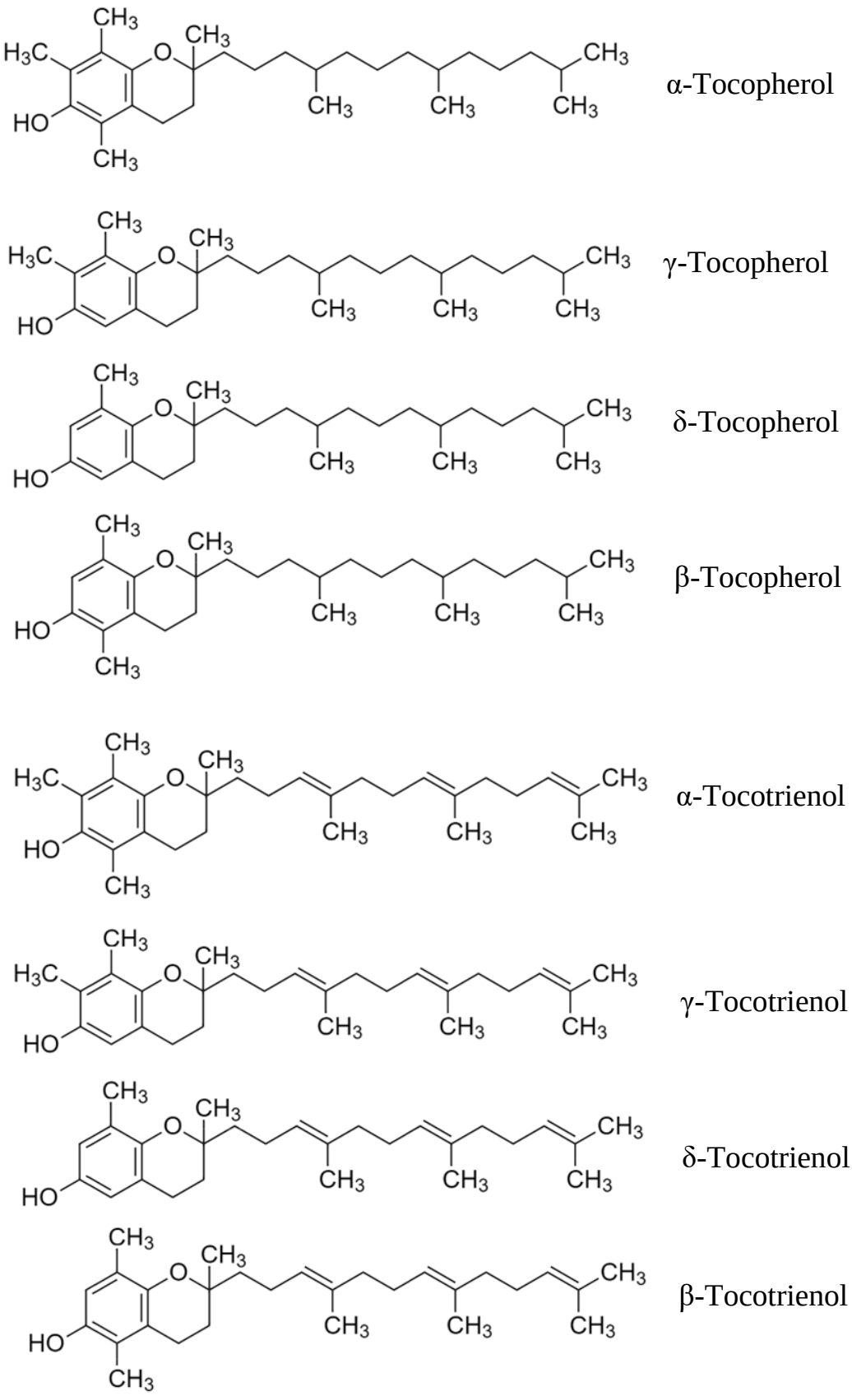

Figure 2. Vitamin E: Chemical structures of all isomers.

VE as an antioxidant has an essential role in metabolism, binding several nutrients, and endogenous factors, where together they form a multi-component system that offers protective effects against reactive types of oxygen, formed during the process of metabolism [52,53]. Moreover, VE increases the regularity of membrane lipid packaging, thus allowing a tighter membrane packaging and, in turn, greater stability for the cell. Enough α-TOH in endothelial cells inhibits platelet aggregation and releases prostacyclin from the endothelium. Several studies have found that VE is very effective in preventing and reversing many different diseases, anti-inflammatory processes, boosting immunity, and anti-carcinogenic. VE deficiency is not common in humans. Rare cases occur mainly in people with an inherited or acquired condition that impairs their ability to absorb vitamins. Some of the symptoms that appear because of insufficient VE include muscle weakness, vision problems, changes in the immune system, numbness, heart disease, and permanent nerve damage. It has been reported that VE deficiency can result in male infertility [9,54,55].

Taking into consideration all the things presented above, monitoring and analysis of the presence and levels of VE is very important, especially for food quality control. Initially, VE was determined with gas chromatography (GC), where tocopherols and tocotrienols must be firstly converted to trimethylsilyl ethers by derivatization. For the determination of VE in food, biological matrices, and cosmetics, liquid chromatography (LC) and mainly HPLC with UV, fluorescent or amperometric detection are used. LC can be performed in reverse or normal phase system [41,44,45,56-60]. The electrochemical behaviour of VE has been described in many studies. Several electroanalytical methods have been developed for the determination of VE [3,16,17,21,62-71]. More details about the electrochemistry and electroanalysis are presented in chapters 1.2.2 and 1.3.2, respectively.

1.1.3 Vitamin K

The first indications of the existence of VK were in 1929 when the Danish scientist Henrik Dam was studying the role of cholesterol in the diet. He conducted this experiment by feeding the chickens a cholesterol-depleted diet. After some time, they developed external haemorrhages and the blood taken from them coagulated slowly. However, it was not the missing cholesterol that was causing the problem. In the chemical process to remove cholesterol from the raw material, another ingredient was also inadvertently removed. When this composition was added again to the raw material, the chickens became normal. Dam realized that the mysterious composition was necessary for blood clotting, and so he called it the

"coagulation vitamin". In 1935, Dam proposed that a healing substance present in vegetables and animal sources was a fat-soluble vitamin which he called vitamin K [72]. There are two natural and three synthetic forms of VK. Vitamin K_1 (phylloquinone, VK_1) and K_2 (menaquinone) make up the bulk of VK. They are polycyclic aromatic ketones, similar in structure; they share a quinone ring but differ in degree of saturation of the carbon tail and the number of repeating isoprene units in the side chain. Their chemical structures are shown in Fig. 3 [73,74].

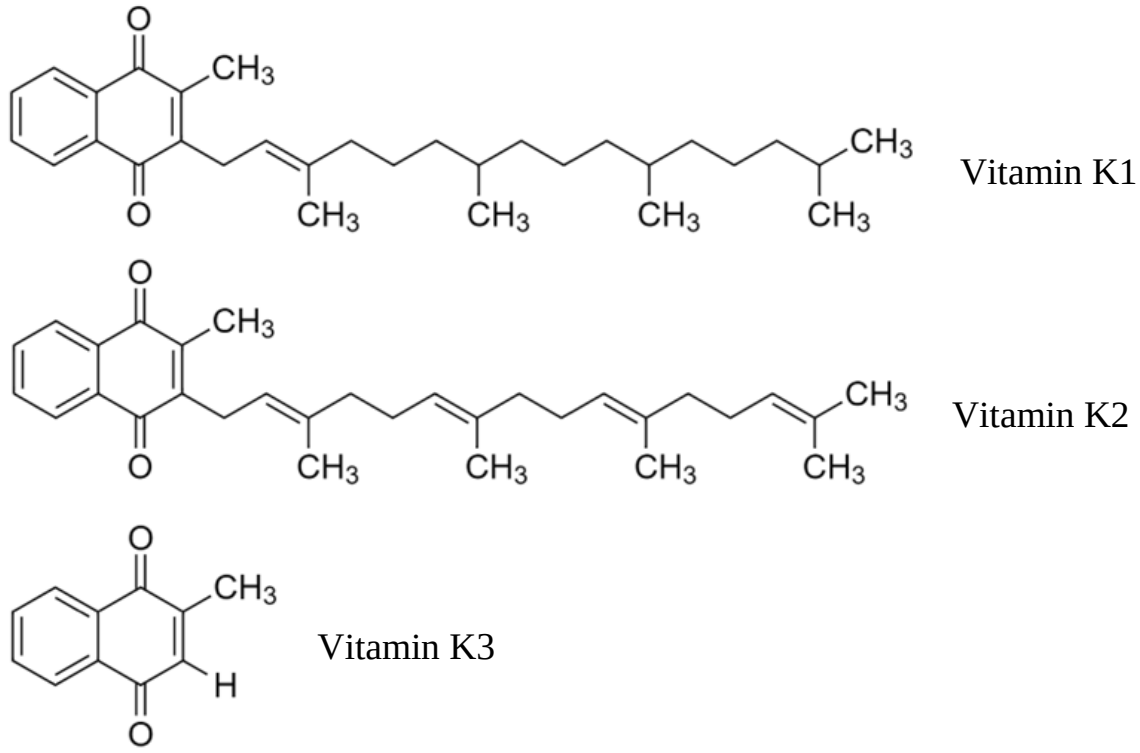

Figure 3. Chemical structures of all forms of vitamin K.

VK_1, also called phylloquinone, phytomenadione, or phytonadione, is a fat-soluble antihemorrhagic vitamin and is synthesized in plants. Good dietary sources of VK_1, where the vitamin is found in sufficient quantities, are green leafy vegetables like kale, lettuce, broccoli, cabbage, and spinach, vegetable oils like soy, sunflower, olive, and canola. The other good source are various fruits like kiwi and avocado. While vitamin K_2 is mainly produced by bacteria and is usually found in animal products or fermented foods. The required amount of VK in our diet per day is 15-30 µg for children, 80 µg for men, and 65 µg for women [75,76]. Various studies have shown that VK has many health benefits for the functioning of the body. VK has an essential role in blood coagulation and its function in cells is to convert glutamate in proteins to gamma-carboxyglutamate. Enough VK_1 in our diet can prevent many diseases

such as cancer, vascular calcification, chronic kidney disease, rheumatoid arthritis, osteoporosis, cancer, dementia, and some skin pathologies. A classic example of VK deficiency as a haemorrhagic disease in the new-born is called premature bleeding from vitamin deficiency. The disease is classified according to the time when it can occur, within the first day in the new-born, in the first week, and from the second week to six months. Low concentration of VK in breast milk, low phylloquinone cell transfer, low level of coagulation factors at birth and a sterile gut all contribute to the disease [10,11]. VK deficiency is not very common in adults, it is usually associated with specific conditions, such as malabsorption, antibiotics, or a diet with a low content of VK. Other cases of vitamin deficiency are bleeding from the nose, intestines,-or fractures, the skin of the cheeks, and other cases of bleeding. A more recent study of adult patients with advanced cancer found that about 75% had some VK deficiency measured in circulating phylloquinone concentrations or PIVKA-II tests. Anticoagulant agents are widely used for the acute treatment of venous thrombosis and arterial thrombosis and the long-term prevention of potentially recurrent thrombosis. Oral anticoagulants are widely used for patients in need of long-term anticoagulation. Warfarin, which acts as an inhibitor of VK epoxy reductase, is the most common oral anticoagulant, while the other two 4-hydroxycoumarins, acenocoumarol and phenprocoumon are used to a lesser extent [10,11,77].

Regarding the analysis of the above-described substances, spectrophotometric methods can be used to determine VK, however, HPLC coupled with UV, fluorescent, electrochemical, chemiluminescent, or mass spectrometry detection is generally used to determine the individual forms of VK [45,78]. The electrochemical behaviour and developed and several electroanalytical methods for the determination of VK have been reported [3,19,79,80-82]. More details about the electrochemistry and electroanalysis are presented in chapters 1.2.3 and 1.3.3, respectively.

1.2 Electrochemical properties lipophilic vitamins A, E and K

1.2.1 Electrochemical properties of vitamin A, its esters, and *β*-carotene

As mentioned above, lipophilic vitamins are electroactive compounds that can be oxidized or reduced. Several studies about the electrochemical properties of retinol, retinyl acetate, and retinyl palmitate have been reported [83-86]. Ziyatdinova et al., based on the observation of an oxidation peak at +0.79V, proposed that all-*trans*-retinol is irreversibly oxidized with the participation of two electrons and two protons to retinal at GCE in ethanol-water mixture (Fig. 4) [85].

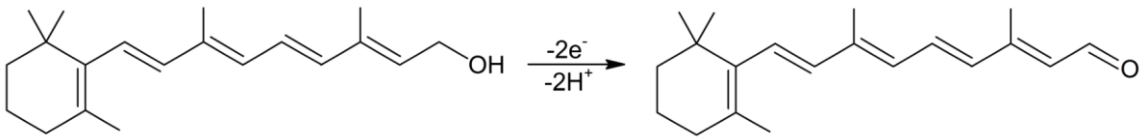

Figure 4. Proposed mechanism of retinol oxidation with formation of retinal [85].

Later, it was confirmed that all esters of vitamin A are oxidized in dichloromethane containing 0.2 mol L^{-1} Bu_4NPF_6 similarly to retinol (87). The electrochemical oxidation of retinyl propionate at the Pt electrode in MeCN containing 0.1 mol L^{-1} Bu_4NClO_4 was studied. Electron density calculations indicated that oxidation probably occurs at the C3 position of the cyclohexene ring involving two electrons and one proton [88]. A detailed study in the oxidation mechanism of all-*trans*-retinol, retinyl acetate, and retinyl palmitate in various working medium such as non-aqueous, aqueous organic mixture, and pure aqueous media was presented by Žabčíková et. al [20]. It was observed that oxidation occurred in several irreversible steps. From the obtained results of theoretical calculation of the electron density, it was indicated that most probably the oxidation is delocalized over carbon atoms of the five conjugated double bonds (C5-C14) as shown in Fig. 5 [20].

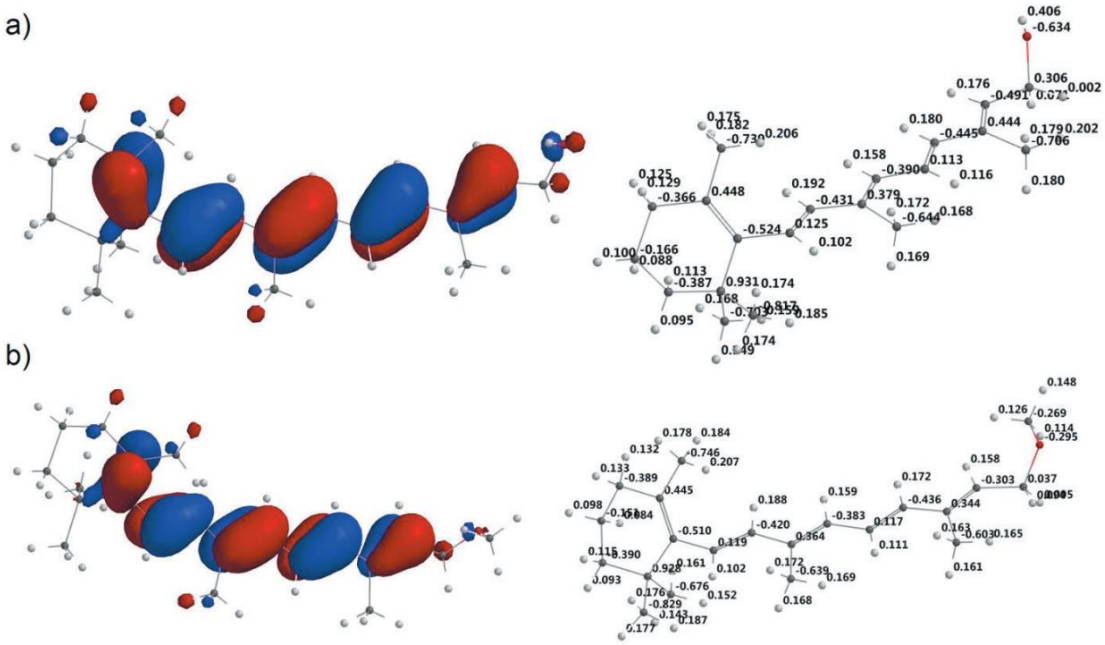

Figure 5. The highest occupied molecular orbital (HOMO) distributions and electron density plots of a) retinol and b) retinyl acetate [20].

β-Carotene, known as the most biologically active carotenoid, represents the main precursor (provitamin) of VA in the human diet. BCA is insoluble in water; thus, its

electrochemical analysis has several challenges [89-91]. The electrochemical behaviour of BCA in different solvents has been studied by several scientists [92-104]. First studies have been focused on reversible electrochemical oxidation of BCA. The oxidation of *β*-carotene in dichloromethane occurs in two reversible steps, with a cation radical as an intermediate (highly reactive) and a dication as the final product [97-99]. However, the oxidation involving single two-electron step was observed in acetonitrile and other polar solvents [100-102].

In the presence of water in the working media (mixed media), the radical β-Car$^{\bullet+}$ undergoes nucleophilic addition of water to be further oxidized in one-electron reaction generating the epoxide ring [99] or diol [103]. Two irreversible steps of *β*-carotene oxidation at 500 and 920 mV at GCE in mixture ethanol-dichloromethane containing 0.1 mol L^{-1} $LiClO_4$ has been observed [104]. Masek et al. found out that the electrochemical behaviour of *β*-carotene at a Pt electrode in non-aqueous solutions proceeds irreversibly. In addition, using the calculations of the molecular orbital energy suggested that the electrochemical oxidation of *β*-carotene is delocalized over the carbon atoms of six conjugated double bonds (C15-C15') with the highest electron density (Fig. 6) [94].

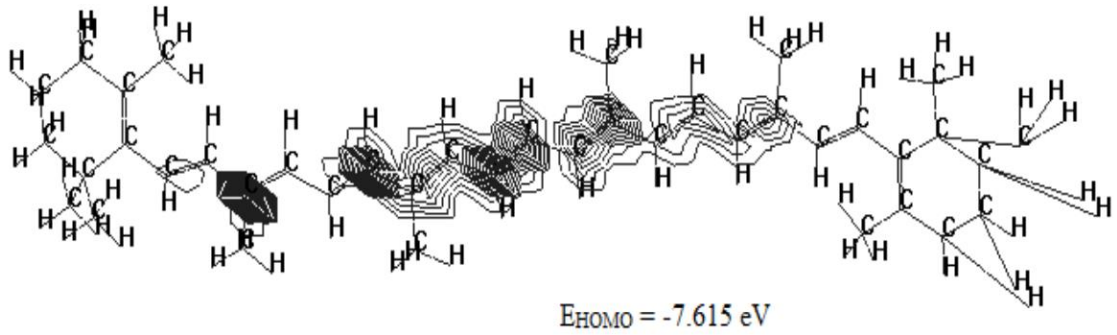

Figure 6. Electron density and probable sites in a β-carotene molecule susceptible to electrooxidation [94].

1.2.2 Electrochemical properties of vitamin E

Vitamin E, which includes several forms of tocopherols and tocotrienols, exhibits electrochemical properties due to its redox-active nature. In general, vitamin E can undergo redox reactions, where it can be oxidized or reduced. The redox behaviour of vitamin E can be affected by several factors, including the solvent used, the pH of the solution, and the presence of other compounds [3,105,106]. The electrochemical behaviour of vitamin E (especially α-tocopherol) has been studied for many years. In organic solvents such as acetonitrile and dichloromethane, the electrochemical mechanism for α-TOH (where it is also applied to other tocopherols such as β-, γ- and δ-TOHs) have been investigated [107-112]. The reaction pathway

depends on whether the oxidation occurs in the presence or absence of acids and bases soluble in organic solvents. The hydrophobic chain does not affect the electrochemical properties.

In pure acetonitrile or dichloromethane, the α-TOH is oxidized by one electron at about +0.5 (±0.1) V *vs* Fe/Fe^{+} and a radical cation $\alpha\text{-TOH}^{\bullet+}$ is formed, which quickly deprotonates to neutral radical, $\alpha\text{-TO}^{\bullet}$. This radical is immediately further oxidized on the surface of the electrode and a diamagnetic cation, $\alpha\text{-TO}^{+}$, is formed (Fig. 7) [110,112-114]. The oxidation mechanism can be considered as ECE mechanism (where E represents the electron transfer, and C the chemical step). There is another possibility where a second electron transfer step occurs through a homogeneous mechanism of disproportionation, leading to the formation of diamagnetic cation ($\alpha\text{-TO}^{+}$). Regardless of the exact pathway, the oxidation in acetonitrile and dichloromethane is completely chemically reversible [115,116]. The counter ions for the charged species are the supporting electrolyte anions [PF6], and protons likely exist coordinated to the organic solvent (or with trace water). [110,114,115].

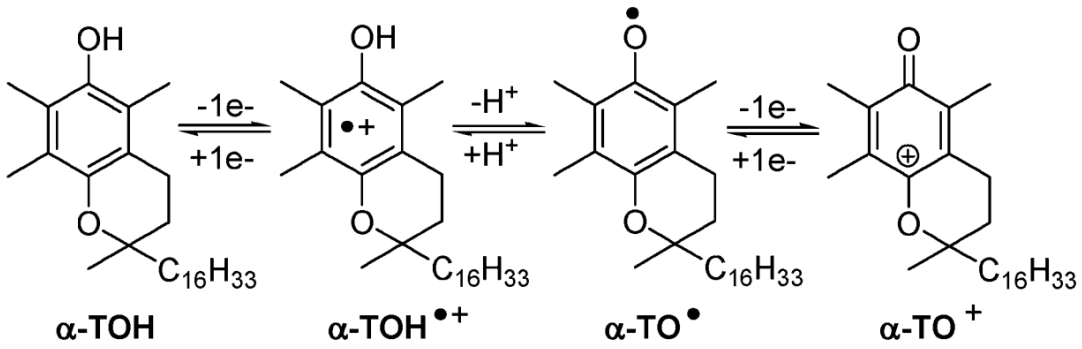

Figure 7. Electrochemically induced transformations of a-tocopherol in CH_3CN or CH_2Cl_2. One resonance structure is displayed for each compound.

1.2.3 Electrochemical properties of vitamin K

Vitamin K and its derivatives have been studied in the context of their redox properties, which relate to their ability to gain or lose electrons. Several studies on the electrochemical properties of vitamin K_1 (phylloquinone) in aqueous and organic solvents have been reported [3,105,116-121]. The same process of the reduction of quinones have been observed for vitamin K. Vitamin K is reduced in two steps with one electron to form first a radical anion and then a dianion when measurements are performed in aprotic organic solvents [118,119]. The electrochemical behaviour of phylloquinone in aqueous-organic mixture consist in the reduction by two electrons and two protons step ($+2e^{-}/+2H^{+}$) to form the phyllohydroquinone (VK_1H_2) form, and possible re-oxidation to the phylloquinone using anodic scan (Fig. 8) [3, 117,120,121].

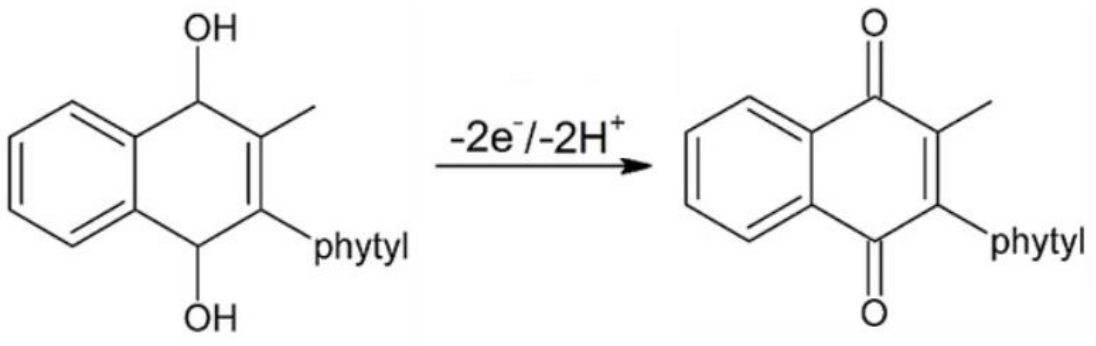

Figure 8. Electrochemical behaviour of phylloquinone in aqueous supporting electrolyte.

1.3 Electroanalysis of vitamin A, E and K

Based on the physicochemical properties of lipophilic vitamins, which are insoluble in water, their electrochemical determination can be achieved in two ways: by extractive stripping voltammetry (ExSV) or using direct pulse voltammetric techniques in organic solvents or their aqueous mixtures without a preconcentration step. Stripping voltammetry, which requires a three-electrode system, involves two steps: the first step is preconcentration, where the required substances are deposited on an electrode surface from the sample solution, and the second, stripping step is a removing of the deposited compounds from the electrode surface into the solution (usually a buffer) by a potential scan [16-19]. There are several stripping voltammetric techniques utilizing various way of accumulation of the analyte and anodic or cathodic detection step.

During the deposition, the analytes are accumulated on the working electrode under a continuous mixing at a constant potential for a given time. After the deposition step, the stirring of solution is stopped, and after a short waiting period the potential is scanned to positive values that cause oxidation of deposited species (e.g., lipophilic vitamins). The recorded peak current (I_p) is proportional to the concentration of analytes. Such a scan can be performed using various voltammetric modes such as SWV, differential pulse voltammetry (DPV), and linear sweep voltammetry (LSV) [112,123].

Anodic stripping voltammetry (ASV) is one of the most used techniques and quantitative analysis of trace and ultra-trace level of analytes in complex environmental, food, clinical, and industrial samples can be achieved. Cathodic stripping voltammetry (CSV) works similarly as ASV but in the inverse regime, when the potential is scanned cathodically to more negative potentials, i.e., the analyte deposited at the electrode is electrochemically reduced (e.g., determination of VK using CSV) [19,82,124,125]. The second way to determine lipophilic compounds is using direct pulse voltammetric techniques (without a pre-concentration step).

The measurements are performed in pure organic solvent or aqueous-organic mixture at different working electrodes. Detection levels are sufficient for the analysis of food, pharmaceutical, and clinical products.

1.3.1 Electroanalysis of vitamin A and related compounds

A lot of research has been done on the development of electroanalytical methods for the determination of vitamin A in food, clinical and pharmaceutical analysis. In 1974, the first study was made for the development of an electrochemical method for the determination of VA in a mixture of 75% ethanol containing 0.01 mol L^{-1} sulphuric acid at a carbon paste electrode using linear sweep voltammetry [83]. Budnikov et al., studied the response of retinol and α-TOH at a stationary platinum microelectrode in 0.1 mol L^{-1} perchloric acid and 0.1 mol L^{-1} sodium acetate in MeCN [84]. Michalkiewicz et al., proposed an electroanalytical method for the determination of α-TOAc using platinum microelectrodes [62]. Later, two other approaches using GCE for the determination of α-TOAc were utilized [63,64].

In 2010, Ziyatdinova et al., developed a method for the determination of retinol in an aqueous solution, in the presence of surfactant media at GCE [85]. Two years later a modification of the graphite electrode by multi-walled carbon nanotubes was proposed for the determination of α-TOH and retinol in 0.1 mol L^{-1} $HClO_4$ in acetonitrile [86]. GCE modified with poly(2,2'-(1,4-phenylene vinylene)-bis-8-hydroxy-quinoline)/MWCNTs was used as working electrode for simultaneous determination of α-TOH and retinol (VA) [65]. Adsorptive stripping differential pulse voltammetry for the simultaneous electrochemical detection of lipophilic vitamins, retinol, cholecalciferol (vitamin D_3), α-TOH (VE), and phylloquinone (VK_1) was studied by Sýs et al. in 2016 [17]. The method is based on the accumulation of lipophilic vitamins on the surface of the GCE in MeCN-water (1:1) medium with subsequent voltammetric determination in acetate buffer.

Electrochemical study and determination of all-*trans*-retinol at CPE modified by surfactant, and electroanalytical method based on anodic oxidation of the α-TOAc at CPE/SDS by SWV performed in pure organic electrolyte 99.8% MeCN containing 0.1 mol L^{-1} $LiClO_4$ was published by Žabčíková et al. in 2018 [20]. An overview of main parameters (type of electrode material, electrochemical technique, electrolyte, linear range, and limit of detection) of developed electrochemical methods for determination of vitamin A (retinol) are presented in Table 1. To date, no electrochemical method is reported for the determination for all-*trans*-retinol esters.

Table 1. Overview of developed electrochemical methods for determination of vitamin A.

Electrode	Technique	Supporting electrolyte	Linear range / mol L^{-1}	LOD / mol L^{-1}	Ref.
CPE/CW/SO	LSV	75% EtOH/0.01 M H_2SO_4	$5.0 \cdot 10^{-5}$–$1.0 \cdot 10^{-3}$	—	[83]
PtE	LSV	0.1 M $HClO_4$/MeCN	$8.2 \cdot 10^{-5}$–$1.0 \cdot 10^{-3}$	—	[84]
PtE	LSV	0.1 M CH_3COONa/MeCN	$7.5 \cdot 10^{-4}$–$1.6 \cdot 10^{-3}$	—	[85]
GCE	CV	0.1 M $LiClO_4$ / 0.1 mM SDS	$2.9 \cdot 10^{-5}$–$9.8 \cdot 10^{-4}$	$1.5 \cdot 10^{-5}$	[85]
GCE	LSV	0.1 M $HClO_4$ / MeCN	$1.3 \cdot 10^{-4}$–$1.2 \cdot 10^{-3}$	$9.5 \cdot 10^{-5}$	[86]
GCE/MWCNTs	LSV	0.1 M $HClO_4$ / MeCN	$6.5 \cdot 10^{-5}$–$1.5 \cdot 10^{-3}$	$4.0 \cdot 10^{-5}$	[86]
GCE/MWCNTs /PPH	SWV	Triton X-100 / Acetate buffer	$5.0 \cdot 10^{-6}$–$2.0 \cdot 10^{-4}$	$8.0 \cdot 10^{-7}$	[65]
GCE	DPV	0.1 M $LiClO_4$ / MeCN	$4.4 \cdot 10^{-6}$–$7.0 \cdot 10^{-4}$	$1.3 \cdot 10^{-6}$	[20]
CPE/SDS	DPV	0.1 M $LiClO_4$ / MeCN	$1.5 \cdot 10^{-6}$–$1.8 \cdot 10^{-4}$	$4.6 \cdot 10^{-7}$	[20]

CPE carbon paste electrode, CV cyclic voltammetry, CW ceresin wax (5%), DPV differential pulse voltammetry, GCE glassy carbon electrode, LOD limit of detection, LSV linear sweep voltammetry, MWCNTs multi-walled carbon nanotubes, PPH poly(2,2′-(1,4-phenylenedivinylene)-bis-8-hydroxyquinaldine), PtE stationary platinum electrode, SDS sodium dodecyl sulfate, SO silicone oil, SWV square wave voltammetry.

Although several research articles reported on the electrochemical behaviour of *β*-carotene [92-104], only three voltammetric methods have been developed for the determination of BCA so far [104,126,127]. In 1992, a voltammetric procedure for determining *β*-carotene at hanging drop mercury electrode, involving extraction from brine into dichloromethane, followed by direct determination in dichloromethane with 0.1 mol L^{-1} Bu_4NBF_4 as the electrolyte was reported [126]. The anodic oxidation of BCA using cyclic voltammetry at the GCE in a mixture of 10 mmol L^{-1} Triton X100 + 0.1 mol L^{-1} $LiClO_4$ in ethanol containing 10% of dichloromethane was used to determine the BCA in raw vegetables and berries [104]. Thompson et al. presented a new approach using cyclic voltammetry at the glassy carbon electrode modified with *β*-cyclodextrin for determination of the *β*-carotene in 0.5% Tween20 in phosphate buffer saline [127].

1.3.2 Electroanalysis of vitamin E

There are many electroanalytical approaches developed for the determination of vitamin E. Li et al. developed a method for the determination of α-tocopherol based on its

oxidation at polypyrrole-modified Pt electrode in dichloroethane-ethanol mixture using differential pulse voltammetry. The method was applied for analysis of several vegetable oils [128]. A voltammetric method for detection of α-tocopherol using square wave stripping voltammetry was proposed. The working electrode was made by mixing carbon nanotubes with double-stranded calf thymus DNA and mineral oil. Under optimized stripping conditions, the method was used to assess the quality of soybean oil samples [129]. Graphite electrode modified with multi-walled carbon nanotubes was prepared for the determination of α-tocopherol and retinol monocomponent pharmaceuticals. The method was based on the oxidation of α-tocopherol at prepared working electrode in 0.1 mol L^{-1} $HClO_4$ in acetonitrile using linear sweep voltammetry [86].

A voltammetric method for α-tocopherol determination at glassy carbon electrode in acetonitrile and its aqueous mixture was proposed. The effect of surfactants (*N*-dodecylpyridinium bromide, Triton X-100, and *N*-cetylpyridinium bromide) on the current of α-tocopherol oxidation were investigated. The presence of surfactants reduced the detection limit and extended the analytical range [130]. The electrochemical detection of α-tocopherol in methanol and in methanol/hexane mixture solutions was investigated at boron-doped diamond (BDD) electrodes by cyclic voltammetry and flow-injection electrochemical measurements. It was found that the electron transfer for electrochemical reaction of α-tocopherol was faster at hydrogenated BDD than oxidized BDD. A hydrogenated BDD electrode was used as an electrochemical detector for HPLC systems for detection of vitamin E using a flow-injection electrochemical system [131]. Square wave voltammetry at ultramicroelectrodes was presented by Robledo et al for the determination of vitamin E in edible vegetable oils. The method was based on the oxidation of α-tocopherol in benzene/ethanol (1:2) containing 0.1 mol L^{-1} H_2SO_4 at a carbon fiber disk ultramicroelectrode [132]. A method for the simultaneous electrochemical determination of α-tocopherol and retinol using a poly(2,2′-(1,4-phenylenedivinylene)-bis-8-hydroxyquinaldine)/multi-walled carbon nanotubes-modified glassy carbon electrode was developed. Triton X-100 was used to solubilize the analytes in the absence of organic solvent. The developed method was applied in the analysis of pharmaceutical products [65].

A nanocomposite Nafion®/graphene electrode showed excellent electrochemical reactivity toward the oxidation of α-tocopherol in acetone/acetate buffer solution and acetone/room temperature ionic liquid solution. The developed procedure was applied for the quantification of α-tocopherol in different pharmaceutical formulations and vegetable oil samples [133]. A new electroanalytical method for determination of vitamin E in the form of the total content of tocopherols present in margarines and edible oils has been developed by

Sýs et al. in 2017. The method was based on extraction of α-tocopherol into silicone oil, acting as lipophilic binder of glassy carbon paste electrode, with subsequent electrochemical detection by square wave anodic stripping voltammetry in 0.1 mol L^{-1} HNO_3. Using the extraction step, the method shown high sensitivity for the determination of vitamin E [16]. A comparison on the linear range and limit of detection of above-mentioned methods is presented in Table 2.

Table 2. Comparison of electroanalytical methods for determination of α-tocopherol.

Electrode	Technique	Linear range (mol L^{-1})	LOD (mol L^{-1})	Reference
PtE/PPy	DPV	5.0×10^{-6}–3.0×10^{-4}	1.5×10^{-6}	[128]
CNTPE/DNA	SWV	1.2×10^{-9}–9.3×10^{-9}	1.3×10^{-10}	[129]
GE/MWCNTs	CV	6.5×10^{-5}–2.0×10^{-3}	5.0×10^{-5}	[86]
GCE in DDPB	CV	2.0×10^{-6}–1.4×10^{-4}	1.0×10^{-6}	[130]
H-BDD	CV	5.0×10^{-7}–1.0×10^{-4}	4.1×10^{-8}	[131]
CFD-UME	SWV	3.4×10^{-5}–2.2×10^{-4}	1.2×10^{-5}	[132]
GCE/MWCNTs/PBHQ	SWV	8.0×10^{-6}–1.0×10^{-4}	1.0×10^{-7}	[65]
Graphene/Nafion	SWV	5.0×10^{-7}–9.0×10^{-5}	6.0×10^{-8}	[133]
GCPE with 10% SO	SWASV	5.0×10^{-8}–1.0×10^{-5}	3.3×10^{-9}	[16]

CFD-UME – carbon fiber disk ultramicroelectrode, CNTPE/DNA – carbon nanotube paste electrode modified by DNA, DDPB – *N*-dodecylpyridinium bromide, GCE – glassy carbon electrode, GE – graphite electrode, GCPE – glassy carbon paste electrode, H-BDD – hydrogenated terminated boron-doped diamond electrode, LOD – limit of detection, MWCNTs – multiwalled carbon nanotubes, PBHQ – poly(2,2′-(1,4-phenylenedivinylene)-bis-8-hydroxyquinaldine), PtE/PPy – polypyrrole modified Pt electrode, SO – silicon oil.

1.3.3 Electroanalysis of vitamin K

Several electroanalytical methods for determination of vitamin K were developed. The three first reported approaches for the determination of vitamin K, were based on non-specific adsorption onto the surface of mercury drop [79,80,134]. The accumulation behaviour of vitamin K_1 at carbon paste electrodes prepared with different types of graphite and pasting agents was studied using linear sweep voltammetry. The optimum accumulation time was 15 min at an open circuit at carbon paste electrode (Nujol-Ultra Carbon Ultra Superior Purity graphite (25 + 75 m/m), and adsorptive stripping voltammetry gave the highest sensitivity for the determination of vitamin K_1 in plasma [134]. A simultaneous electrochemical detection of vitamin K_1 and vitamin D_3 at poly(alizarin red S)/multi-walled carbon nanotubes film on glassy carbon electrode in sodium dodecyl sulphate (SDS)-containing buffer solution was proposed.

The method has been tested for the simultaneous determination of VK_1 and VD_3 in plant and in milk samples [135].

In 2017, Sýs et al. developed a new electroanalytical method for determination of vitamin K_1. The presented method was based on adsorptive accumulation of this vitamin onto the surface of solid glassy carbon electrode with subsequent electrochemical detection using square wave adsorptive stripping voltammetry in 0.1 mol L^{-1} HCl. The method was applied in the analysis of olive oil and food supplement samples [19]. A summary of main parameters, such as a working electrode, electroanalytical technique used, linear range and limit of detection is presented in Table 3.

Table 3. Overview of electroanalytical methods for the determination of vitamin K_1.

Electrode	Technique	Linear range (mol L^{-1})	LOD (mol L^{-1})	Ref.
HMDE	DPP	2.2×10^{-8}–2.2×10^{-7}	—	[81]
HMDE	SWAdSV	1.0×10^{-9}–1.0×10^{-6}	—	[80]
HMDE	SWAdSV	2.0×10^{-10}–5.0×10^{-7}	1.3×10^{-10}	[79]
CPE	AdSV	6.7×10^{-7}–4.4×10^{-6}	4.0×10^{-7}	[134]
Poly(ARS)/MWCNTs	SWV	5.0×10^{-7}–8.0×10^{-5}	6.0×10^{-8}	[135]
GCE	SWAdSV	5.0×10^{-6}–1.0×10^{-4}	5.1×10^{-8}	[19]
		1.0×10^{-8}–1.0×10^{-6}	8.9×10^{-9}	

AdSV – adsorptive stripping voltammetry with linear scan, CPE - carbon paste electrode, DPP – differential pulse polarography, GCE – solid glassy carbon electrode, HMDE – hanging mercury drop electrode, LOD - limit of detection, Poly(ARS)/MWCNTs) – Poly (Alizarin red S)/multi-walled carbon nanotubes, SWV – square wave voltammetry.

1.4 Physicochemical properties of nitrites

Nitrites, such as sodium nitrite or potassium nitrite, are salts of nitrous acid (HNO_2). Nitrites can be produced by the oxidation of ammonia or by the reduction of nitrate. The most common nitrite salt is sodium nitrite ($NaNO_2$), which is a white to slightly yellowish crystalline powder. Nitrites are highly soluble in water and have a slightly acidic taste [136,137]. Nitrites are used to prevent the growth of bacteria and to enhance the colour and flavour of processed meats, especially in the production of sausages and cheeses [138,139]. Nitrites are also used in medicine as vasodilators to treat certain medical conditions, such as angina. Nitrite toxicity can be manifested in two ways: in the stomach, nitrites can react with amines and amides to form *N*-nitrosamine compounds, which are known to be highly carcinogenic, and in combination

with blood pigment nitrites produce methaemoglobin in which oxygen is no longer available for tissues [140,141]. Due to their wide use as food additives, their quantification is important in many industries. Therefore, many methods for nitrite determination have been developed in recent years, including chemiluminiscence [142,143], capillary electrophoresis [144], chromatography [145], spectrophotometry [146,147] and electrochemical techniques [139,148-150].

1.4.1 Electrochemical behaviour and electroanalysis of nitrites

Nitrite ions (NO_2^-) can undergo various electrochemical reactions, depending on the electrode material, pH, and the presence of other chemical species in the solution. Nitrites can be oxidized or reduced at appropriate electrode potentials. The mechanism of nitrite oxidation in various working media, pure alkali nitrite melts [151], sodium nitrate–potassium nitrate eutectic melts [151-153], water [154-155], and the aprotic solvent [156] have been reported. The electroreduction of nitrite has also been reported in protic solvents [157]. No reduction peaks have been found in aprotic solvents or melts. The oxidation of nitrite results in the formation of nitrogen dioxide gas. Depending on the used solvent, different reaction appears. In water working medium NO_2 (or its dimer N_2O_4) is supposed to undergo a disproportionation reaction to form nitrate ions (NO_3^-) [154,155]. In DMSO, the mechanism is considered to involve the reaction of NO_2 with NO_2^- to form N_2O_4 [156].

Various analytical methods are used for their determination, but the advantages of electrochemical methods are speed, simplicity, and low cost. Nitrites are electroactive but unfortunately their oxidation usually occurs at high potentials. Several approaches including amperometric techniques [158-162], differential pulse voltammetry and square-wave voltammetry were reported [149,163-170]. Due to high overpotential, several investigations have been carried out to find the most suitable electrode modification to decrease the anodic peak potential. Numerous scientific reports on the voltammetric determination of nitrites in various water samples were published [166-170].

Much research has been devoted so far to improve the sensitivity and selectivity of electroanalytical methods (DPV or SWV) for the determination of nitrites in meat products. Various electrode modifications have been reported, namely Au electrode modified with Pt nanoparticles embedded into layer of electroactive polymer poly(2-aminothiophenol) [149], a thionine-modified aligned carbon nanotubes at GCE electrode [163], Au electrode functionalized with *p*-aminothiophenol and gold nanoparticles [164], and CPE modified with

polyvinylimidazole [165]. An overview of the main parameters of these voltammetric sensors is shown in Table 4.

Table 4. Overview of voltammetric methods for determination of nitrite in the meat products.

Technique	Electrode / modification	Linear range (μmol L^{-1})	LOD (μmol L^{-1})	E_p (V)	Ref.
DPV	AuE/PATP-PtPs	3.0–1000	1.00	0.75	[149]
DPV	GCE/Thionine-ACNTs	3.0–500	1.12	0.80	[163]
SWV	AuE/p-ATP-AuNPs	10–1086	2.61	0.76	[164]
DPV	CPE/PVI	0.5–100	0.09	0.83	[165]

Note: ACNTs; aligned carbon nanotubes; AuE; gold electrode, AuNPs; gold nanoparticles, GCE; glassy carbon electrode, p-ATP; p–aminothiophenol, PtPs; platinum nanoparticles; PVI; polyvinylimidazole, and PATP; poly(2-aminothiophenol).

1.5 Physico-chemical properties of ethanol

Ethanol is the type of alcohol commonly consumed by humans in alcoholic beverages. The amount of ethanol in alcoholic beverages is very important, ranging from 7 ~ to 21% (v/v) for wines, liqueurs, and beers [171]. The alcohol used in alcoholic beverages is obtained by fermentation of carbohydrates such as glucose, fructose, or sucrose from plant origin. It is mainly metabolized in the liver. Ethanol plays the role of a depressant in the central nervous system, which means it can slow down brain activity. The association of ethanol consumption in small amounts with the reduction of the risk of cardiovascular disease in the population continues to be controversial [172,173], the last study showed that there are no significant reductions in the risk of mortality at low levels of consumption compared to life with those who do not consume alcohol [174]. Excessive ethanol consumption can lead to several negative health effects, such as liver damage, gastrointestinal problems, and cognitive impairment. Chronic alcohol abuse can lead to alcoholism, which can cause long-term damage to the body and brain. Ethanol is also used as a solvent in many industries, such as pharmaceuticals, perfumes, and cosmetics [171,175,176].

1.5.1 Electrochemical analysis of ethanol

Starting from what was said in the paragraph above, it is obvious that the determination of ethanol is of great importance in many fields such as processing of alcoholic beverages, food industry, and medicine. Some of the most common methods for the determination of ethanol in the analysis of alcoholic beverages are gas chromatography with quantification through internal

standards [176], infrared spectroscopy [177,178], and Raman spectroscopy [179]. When considering electrochemical methods, sensitive amperometric enzyme biosensors are generally used for the determination of ethanol providing low costs, rapid analysis, and the possibility of miniaturization and integration into portable systems. Nickel-based electrocatalysts have been used for the electrooxidation of alcohols, such as ethanol and methanol in alkaline medium, due to their electrocatalytic activity [180,181].

Two approaches in enzyme biosensing of ethanol are reported. The first relies on alcohol oxidase (AOX) [182-185], where oxygen consumption or H_2O_2 production is measured. The second type is utilizing alcohol dehydrogenase (ADH) [186-190], which catalyses the conversion of ethanol to acetaldehyde in the presence of the coenzyme nicotinamide adenine dinucleotide (NAD^+). Fabrication of alcohol sensors with ADH is of greater interest than those with AOD, because oxygen is not interfering in that case. Furthermore, ADH belongs to a large group of dehydrogenase enzymes that depend on the same cofactor. One of the problems in the use of dehydrogenase is the irreversibility of NADH oxidation. The oxidation of reduced nicotinamide adenine dinucleotide requires high overpotentials at unmodified electrodes [191]. An overview of the amperometric biosensors based on alcohol oxidase and alcohol dehydrogenase, compatible with the flow injection analysis, is presented in Table 5.

Table 5. Amperometric enzyme biosensors used in the flow injection analysis.

Bioelectrode	**Potential mV**	**Linear range mM**	**LOD mM**	**Storage stability (days)**	**Ref.**
AOX/nAuCePt/GE	−200	0.005–0.10	0.001	14	[185]
AOX/PO/GE	−50	0.130–0.90	0.039	—	[185]
AOX/nCuCe/GE	−50	0.050–2.10	0.015	—	
AOX/nCuCe/npAu/GE	−50	0.033 – 0.50	0.010	—	
AOX/nPtRu/GE	−100	0.020 – 0.60	0.002	14	[183]
AOX-PO/Os polymer	−50	0.50 – 2.00	0.015	16	[182]
AOX-CF-hemin-Au	−100	0.010 – 0.15	0.005	—	[182]
CPE/TBO-NAD^+-ADH	+50	0.02 – 0.24	0.0087	1	[186]

Bioelectrode	Potential mV	Linear range mM	LOD mM	Storage stability (days)	Ref.
CPE-yeast/PVP−$Fe(CN)_6^{-3}$	+600	up to 0.3	0.002	75	[187]
COE/CNBr-AS4/NAD^+-ADH	−700	0.25 – 1.0	—	30	[188]
GE/PVI_{13}dmeOs-PQQ-ADH	+200	0.0025 – 0.25	0.0012	30	[189]
CPE/FS-NAD^+-ADH	+800	—	0.08	1	[190]

Note: AS4; activated Sepharose 4, COE; Clark-type oxygen electrode, CPE; carbon paste electrode, FS; fumed silica, GE; graphite electrode, PQQ; pyrroloquinoline quinone, PVI_{13}dmeOs; poly(1-vinylimidazole) complexed with [Os(4,4′-di-methylbipyridine)$_2$Cl]$^{+/2+}$, PVP; poly (4-vinylpyridine), TBO; Toluidine Blue O.

2. Results and discussion

All the experimental results of this dissertation were published, see in refs. [22-29], and the corresponding articles are available in appendices as Publications I to VII.

2.1. Characterization of carbon paste electrode with various surfactants

During this doctoral research, the main subject of interest was the development of electroanalytical methods based on pulse voltammetric techniques that can be used for the simultaneous detection of lipophilic vitamins. The selection of working electrode material has an important role in electrochemical methods. Various electrode materials including metal, carbon-based, and modified electrodes have been used for electroanalysis of lipophilic compounds. Carbon-based electrodes are the most widely used working electrodes for electrochemical sensing applications. They possess many advantages such as low cost, wide potential window, low background current, good stability, and it is very easy to modify them.

Carbon paste electrodes made of mixture of carbon powder and pasting liquid (binder) represent one of major types of carbon-based electrodes. Considering that carbon pastes have some drawbacks that limit their use in certain experiments, the most mentioned problem is their low stability in many organic solvents, where generally used mixtures of carbon pastes undergo a rapid and usually total dissolution. To circumvent this unwanted behaviour, various modifications using different -binders and surfactants can increase the stability of carbon paste material. In case of surfactants, an explanation can be found in the specific molecular structure of surfactant containing both hydrophobic and hydrophilic groups. Surfactant molecules are dissolved via their hydrophobic “tails” in carbon paste binder that links together the individual carbon particles, whereas hydrophilic “heads” are directed onto the electrode surface, representing the interfacial layer.

In ongoing research of electrochemistry group at the Department of Analytical Chemistry, carbon paste electrode modified with sodium dodecyl sulphate (so called “non-aqueous” CPE) was used for the determination of α-TOAc in cosmetics [21] and electrochemical study and determination of all-*trans*-retinol [20], and the results confirmed applicability and stability of such an electrode in organic media. Therefore, a detailed characterization of CPE modified with surfactants was performed focusing on individual aspects of the composition of the carbon paste, the effect of the surfactant according to its type, structure, and content with respect to the resulting analytical performance of the electrode in electrochemical measurements. The purpose of such a study was to fill a gap in the knowledge

on the properties and behaviour of carbon pastes in non-aqueous environments and to provide a guide for the preparation of electrodes applicable in studies of water-insoluble electroactive substances.

Carbon pastes made of natural or synthetic graphite particles can absorb a higher ration of binder than a compact glassy carbon or carbon nanotubes powder. The relationship between the type of carbon material including five different CPEs and the amount of surfactant at constant mineral oil (MO) content (20% w/w) was investigated. Obtained results from CPEs prepared with chemically purified natural graphite powder and glassy carbon Sigradur are presented as dependence of the ohmic resistance on the content of SDS (w/w) and the types of CPEs containing 20% MO (see Fig. 1, Publication 1). The CPEs prepared from raw shungite (mineralized carbon) and multi-wall carbon nanotubes (MWCNTs) with the same amount of 20% MO and 10% SDS were unstable in tested organic solvent. Table 1, Publication 1., shows electrochemical characterization of all prepared CPEs using cyclic voltammetry (CV) of ferrocenium/ferrocene redox couple measured in nonaqueous media. From the presented data it was found that the optimal composition of non-aqueous carbon paste depends on the type of carbon material used, and the amount and type of non-electroactive surfactant. Chemically purified natural graphite powder can accommodate the largest amount of SDS (20-50%), contrary to glassy carbon, spectroscopic graphite powder (RWB) carbon, and MWCNT (only 10% of SDS at constant content of 20% MO).

Next step was to test various carbon paste binders, such as MO, silicone oil (SO), paraffin wax (PW), vaseline, atactic polypropylene (APP), and tricresyl phosphate (TCP), mixed at the same amount of 20% w/w with graphite, and a constant amount of 30% SDS. Using LSV mode, the baseline of all prepared CPEs was recorded in pure acetonitrile (MeCN) containing 0.1 mol L^{-1} $LiClO_4$. Results shown excellent polarisation capabilities of CPEs for all binders except TCP (see Fig. 3, Publication 1). Moreover, the anodic and cathodic potential limits were compared, and vaseline, MO, and PW provided wider potential window. In order to select the most suitable surfactant, several types of surfactants such as anionic SDS, cationic cetylpyridinium chloride, benzethonium chloride, 1,3-didecyl-2-methylimidazolium chloride, cetyltrimethylammonium bromide, didodecyldimethylammonium bromide, amphoteric sodium lauroamphoacetate (SLAA), and neutral Triton^{TM} X-100 were investigated and compared in baseline measurements. The results obtained using CV in pure MeCN with 0.1 mol L^{-1} $LiClO_4$ showed that CPE modified with SDS provided no signal due to the electrochemically inert sulfonic group (Fig. 5, Publication 1). On the other hand, all types of cationic surfactants were inapplicable, because of their electroactivity in the interested potential range. TritonTM X-100

and sodium lauroamphoacetate were unstable in MeCN working media. SDS was confirmed as a suitable surfactant modifier with excellent stabilizing effect. Electrochemical studies for the redox pair ferrocenium/ferrocene obtained for CPEs with varying SDS amounts ranged from 20 to 40% (w/w) at a constant ratio with the binder (20% MO w/w) and the respective carbon constituent were compared in peak currents. Obtain results show almost comparable peak currents. From the experimental data it was found that the amount of surfactant in natural and synthetic graphite can be in higher content (Fig. 9), than in the compact carbon materials, such as glassy carbon powder and carbon nanotubes (Fig. 4, Publication 1).

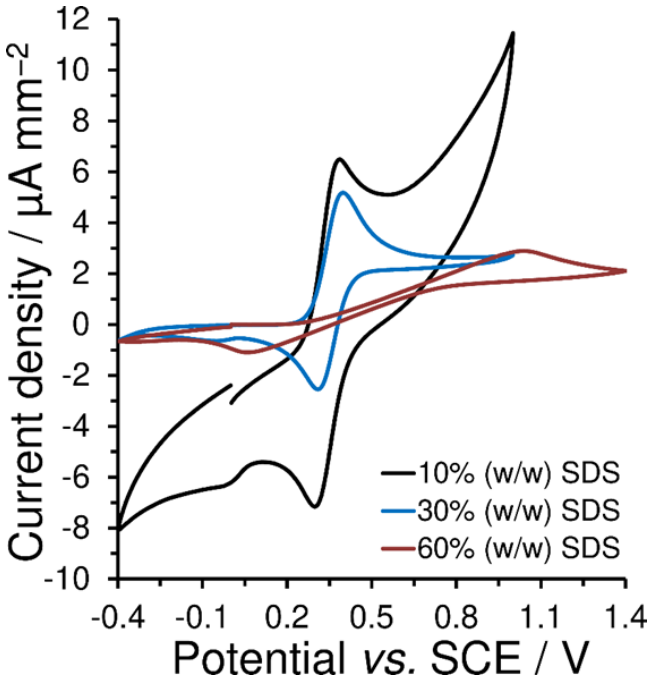

Figure 9. Cyclic voltammograms of 500 µmol L^{-1} ferrocenium/ferrocene redox pair recorded on CPEs containing 20% (w/w) MO and modified with different content of SDS in pure MeCN with 0.1 mol L^{-1} $LiClO_4$ at E_{step} = 2.5 mV and ν = 50 mV s^{-1}.

Several organic solvents such as MeCN, methanol (MeOH), tetrahydrofuran, acetone (ACE), *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide, and isopropyl alcohol, all containing 0.1 mol L^{-1} $LiClO_4$, were compared in baseline measurements at CPE made of 40% (w/w) MO and SDS using LSV. From the experimental data it was found that a significant increase of the background currents and shorter potential ranges were obtained for all tested solvents, except for MeCN (Fig. 6, Publication 1). In addition, three most commonly used salts for electrochemical experiments in organic media, namely $LiClO_4$, tetrabutylammonium hexafluorophosphate, and tetrabutylammonium perchlorate in concentration 0.1 mol L^{-1} were evaluated for their effect on the electrochemical behaviour of ferrocenium/ferrocene. Almost the same cyclic voltammograms were obtained for this redox pair in all three electrolytes. Nevertheless, $LiClO_4$ salt was used in further experiments. The presence of water up to 10%

does not cause damage in the morphological structure of the electrode surface, while the presence of water in a percentage greater than 1% causes an increase in background current. Moreover, a comparison of nonaqueous CPEs modified with SDS with a commercial GCE has been carried out using CV in pure MeCN, when the electrochemical behaviour of 500 μmol L^{-1} α-TOH, eugenol, and dopamine was examined. Comparable results of peak height and overall shape of the respective current responses to those obtained at the GCE were obtained (Fig. 10).

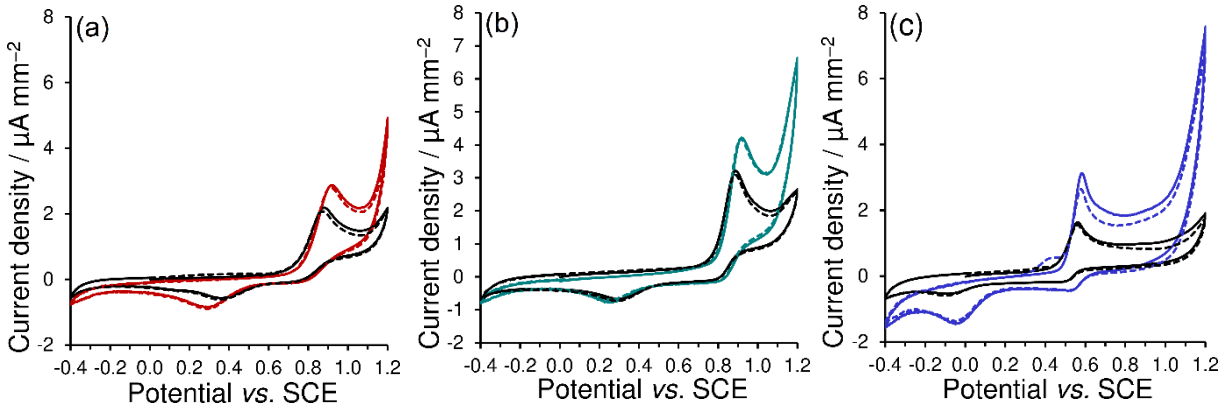

Figure 10. Repetitive cyclic voltammograms (two cycles) 500 μmol L^{-1} dopamine (a), eugenol (b) and α-tocopherol (c) recorded on CPE containing 40% (w/w) MO and modified with 40% (w/w) SDS (coloured) and GCE (black lines) in MeCN with 0.1 mol L^{-1} $LiClO_4$ at E_{step} = 2.5 mV and ν = 100 mV s^{-1}, where dotted lines represent the second repetitions.

From this detailed study, it has been demonstrated that the optimal composition of the nonaqueous carbon paste depends mainly on the type of carbon material, and amount of non-electroactive surfactant, whereas the type of organic binder is less important. MeCN as optimal working medium offered a wider potential range with low background current over other organic solvents. The comparisons of the surfactant-modified CPE with the GCE on the model redox pairs and selected biological compounds have proved that the CPE/SDS could find its application in nonaqueous electrochemistry, especially for nonpolar electroactive organic compounds. Moreover, nonaqueous carbon paste electrode can offer interesting application in food and pharmaceutical analysis.

2.2. Electrochemical determination of vitamin A and related compounds

2.2.1. Simultaneous determination of lipophilic vitamin esters

From all above-mentioned studies it is evident that no method has been developed yet for the simultaneous determination of RAc or RPa and α-TOAc, while three methods have been published focusing only on the determination of α-TOAc in pharmaceutical and cosmetic preparations [20,21,62-64]. The literature and experiments to date have provided sufficient proof that a simultaneous determination of RAc or RPa and α-TOAc would be important due to their presence in cosmetic products. The majority of cosmetic preparations contains at least one retinol form (RAc or RPa) and α-TOAc in recommended amounts based on the type of product.

Starting from separate studies for all-*trans* retinol [20] and α-TOAc [21,62-64] and considering that these compounds are present together in cosmetic products, a completely new voltammetric approach for simultaneous determination of RAc or RPa and α-TOAc is presented in this thesis. Many conditions such as various working electrode materials, the selection of suitable organic solvent, and the parameters of the voltammetric technique were investigated. Firstly, the electrochemical behaviour of RAc, RPa and α-TOAc was studied at GCE using CV in pure ACE. From the obtained data it was observed that RAc and RPa provided single oxidation peak at +0.85 V, accompanied with some other broad and overlapped signals at more positive potentials. On the other hand, α-TOAc gave only a single oxidation peak at +1.4 V. The distance between the oxidation peaks indicates the possibility for a simultaneous determination of these analytes. In addition, the effect of scan rate on the electrode behaviour of 500 μmol L^{-1} of all analytes was investigated. The relationships obtained by plotting the I_p against square root of scan rate revealed that the oxidation of RAc and RPa are diffusion-controlled processes unlike the oxidation of α-TOAc, which is governed by the adsorption.

Two metal-based electrodes, gold (AuE) and platinum electrode (PtE), and GCE were compared for the simultaneous detection of 50 μmol L^{-1} RAc and α-TOAc in pure MeCN. From the results it was shown that the electrode material had not any significant effect on the peak potential. The current response for RAc at all tested electrodes was nearly identical, whereas α-TOAc provided twice higher response at GCE. Nonaqueous CPE/SDS was also tested but was not further considered due to a high background current. To select the most suitable working medium, several organic solvents, such as methanol, isopropyl alcohol, acetonitrile, acetone, and *N*,*N*-dimethylformamide, retinyl acetate and α-tocopheryl acetate were compared

in terms of peak potential position and current response (Table 6). No effect on the peak position and higher peak current was obtained in acetone, therefore it was chosen as an optimum.

Table 6. Comparison of anodic peak potential and current response in various solvents at the GCE.

Solvents	**Retinyl acetate**		**α-Tocopheryl acetate**	
	E_p^a **(V)**	I_p^a **(μA)**	E_p^a **(V)**	I_p^a **(μA)**
Acetonitrile	0.811	1.046	1.294	2.356
Acetone	0.871	2.534	1.380	1.657
Methanol	0.806	1.825	1.309	1.131
Dimethylformamide	0.836	1.369	1.279	0.516
Isopropanol	0.851	1.305	1.399	0.274

Values are given as arithmetic mean of five repetitions.

The influence of the content of water (0, 10, 20, 30, 40, and 50 % v/v) in ACE on RAc and α-TOAc detection was studied. In presence of water, the oxidation peak of RAc has decreased contrary to α-TOAc. After all optimization steps, the proper procedure is based on direct anodic oxidation of RAc or RPa and α-TOAc at GCE in pure ACE containing 0.1 mol L^{-1} $LiClO_4$. Two linear ranges for RAc in intervals from 3.1 to 140 μmol L^{-1} and from 140 to 400 μmol L^{-1}, one linear range 2.8–180 μmol L^{-1} for RPa, and one linear range 5.3–400 μmol L^{-1} for α-TOAc were attained. Detection limits (LOD) of 0.9 μmol L^{-1} RAc (or 0.8 μmol L^{-1} RPa) and of 1.6 μmol L^{-1} α-TOAc were calculated. The repeatability for level of significance $\alpha = 0.05$ is presented as the relative standard deviation (RSD) and values of 2.27 % and 2.19 % were calculated for ten replicates of a model mixture of 50 μmol L^{-1} RAc and α-TOAc, respectively. Additionally, it was found that there is no mutual influence on the peak potential and height during simultaneous increase of concentration of both analytes up to 900 μmol L^{-1}. A model sample analysis for different concentration ratios of both analytes was used to determine the accuracy as a recovery. Acceptable recoveries in range 97.8–106.1 % were achieved. The developed method was applied in three types of cosmetic products such as hand cream, refreshing cleaning body milk, and face. The samples were dissolved directly in the working medium without any treatment. The presence of α-TOAc was declared by all producers, presence of RPa was mentioned only for face cream, whereas the presence of RAc not. Moreover, analyses of mixed samples of two cosmetic products were possible. The results of all analyses and the calculated amounts are presented in Table 2, Publication 2. The voltammograms of sample face cream, and hand cream analysis are shown in Fig. 11.

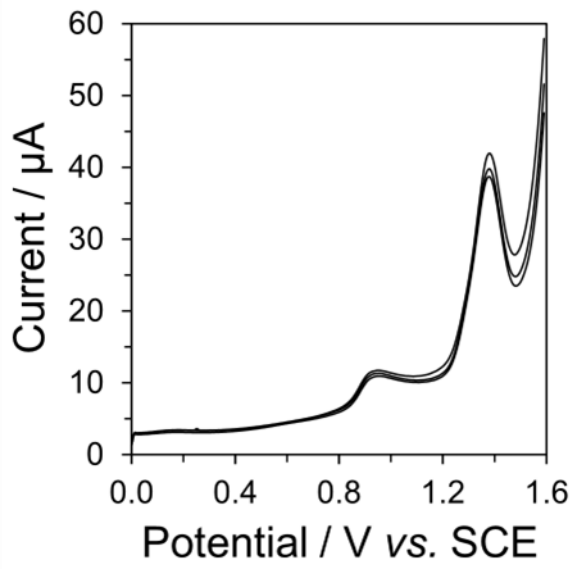

Figure 11. Voltammograms of sample face cream δ hand cream. SWV at GCE in 99.7% acetone containing 0.1 mol L^{-1} $LiClO_4$, E_{step} = 10 mV, E_{ampl} = 40 mV and f = 30 Hz.

We can conclude that the main advantages achieved were the simplicity of preparation of cosmetic samples, using less hazardous ACE as the working medium, elimination of steps like saponification and extraction/re-extraction into organic solvent which are quite complex, and the possibility of monitoring RAc (or RPa) and α-TOAc simultaneously.

2.2.2. Extractive stripping voltammetry of lipophilic vitamins in cow's milk and cream

Milk and all its products are considered as very complex sample for analysis and often require time-consuming sample preparation. Several steps, such as alkaline hydrolysis, liquid-liquid extraction, filtration, and evaporation of the used solvent, are usually performed before the analysis. This research offers a simple voltammetric approach for monitoring VA as a sum of retinoids and carotenoids in cow's milk and cream product without sample preparation steps. The method is based on two steps: the first step is the direct extraction of analytes from the milk into a pasting liquid (nonpolar binder) of GCPE and, after transferring the working electrode to the electrochemical cell, subsequent electrochemical detection by SWV in 0.1 mol L^{-1} Britton-Robinson buffer with pH 4.5 in second step.

Since the procedure for extracting lipophilic vitamins from cow's milk usually involves an alkaline hydrolysis step, this step could be eliminated by extraction into electrode material of GCPE. Five GCPEs containing always 20% (w/w) portion of the organic binder, differing only in the type of used organic binder (APP, paraffin oil, PW, SO, and vaseline), were investigated to find the most suitable composite for the extraction of lipophilic vitamins. The silicon oil as an organic binder provided better extraction efficiency of lipophilic vitamins into GCPEs from a sample of cow's milk (3.5% of fat) for 10 minutes of accumulation time and stirring rate of 400 rpm (Table 1, Publication 3).

In addition, the SO content (5, 10, 15, 20, and 25 % w/w) was optimized in order to increase the amount of extracted lipophilic vitamins into GCPEs. From the results it was found that the current response of vitamins was higher at lower content of SO. The content of 15% (w/w) SO was chosen as a compromise of current response and lower background current (Table 2, Publication 3). The electrochemical detection parameters were as follow: detection medium 0.1 mol L^{-1} Britton-Robinson buffer (pH 4.5), potential scan from 0 to +1.4 V, step potential (E_{step}) of 5 mV, potential amplitude (E_{ampl}) of 25 mV, and frequency (*f*) of 10 Hz.

The accumulation time and stirring rate are two important parameters for increasing the extraction efficiency of lipophilic vitamins into GCPEs. The extraction equilibrium has been achieved after 10 minutes; longer period did not cause any change in peak response (Fig. 2, Publication 3). The stirring rate faster than 300 rpm did not have any impact on the final peak current signal, therefore it was chosen as optimum for subsequent experiments.

Other factors which affect the detection of the lipophilic vitamins are the composition and pH of detection medium and parameters of square-wave voltammetry. A study from pH 2 to pH 7 for 0.1 mol L^{-1} Britton-Robinson buffer was carried out. The results shown that the

peak potential shifted to more negative values with increased pH. The highest current response was obtained in the pH values 4 and 5. Britton-Robinson buffer could be replaced with acetate buffer pH 4.5 as a medium with a simpler composition. Parameters of SWV, namely E_{ampl} and *f*, were optimized. The intensity of signal response increased up to E_{ampl} value of 25 mV with a slight increase of background current. Higher values than 25 mV resulted in significantly increased background current. The same effect was observed with increasing the value of *f*. Thus, a value of 50 Hz was taken as compromise between peak current response and background current.

From the obtained results, it was observed that milk fat globules (MFG) mainly contain VA (carotenoids and retinoids), especially all-*trans*-retinol, which can serve as an important marker of fat content. However, peaks of individual compounds cannot be distinguished due to the significant broadening and intensive overlapping of the oxidation signals (Fig. 3, Publication 3). Analysis of several samples of milk and cream with different percentages of fat revealed that the voltammetric signal was higher in samples with the lowest percentage of fat, even though it is assumed that samples with higher amounts of fat have a higher content of lipophilic vitamins. This can be explained by the fact that the lipophilic vitamins in the samples are evenly distributed between the pasting liquid, and milk fat during extraction ('liquid-liquid') and these vitamins are more isolated in the creams (12-31% fat) than in milk (0.5-3.5% fat) due to the much higher fat content (Fig. 5, Publication 3). The advantage of this method is attributed to the simplicity of sample preparation without the need for a complicated step. However, the direct extraction of lipophilic vitamins (mainly VA) can be used only for the semi-quantitative determination of milk fat at this stage of development.

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EXTRACTIVE STRIPPING VOLTAMMETRY AT A GLASSY CARBON PASTE ELECTRODE FOR ANALYSIS OF COW'S MILK AND CREAM

Granit Jashari, Michaela Frühbauerová, Milan Sýs, Libor Červenka

ABSTRACT

In this paper, a procedure based on extractive accumulation of milk fat globules (MFGs) into a pasting liquid (lipophilic binder) of glassy carbon paste electrode (GCPE) with subsequent electrochemical detection by square-wave voltammetry (SWV) in 0.1 mol L^{-1} Britton-Robinson buffer of pH 4.0 has been tested to find out whether it can be utilized as a simple screening analytical method for cow's milk and cream nutrition control. Since there is assumption that the necessary alkaline hydrolysis of cow's milk and subsequent extraction of lipophilic vitamins into an organic solvent could be avoided, several GCPEs differing in type (atactic polypropylene, paraffin oil, paraffin wax, silicone oil, and vaseline) and content (5, 10, 15, 20, and 25%; w/w) of pasting liquid used were tested as part of complex optimization. The obtained results show that MFGs contain predominantly vitamin A (carotenoids and retinoids), especially all-*trans*-retinol, which could serve as significant marker of the fat content. However, their individual forms were not possible to distinguish due to the considerable anodic peak broadening (overlapping).

Keywords: carbon paste electrode; cow's milk; extraction; milk fortification; nutrition control; voltammetry

INTRODUCTION

In the mammary glands, milk fat globules (MFGs), ranging in size from 0.1 to 15 μm in diameter **(Logan et al., 2014)**, originate as fat droplets composed largely (>98%) of triacylglycerols (TCGs). These fat droplets are evenly emulsified throughout the volume and contain lipophilic (fat-soluble) vitamins dissolved in them **(Heid and Keenan, 2005)**. Losses of naturally occurring lipophilic vitamins are significant after mechanical separating the milk fat (cream) from the raw milk. Obtained skimmed milk is then homogenized that is a process of breaking down the large fat droplets under high pressure so that they stay together and do not separate as cream. To improve the nutritional values, the homogenized milk is usually fortified by extra vitamins (retinyl palmitate and cholecalciferol) and minerals that are not naturally found in milk in significant amounts **(Trinidad et al., 2015)**.

The cow's milk and products made from it are considered as very complex sample matrixes and their analysis is often complicated and time-consuming **(Trenerry et al., 2011)**. Valid reference analytical methods used for lipophilic vitamins determination in foodstuffs in laboratories of the Czech Agriculture and Food Inspection Authority 211/2004 Coll4. utilize a HPLC with UV detection, known as standard: ČSN EN 12823 (vitamin A), ČSN EN 12821 (vitamin D), ČSN EN 12822 (vitamin E) and ČSN EN 14148 (vitamin K). In addition, a gravimetric method (EN 1211) is used to determine milk fat content.

Evaluation of the lipophilic vitamins content in milk (also dairy produce) has its substantiation, especially in case of human nutrition which deals on provision of essential nutrients in food necessary to support human life and health **(Haug et al., 2007)**. Moreover, analytical methods for simultaneous determination of lipophilic vitamins and their provitamins in milk using microcolumn **(Gomis et al., 2000)**, narrow-bore column **(Blanco et al., 2000)** and two-dimensional liquid chromatography **(Zhang et al., 2015)** have been developed.

Time-consuming sample preparation is the most challenging step in the analysis as it involves several steps (alkaline hydrolysis, liquid-liquid extraction, filtration and evaporation of organic solvent) in which the analytes may be lost **(Trenerry et al., 2011)**. To avoid degradation of analytes, the alkaline hydrolysis shoud be carried out in presence of an antioxidant, under an inert atmosphere, and in absence of light.

A simple semiquantitative method for the determination of vitamin D in skim milk is worth mentioning **(Michlová et al., 2012)** when a sample is diluted with water, ethanol, and an aqueous ammonia solution. Vitamin D is subsequently extracted with a mixture of ether and hexane

for 4 hours. After evaporation of the organic solvent, vitamin D is transferred to the appropriate solvent (usually methanol or acetronitrile). The advantage of this procedure is that the sample does not need to undergo alkaline hydrolysis.

This paper offers a simple screening voltammetric method for monitoring vitamin A content (sum of retinoids and carotenoids) in cow's milk and cream samples without the need for a use complicated sample preparation. Since all lipophilic vitamins are electrochemically active organic compounds that undergo oxidation (vitamins A, D and E) or reduction (vitamin K) electrode reactions **(Lovander et al., 2018)**, they can be directly extracted from the milk into a pasting liquid (nonpolar binder) of glassy carbon paste electrode (GCPE). After transferring GCPE into an aqueous working medium (medium−exchange approach), the electrochemical detection of accumulated vitamins can be performed using a pulse voltammetric technique **(Sýs et al., 2019)**, namely square-wave voltammetry (SWV).

Scientific hypothesis

In this work, an effort was to find out whether square-wave anodic stripping voltammetry at GCPE can represent a suitable method for rapid determination of vitamin A.

MATERIAL AND METHODOLOGY

Chemicals and reagents

All-*trans*-retinol (≥95%) and ethanol (≥99.5%) were purchased from Sigma-Aldrich. Universal 0.1 mol.L^{-1} Britton-Robinson buffer (BRB), prepared by mixing of appropriate amounts of boric acid, glacial acetic acid, 85% phosphoric acid, and sodium hydroxide all from the aforementioned company, was used in selection of suitable detection medium. BRB was prepared using deionized water (minimum electric resistivity 18.2 MΩ cm, maximum 3 µg L^{-1} of total organic carbon) made in a Milli-Q® ultrapure water system from Merck Millipore (Burlington, USA).

Instrumentation

Voltammetric detection of accumulated lipophilic vitamins into the pasting liquid was performed in a conventional three-electrode arrangement containing always GCPE (working), silver chloride electrode with 3 mol.L^{-1} KCl salt-bridge (reference) and platinum sheet (auxiliary electrode). These electrodes were connected to the potentiostat Autolab PGSTAT101 controlled by software Nova (Version 1.11.0), both from Metrohm (Prague, Česká republika).

Preparation of carbon paste electrode

Glassy carbon powder of type Sigradur G (mixture 5 – 20 µm, HTW Maintingen, Germany) and one of randomly selected pasting liquids were mixed in a ceramic mortar for 15 min to create homogenous glassy carbon paste. The amount of tested pasting liquid differed from 5 to 30% (w/w). The resulting glassy carbon paste was packed into the cavity of the Teflon® piston-driven electrode holder with an end-hole of 3 mm in diameter. It is necessary to mention that the height of column in the cavity must be less than 2 cm due to difficult extrusion of glassy carbon paste. It is recommended that freshly prepared GCPEs should not be employed in any experiments due to their rather unstable electrochemical behaviour attributed to an incomplete homogenization. Consequently, freshly prepared GCPEs were left at the laboratory conditions for one day. After this self-homogenization process, GCPEs can be used for following voltammetric measurements **(Sýs et al., 2017)**.

Methods

Principle of medium-exchange extractive stripping voltammetry is illustarted in Figure 1. The extraction of lipophilic vitamins into the pasting liquid was carried out from 10 mL non-treated milk and cream samples (available in Czech stores) without need to apply a potential in the electrode cell (nonelectrolytic preconcentration), which is an approach known as "open circle procedure". After 10 min, GCPE enriched with analytes was rinsed with a stream of deionized water and immersed together with others electrodes into 0.1 mol.L^{-1} BRB. Final voltammetric detection was performed using square-wave voltammetry at potential range from 0 to +1.4 V, potential step (E_{step}) of 5 mV, potential amplitude (E_{ampl}) of 25 mV and frequency (f) of 50 Hz.

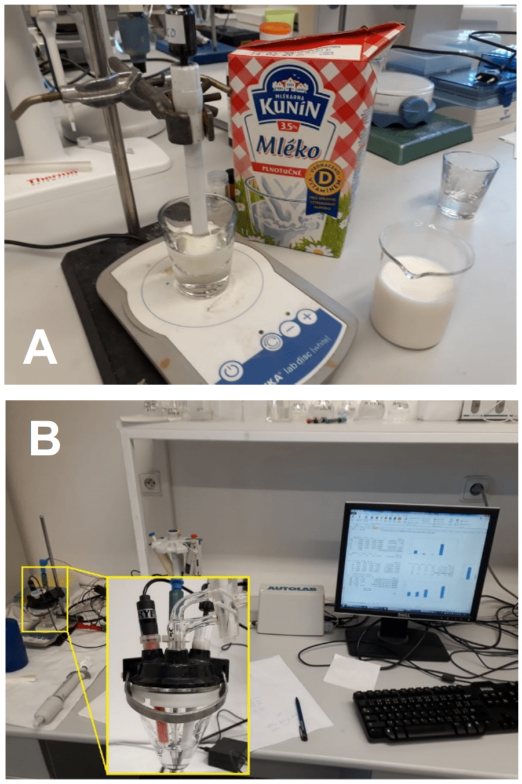

Figure 1 Individual steps of extractive stripping voltammetry with medium-exchange (A; accumulation and B; electrochemical detection using SWV).

Statistic analysis

Extraction repeatability

Generally, the repeability may be expressed by several indexes, nemaly coefficient of repeatability (CR), coefficient of variation (CV) and intra-class correlation

coefficient (ICC). CR defined by formula (1) is a precision measure which represents the value below which the absolute difference between two repeated test results may be expected to lie with a probability of 95%. The standard deviation (σ) under repeatability conditions is part of precision and accuracy.

$$CR = 1.96\sqrt{2\sigma^2} \quad (1)$$

However, the repeatability is more often given by CV defined as the ratio of the standard deviation (σ) and to the mean (μ). If this ratio is expressed as a percentage (see Eq. 2) then it will be referred to as relative standard deviation (RSD).

$$RSD = \frac{\sigma}{\mu} * 100 \quad (2)$$

Sufficient extraction repeatability constitutes the main criterion for development of voltammetric methods utilizing extractive accumulation to be able to use them for analytical purposes. ICC could not be used because units of two physical quantities (variables) were statistically tested only. Therefore, using RSD can be probably expected to be sufficient.

RESULTS AND DISCUSSION

Selection of pasting liquid type

Several GCPEs differing in the type of pasting liquid and containing always 20% (w/w) portion were investigated in SWASV of cow's milk (3.5% fat) to choose the optimum one. Working conditions for this experiment were as follows: accumulation for 10 min, stirring at 400 rpm, electrochemical detection in 0.1 mol.L^{-1} BRB (pH 4.5) at E_{start} = 0 V, E_{end} = +1.4 V, E_{step} = 5 mV, E_{ampl} = 25 mV and f = 10 Hz. Due to relatively high current response and required reproducibility (Table 1), silicone oil should be taken for optimum extraction of lipophilic vitamins.

Table 1 Comparison of glassy carbon paste electrodes.

Pasting liquid	R (Ω)	E_p (V)	I_p (μA)
Atactic polypropylene	10.5 ±0.8	0.831	0.075
Paraffin oil	7.1 ±0.2	0.851	3.69 ±1.6
Paraffin wax	4.7 ±0.3	0.836	0.24 ±0.1
Silicone oil (8000 cSt)	8.0 ±0.2	0.844	1.14 ±0.2
Vaseline	17.4 ±1.0	0.829	0.013

Note: Values (R; ohmic resistance; E_p; peak potential, I_p; peak current response) given as $\mu \pm 2\sigma$ (95% probability) for five repetitions.

Ratio between carbon powder and pasting liquid

Under the prediction, an amount of extracted lipophilic vitamins would increase with a higher content of paste liquid in GCPE. However, electrochemical properties of GCPE are affected by ratio between glassy carbon powder and paste liquid. In principle, it can be stated that carbon particles remain in intimate contact (electrically conductive) until the amount of paste liquid exceeds 30% (w/w) **(Švancara and Schachl, 1999)**.

Surprisingly, it was found that the highest peak current response (unfortunately, background current (I_b) as well) was obtained at GCPE containing 5% (w/w) silicone oil, as demonstrated in Table 2. Despite high current yield, the content of 15% (w/w) silicone oil was chosen as optimum, thanks to the high reproducibility of accumulation. A non-specific adsorption of milk fat onto electrode surface, which acts as an electrical insulator causing a significant increase of background current (baseline signal), can be considered as possible explanation.

Table 2 Effect of silicone oil content in GCPE.

Content (%)	R (Ω)	I_p (μA)	I_b (μA)
5	6.7 ±0.2	10.0 ±0.3	66.4 ±3.50
10	7.0 ±0.3	4.7 ±0.3	8.9 ±0.90
15	4.8 ±0.2	5.1 ±0.3	4.4 ±0.50
20	8.0 ±0.2	1.1 ±0.2	0.9 ±0.02
25	6.0 ±0.1	0.9 ±0.1	0.7 ±0.02

Note: Values (R; ohmic resistance; I_p; peak current response; I_b; background current) given as $\mu \pm 2\sigma$ (95% probability) for five repetitions.

Effect of accumulation time

Principally, the optimum value of accumulation time is defined as a period required for reaching the equilibrium of lipophilic vitamins distribution between a nonopolar pasting liquid of GCPE and used milk sample. The cow's milk can be considered as a direct emulsion (so-called the first type emulsion) because a small amount of fat droplets (organic phase) are uniformly distributed throughout the milk volume (aqueous phase).

Resulting saturation curve describing dependence of current peak height on accumulation time showed a typical extraction equilibrium isotherm, as shown in Figure 2. The extraction equilibrium has been achieved after 600 s because using accumulation for longer period did not cause any significant increase in peak current response. Hence, accumulation time of 10 min was chosen as optimum.

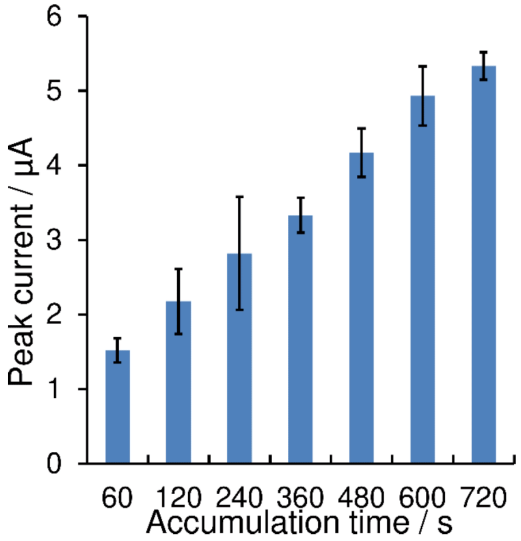

Figure 2 Effect of extraction time on extraction yield of lipophilic vitamins from the cow's milk (3.5% fat).
Note: Data obtained from SWV at GCPE containing 15% (w/w) silicone oil, E_{step} = 5 mV, E_{ampl} = 25 mV, and f = 20 Hz.

Effect of stirring speed

Stirring speed affects the rate of fat droplets transport to the electrode surface where these droples containing lipophilic vitamis are then extracted into the pasting liquid of GCPE. Under this study, it was found that setting the rate of magnetic stir bar higher than 300 rpm did not have any significant effect on increase in final peak current response. Therefore, the above mentioned value can be considered as an optimum for subsequent experiments.

Identification of lipophilic vitamins in cow's milks

The fat dispersed in cow's milks and creams is formed by non-polar TCGs that are surrounded by phospholipids and membrane lipoproteins **(Heid and Keenan, 2005)**. At the natural pH of the cow's milk, they carry a negative charge and thus prevent bonding of MFGs. It is worth considering whether whole MFGs are extracted into the pasting liquid or a further equilibrium distribution of present lipophilic vitamins between the TCGs and the pasting liquid cannot exist. It can be assumed that both processes take place simultaneously. TCGs extracted (adsorbed) block surface of GCPE and therefore cause a dramatic increasing the background current.

From the literature **(Indyk and Woollard, 1997; Hulshof et al., 2006; Trenerry et al., 2011; Musara and Nyagura, 2017)**, lipophilic vitamins are found primarily in the milk fat. Unlike cholecalciferol (vitamin D3) and phylloquinone (vitamin K1) present in limit amounts (0.1 µg per 100 g), α-tocopherol (vitamin E), and retinol together with its provitamines (carotenoids) such as β-carotene, zeaxanthine and luteine (vitamin A) are the major representatives (40-110 µg per 100 g). Moreover, an artificially added retinyl palmitate **(Jensen et al., 1991)** can be present as well.

Generally, most extracted lipophilic vitamins and their provitamins usually provide very broad sensitive oxidation/reduction peaks (up to 250 mV) due to slow kinetic of corresponding electrode reactions occurred at liquid-liquid interface **(Sýs et al., 2019)**. As confirmation, a broad anodic peak beginning +0.705 V at and ending at +1.007 V was obtained for all investigated cow's milks and creams.

It is therefore impossible to distinguish and determine the individual forms of retinoids and carotenoids **(Žabčíková et al., 2018)**. Nevertheless, a number of published scientific papers suggest that the all-*trans*-retinol occupies a dominant position **(Jensen, 1994; Hulshof et al., 2006; Hodulová et al., 2015)**. Thus, it can be assumed that the peak obtained most likely corresponds to the anodic oxidation of all-*trans*-retinol a +0.852 V (compare with (overlapping peak at +0.886 V for cow's milk), as shown in Figure 3.

It seems that proposed extractive stripping voltammetry (ExSV) based on direct immersing of GCPE into continuously stirred cow's milk (3.5% fat) and subsequent electrochemical detection using SWV provides the desired sensitivity for detecting the sum of retinoids and carotenoids. A quantitative or at least semi-quantitative determination of vitamin A in cow's milk and cream samples was not the aim of this study. Voltammetric analysis of cow's milk enriched by differently defined amounts of all-*trans*-retinol could be probably considered as semi-quantitative analytical method.

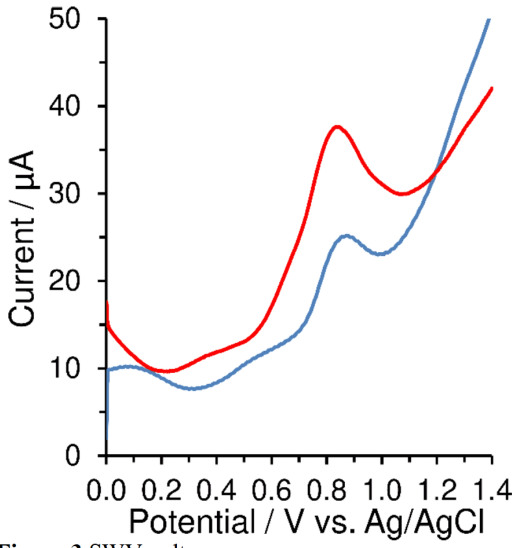

Figure 3 SWV voltammogram.
Note: SWV voltammogram of extracted (at 400 rpm for 10 min) cow's milk (3.5% fat) into GCPE containing always 15% (w/w) silicone oil with subsequent voltammetric detection in 0.1 mol.L^{-1} BRB (pH 4.5) at E_{step} = 5 mV, E_{ampl} = 25 mV, and f = 50 Hz (blue). SWV voltammogram of all-*trans*-retinol extracted (at 400 rpm for 5 min) from its (500 µmol.L^{-1}) 60% ethanolic solutions into GCPE containing always 20% (w/w) silicone oil with subsequent voltammetric detection in 0.1 mol.L^{-1} BRB (pH 4.5) at E_{step} = 1 mV, E_{ampl} = 25 mV, and f = 25 Hz (red line).

Selection of detection medium and supporting electrolyte

Optimisation consisted in finding out proper working conditions for an anodic oxidation of lipophilic vitamins which are presented in MFGs accumulated into silicone oil. At first, the electrochemical detection has been subjected to pH study which was investigated for 0.1 mol.L^{-1} RBRs of pH values from 2 to 7. A linear relationship between peak potential and pH values of used supporting electrolytes, statistically evaluated as E_p = -0.0558 pH + 1.0891 (R^2 = 0.9978), was observed. The peak potential was shifted to more negative values with increased pH of used BRBs. This phenomenon probably occurs due to lowering the energy barrier and easier deprotonation of present lipophilic vitamins. The value of slope 0.0558 indicates the transition of electrons together with protons in a 1:1 ratio. The most sensitive peak current response was achieved using BRBs of pH values 4 and 5. It seems that BRB would be replaced by an acetate buffer of pH 4.5, more simple in composition.

Optimization of square-wave voltammetry

Parameters of SWV, potential amplitude and frequency, were optimised at constant potential step of 5 mV. Extracted lipophilic vitamins provided a broad anodic peak which height increased with increasing potential amplitude up to value of 25 mV. Therefore, potential amplitude of 25 mV was chosen for following analysis of cow's milks and creams. As shown in Figure 4, the height of anodic peak linearly increased with higher frequency. However, it

was observed that background current (baseline) increased as well. Hence, a value of 50 Hz representing a compromise was chosen as optimum.

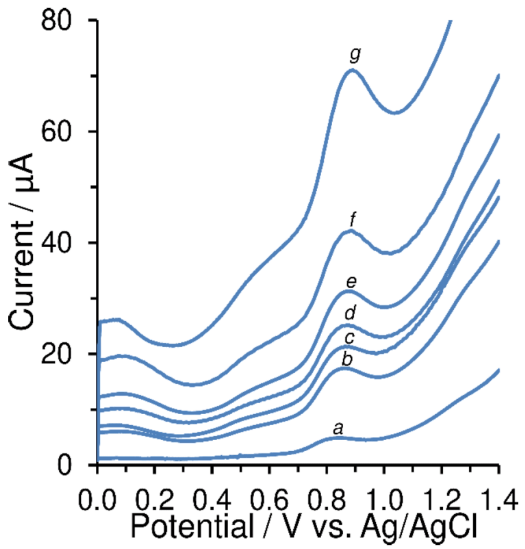

Figure 4 Voltammograms of lipophilic vitamins.
Note: Voltammograms of lipophilic vitamins sum (predominantly all-*trans*-retinol and β-carotene) extracted from cow's milk (3.5% fat) in GCPE containg 15% (w/w) silicone oil at 300 rpm and for 10 min. After rinsing with distilled water, subsequent voltammetric detection was performed in 0.1 mol.L^{-1} BRB (pH 4.5) at E_{step} = 5 mV, E_{ampl} = 25 mV, and f = 10 (*a*), 20 (*b*), 30 (*c*), 40 (*d*), 50 (*e*), 60 (*f*) and 100 (*g*) Hz.

Analysis* of *cow's milks and creams

At first, it is necessary to mention that a sample of whipped cream (40% fat content) could not be analysed using designed protocol due to high viscosity **(Van Vliet and Walstra, 1980)**. The whipped cream completely covered GCPE used and did not allow rinsing the surface with distilled water. At first glance, someone may think that peak current will be higher for creams (*'more milk fat more vitamins'*) **(Gaucheron, 2011)**. According to Figure 5, demonstrating a dependence of anodic peak current on milk fat content in selected samples, it seems that the assumption is misleading. An explanation could be summarized as follows: lipophilic vitamins present in samples are equally distributed between pasting liquid and milk fat during (*'liquid-liquid'*) extraction and these vitamins are more detained in creams (12 - 31%) than in milks (0.5 - 3.5%) due to many times higher fat content (*'like dissolves like'*).

Extraction repeatability

Assuming that MFGs are homogeneously dispersed throughout the volume of cow's milks and creams and their diameter is not higher than 1 µm **(Robin and Paquin, 1991; Michalski et al., 2004)**, an extraction repeability will be affected only by homogeneity of glassy carbon paste used **(Sýs et al., 2017)**. If recovery of developed HPLC-based methods ranging approximately from 85 to 110% **(Blanco et al., 2000; Gomis et al., 2000)** is taken to account, satisfactory repeatability of extraction characterized by RSD lower than 9% was achieved. Even despite the relatively short error bars (see Figure 5), it is not possible to determine, on the basis of the peak heights received, whether it is cream or milk with a 3.5% fat content.

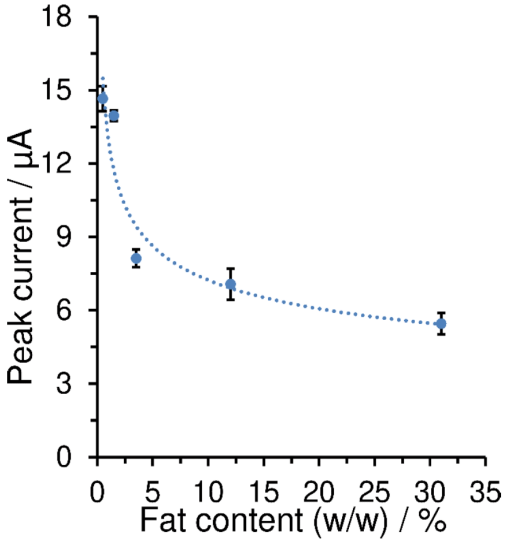

Figure 5 Dependence of peak current response (anodic oxidation of present lipophilic vitamins) on the content of fat in cow's milks and creams.

CONCLUSION

Everyone agrees that the most important part of whole analysis represents a sample preparation at which significant losses of analytes may occur and cannot be detected during the final analysis. Because the extractive stripping voltammetry requires a minimum sample preparation, it was tested as suitable analytical tool for cow's milks and creams quality control. However, the results obtained suggest that direct extraction of lipophilic vitamins (dominantly all-*trans*-retinol) from a continuously stirred sample and subsequent voltammetric detection using square-wave voltammetry could only be used for semi-quantitative determination of milk fat, at this stage of development. Finally, it can be concluded that the scientific hypothesis was refuted.

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Conflict of interest:

All authors declare no conflict of interest.

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2.2.3. A new voltammetric approach for the determination of *β*-carotene

The aim of the research was to propose a new voltammetric approach for the determination of BCA in foodstuffs and pharmaceutical supplements. The focus of the work was to avoid hazard organic solvents and to simplify sample preparation. The first step was to find the suitable working medium. Various organic solvents, such as MeCN, mixture of MeCN and toluene (1:1), mixture of MeCN and methyl tert-butyl ether (1:1), and pure ACE, all containing 0.1 mol L^{-1} $LiClO_4$, were tested. BCA provided the highest oxidation current at GCE using CV in the mixture of MeCN and toluene (Fig. 1, Publication 4). Nevertheless, ACE was preferred due to its high extraction efficiency for foodstuffs being a less hazardous solvent. Two metal-based electrodes (Aue and PtE), and GCE were compared in voltammetric measurement of 200 μmol L^{-1} BCA in pure ACE with 0.1 mol L^{-1} $LiClO_4$ using SWV. GCE provided almost twice broader anodic peak at +0.499 V in comparison with metallic electrodes (Fig. 12).

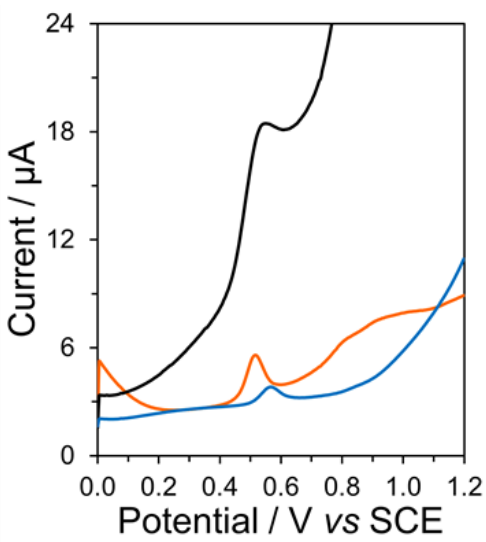

Figure 12. Comparison of square-wave voltammetric records for 200 μmol L^{-1} BCA obtained at GCE (black), AuE (orange), and PtE (blue line) in pure acetone.

In addition, a decrease in the peak of interest with the increasing number of consecutive measurements (RSD = 12.73%) was observed for GCE, unlike in the case of AuE (RSD = 1.33%). This could be explained by the strong passivation of the GCE surface with some product(s) formed by oxidation of BCA (Fig. 2, Publication 4). Two SWV parameters (*f* and E_{ampl}) were optimized at constant E_{step} = 5 mV. BCA oxidation peak current increased when value of *f* was stepped up to 220 Hz, unfortunately, background current increased significantly when frequency was higher than 80 Hz. Thus, the value of 80 Hz was chosen as optimum. In case of E_{ampl}, the BCA peak current increased up to 25 mV, therefore this value was selected for further measurements.

The analytical performance of the new method for the voltammetric determination of BCA in the optimized conditions has been tested. The method is based on the oxidation of BCA at AuE in ACE as an organic working medium with the possibility to perform analysis at the same electrode surface. Compared to previously reported methods, a wider linear range within 5–600 μmol L^{-1} BCA and LOD of 1.6 μmol L^{-1} were obtained (Table 1, Publication 4). Accuracy was calculated from the analysis of a model sample and a recovery of 95.4% (error less than 5%) was attained.

The interference study was performed before the real samples analysis. The common compounds accompanying BCA in the pharmaceutical supplements are VE, glycerol, soybean, gelatine, lecithin, microcrystalline cellulose, sodium carboxymethylcellulose, magnesium stearate, and purified water. From all above-mentioned substances, only VE is electroactive compound. Anodic oxidation of VE occurs around +0.65 V and no interference with the BCA signal at +0.505 V was observed. Moreover, the analysis of 100 μmol L^{-1} BCA in the presence of the potentially interfering species at 10-fold concentration excess, except for water a sixty-times higher content than BCA have been tested. The current response of BCA was reduced to 80% in the presence of glycerol and water due to insolubility, see Fig. 13. This negative effect of water could be eliminated by drying the vegetable samples before the extraction step with acetone. Glycerol effect in pharmaceutical formulation could be supressed by appropriate dilution of the sample in pure acetone.

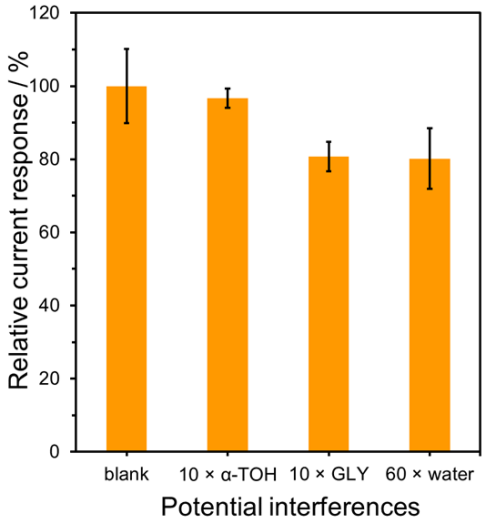

Figure 13. Effect of potential interferences (α-tocopherol; α-TOH and glycerol; GLY) on voltammetric determination of BCA where the blank represents the relative peak current response of 100 μmol L^{-1} BCA. Error bars in all graphs shown are also presented as confidence intervals.

It is known that BCA in the presence of atmospheric oxygen undergoes the oxidation, forming colourless reaction products. The effect of oxygen was investigated by SWV and the

reference method UV-Vis spectrophotometry. It has been confirmed that the stability of BCA depends on the duration of the analysis (Fig. 5, Publication 4). However, it was found that if the procedure for analysis including the sample preparation and the voltammetric measurements take less than three hours, satisfactory results can be obtained (RSD $\leq$ 5%).

Analysis of several raw vegetables and pharmaceutical capsules was conducted using the newly developed approach. The results were in good agreement with those acquired by UV-Vis spectrophotometry reference method (Table 2, Publication 4). Several advantages were achieved compared to already reported methods. Real samples were directly extracted using acetone as a solvent, which is not as toxic as the chlorinated hydrocarbons used before. Analytical performance of proposed method provides wider linear range as well as a lower detection limit than already published approaches. In addition, the same procedure can be applied to assay both food and pharmaceutical samples. In conclusion, it can be stated that this method can find applicability in many laboratories equipped with essential electroanalytical instruments as low time-consuming procedure for the determination of BCA.

2.3. Simultaneous determination of vitamin E and K

In two earlier studies it was shown that it is possible to utilize SWAdSV and GCE with good results in the determination of lipophilic vitamins [17], and vitamin K_1 [19]. By combining these two investigations, a simple and fast procedure has been developed for the simultaneous determination of VK and VE in food and pharmaceutical samples using SWAdSV.

The working methodology was based on three steps: the first step was the accumulation of lipophilic vitamins in open circuit on the nonpolar surface of the GCE in an aqueous-organic mixture, followed by the application of a negative potential for a certain period for electrochemical reduction of phylloquinone (VK_1) to phyllohydroquinone (H_2VK_1), and finally sequential electrochemical oxidation of reduced compounds providing current signals according to their various redox potentials. Usually, SWAdSV method requires much more optimization steps than direct voltammetric approaches. The work on this study is described in four main sections: the optimization of adsorption of vitamins, optimization of their voltammetric detection, analytical performance of the developed voltammetric method and analysis of food supplements.

The adsorption of the analytes to the working electrode is affected by several factors, such as type of organic solvent, accumulation time, stirring rate and ionic strength of the solution. Since VE and VK_1 are fat-soluble compounds, they are soluble in polar organic solvents and their aqueous mixtures. The effect of MeCN content (30 to 70% v/v) on the current signal response for both analytes was investigated using stirring rate of 300 rpm and accumulation time of 5 minutes. It was observed that MeCN content higher than 50% did not have any effect on the peak current response, therefore the mixture MeCN-water in ratio 1:1 was taken as an optimum (Fig. 14A). In addition, absence and presence of 0.0001, 0.001, 0.01, and 0.1 mol L^{-1} KCl in 50% MeCN was investigated. No significant increase in peak heights was found (Fig. 14B). Based on the obtained results, 0.1 mol L^{-1} KCl was used in further experiments to lower the ohmic resistance of the solution. The stirring rate affects the adsorption of the analytes to the working electrode, thus different speeds (100, 200, 300, 400, and 500 rpm) were tested. Setting the stirring rate faster higher than 400 rpm did not cause any significant increase in peak current responses, therefore this value was taken as optimum. Basically, the adsorption of the nonpolar analytes onto a nonpolar solid substrate (working electrode) is an equilibrium process. Hence, the accumulation time is one of the main parameters to be optimized in order to achieve a sufficient adsorption of the analyte. The accumulation time from 30 s to 900 s was investigated and the results shown that the adsorption

of the analytes intensified up to 300 s, afterwards no significant increase in peaks heights was observed. The electrode surface became probably already saturated at higher accumulation times, therefore the value of 300 s was considered as an optimum (Fig.1, Publication 5).

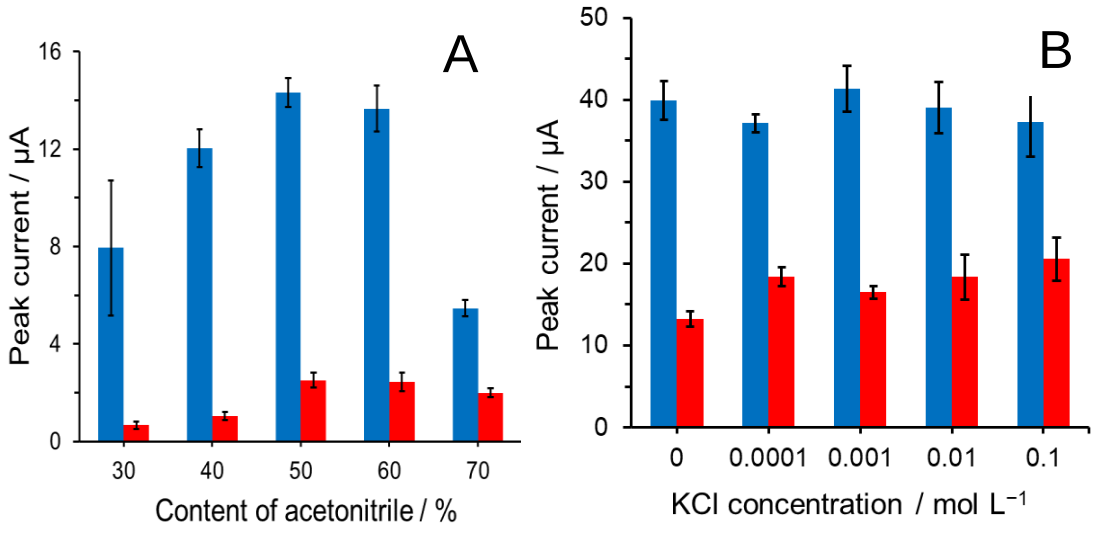

Figure 14. Dependence of anodic peak current on different content of acetonitrile (30 to 70 % v/v of 50 µmol L^{-1} vitamin K_1 (red) and α-tocopherol (blue), adsorbed at 300 rpm for 300 s. Voltammetric detection was carried out in 0.01 mol L^{-1} HNO_3 containing 0.1 mol L^{-1} KCl (pH 2.08) at E_{dep} = −0.1 V, t_{dep} = 60 s, E_{step} = 5 mV, E_{ampl} = 25 mV and f = 20 Hz (A). The effect of ionic strength on peak current response in 50 % acetonitrile content of 50 µmol L^{-1} vitamin K_1 (red) and α-tocopherol (blue), adsorbed at 300 rpm for 300 s. Voltammetric detection was carried out in 0.01-mol L^{-1} HNO_3 containing different content of KCl (pH 2.08) at E_{dep} = −0.1 V, t_{dep} = 60 s, E_{step} = 5 mV, E_{ampl} = 25 mV and f = 20 Hz (B).

Subsequently, the proper detection medium for the voltammetric detection was selected. Three monobasic acids (HCl, HNO_3, and $HClO_4$) with concentration 0.01 mol L^{-1} and dibasic 0.005 mol L^{-1} H_2SO_4 always containing 0.1 mol L^{-1} KCl to lower the ohmic resistance of the supporting electrolyte were tested. Higher current responses of VK_1 and α-TOH were obtained in nitric acid. For optimization of SWV parameters, E_{ampl} from 5 mV to 50 mV and f from 5 Hz to 100 Hz at constant E_{step} of 5 mV were investigated. The optimum value of 30 mV for E_{ampl} and 80 Hz for f were used (Fig. 2, Publication 5). In all cases, a reduction of accumulated VK_1 to H_2VK_1 was always performed by applying −0.1 V for 60 s prior the SWV detection in anodic mode [32].

The memory effect was studied for each vitamin separately and their voltammetric detection was repeated five times. In case of VK_1, no change was observed in the oxidation peak of H_2VK_1 (Fig. 3A, Publication 5). On the other hand, peak current of α-TOH decreased after consecutive measurements (Fig. 3B, Publication 5). It is evident that the GCE surface without renewing significantly affects the analysis of real samples. Therefore, a blank

measurement with a freshly polished GCE was included before each analysis. Two linear ranges were obtained, the short linear ranges for VK_1 and α-TOH determination were relatively short, 77–1000 nmol L^{-1} for VK_1 and 29–1000 nmol L^{-1} for α-TOH, with detection limits (LOD) of 25 and 10 nmol L^{-1}, respectively.

Additionally, linear ranges for higher concentrations were found: 1.0–7.0 μmol L^{-1} VK_1 and 1.0–10 μmol L^{-1} α-TOH described by regression equations I_p (μA) = 1.964 c (μmol L^{-1}) − 1.046 with R^2 = 0.9997 and I_p (μA) = 3.416 c (μmol L^{-1}) + 1.4105 with R^2 = 0.9973, respectively. Due to low value of intercept, standard addition method can be used for quantitative analysis. The precision was taken as recovery of measurements for eight replicates and the RSD values of 4.7% and 6.6% for VK_1 and α-TOH, respectively, were achieved. Two food supplements were analysed, and the results obtained by developed voltammetric method were in good agreement with HPLC (Table 2, Publication 5). The main advantages of the method are the possibility of simultaneous determination of VK_1 and α-TOH, simple preparation for samples (especially in case of pharmaceutical sample, which can be directly dissolved in working media), and minimal interference of accompanying substances. The developed analytical method is very simple and low-cost for analyses of food supplements. Furthermore, this method could be possibly utilized in clinical analysis since the contents of VE and VK in human plasma range from 12 to 30 μmol L^{-1} and from 0.4 to 7.1 μmol L^{-1}, respectively.

applied sciences

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Article

Simultaneous Determination of Vitamin E and Vitamin K in Food Supplements Using Adsorptive Stripping Square-Wave Voltammetry at Glassy Carbon Electrode

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Abstract: A new voltammetric method for the simultaneous determination of vitamin E and vitamin K present in different types of commercially available food supplements has been developed. This electroanalytical method is based on the ex situ adsorptive accumulation of these biologically active compounds onto the surface of a solid glassy carbon electrode (GCE) with subsequent electrochemical detection by square-wave adsorptive stripping voltammetry in 0.01-mol L^{-1} HNO_3 containing 0.1-mol L^{-1} KCl at pH 2.08. Due to reversible electrochemical reactions of phylloquinone, a subsequent voltammetric detection of both vitamins in anodic mode can be performed. Since individual forms of vitamins E and K usually exhibit nearly identical electrochemical behavior, it is therefore impossible to distinguish individual forms (quinones and tocopherols) and determine their molar concentrations in this way. Thus, the values of vitamin content were expressed as mass equivalent of phylloquinone and α-tocopherol as they are the most biologically active forms. Despite the high sensitivity, relatively short linear ranges were obtained due to the interaction (competition) of both vitamins during adsorption onto the freshly polished surface of the GCE from a 50% aqueous–acetonitrile mixture. The obtained results showed that the voltammetric approach is a very simple and low-cost analytical method that can be used in analyses of food supplements.

Keywords: vitamin E; vitamin K; adsorptive stripping voltammetry; glassy carbon electrode; food supplements

1. Introduction

Vitamin E and K belong to a group of fat-soluble vitamins [1–4], which are classified as non-polar organic compounds [5]. Vitamin E (VE), known as the most active lipophilic antioxidant, exists as four tocopherol and tocotrienol isomers [3,4,6,7] whose sources include vegetable oils, nuts and seeds of plants [8,9]. Vitamin K present in green plants (phylloquinone; VK1) and produced by bacteria (menaquinone; VK2) [10–14], is widely used in the diet for its anti-hemorrhagic properties [11,12].

VE is essential in the protection of fatty acid chains in the lipoprotein bilayer in the cytoplasmic membrane [3,4], because it preferentially reacts with peroxy radicals to form harmless oxidation

products which are reduced back by the reduction properties of ascorbic acid (vitamin C) [15]. Due to VE's significant physiological effects, avitaminosis may contribute to an increased risk of developing several serious diseases of civilization, such as fertility disorder [16], cardiovascular diseases (heart attack and stroke) [1], Alzheimer's disease [17] and renal impairment [18].

VK is essential for the carboxylation reaction of glutamic acid, which is known to be a precursor of blood-clotting factors [19]. Its deficiency results in a deactivation of prothrombin, causing hemorrhage [20], prosthetic valve failure [21] and bone formation disorders [11].

From all of the above information, it is evident that analysis of the presence and levels of these vitamins is justified, especially for food quality control. For Czech legislation based on that from the European Union, the reference analytical method used for the determination of VE and VK in foodstuffs is high-performance liquid chromatography (HPLC) in either normal-phase or reversed-phase systems [22–25] with spectrophotometric detection, known as ČSN EN 12822 (560055) and ČSN EN 14148 (560053), respectively. Moreover, it is worth mentioning that a recently investigated electrochemical detection method for both vitamins could be potentially used [1,2], however, this electrochemical detection has not been implemented for the simultaneous determination of VE and VK.

Several scientific papers suggest that there is a real chance to simultaneously determine VE and VK in various foodstuffs using adsorptive stripping voltammetry (AdSV) [2]. Principally, all the lipophilic vitamins present in a sample can be accumulated onto the nonpolar surface of a freshly polished glassy carbon electrode (GCE) from an optimum aqueous–organic mixture, and after applying a negative deposition potential over a period of time (electrochemical reduction of phylloquinone to phyllohydroquinone; H2VK1), they can be anodically oxidized sequentially in one step according to their different standard redox potentials [4]. Compared to the standard HPLC method, its relatively high sensitivity and fast and easy sample preparation for analysis can be seen as a great advantage. However, AdSV is unable to recognize individual vitamin forms due to overlap of their peak current signals. Hence, a sum of individual forms is usually expressed as the concentration equivalent of the most biologically active form [1].

In this study, a simple and rapid AdSV procedure is presented with square-wave voltammetry (SWV) as an electroanalytical technique for the simultaneous determination of VE and VK in commercial food supplements. The developed analytical method is based on an adsorptive accumulation of these biologically active compounds onto the surface of a GCE with subsequent electrochemical detection using SWV in 0.01-mol L^{-1} HNO_3 containing 0.1-mol L^{-1} KCl (pH 2.08). Special attention is paid to the optimization of all working conditions, such as the content of MeCN in the accumulation medium, the effect of ionic strength (presence of salt), the accumulation time during adsorption, speed of stirring (effect of mass transport), the interaction of analytes (simultaneous calibration) and the working parameters of electrochemical detection mediated by SWV. Finally, the presented square-wave adsorptive stripping voltammetry (SWAdSV) method is assessed in terms of analytical parameters, namely the calibration range, limit of detection, precision (repeatability), accuracy (recovery) and feasibility of using the standard addition method in a real analysis.

2. Materials and Methods

2.1. Reagents and Chemicals

Analytical standards of (+)-α-tocopherol (α-TOH), γ-tocopherol (γ-TOH), δ-tocopherol (δ-TOH), phylloquinone (VK1), menaquinone (VK2) and menadione (VK3) together with pure acetonitrile (MeCN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade purity from Lach-Ner, Ltd. (Neratovice, Czech Republic): hexane for the cleaning surface of the GCE, 65% nitric acid, 35% hydrochloric acid, 96% sulfuric acid, 68% perchloric acid, glacial acetic acid and potassium chloride for the preparation of the aqueous detection medium.

Ultrapure water (ρ = 18.3 MΩ cm) obtained with a Milli-Q® water purification system from Merck (Darmstadt, Germany) was used for the preparation of all solutions.

2.2. Pretreatment of Glassy Carbon Electrode

Before each measurement, a solid GCE (type 6.1204.300) with a surface diameter of 3 mm from Metrohm (Herrisau, Switzerland) had to be polished using a water dispersion of Al_2O_3 powder (particle size of 0.3 µm) for 30 s. After this treatment, the electrode was dried with wood pulp paper, then immersed in 95% hexane and ultrasonicated for 5 min. All these cleaning steps were necessary since it was known that the targeted analytes (or their electrode reactions products) remain on the GCE surface due to their high abilities to be adsorbed [1,2]. Before each voltammetric analysis, a baseline measurement was done to checked whether the surface of the GCE was appropriately renewed.

2.3. Apparatus

All voltammetric measurements were performed in a conventional electrochemical glass cell with a three-electrode system consisting of a GCE (working), a silver chloride electrode with 3.0-mol L^{-1} KCl (reference) and a platinum wire (counter electrode) all from Metrohm (Prague, Czech Republic). These electrodes were connected to a potentiostat/galvanostat (type Autolab/PGSTAT101) from Metrohm (Prague, Czech Republic) operated with NOVA 1.11 software.

2.4. Sample Preparation for Voltammetric Analysis

Two different food supplements commercially available in Czech pharmacies, namely VITAMIN K2 MK 7 + D3 FORTE (Sample 1) and VITAMARIN 90 cps (sea fish oil from *Engraulis japonicus*) (Sample 2), were analyzed to verify the developed analytical method. The simultaneous voltammetric determination of vitamin E and K enables the analysis of two different food supplements (containing only one investigated lipophilic vitamin), which can minimize chemical consumption and significantly reduce analysis time. Therefore, one tablet of Sample 1 containing 100 µg VK and one capsule of Sample 2 containing 2.5 mg vitamin E were dissolved with 50% MeCN in 100-mL voltammetric flasks using ultrasonication for 20 min. For voltammetric analysis, 6-mL of Sample 1 solution, 1 mL of Sample 2 solution, and 4 mL of 50% MeCN were mixed in an accumulation glass cell.

2.5. Methods

2.5.1. Square-Wave Adsorptive Stripping Voltammetry

Generally, ex situ adsorptive stripping voltammetry (AdSV)—utilizing the adsorption of lipophilic analytes onto a nonpolar electrode surface (a freshly polished GCE)—is divided into two steps, which guarantees higher selectivity than with AdSV in situ mode [26,27]. In this study, the adsorption of analytes started when the GCE was immersed into a continuously stirred (400 rpm) 10 mL of 50% MeCN for 300 s. Electrochemical detection using SWV as the electroanalytical technique was performed in 10 mL of 0.01-mol L^{-1} HNO_3 containing 0.1-mol L^{-1} KCl (pH 2.08) with the following working parameters: deposition potential (E_{dep}) −0.1 V, deposition time (t_{dep}) of 60 s, equilibrium time (t_{eq}) 5 s, potential range from −0.1 to +0.8 V, potential step (E_{step}) 5 mV, potential amplitude (E_{ampl}) 30 mV and frequency (f) 80 Hz. The method of standard addition was used for evaluating VK1 and VE content, where three additions of 2 µL of 0.01-mol L^{-1} stock solutions of VK1 and VE were subsequently added to the mixed solution of food supplements. Each analysis was repeated at least eight times (n = 8). Unless stated otherwise, all changes in the experimental conditions are described in the legends of the corresponding figures.

2.5.2. High-Performance Liquid Chromatography

A liquid chromatograph, consisting of a degasser of mobile phase DG 3014 from Ecom (Prague, Czech Republic), pump Spectra System P2000, autosampler Spectra Series AS100 and detector Spectra System UV3000, all from Thermo Separation Products (Waltham, MA, USA), was coupled with a commercial Kinetex™ PFP 100 Å column (150 × 3 mm, the particle size of 2.6 µm) from Chromservis Ltd. (Prague, Czech Republic). The detection wavelength was set to 246 nm. A mixture of water/methanol (10/90 *v/v*) was used as the mobile phase at a flow rate of 0.3 mL min^{-1} with isocratic elution at a temperature of 40 °C. For a sample volume of 10 µL, the direct comparison approach was used for the determination of lipophilic vitamins in the food supplements.

The gelatin capsule of fish oil was pierced with a needle, and the total content was transferred using a syringe into 10 mL of pure methanol. Five pills of a food supplement containing VK2 was dissolved in 10 mL hexane. After filtration through a folded filter paper, the hexane was evaporated with nitrogen at 40 °C. The resulting residue was dissolved in 1 mL methanol. The resulting sample solutions were spiked with 10 mg L^{-1} standards of α-TOH and VK1 to verify the recovery of the sample preparation.

2.6. Statistical Evaluation

The final results evaluated from eight repetitions are presented as confidence intervals $x \pm st_{1-\alpha}$, where x is the arithmetic mean, s the standard deviation and $t_{1-\alpha}$ the critical value of Student's t-distribution (2.365) for a given number of determinations (two-sided distribution) at a significance level α of 0.05 (95% probability). The feasibility of using the standard addition method was determined by testing the significance of intercepts of the corresponding calibration curves using the statistical software QC Expert version 2.5 from TriloByte Statistical Software (Pardubice, Czech Republic).

3. Results and Discussion

The results are described within four main sections: the optimization of adsorption, optimization of subsequent voltammetric detection, analytical performance of the developed voltammetric method and analysis of food supplements. In comparison with direct voltammetric approaches, the presented SWAdSV method requires much more optimization. Nevertheless, the results showed that this voltammetric method is significantly more selective and sensitive, which is consistent with the literature [28].

3.1. Optimization of Adsorption

An adsorptive accumulation of lipophilic vitamins onto the freshly renewed surface of GCE (nonpolar substrate) was already used as a non-electroplating step (a non-electrolytic preconcentration) in the development of electroanalytical stripping methods [2,26], unfortunately not for their simultaneous voltammetric determination. The efficiency of adsorptive accumulation (the sensitivity of the final voltammetric method) usually depends on several factors that must be optimized. Among these factors are the selection of the water–organic solvent mixture, the presence of salts, stirring speed of the magnetic stirrer bar and accumulation time.

3.1.1. Effect of Organic Solvent Content

Since VE and VK1 are fat-soluble compounds, they are soluble in low-polar organic solvents and relevant aqueous mixtures. Generally, polar aprotic organic solvents with a simple linear structure, good solvation properties and sufficiently high boiling point while maintaining the same physical conditions during adsorption are preferred. Moreover, it is well known that the presence of water in MeCN enables the formation of hydrogen bonds [29] that provide interactions to increase the adsorption of vitamins onto the electrode surface. The effect of MeCN content on the peak current responses of lipophilic vitamins was investigated from 30% to 80% (*v/v*) at a stirring rate of 300 rpm for

300 s. No significant increase in both vitamins peak current responses was observed for a MeCN content higher than 50% (*v*/*v*), and therefore 50% aqueous–acetonitrile mixture was accepted as the optimum.

3.1.2. Effect of Ionic Strength

Another parameter tested in this research was the effect of ionic strength on peak current response. The presence of 0, 0.0001-, 0.001-, 0.01- and 0.1-mol L^{-1} KCl in 50% MeCN was investigated. No statistically significant increase in peak heights was found. Based on the obtained results, we did not use an excess of salt in further experiments.

3.1.3. Speed of Stirring

The stirring speed of the magnetic bar affects the rate of lipophilic vitamin transport to the electrode surface where these analytes are adsorbed. Here, it was observed that setting the rate of the magnetic stirrer higher than 400 rpm did not cause any significant increase in peak current responses. Therefore, the above-mentioned value was considered to be the optimum for subsequent measurements.

3.1.4. Accumulation Time

Fundamentally, adsorption of the nonpolar analyte onto a nonpolar solid substrate (working electrode) is an equilibrium process. Curves characterizing the dependencies of accumulation time on peak current responses have the typical shape of adsorption isotherms [30] that express the variation in the amount of analyte adsorbed by the adsorbent within the time period at a constant temperature. As is shown in Figure 1, peak heights increased with increasing accumulation (adsorption) time up to 300 s. For longer accumulation periods, no significant increase in peaks intensity (saturation) was found based on the comparison of standard deviation values (error bars). For this reason, the value of 300 s was chosen as the optimum to achieve the equilibrium.

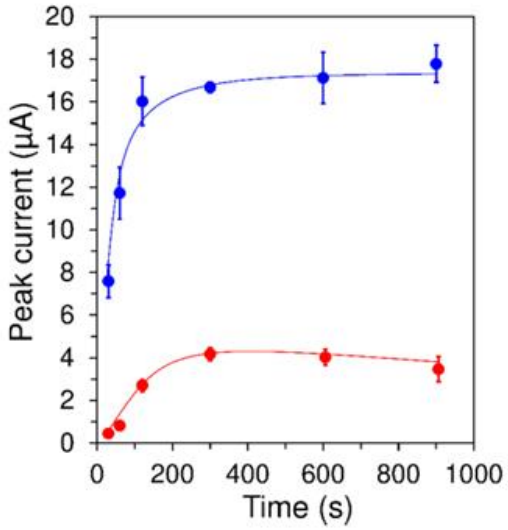

Figure 1. Dependence of anodic peak current phylloquinone (VK1) (red) and of (+)-α-tocopherol (α-TOH) (blue line) on adsorption time. square-wave adsorptive stripping voltammetry (SWAdSV) of 50-μmol L^{-1} α-TOH and 50-μmol L^{-1} VK1 adsorbed from its 50% aqueous–acetonitrile mixture onto a glassy carbon electrode (GCE) at stirring rate 400 rpm for different time durations. Voltammetric detection was carried out in 0.01-mol L^{-1} HNO_3 containing 0.1-mol L^{-1} KCl (pH 2.08) at E_{dep} = −0.1 V, t_{dep} = 60 s, t_{eq} = 5 s, E_{step} = 5 mV, E_{ampl} = 25 mV and f = 20 Hz.

3.2. Optimization of Subsequent Voltammetric Detection

From a chemical point of view, H2VK1 and α-TOH are phenolic compounds, and thus they can be considered to be weak organic acids [31,32]. Therefore, it can be assumed that the pH of the working (detection) medium has a fundamental influence on the position and height of the oxidation peaks. The electrochemical reduction of adsorbed VK1 to H2VK1 had to be optimized to achieve the maximum current yield of the electrode reaction.

SWV is a commonly used pulse voltammetry technique that exhibits a greater capacity to discriminate the influence of capacitive current. Essentially, the size of the potential amplitude affects this discrimination, and the frequency determines the scan rate (ν). Therefore, it was clear that these two working parameters can significantly influence the final sensitivity of the developed voltammetric method.

3.2.1. Effect of Detection Media

In the literature, the highest peak currents were recorded at carbon-based electrodes in aqueous solutions of strong inorganic acids [1,2,33,34]. Here, 0.005-mol L^{-1} dibasic (H_2SO_4) and 0.01-mol L^{-1} monobasic (HCl, HNO_3 and $HClO_4$) strong mineral acids, always containing 0.1-mol L^{-1} KCl to increase the electric conductivity, were tested as a potential detection medium. Slightly higher current responses of VK1 and α-TOH were obtained for 0.01-mol L^{-1} HNO_3 in the presence of 0.1-mol L^{-1} KCl. As a result, this aqueous solution of HNO_3 was chosen as optimal.

3.2.2. Effect of Square-Wave Voltammetry Working Parameters

From previously reported papers, it is known that applying a potential of −0.1 V for 60 s is sufficient to reduce VK1 to H2VK1 [2,33]. Hence, it was necessary to determine the optimal working parameters of SWV. At the constant potential step of 5 mV, an effect of other two working parameters for α-TOH and VK1 peak current responses was investigated: potential amplitude from 5 to 50 mV and frequency from 5 to 100 Hz (Figure 2).

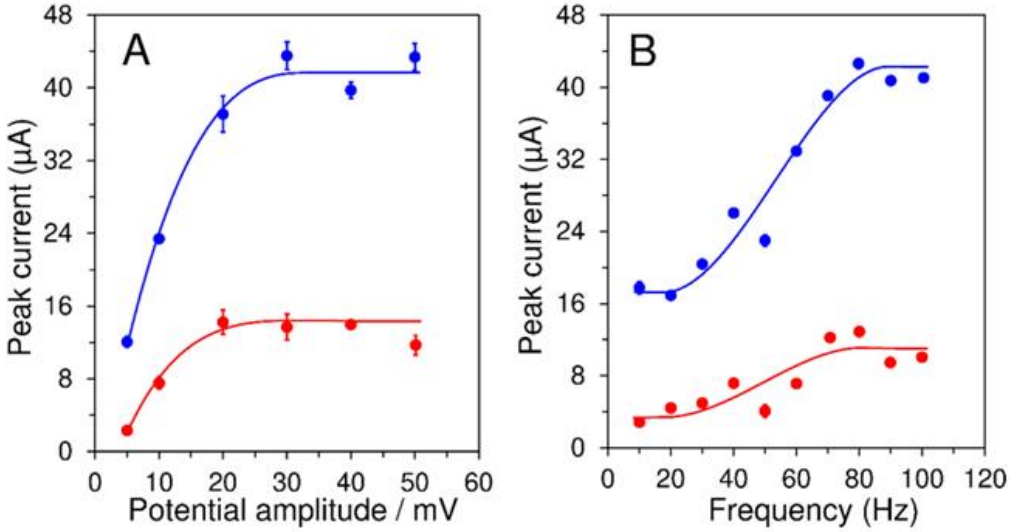

Figure 2. Dependence of anodic peak current VK1 (red) and α-TOH (blue line) on potential amplitude (**A**) and frequency (**B**). SWAdSV of 50-μmol L^{-1} α-TOH and 50-μmol L^{-1} VK1 adsorbed from its 50% aqueous–acetonitrile mixture onto GCE at stirring rate of 400 rpm for 5 min. Voltammetric detection was carried out in 0.01-mol L^{-1} HNO_3 containing 0.1-mol L^{-1} KCl (pH 2.08) at E_{dep} = −0.1 V, t_{dep} = 60 s, t_{eq} = 5 s and E_{step} = 5 mV. The effect of potential amplitude was examined at a constant frequency of 80 Hz and the effect of frequency was studied at a constant potential amplitude of 25 mV.

3.2.3. Memory Effect

Generally, one of the main criteria affecting the precision of analytical methods is the repeatability of measurements. To be more specific, the previous measurement should not affect the next one. This negative phenomenon is usually referred to as the memory effect. In this study, each vitamin was accumulated separately and their voltammetric detection was repeated five times (Figure 3). Due to the reversible electrochemical behavior of VK1 with the precipitation of two electrons and protons (hydroquinone/benzoquinone redox couple), no change was observed in the oxidation peak of H2VK1 at +0.202 V (see Figure 3A). In contrast, α-TOH is anodically oxidized at +0.444 V to form a dienone cation [31,35] which nucleophilically reacts with water to form α-tocohydroquinone (α-TQ). This α-TQ is subsequently electrochemically reduced by applying a negative deposition potential (−0.1 V) for 60 s to form α-tocohydroquinone (α-TQH2). In the subsequent measurements, the α-TQH2 is anodically oxidized at +0.247 V with the precipitation of two electrons and protons (α-TQ/α-TQH2 redox couple), as shown in Figure 3B.

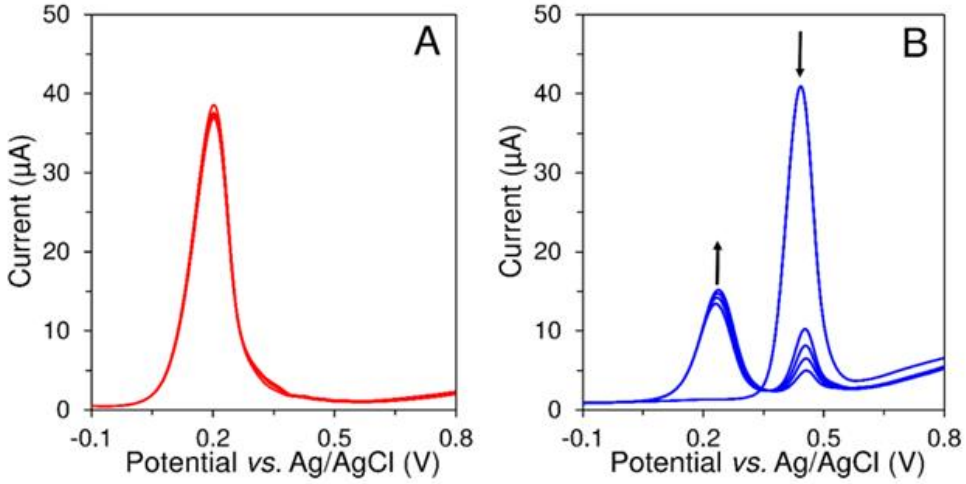

Figure 3. Repetitive SWV of VK1 (red, **A**) and α-TOH (blue records, **B**) adsorbed onto GCE surface from their 50-μmol L^{-1} solution (accumulated from 50% MeCN at stirring speed of 400 rpm for 5 min) in 0.01-mol L^{-1} HNO_3 containing 0.1-mol L^{-1} KCl (pH 2.08) at E_{dep} = −0.1 V, t_{dep} = 60 s, t_{eq} = 5 s, E_{step} = 5 mV, E_{ampl} = 30 mV and f = 80 Hz.

It was evident that an imperfectly renewed GCE surface significantly affects the analysis of real samples, especially the overlapping of anodic peaks of H2VK1 and α-TQH2. For this reason, a blank measurement with a freshly polished GCE was included before each analysis.

3.3. Analytical Performance of the Developed Voltammetric Method

From previous paragraph, it seems that VK1 and α-TOH gave nearly the same peak current response (I_p) for equal concentration level (c). This fact applies only if they are determined separately using SWAdSV. The separate measurements on SWAdSV of VK1 and VE showed different behavior from the simultaneous ones, based on peak current responses (I_p), as it is shown in Figure 4. The α-TOH gave significantly higher peak current response than VK1. An explanation could be found in preferable adsorption of α-TOH onto the surface of the GCE from 50% MeCN. It was found that this phenomenon negatively affected the final linear ranges. The peak height of the VK1 decreased above a concentration of 8 μmol L^{-1} VK1, while the oxidation peak of α-TOH still increased linearly with increasing content up to 10-μmol L^{-1} α-TOH (Figure 4A, dashed and dotted lines).

The linear ranges for VK1 and α-TOH determination were relatively short, 77–1000 nmol L^{-1} for VK1 and 29–1000 nmol L^{-1} for α-TOH, with detection limits (LOD) of 25 and 10 nmol L^{-1}, respectively.

Values of LODs were calculated as three times the standard deviation (*s*) of ten replicate measurements (200-nmol L^{-1} VK1 and 50-nmol L^{-1} α-TOH) divided by the slopes of corresponding regressions (*k*). Limits of quantification (LOQ), presented as the first values of corresponding linear ranges, were calculated as 10 *s*/*k*. The above-mentioned linear ranges are described by equations of calibration curves as follows: I_p (μA) = 0.611 *c* (μmol L^{-1}) − 0.080 with coefficient of determination (R^2) 0.9920 for VK1 and I_p (μA) = 6.333 *c* (μmol L^{-1}) − 0.012 with R^2 = 0.9993 for α-TOH. Statistically insignificant (negligible) values of intercepts (*q*) allow the use of the standard addition method for the simultaneous voltammetric determination of VK1 and α-TOH in selected food supplements. Moreover, additional linear ranges for higher concentrations were found: 1.0–7.0-μmol L^{-1} VK1 and 1.0–10-μmol L^{-1} α-TOH described by regression equations I_p (μA) = 1.964 *c* (μmol L^{-1}) − 1.046 with R^2 = 0.9997 and I_p (μA) = 3.416 *c* (μmol L^{-1}) + 1.4105 with R^2 = 0.9973, respectively.

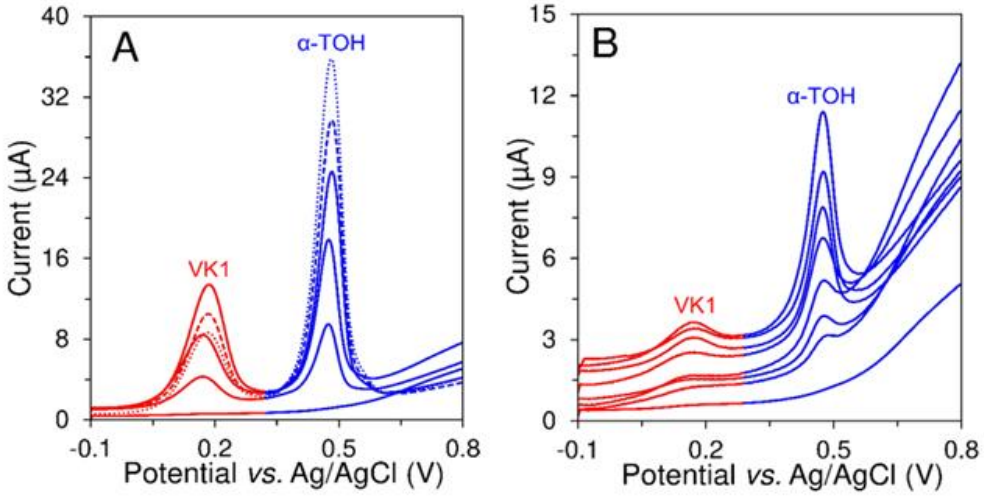

Figure 4. SWAdSV voltammograms of calibration curves of VK1 and α-TOH at optimized working conditions: (**A**) 0 (blank), 2, 4, 6, 8 (dashed) and 10 (dotted line); (**B**) 0 (blank), 0.05, 0.1-, 0.2-, 0.4-, 0.6-, 0.8- and 1.0-μmol L^{-1} VK1 and α-TOH.

Comparable analytical parameters were obtained with previously reported adsorptive stripping voltammetric methods (Table 1). The precision of the developed SWAdSV, referring to the mutual agreement between repeated measurements, was calculated for eight replicate measurements of equal vitamin content (50-μmol L^{-1}) and relative standard deviations (RSD) of 4.7% and 6.6% for VK1 and α-TOH were achieved, respectively.

Table 1. Overview of stripping voltammetric methods for the determination of VK and vitamin E (VE).

Electrode	Analyte	Method	Linear Range (mol L^{-1})	LOD (mol L^{-1})	Reference
HMDE	VK1	DPP	2.2×10^{-8}–2.2×10^{-7}		[36]
HMDE	VK1	SWAdSV	1.0×10^{-9}–1.0×10^{-6}		[37]
HMDE	VK3	SWAdSV	2.0×10^{-10}–5.0×10^{-7}	1.3×10^{-10}	[33]
CPE	VK1	LSAdSV	6.7×10^{-7}–4.4×10^{-6}	4.0×10^{-7}	[26]
GCE	VK1	SWAdSV	5.0×10^{-6}–1.0×10^{-4}	5.1×10^{-8}	[2]
GCE	VK1	SWAdSV	1.0×10^{-8}–1.0×10^{-6}	8.9×10^{-9}	[2]
GCE	VK1	SWAdSV	7.7×10^{-8}–1.0×10^{-6}	2.5×10^{-8}	This work
GCPE	VE (α-TOH)	SWASV	5.0×10^{-7}–4.0×10^{-5}	1.0×10^{-7}	[1]
GCE	VE (α-TOH)	SWAdSV	2.9×10^{-8}–1.0×10^{-6}	1.0×10^{-8}	This work

Notes: CPE—carbon paste electrode; GCPE—glassy carbon paste electrode; DPP—differential pulse polarography; HMDE—hanging mercury drop electrode; LSAdSV—linear sweep adsorptive stripping voltammetry and VK3—menadione. LOD—detection limits.

3.4. Analysis of Food Supplements

Common food supplements available in Czech stores usually do not contain lipophilic vitamins in their natural biologic form, but instead their synthetic analogs (menadione; VK3 and α-tocopheryl acetate), due to their higher chemical stability. Nowadays, food supplements based on vegetables or fish oils [38] encapsulated in gelatin capsules are rapidly growing in popularity. These good supplements usually contain a mixture of natural lipophilic vitamins. Here, a mixture of two of these types of food supplements was analyzed using the developed SWAdSV.

From Figure 5 it is evident that all the commonly occurring forms of VE are present in the capsule of VE because three overlapping peaks attributed to the anodic oxidation of α-TOH, γ-TOH and δ-TOH at +0.464 V, +0.539 V and +0.604 V were observed, respectively. Based on the position of the oxidation peaks reflecting the number of methyl groups (+I effect) in the chromanol ring, the order of the individual forms of vitamin E was determined [38,39]. Due to this unsymmetrical overleaping peak, an evaluation of peak height was not used and peak area (A_p) was used instead (Figure 5, inserted graph).

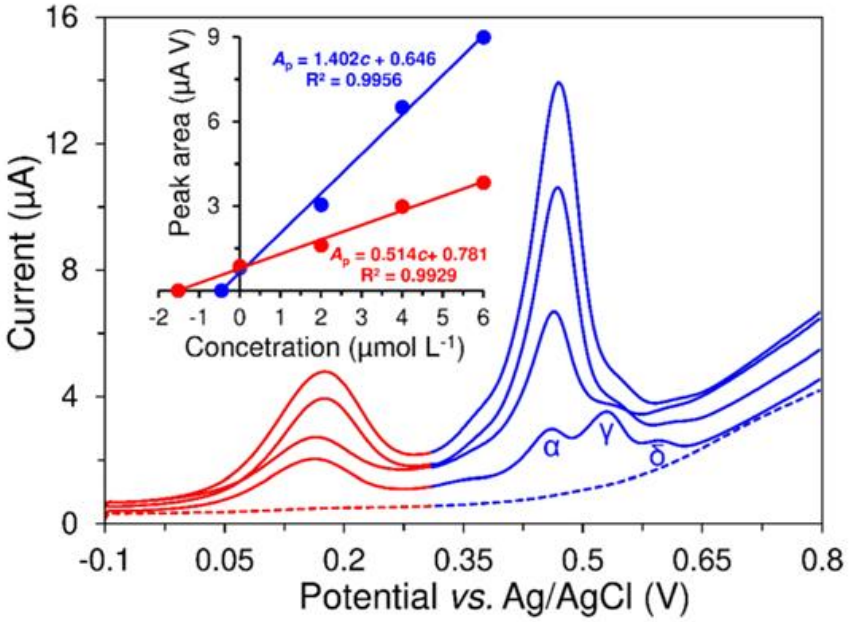

Figure 5. SWAdSV voltammograms of mixture of two different food supplements analyzed by standard addition method (inset).

As was mentioned above, the recognition of individual forms of VE in the real sample was also possible using SWAdSV. Unfortunately, this did not apply for VK, because the HPLC analysis showed (Figure 6) that the food supplement (Sample 1) did not only contain only VK2 (retention time of 6.53 min), as the manufacturer claimed, but also VK1 (10.73 min) and menadione (VK3; 2.60). Thus, it can be concluded that SWAdSV is not a suitable electroanalytical tool for determining individual forms, but only their sums. By spiking sample solutions with a known concentration of individual forms of vitamins, the rate of recovery for HPLC was found to be in the range of 91–105%.

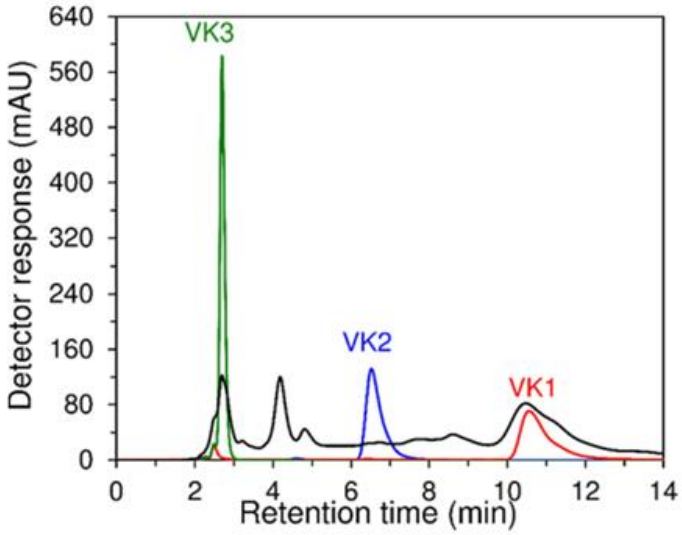

Figure 6. HPLC analyses of food supplement VITAMÍN K2 MK 7 + D3 FORTE (black line) and 3 standard solutions of 10 mg L^{-1} VK1, VK2 and VK3. KinetexTM PFP 100 Å column (150 × 3 mm, particle size of 2.6 µm), mobile phase of water/methanol (10/90 *v/v*), flow rate of 0.3 mL min^{-1}, sample volume of 10 µL, temperature 40 °C, detection at 246 nm.

Table 2 summarizes the results obtained by both SWAdSV and HPLC methods of analysis of food supplements. For food supplements based on natural oils, the hydrolysis of fats was necessary and a simple dissolution in an organic solvent alone was insufficient to achieve precise (declared) results. Nevertheless, the developed SWAdSV could find a direct utilization (without complicated sample preparation) in the analysis of many other food supplements, especially in the form of tablets. Due to its high sensitivity, SWAdSV could represent a suitable analytical tool for the determination of lipophilic vitamins, e.g., in human plasma. Future research should be directed towards the possibility of the direct absorption of lipophilic vitamins bound with carrier lipoproteins [26] or also include a hydrolysis step with specific lipoprotein lipases [40].

Table 2. Comparison of SWAdSV with HPLC for the analysis of selected food supplements.

Food Supplement	SWAdSV		HPLC		Declared Content	
	VK	VE	VK	VE	VK	VE
Model sample	4.72 ± 0.28	42.86 ± 1.94	4.8 ± 0.13	45.3 ± 1.6	4.51	43.71
Sample 1	0.11 ± 0.02	–	0.12 ± 0.01	–	0.10	–
Sample 2	–	0.20 ± 0.02	–	0.21 ± 0.01	–	2.50

Note: Values are presented as mg per capsule (µg per 100 mL in case of the model sample) and given as confidence intervals $x \pm st_{1-\alpha}$, where x is the arithmetic mean, s the standard deviation and $t_{1-\alpha}$ the critical values (2.365) of Student's t-distribution for 8 repetitions of each analysis at $\alpha = 0.05$.

4. Conclusions

In this study, it was demonstrated that the simultaneous determination of vitamin E and K in food supplements is possible using the developed square-wave adsorptive stripping voltammetry on the glassy carbon electrode. Due to the similar electrochemical properties of individual forms, the voltammetric method presented here is suitable for determining their sums, expressed as the concentration equivalents of the most biologically active forms (α-tocopherol and phylloquinone). However, relatively short linear calibration ranges were achieved because of the limited size of the working electrode surface. Thanks to the ex situ type of accumulation, the main advantage of this voltammetric method can be seen in the easy sample preparation consisting of dissolving the sample

in the accumulation medium and minimal interference of accompanying substances. The benefits of the developed method may also lead to its application in clinical analysis because the contents of VE and VK in human plasma range from 12 to 30 μmol L^{-1} and from 0.4 to 7.1 μmol L^{-1}, respectively. This assumption can be considered to be a continuation of this study.

Author Contributions: Conceptualization, L.K. and M.S.; methodology, M.S. and R.M.; formal analysis, G.K., B.Š. and G.J.; authors of the manuscript, G.K., M.S. and L.K.; final correction, M.S. and L.K.; supervising, T.A. and Z.B. All authors have read and agreed to the published version of the manuscript.

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2.4. Possibilities of simultaneous voltammetric determination of individual tocopherol isomers

During the research of simultaneous determination of VK and VE in food supplements, it was observed that in the obtained voltammograms of food supplement sample there several signals indicating the presence of forms of VE. These three overlapping peaks were attributed to the anodic oxidation of α-TOH, γ-TOH, and δ-TOH at +0.464 V, +0.539 V, and +0.604 V, respectively. Based on this finding, a detailed study on the possibility of simultaneous determination of three forms of VE have been carried out.

Several works on the simultaneous voltammetric determination of tocopherols have been reported [66-71]. The initial electrochemical study of individual tocopherols using linear sweep voltammetry for the determination of the individual tocopherols in vegetable oils dissolved in the mixture of ethanol and benzene was conducted by McBride and Evans in 1973 [70]. Clough with his team reported on the possibility of simultaneous electrochemical detection of tocopherols by SWV in several vegetables' oils in the mixture of ethanol and toluene (ratio 1:1) containing 0.1 mol L^{-1} H_2SO_4 in 1992 [66]. Analysis of tocopherol mixtures in vegetable oils and fats mixed in *N*-methyl-pyrrolidone containing 0.01 mol L^{-1} quaternary ammonium salt at platinum microelectrode using differential pulse voltammetry (DPV), was reported by Coatena et al. However, only one broad oxidation peak was obtained due to presence of sample matrix (edible oils) [71].

Unfortunately, no improvement was achieved in the peak separation of individual tocopherols using voltammetric approach. For the first time, Diaz et al., utilized voltammetric techniques combined with chemometric methods with the aim to resolve the overlapping voltammetric signals of tocopherols. The proposed method was based on the determination of tocopherol forms in olive oil samples with pre-cleaning step by solid-phase extraction on silica cartridges in hexane-ethanol solution at GCE using DPV [68]. In all cases mentioned above, an overlap of their respective anodic peaks was observed.

To find the most optimal conditions for sufficient peak separation of all three forms of tocopherols, many factors have been studied. Various working electrode materials, presence of water in organic solvent, and the effect of non-ionic, cationic, and anionic surfactants were tested. AuE, PtE, GCE, boron doped diamond electrode, pyrolytic graphite electrode, and GCE/MWCNTs were tested for the simultaneous voltammetric detection of 50 μmol L^{-1} tocopherol forms in pure MeCN containing 0.1 mol L^{-1} $LiClO_4$ (Fig. 2, Publication 6). Only GCE provided the widest peak separations with peak potentials at +0.645 V for α-TOH, +0.752 V for γ-TOH, and +0.857 V for δ-TOH. The reason of such observation is most probably due

to slower charge transfer at GCE comparing to metal-based electrodes (AuE and PtE) and other carbon electrodes, where peaks were strongly overlapped. Hence, GCE was chosen as working electrode for further experiments.

Since tocopherols are soluble in polar organic solvents, DMF, propylene carbonate, ethanol (EtOH), and MeCN were examined to find the most compatible working medium. Two low current response and broad anodic peaks of the three tocopherol forms were observed in DMF and propylene carbonate, on the other hand, three overlapping peaks with higher current signal were obtained in EtOH and MeCN (Fig. 3A, Publication 6). Considering that three well-defined voltammetric signals from individual tocopherol forms were obtained only in MeCN, the mixture of MeCN with water in ratio (90:10 % v/v) was further studied. The presence of water caused a shift of anodic peaks to more negative potentials and a significant decrease of peak currents (Fig. 3B, Publication 6). In addition, the effect of different contents (0.1, 0.01, and 0.001 mol L^{-1}) of nonionic (Triton X-100), anionic (SDS), and cationic (cetylpyridinium chloride and cetyltrimethylammonium bromide) surfactants on peaks separation was investigated. No improvement on the overlapped peak signals was observed (Fig. 15).

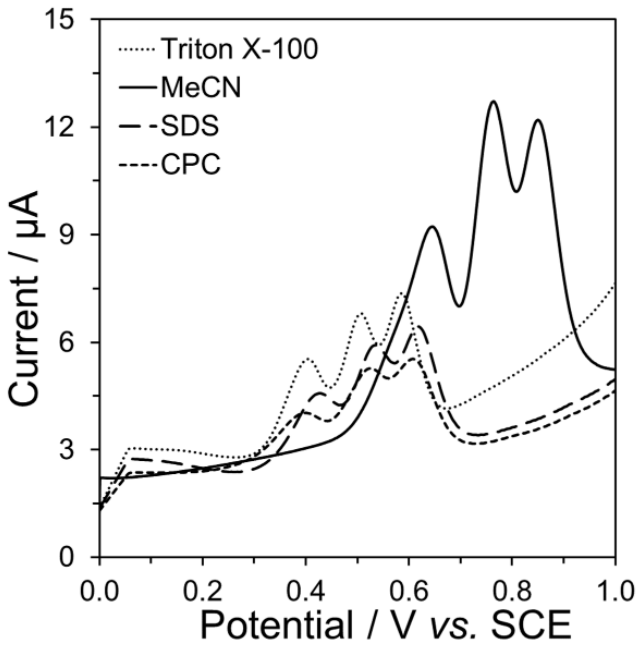

Figure 15. Voltammograms of 50 µmol L^{-1} α-TOH, γ-TOH and δ-TOH at GCE in pure MeCN containing 0.1mol L^{-1} $LiClO_4$ and 0.001 mol L^{-1} CPC, SDS or Triton X-100 at E_{step} = 1 mV, E_{ampl} = 25 mV and f = 20 Hz.

Three voltammetric techniques (LSV, DPV, and SWV) were compared in simultaneous electrochemical detection of tocopherols at scan rate of 25 mV s^{-1}. LSV showed a low background current, but it was almost impossible to distinguish the individual peaks. On the other hand, the peak separation was similar in both pulse voltammetric techniques and SWV

was selected due to higher peak current responses. The main parameters of SWV (E_{step}, E_{ampl}, and f) were optimized. It was found that E_{step} has a significant effect on peak separation; values higher than 1 mV caused peaks overlapping (Fig. 16A). The E_{ampl} and f did not have any effect on the peak separation, therefore, 25 mV for E_{ampl} potential step, and 25 Hz for f were taken as optimum values (Fig. 16).

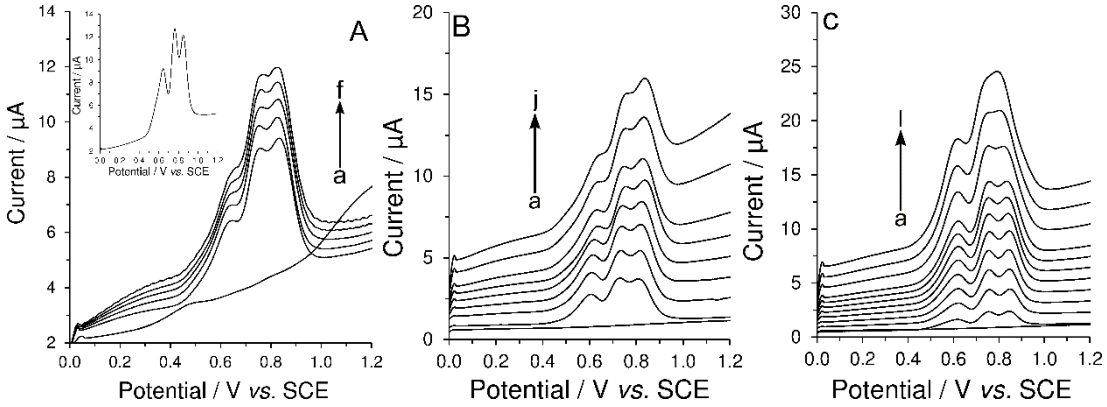

Figure 16. Voltammograms of 50 μM α-TOH, γ-TOH and δ-TOH at GCE in 99.9% MeCN containing 0.1 mol L^{-1} $LiClO_4$ at E_{step} = 1 – 15 mV, E_{ampl} = 25 mV and f = 20 Hz (A), f = 5 – 50 Hz, E_{step} = 2.5 mV and E_{ampl} = 25 mV (B), E_{ampl} = 5 – 80 mV, E_{step} =2.5 mV and f = 20 Hz, (C).

The peak height and width increased with a higher concentration of analyte. Hence, it was necessary to find out if the concentration ratio can distort the recognition of individual tocopherols. The obtained voltammograms showed that overlapping of γ-TOH and δ-TOH anodic peaks is evident if one of these forms has a higher concentration than the other, while α-TOH and δ-TOH peaks increased with a higher concentration of both forms. Overlapping was suppressed in case when concentrations of all three forms were mutually increased (Fig. 4, Publication 6).

Furthermore, a comparison of four different methods for evaluation of current signals, obtained during the voltammetric analysis of tocopherols, has been carried out. The main analytical parameters such as, limit of detection, limit of quantification, linear range and the correlation coefficient are shown in Table 2, Publication 6. The precision, defined as the result of variability found for 10 repeated measurements, was calculated for the tocopherols mixture (50 μmol L^{-1} of each form) and presented as RSD. RSD values lower than 5% were obtained for evaluation using linear baseline, zero base, and deconvolution of signals, contrary to polynomial baseline evaluation with RSD over 5%. The accuracy was presented as recovery values in percentage. Acceptable accuracy values ranging from 72% to 119% were attained only for the deconvolution method, (see Table 3, Publication 6). Compared to previously reported scientific papers [66-71], simultaneous voltammetric detection of tocopherols at GCE in the pure MeCN using SWV was simplified, and significantly higher peak separations were

achieved. However, it is important to note that evaluation of voltammetric signals is not possible without the use of the mathematical deconvolution of overlapping peaks. The current electrochemical study includes detailed and relevant information for the possible development of a direct voltammetric method for the simultaneous determination of α-TOH, γ-TOH, and δ-TOH in foodstuffs or pharmaceutical preparations.

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Electrochemical Behaviour of Tocopherols: Possibilities of Their Simultaneous Voltammetric Detection

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Abstract: An electroanalytical study for possible simultaneous detection of three naturally occurring isomers of vitamin E (α, γ, and δ-tocopherol) was performed. This research includes several optimization steps, such as selection of electrode material, composition of working medium, selection of electrochemical technique, and parameters of square-wave voltammetry (SWV), to reach a well-defined recognition of peaks. A glassy carbon electrode, 99.9% acetonitrile containing 0.1 mol L^{-1} lithium perchlorate, SWV at the potential step of 1 mV, potential amplitude of 25 mV, and frequency of 25 Hz were decided as the most suitable working conditions. Nevertheless, the corresponding anodic peaks were not sufficiently separated due to their overlapping. Thus, four standard evaluation methods (polynomial or linear baseline, zero base, and deconvolution) were compared, and the last-mentioned method was chosen as optimum. Similar linear ranges from 3.0×10^{-6} to 1.0×10^{-5} mol L^{-1} were obtained for α, γ, and δ-tocopherol, characterized by determination coefficient of 0.998, 0.985, and 0.994, quantification limits of 11.28, 2.70, and 3.67×10^{-6} mol L^{-1} and detection limits of 3.72, 0.89, and 1.21×10^{-6} mol L^{-1}, respectively. A recovery from 72.0 to 128.5% for different concetration ratios of tocopherols has been achieved. This recovery range is in the accordance with values reported for liquid chromatography.

Keywords: evaluation methods; glassy carbon electrode; simultaneous detection; square wave voltammetry; tocopherols

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1. Introduction

Tocopherols as major forms of vitamin E (VE) are considered as naturally occurring lipophilic phenolic antioxidants, especially in vegetable oils such as corn, soybean, cottonseed, and sesame [1]. Often, they are found in pharmaceuticals and cosmetics products due to their biological activity [2–4], which consists of polyunsaturated fatty acids and their esters, renowned for inhibiting free-radical chain auto-oxidation [5–9]. The dosage of tocopherols for dietary intake is important because they protect the phospholipid bilayer from free radicals. It has been suggested that the biggest role of VE in the human body is the protection from different aggressive processes such as cancer, especially hepatocellular carcinoma (HCC), and prevention of the progression of pre-cancerous lesions, cataracts, and different cardiovascular diseases. Its deficiency may contribute to the most common circulatory disorders [3,10–13], such as fertility disorder, Alzheimer's disease, etc. [14].

From the chemical point of view, it is known that VE comprises two main groups, namely tocopherols and tocotrienols. They are usually assorted based on their numbers of methyl groups on the chromanol ring, which has an extended alkyl (phytyl) chain in the

2-position responsible for their lipophilic properties [5,15,16]. Classification of α-, β-, γ-, and δ-forms of tocopherols is based on the degree of methylation.

Common samples with natural origin are a mixture of these forms. It is worth mentioning that β-tocopherol presenting negligible content [17] is not analytically important, while pharmaceutical and cosmetic products are rich in α-tocopherol (α-TOH) and α-tocopheryl acetate (α-TOAc) content. Generally, high-performance liquid chromatography (HPLC) coupled with ultraviolet or fluorescence detector is used as a standard analytical method for the analysis of tocopherols isomers [4,18]. The sample preparation, including saponification with the presence of an antioxidant and subsequent extraction into an organic solvent can cause significant losses of vitamin E [12,19,20].

Hence, a voltammetric approach in the analysis of tocopherols is proposed as an interesting alternative due to easier sampling, less time-consuming analysis, and low operating costs [19]. As fat-soluble antioxidants (occurring in reduced forms), they are considered as electroactive compounds [21,22], which can be anodically oxidized at various working electrodes (glassy carbon [23], platinum electrode [24], and glassy carbon paste electrode [12] in pure organic solvents or in their aqueous mixtures). Voltammetric determination of VE in pure aqueous solution is possible to be performed after their adsorptive/extractive accumulation onto the surface of the working electrode (medium-exchange procedure) [12].

Several studies have been reported on the simultaneous voltammetric determination of tocopherols. In all cases, an overlapping of their corresponding anodic peaks was observed [21–26]. In 1973, an initial electrochemical study of individual tocopherols was reported by McBride and Evans [25]. In 1992, a scientific group around Clough began to deal with their simultaneous electrochemical detection [21]. Unfortunately, no improvement in their separation using voltammetric approach has been reached. Later, Coatanea performed an analysis of tocopherols mixtures at platinum microelectrode using differential pulse voltammetry (DPV). However, a broad oxidation peak was observed due to the influence of matrix (edible oils) [26]. Later, Diaz utilized various voltammetric techniques combined with chemometric methods with the aim to resolve the overlapping voltammetric signals of individual tocopherols [23].

In comparison with the above-mentioned studies, the effects of organic solvent, water content, and the presence of surfactant on overlapping signals of tocopherols have not been studied yet. These and many other factors, such as working electrode materials, surface modifications, selection of voltammetric techniques, and parameters of voltammetric techniques are investigated in the present study. Moreover, different ways of performing the evaluation of peaks in voltammetric analysis of tocopherols are compared. The obtained results suggest that an approach utilizing a deconvolution of signals seems to be viable for the simultaneous determination of tocopherols in various food samples.

2. Materials and Methods

2.1. Reagents and Chemicals

The lithium perchlorate, 99.99% surfactants such as Triton X-100, 98% sodium dodecyl sulfate (SDS), 99–102.0% cetylpyridinium chloride (CPC), 99% cetyltrimethylammonium bromide (CTAB), and analytical standards of (+)-α-tocopherol, (+)-γ-tocopherol and (+)-δ-tocopherol were purchased from Merck KGaA (Darmstadt, Germany). Organic solvents, namely 99.9% acetonitrile (MeCN), 96% ethanol (EtOH), 99.8% dimethylformamide (DMF), and 99.9% propylene carbonate (CP), were obtained from Lach-Ner, s.r.o. (Neratovice, Czech Republic). Multi-walled carbon nanotubes (MWCNTs) of diameter 10–30 nm, length 5–15 μm, and specific surface area 40–300 m^2 g^{-1} were from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China). An alumina powder with particle size of 1.0 μm was obtained from Metrohm Česká republika s.r.o. (Prague, Czech Republic). Ultrapure water with resistivity lower than 18.3 MΩ cm was prepared by Milli-Q® system from Merck Millipore (Burlington, NJ, USA).

2.2. Instrumentation

Different electrodes from Metrohm with the same diameter (3 mm) have been used for this study, namely glassy carbon electrode (GCE), gold disk electrode (AuE), platinum disk electrode (PtE), and boron-doped diamond electrode (BDDE). In addition, GCE was modified with a thin layer of MWCNTs (GCE/MWCNTs). Pyrolytic graphite electrodes (PGE) prepared at the Institute of Biophysics of the Czech Academy of Sciences were also tested. All voltammetric measurements were carried out through a common three-electrode system consisting of one of the tested working electrodes, saturated calomel electrode (SCE) with 99.9% MeCN containing 0.1 mol L^{-1} $LiClO_4$ as a salt bridge (reference) and platinum wire (auxiliary electrode) in a conventional electrochemical glass cell. The electrode system was connected to a potentiostat/galvanostat PGSTAT101 operated with Nova 1.11 software from Metrohm. This software enables evaluation of peak-shaped electrochemical signals with several evaluation methods, such as linear and polynomial baseline and reading of peak current from zero base. Moreover, a deconvolution method was performed by OriginPRO® software version 9.0 from OriginLab Corporation (Northampton, MA, USA). The instrumentation used together with evaluation methods are shown in Figure 1.

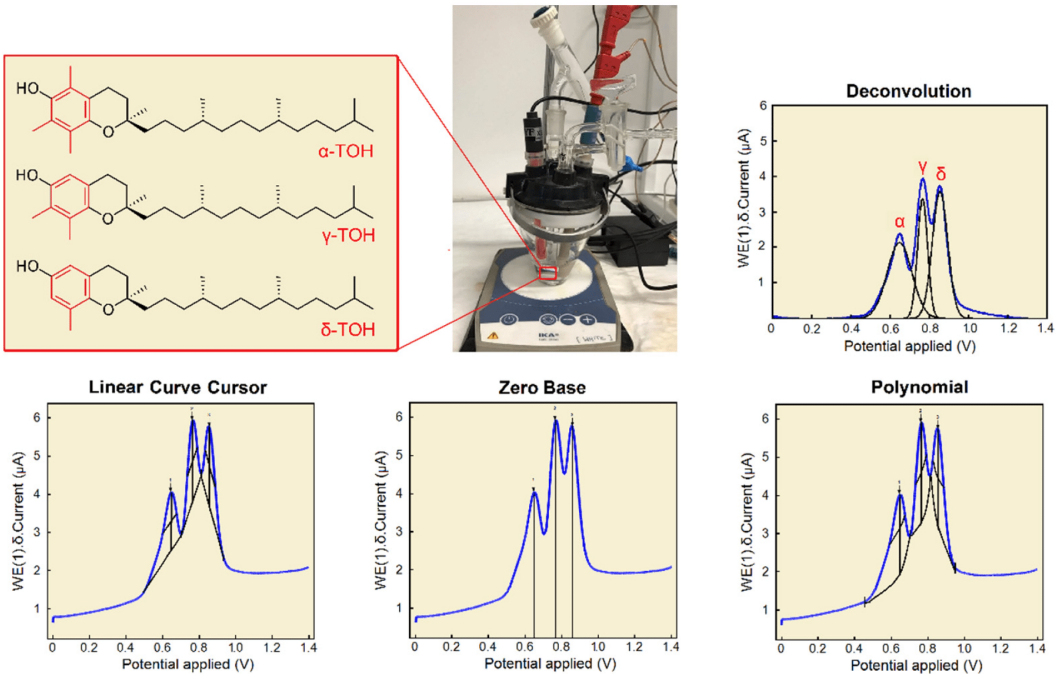

Figure 1. Instrumentation and principles of evaluation methods for simultaneous electrochemical detection of different tocopherols.

2.3. Pretreatment and Preparation of Working Electrodes

Surfaces of solid electrodes were renovated on a polishing pad with water suspension of Al_2O_3 powder (particle size 1.0 μm) for 30 s. Then, the polished surfaces were washed by stream of pure ethanol and dried using a pulp paper. To prepare GCE/MWCNTs, an aliquot of 10 μL MWCNTs dispersion in DMF (2 mg mL^{-1}) was applied on pre-treated GCE surface and left to dry at laboratory conditions for one hour [27].

2.4. Procedures

Linear sweep voltammetry (LSV) was applied as the initial voltammetric technique for a mixture containing 50 μmol L^{-1} of each tocopherol at GCE with a potential range from 0

to +1.0 V, potential step (E_{step}) 2.5 mV, and different scan rates (ν). Due to low sensitivity and high overlapping of peaks in LSV, two more useful pulse voltammetric techniques were tested. Differential pulse voltammetry (DPV) of 10 µmol L^{-1} tocopherols at GCE with a potential range from 0 to +1.2 V, different potential step (E_{step}), potential amplitudes (E_{ampl}), and scan rates were compared with square-wave voltammetry (SWV).

The last-mentioned voltammetric technique was investigated for simultaneous electrochemical detection of 1–900 µmol L^{-1} tocopherols at GCE within a potential range from 0 to +1.2 V using different E_{step}, E_{ampl}, and frequencies (f) with the intent to obtain optimum peaks separation. Each voltammetric measurement was repeated minimally three times. All additional changes in working parameters are shown in the legends of the corresponding figures.

3. Results and Discussion

3.1. Effect of Electrode Material

Tocopherols represent phenolic compounds differing in the number of methyl groups in the chromanol ring. An electrochemical oxidation of chromanol ring can be carried out at different electrode materials in pure MeCN, as shown in Figure 2. GCE provided the most suitable peaks separation, namely α-TOH at 0.645 V, γ-TOH at 0.752 V, and δ-TOH at 0.857 V, due to slower charge transfer at carbon-based over metal-based electrodes [28]. Hence, other carbon-based electrode materials were compared with GCE; however, no improvement was observed.

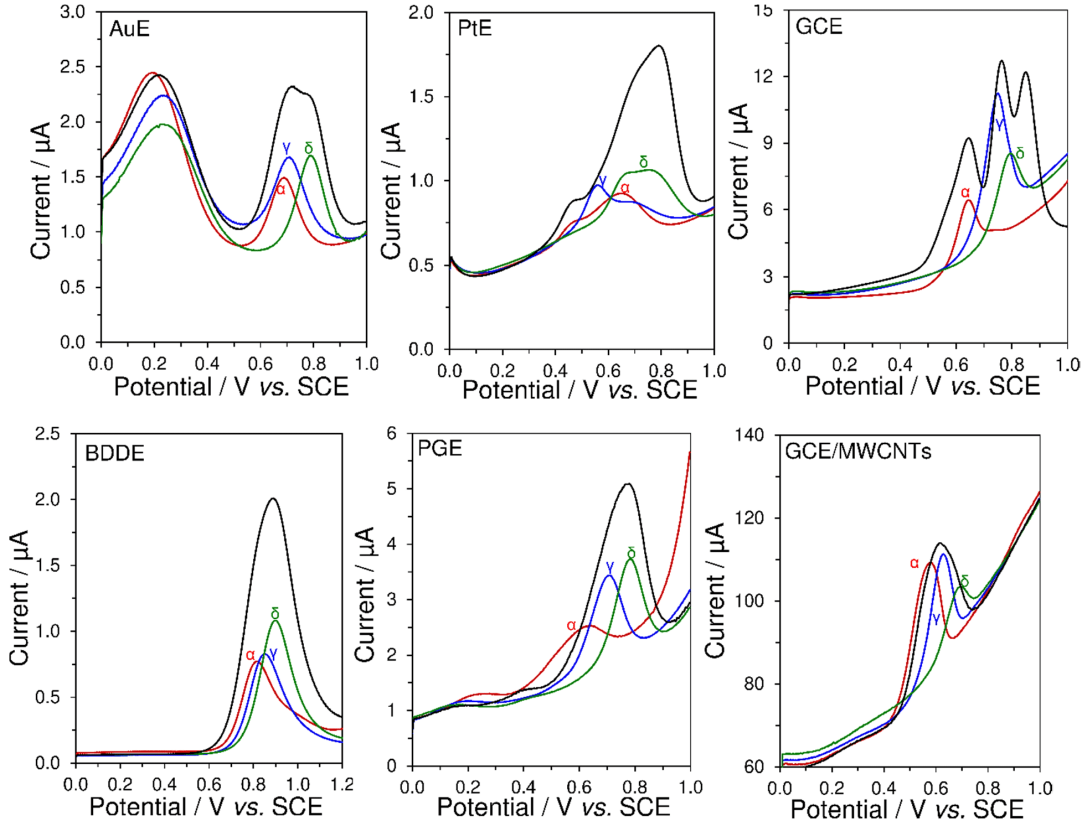

Figure 2. SWV of 50 µmol L^{-1} α-TOH, γ-TOH, and δ-TOH at different tested working electrodes. The corresponding experiments were performed in 99.9% MeCN containing 0.1 mol L^{-1} $LiClO_4$ at E_{step} = 5 mV, E_{ampl} = 25 mV, and f = 20 Hz.

In 2006, Richard et al. explained the electrochemical behaviour of all tocopherol forms and proposed ECE mechanism [5]. Furthermore, they observed a dependence of their anodic peak potentials on numbers of methyl groups on the chromanol ring [5]. Fundamentally, methyl groups have a positive inductive effect, and therefore they release electron density into the benzene ring since these methyl groups, being sp3 hybridized, are less electronegative than the sp2 hybridized aromatic carbons [5]. Accordingly, investigated tocopherols are anodically oxidized in the following order: α-TOH (three), β-TOH (two), γ-TOH (two), and δ-TOH (one) methyl groups [24].

3.2. Effect of Working Medium

Since tocopherols and tocotrienols are lipophilic compounds, they are soluble in polar organic solvents (DMF, PC, EtOH, and MeCN). The above-mentioned solvents and their aqueous/organic mixtures containing 0.1 mol L^{-1} $LiClO_4$ were examined to find a compatible working medium in which the anodic peaks will be well-defined. According to the results shown in Figure 3A, two broad anodic peaks with low current responses were observed in DMF and PC. In contrast, three overlapping peaks were observed in the case of using EtOH and MeCN, where the latter represents the best choice due for high resolution of individual peaks. Figure 3B points to the fact that the presence of water in organic solvents caused a shift of anodic peaks to more negative potentials and significantly decreased their current responses. This phenomenon can be attributed to easier deprotonation of hydroxyl group in aqueous environment than in pure organic solvents [29].

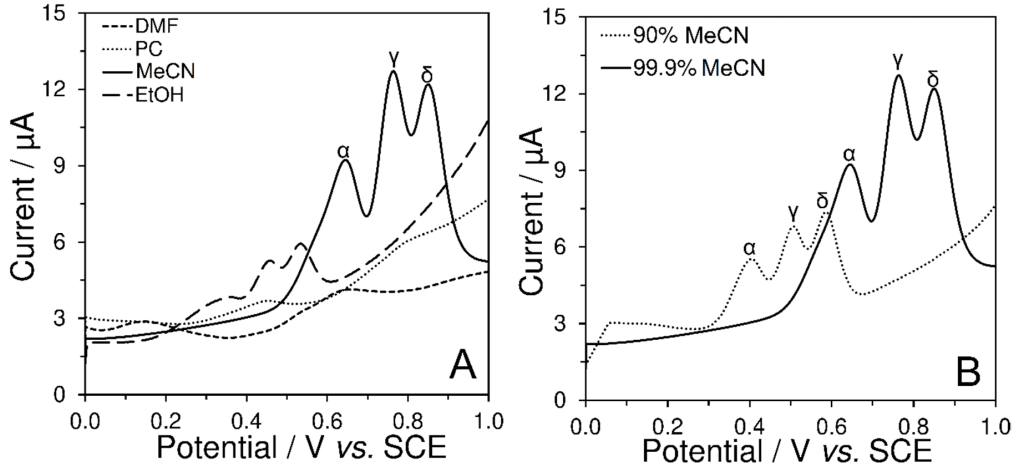

Figure 3. SWVs of 50 µmol L^{-1} tocopherols obtained at GCE in different organic solvents (**A**) and different content of water (**B**) containing 0.1 mol L^{-1} $LiClO_4$ at E_{step} = 1 mV, E_{ampl} = 25 mV and f = 20 Hz, the potential was scanned from 0.0 to 1.0 V.

Generally, it is known that the presence of surfactants increases the solubility of non-polar substances in polar organic solvents and facilitates the charge transfer at the working electrode due to their ability to reduce surface tension. For that reason, the effect of different contents (0.1, 0.01, and 0.001 mol L^{-1}) of nonionic (Triton X-100), anionic (SDS), and cationic (CPC and CTAB) surfactants on peaks separation was investigated. It was observed that presence of surfactant did not improve overlapping of adjacent signals and rather caused a deterioration in peak current responses (see Figure S1 in the Supplementary Materials). Finally, the effect of electrode material and composition of working medium on anodic peak potentials of tocopherols are included in Table 1.

Table 1. Comparison of electrode material and working medium on anodic potential peaks.

Electrode	Method	Working Medium	Anodic Peak Potential (V)			Reference
			α-TOH	γ-TOH	δ-TOH	
GCPE	ExSV-SWV	H_2O and acetone (40:60)	0.475	0.520	0.540	[12]
GCE	DPV	Benzene and MeOH (1:2)	0.644	0.714	0.804	[19]
GCE	DPV	Hexane and EtOH (40:60)	0.475	0.550	0.665	[23]
PtE	DPV	Glacial acetic acid	0.692	0.755	0.889	[24]
GCE	SWV	MeCN	0.645	0.753	0.857	This work

Notes: GCPE; glassy carbon paste electrode and ExSV; extractive stripping voltammetry.

3.3. Selection of Voltammetric Technique

A comparison between LSV, DPV, and SWV in simultaneous electrochemical detection of tocopherols at scan rate of 25 mV s^{-1} is shown in Figure S2. Despite the low background current for LSV, it was almost impossible to distinguish the individual oxidation signals. The peak separation was similar in both other pulse voltammetric techniques. SWV was selected and preferred for further optimisation steps due to higher peak current responses.

3.4. Optimisation of Square-Wave Voltammetry

Herein, it was observed that finding the optimum working parameters for peak separation is crucial. These parameters included potential step (E_{step}), frequency (f), and potential amplitude (E_{ampl}). For SWV, it is known that the setting of E_{step} and f defines the final scan rate (v), which may have the main effect on the peak separation. The whole optimisation of SWV parameters is presented in Figure S3. It was found that the potential step has the main effect on peak separation when the values higher than 1 mV caused significant peaks overlapping. The other two parameters did not have any effect on peak separation. The peak current responses only increased with higher values of these two parameters. For E_{ampl} values higher than 25 mV, frequency 25 Hz, and potential step 1 mV, there was no effect found on peak separation. Therefore, these values were chosen as optimum for the construction of calibration curves for different concentration ratios.

3.5. Effect Concentration Ratios and Evaluation Methods on Analytical Performance

In general, peak height and width increase with a higher concentration of analyte. It follows that concentration ratio can significantly distort the recognition of individual tocopherols. Hence, it was necessary to find out if investigated tocopherol compounds overlap within the scope of calibration measurement, which could affect the main analytical characteristics, namely linear range, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy.

Figure 4A–C represents calibration voltammograms (20–200 µmol L^{-1}) of a selected form of tocopherols, whereas two others had a constant concentration of 50 µmol L^{-1}. Additionally, calibration measurements were performed to find out mutual influencing, where the concentration of two forms was simultaneously increased, as shown in Figure 4D–F. Resultant voltammograms showed that overlapping of γ-TOH and δ-TOH anodic peaks is more evident if one of these forms has a higher concentration than the other, whereas the mutual recognition of α-TOH and δ-TOH peaks increased with a higher concentration of both forms. Surprisingly, the overlapping was suppressed if concentrations of all three forms (3–300 µmol L^{-1}) were mutually increased (see Figure 4G).

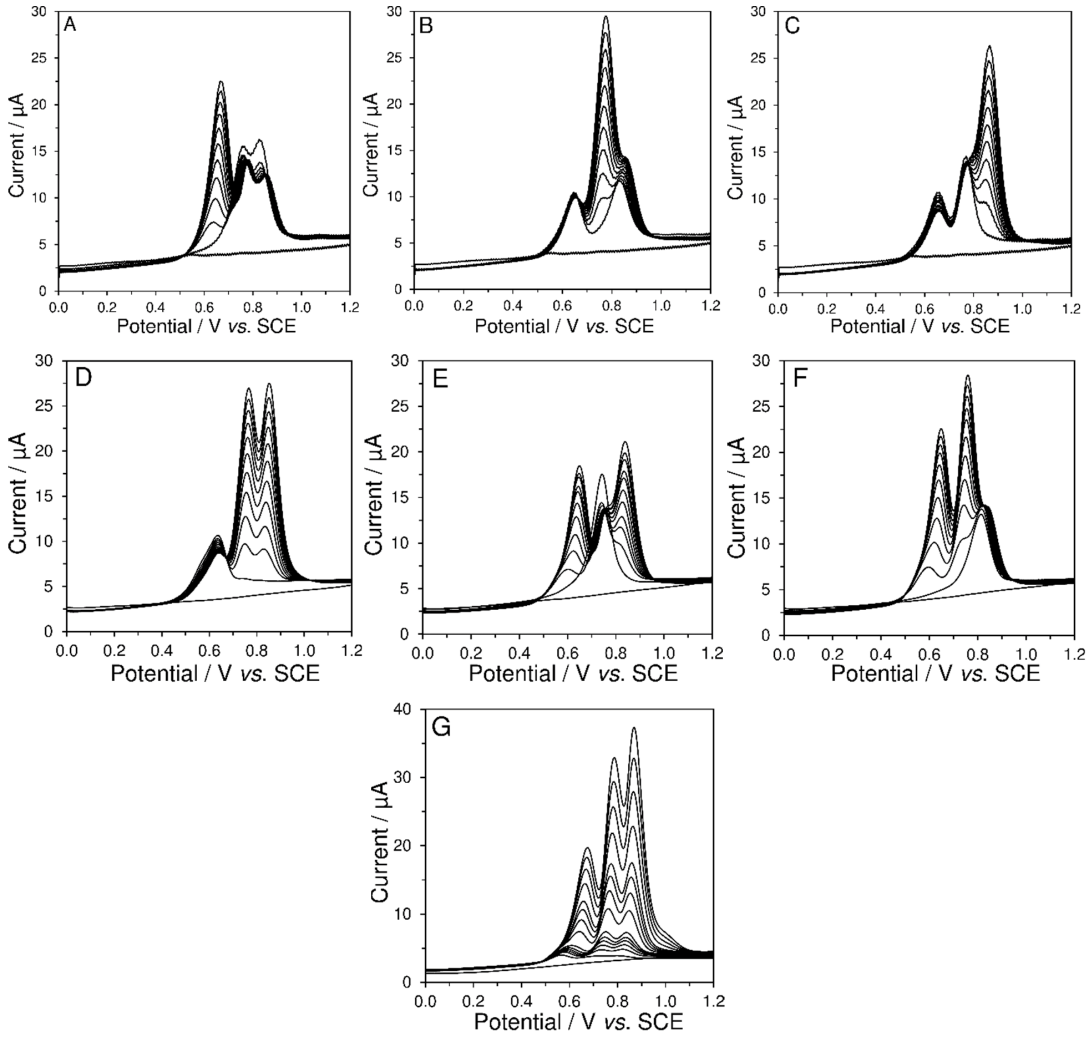

Figure 4. SWVs of 20–200 µmol L^{-1} of one tocopherol form when two others had a constant concentration of 50 µmol L^{-1} (varying α concentrations); (**A**), varying γ concentrations; (**B**) and varying δ; (**C**). SWVs of 20–200 µmol L^{-1} of two tocopherols when one had a constant concentration of 50 µmol L^{-1} (varying γ and δ concentrations); (**D**) varying α and δ concentrations; (**E**) and varying α and γ concentrations;(**F**). Simultaneous voltametric detection of 3–300 µmol L^{-1} of all tocopherol forms (**G**). All voltammograms were obtained at GCE in pure MeCN containing 0.1 mol L^{-1} $LiClO_4$, E_{step} = 1 mV, E_{ampl} = 25 mV, and f = 25 Hz. The potential was scanned from 0.0 to 1.2 V.

Overview of all analytical parameters obtained during simultaneous voltammetric detection of investigated tocopherols is presented in Table 2. To achieve the coefficient of determination (R^2) in the accepted value higher than 0.9900, calibration ranges from 3 to 100 µmol L^{-1} for all tocopherol forms can be taken into account. High positive y-intercept (q) values and low sensitivity (k) of appropriate equations of the calibration curves (q > 0.2 µA and k < 0.09 µA mol^{-1} L) were obtained when polynomial, linear curve cursor, and zero base evaluation methods were used. On the other hand, if the deconvolution evaluation method is preferred, values of q < 0.1 µA and k > 1.0 µA mol^{-1} L will be obtained. Therefore, the deconvolution should be preferred. From all LOQ values shown in Table 2, it seems that the present study could help to develop a simple voltammetric

method for simultaneous determination of tocopherols in margarines [12], edible vegetable oils [17], nuts, and seeds [30].

Table 2. All analytical parameters with four different methods for evaluation of signals.

	Polynomial (μmol L^{-1})			Linear Curve Cursor (μmol L^{-1})			Zero Base (μmol L^{-1})			Deconvolution (μmol L^{-1})		
	α-TOH	γ-TOH	δ-TOH	α-TOH	γ-TOH	δ-TOH	α-TOH	γ-TOH	δ-TOH	α-TOH	γ-TOH	δ-TOH
LOD	2.30	0.42	0.60	2.09	0.28	0.49	3.39	1.21	1.53	3.72	0.89	1.21
LOQ	6.99	1.30	1.83	6.34	0.85	1.50	10.27	3.68	4.65	11.28	2.70	3.67
Linear range	3–100	3–100	3–100	3–100	3–100	3–100	3–100	3–100	3–200	3–100	3–100	3–100
R^2	0.996	0.994	0.993	0.997	0.991	0.996	0.995	0.982	0.990	0.998	0.985	0.994

From the analytical point of view, determination of precision and accuracy is necessary to obtain relevant results. The precision, defined as the result of variability found with repeated measurements, was calculated for tocopherols mixture (50 μmol L^{-1} of each form) measured ten times and presented as relative standard deviation (RSD). Values of 5.48%, 4.88%, and 7.87% RSD for polynomial baseline; 4.78%, 0.11%, and 0.20% RSD linear baseline; 2.01%, 0.15%, and 0.05% RSD for zero base; and 1.66%, 2.75%, and 1.94% RSD for deconvolution were calculated. For a significance level of 5%, it is evident that only the polynomial evaluation method is not satisfactory for analytical purposes.

Table 3 shows the comparison of evaluation methods used in determination of accuracy for different model samples having the randomly chosen contents of investigated tocopherols. In this case, the accuracy is presented as recovery values (%). Acceptable accuracy values ranging from 72% to 119% can be accepted only for the deconvolution method, which is comparable with recovery ranges from 71% to 116% and from 77% to 85% recommended for tocopherols determination in nuts and virgin olive oils using QuEChERS-liquid chromatography and reversed-phase HPLC with spectrophotometric detection, respectively [30].

Table 3. Comparison evaluation methods in determination of accuracy presented as recovery values.

Concetration Ratios (μmol L^{-1})	Polynomial (%)			Linear Curve Cursor (%)			Zero Base (%)			Deconvolution (%)		
	α-TOH	γ-TOH	δ-TOH	α-TOH	γ-TOH	δ-TOH	α-TOH	γ-TOH	δ-TOH	α-TOH	γ-TOH	δ-TOH
30:60:45	58.33	48.19	46.24	45.78	49.07	48.08	54.54	76.18	101.60	105.80	102.2	105.50
45:75:60	44.10	58.90	35.56	47.49	55.75	43.20	53.36	74.49	91.35	85.95	97.75	99.07
60:90:75	47.70	54.70	33.97	49.12	58.31	40.63	54.90	72.81	86.07	80.00	97.84	99.65
45:60:30	26.38	65.28	52.15	30.94	70.77	55.52	19.52	62.80	82.41	81.34	95.18	118.50
60:75:45	34.77	69.99	51.65	33.10	70.19	54.83	31.35	65.21	85.32	90.81	102.70	111.50
75:90:60	39.80	66.11	70.97	32.86	69.92	61.25	40.30	65.30	86.73	79.52	93.64	106.90
60:30:45	41.91	55.82	83.20	43.29	28.60	88.21	24.57	68.49	67.75	73.49	111.70	101.40
75:45:60	37.89	45.90	68.47	41.36	36.46	75.64	32.34	69.62	71.37	73.86	109.70	71.37
90:60:75	38.84	44.02	61.62	40.54	39.66	65.31	36.01	67.25	69.68	72.03	100.50	95.57

Obtained results showed that it is not yet possible to perfectly split the anodic peaks of α-TOH, γ-TOH, and δ-TOH differing only in the number of methyl groups in chromanol ring. Nevertheless, it was found that the type of working electrode material, composition of detection medium, set parameters of electrochemical technique, and selection of evaluation method have a significant effect in their sufficient resolution so that the individual signals can be quantified. The distance around 100 mV of individual oxidation peaks (0.645 V for α-TOH, 0.753 V for γ-TOH and 0.857 for δ-TOH) has been achieved at optimum working conditions.

4. Conclusions

In this contribution, an electrochemical behaviour of naturally occurring tocopherols (α-TOH, γ-TOH, and δ-TOH) was investigated to find out optimum working conditions for their simultaneous voltammetric detection. In comparison with the previously reported scientific papers [12,19,23,24], simultaneous voltammetric detection of investigated

tocopherols was simplified and significantly higher peaks separation was reached using SWV in pure MeCN at GCE and necessary low potential step of 1 mV. However, it seems that analysis of voltammetric signals is not possible without the use of mathematic decomposition (deconvolution) of overlapping peaks because standard evaluation methods included in Nova 1.11 software (polynomial and linear baseline and zero base reading) are not sufficient. Due to high precision and comparable accuracy with standard HPLC methods, it can be assumed that the present electrochemical study includes appropriate knowledge and instructions useful for the development of a direct voltammetric method for simultaneous determination of α-TOH, γ-TOH, and δ-TOH in different foodstuffs.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11178095/s1, Figure S1: Voltammograms of 50 μmol L^{-1} α-TOH, γ-TOH, and δ-TOH at GCE in pure MeCN containing 0.1 mol L^{-1} $LiClO_4$ and 0.001 mol L^{-1} CPC, SDS, or Triton X-100 at E_{step} = 1 mV, E_{ampl} = 25 mV, and f = 20 Hz. Figure S2: Comparison of voltammetric techniques in simultaneous determination of 50 μmol L^{-1} α-TOH, γ-TOH, and δ-TOH for LSV and 10 μmol L^{-1} these tocopherols for DPV and SWV. All measurements were carried out at GCE in pure MeCN containing 0.1 mol L^{-1} $LiClO_4$. Conditions of LSV: E_{step} = 2.5 mV and ν = 25 mV s^{-1}; DPV: E_{step} = 2.5 mV, E_{ampl} = 50 mV, and ν = 25 mV s^{-1}; and SWV: E_{step} = 1 mV, E_{ampl} = 25 mV, and f = 25 Hz. Figure S3: Voltammograms of 50 μmol L^{-1} α-TOH, γ-TOH, and δ-TOH at GCE in pure MeCN containing 0.1 mol L^{-1} $LiClO_4$ at (A) E_{ampl} = 5–80 mV, E_{step} = 2.5 mV, and f = 20 Hz; (B) f = 5–50 Hz, E_{step} = 2.5 mV, and E_{ampl} = 25 mV; and (C) E_{step} = 1–15 mV, E_{ampl} = 25 mV, and f = 20 Hz.

Author Contributions: Formal analysis, G.J., G.K.; methodology, L.K., R.M., M.S.; validation and author of manuscript, G.J.; final correction, R.M.; supervision, A.M.A. All authors have read and agreed to the published version of the manuscript.

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2.5. Voltammetric approaches for the determination of selected biologically active compounds

2.5.1 Voltammetric determination of nitrites in meat products after reaction with ranitidine

In this research, a completely new voltammetric method is proposed for the first time based on the cathodic reduction of the product 2-methyl-2H-furan-3-one, after a specific reaction of nitrites with ranitidine (RAN) in an acidic environment [110, 111]. GCPE covered with a thin film of electrochemically reduced graphene oxide and adsorbed SDBS was used for the determination of nitrite ions in 0.1 mol L^{-1} Britton-Robinson buffer at pH 2. Graphene oxide was electrochemically immobilized on GCPE using CV (20 cycles) in 0.1 mol L^{-1} phosphate buffer. Unmodified GCPE, GCPE modified with graphene oxide, graphene oxide and surfactant SDS, and graphene oxide and surfactant SDBS were compared. It was evident that the reduction peak at the GCPE modified with graphene oxide and SDBS provided higher current peak response, probably due to an electrostatic interaction between methylfuran cation and sulphate anion from anionic surfactant (Fig. 2, Publication 7). Therefore, this modification was chosen for further experiments. Surfactant content in 10 μL volume applied on the surface of GCPE/ERGO/SDBS was optimized using different concentration (0.1, 0.5, 1.0, 1.5, and 2.0 mmol L^{-1}). Concentration higher than 1.0 mmol L^{-1} did not influence the current peak reduction, shown in Fig. 17. Thus, the concentration of 1.0 mmol L^{-1} was taken as optimum.

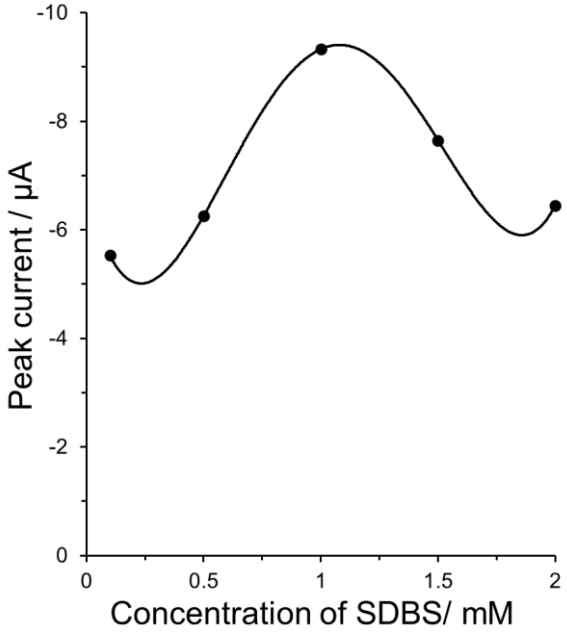

Figure 17. Effect of surfactant concentration (0.1, 0.5, 1.0, 1.5, and 2.0 mmol L^{-1}) on peak current obtained for mixture of 320 μmol L^{-1} ranitidine with 250 μmol L^{-1} nitrite after reaction time of 60 s in 0.1 mol L^{-1} BRB of pH 2.

The pH of working media solution had a key role on the conversion of nitrite ion to nitrosonium cation which reacts with ranitidine. Because the conversion occurs in acidic media, the pH could affect the current response of the cathodic peak. The obtained results shown that the maximum reduction current signal was at the pH 2. In higher pH values, the response was dramatically decreased and over pH 5 no signal was observed (Fig. 3A, Publication 7). The product (nitrosamines) of the reaction of RAN with nitrites in acidic medium was used for the determination of nitrites. Hence, the concentration of RAN within a range from 80 μmol L^{-1} to 540 μmol L^{-1} at constant content of nitrites of 250 μmol L^{-1} in 0.1 mol L^{-1} BRB pH 2 was optimized. A linear dependence of current response was found up to 320 μmol L^{-1} RAN, see Fig. 18. Consequently, the reaction time was optimized. After two minutes, the constant value of the reduction peak current has been achieved (Fig. 3B, Publication 7).

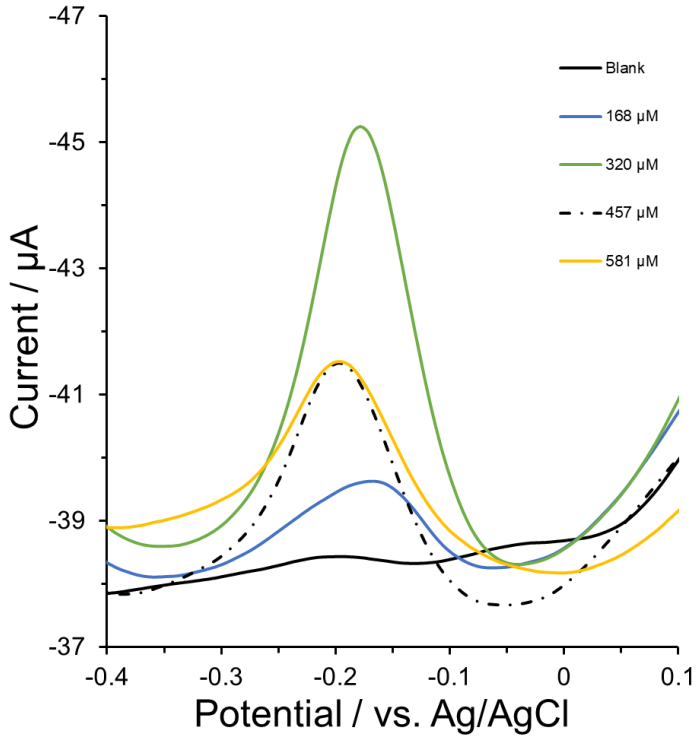

Figure 18. Voltammograms recorded on GCPE/ERGO/ SDBS within a range from 80 μmol L^{-1} to 540 μmol L^{-1} of ranitidine (250 μmol L^{-1} nitrite in 0.1 mol L^{-1} BRB pH 2) when each measurement was carried out after stirring at 400 rpm for 60 s.

In the optimized conditions, two linear ranges from 6.2 μmol L^{-1} to 125 μmol L^{-1} and from 150 μmol L^{-1} to 300 μmol L^{-1} nitrites were found, characterized by R^2 of 0.9991 and 0.9963, respectively, with a detection limit of 1.89 μmol L^{-1} nitrites. Repeatability of analysis of the proposed voltammetric method was determined as RSD of 3.8% using ten repeated measurements. Satisfactory accuracy (difference between determined and true analyte content) characterized with a recovery value of 95.4% has been found. Compared to the methods

mentioned above, one of the advantages is the elimination of multiple interferences because the previous voltammetric methods were based on anodic oxidation in contrast to the cathodic reduction (Ep = –0.2 V) used in this method, where interference of accompanying substances is not expected. The developed method has been successfully applied in the quantification of nitrites in some meat products such as beef sausages, chicken sausages, lunchmeat, and smoked meat where the results have been satisfactory and comparable to reference methods using Griess Reagent Kit (G-7921) (Table 2, Publication 7). Moreover, the results were compared with the maximum allowed content of nitrites (EU legislation: No 1129/2011) in meat samples. Chicken sausages were out of the allowed limit.

2.5.2 Comparison of amperometric biosensor with portable Raman spectrometer determination of ethanol in alcoholic drinks

In this work, an amperometric biosensor and a Mira-DS handheld Raman spectrometer were compared in determining the amount of EtOH in various alcoholic beverages such as rum, vodka, homemade plum brandy, white and red wines, plus two different types of beer. Both analytical approaches have been optimized and compared in the real samples analyses. Three different modifications of screen-printed carbon electrode (SPCE) were tested for higher current yields during NADH anodic oxidation. SPCEs covered by thin layer of reduced graphene oxide, MWCNTs or carbon ink with always 5% RhO_2 (w/w) has been compared in the electrochemical response of NAD^+/NADH redox couple (Fig. 19). Higher peak at a relatively low potential of +0.307 V was obtained at SPCE modified with reduced graphene.

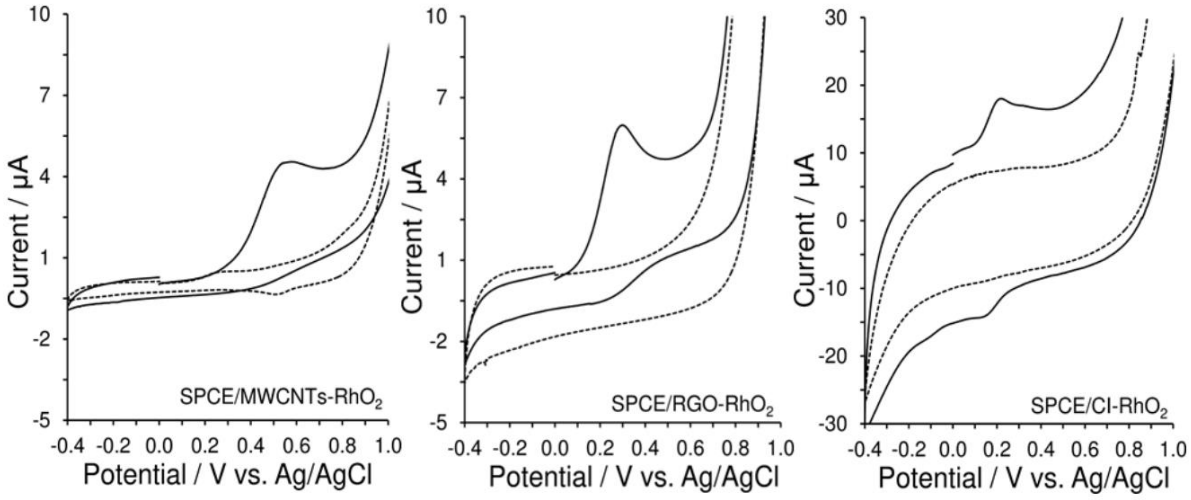

Figure 19. Cut-outs of cyclic voltammograms for 0 (dashed) and 0.5 mmol L^{-1} NADH solid lines) obtained at different transducers containing 5% RhO_2 (w/w). All measurements were performed in 0.1 mol L^{-1} PB of pH 8.5 at scan rate 10 mV s^{-1}.

Two different procedures for fabrication of amperometric ADH biosensors have been compared to choose the most suitable one. Firstly, the consecutive layers of the respective components (RGO, RhO_2, ADH, NAD^+, GTA, and Nafion®; in this order), and second as a composite of all components prepared as follows: in a vial, portions of 1.0 mg reduced single-layer graphene oxide, 50 µg rhodium dioxide, 8.75 mg alcohol dehydrogenase, 1.75 µg *β*-nicotinamide adenine dinucleotide sodium salt, 125 µL 1% glutaraldehyde and 375 µL 1% Nafion® were mixed and homogenized using an ultrasonic bath for 30 minutes. After two hours, a volume of 10 µL of the prepared dispersion was applied onto the surface of screen-printed carbon electrode and left for drying in the room temperature for 1 hour. Amperometric

responses of 40% EtOH (v/v) revealed that the fabrication of the biosensor using the composite procedure shown a stable peak current signal (Fig. 20).

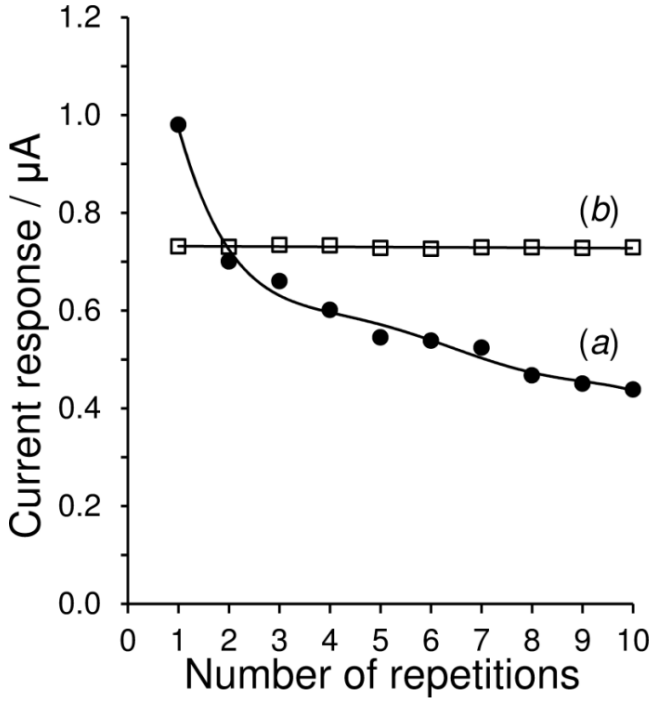

Figure 20. The comparison between ADH biosensor built up in successive layers (a) and ADH biosensor with a composite layer (b) in amperometric response stability of 40% EtOH (v/v). Experimental conditions: injection volume 100 μL, flow rate 1.6 mL min^{-1} of 0.1 mol L^{-1} PB (pH 8.5) and applied potential 0.2 V.

The experimental conditions in flow injection analysis were optimized and 0.1 mol L^{-1} phosphate buffer (pH 8.5) carrier solution, flow rate of 1.6 mL min^{-1}, the detection potential of +0.2 V, and 100 μL injection loop were utilized. Mira-DS was optimized using calibration series of EtOH 5-40% (v/v) and measured in the spectral range from 400 cm^{-1} to 2300 cm^{-1}. Laser output power was set to 50 mW, the other parameters were laser wavelength of 785.0 nm, resolution of 8 cm^{-1}, spot size of 0.04 mm, and raster size of 2.5 mm.

The analytical performance of developed biosensor was studied. However, no significant improvement in the sensitivity comparing to other ADH-based electrochemical biosensors has been achieved. Nevertheless, all analytical parameters indicate that the biosensor can be used in analysis of highly alcoholic drinks. Two linear ranges of 0.25-10% and 10-50% EtOH (v/v) have been obtained and no memory effect was observed (Fig. 2, Publication 8). If the developed biosensor is stored in a freezer at -21 °C, its lifetime would be minimally 7 days. The standard solutions of EtOH and MeOH with content of 20% were investigated for the maximum intensive bands in Raman spectrometer, which were found at 880 cm^{-1} for EtOH and

1020 cm^{-1} for MeOH (Fig. 3, Publication 8). A good linear relation between the height of the Raman band and EtOH content in the range of 5-40 % has been achieved.

The effect of possible interfering substances, such as isopropyl alcohol, n-propanol, ethyl acetate, and MeOH, in amperometric detection with electrochemical biosensor was investigated. It was observed that all tested compounds provided a false positive current signal upon injection (Fig. 4, Publication 8). The low selectivity of the proposed biosensor can be found in low substrate specificity of the enzyme used. On the other hand, it was shown that the ADH-based sensing can be applied in the analysis with high content of EtOH, where the presence of interfering substances is minimal. In case of the Raman spectrometer, it can identify and determine EtOH in the presence of the above-mentioned interfering substances without any problems.

Three real samples of highly alcoholic spirits (home-made plum brandy, commercially vodka, and white rum), were chosen for analysis. The results of determinations in real samples were fully comparable to the stated content of EtOH in vodka and rum (Table 2, Publication 8). However, both analytical methods encountered limits in the determination of EtOH in red wine and darker beers. There is still a need for further improvements in order to achieve better selectivity of the electrochemical biosensor. The comparison between the developed amperometric biosensor and Mira-DS portable Raman spectrometer showed that both tools cannot be used universally, due to some limitations in analysis of samples with complex matrices.

3. Conclusions

In this doctoral thesis, several new electrochemical approaches for the simultaneous determination of lipophilic vitamins in foodstuffs, pharmaceutical preparations, and cosmetic products have been presented. In the first research, the detailed data about the preparation and the possibilities of (bulk-)modified carbon paste electrodes with surfactants were of special interest. It was found that nonaqueous carbon paste electrode depends on the type of carbon material and the amount of non-electroactive surfactant. Acetonitrile containing $LiClO_4$ was found as the most suitable working medium (low background current and wide potential range). Several selected biological compounds analysed with the developed CPE/SDS were compared with commercial GCE, where the results were in a good agreement between both working electrodes. The modified CPE with surfactant offers wide application, in pharmaceutical and food analysis, especially for nonpolar electroactive compounds.

A new, simple, and rapid electroanalytical method for simultaneous determination of retinyl acetate (or retinyl palmitate) and alpha-tocopheryl acetate in cosmetic products was proposed and developed for the first time. The method is based on direct anodic oxidation of analytes at glassy carbon electrode in acetone containing $LiClO_4$. Complicated steps for samples preparation (saponification and extraction into organic solvent), were avoided due to direct dissolution of samples in the supporting electrolyte, unlike in HPLC. It offers satisfactory detection capabilities for routine analysis of cosmetic products and related samples. The results of several samples analysed by the developed method shown statistically identical values. In the future the electroanalytical approach could potentially replace the time-consuming chromatographic methods.

Extractive stripping voltammetry of vitamins at a glassy carbon paste electrode was proposed for the analysis of cow's milk and cream. The procedure is based on two steps: the extractive accumulation of milk fat globules into a glassy carbon paste electrode containing lipophilic binder and transfer of the electrode to electrochemical cell for subsequent detection by square-wave voltammetry in Britton-Robinson buffer (pH 4). The obtained results show that the vitamin A (carotenoids and retinoids), especially all-*trans*-retinol, dominates the milk fat globules. However, the results suggest that direct extraction of lipophilic vitamins (mainly all-*trans*-retinol) from continuously stirred milk and cream samples, and subsequent voltammetric detection could only be used for semi-quantitative determination of milk fat, whereas the individual forms were not possible to distinguish due to overlapping of signals.

A novel approach for the voltammetric determination of β-carotene has been presented. The method is based on anodic oxidation of β-carotene in pure acetone containing $LiClO_4$ at gold electrode using square-wave voltammetry. Compared to the already reported methods, several improvements were attained due to a new sample preparation using acetone (solvent not toxic like previously used chlorinated hydrocarbons) providing wider linear range and lower limit of detection. Moreover, the same method is suitable in food and pharmaceutical analysis. The results from analysis of raw carrots, sweet potatoes, and nutritional capsules were in a good agreement with the reference spectrophotometric assay. The method could find its application in food and pharmaceutical industry laboratories.

The simultaneous voltammetric determination of vitamin E and K in food supplements at glassy carbon electrode using square-wave adsorptive stripping voltammetry was developed. The presented method was suitable for determining the sum of vitamin E (all tocopherol forms) and vitamin K (phylloquinone). The procedure was based on ex situ adsorptive accumulation of the vitamins onto surface of glassy carbon electrode in acetonitrile-water (ratio 1:1 v/v), followed by the transfer of the working electrode to the electrochemical cell with HNO_3 + KCl supporting electrolyte (pH 2.08) for the detection by square wave voltammetry. Short linear calibration ranges were achieved due to the limited size of the working electrode. However, the main advantages of the method are based on the ex-situ accumulation, where the sample preparation consists of the dissolving the sample direct in the accumulation medium, which minimizes interference. Two food supplements were analysed, and the obtained results were comparable with reference HPLC method. The method could find its application in clinical analysis because the contents of the vitamin E and K in human plasma are in the range from 12 $\mu mol\ L^{-1}$ to 30 $\mu mol\ L^{-1}$ and from 0.4 $\mu mol\ L^{-1}$ to 7.1 $\mu mol\ L^{-1}$, respectively.

Possibilities for simultaneous voltammetric detection of tocopherols was investigated in detail, and the optimum parameters were found. In the comparison with the reported scientific papers, the simultaneous voltammetric detection of tocopherols was simplified and well-defined peaks of tocopherols were obtained at GCE in pure MeCN using SWV with potential step of 1 mV. However, the analysis of voltammetric signals is not possible without a deconvolution process for the overlapped signals. Thus, the present electrochemical study includes appropriate information and instructions for the development of the voltammetric approach in simultaneous determination of α-TOH, γ-TOH and δ-TOH in foodstuffs.

Monitoring the content of nitrites in meat products was another subject in focus. The approach is based on cathodic reduction of electroactive product formed after chemical reaction of ranitidine and nitrosonium ion, originated from nitrites. The indirect determination of nitrites

at glassy carbon paste electrode covered with a thin film of electrochemically reduced graphene oxide with presence of adsorbed dodecyl benzene sulfonate in Britton-Robinson buffer (pH 2) was successfully carried out. The analysis of several meat products shown statistically comparable results with commercially available spectrophotometric assay (Griess Reagent Kit G-7921). Prospects seem to be in the working electrode proposed, which could simply be converted to a planar configuration and used as disposable screen-printed sensor.

The immobilization of alcohol dehydrogenase into graphene-based composite was used to set up a simple bioanalytical device suitable for amperometric detection of ethanol in highly alcoholic spirits and white wines using flow injection analysis. However, there is still a need for further improvements in order to achieve better selectivity and simplify the preparation steps of biosensor.

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5. List of publications

5.1 Publications in impacted journals

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2. Jashari, G., Sýs, M., Mikysek, T., Metelka, R., Švancara, I. Electrochemical behaviour of *β*-Carotene at different working electrodes. In XL. Moderní elektrochemické metody : sborník přednášek. Ústí nad Labem: BEST Servis, 2021. s. 98-101 s. ISBN 978-80-905221-8-3.
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2. Jashari G., Musliu A., Sýs M., Arbneshi T., Metelka R., Mikysek T., Švancara I. Optimization of working conditions in voltammetric simultaneous determination of retinyl acetate and α-tocopheryl acetate, XVIII. Vedecká konferencia s medzinárodnou účasťou, Piešťany, Slovakia, 22. – 24. September 2021.
3. Jashari G., Muriqi S., Sýs M., Arbneshi T., Švancara I., Metelka R. Optimization of working conditions in voltammetric detection of β-carotene, 3rd Cross-Border Seminar on Electroanalytical Chemistry, Virtual Seminar, 8. - 9. April 2021
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