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Ph.D. Thesis

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Utilization of potential programs in flow electrochemical determination of biologically active organic compounds

Využití potenciálových programů při průtokovém elektrochemickém stanovení biologicky aktivních organických látek

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Prohlášení

Prohlašuji, že jsem tuto závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze 02. 07. 2018 Mgr. Dmytro Bavol

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Contents

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Klíčová slova:

Antioxidanty
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Průtokový systém
Elektroda ze skelného uhlíku
Vysokoúčinná kapalinová chromatografie
Metody
Multi-pulsní amperometrie

List of symbols and abbreviations:

α	significance level (95%)
BHA	butylated hydroxyanisole
B-R	Britton-Robinson
c	molar concentration (mol L^{-1})
CA	caffeic acid
CE	capillary electrophoresis
CF	correction factors
CPE	carbon paste electrode
CV	cyclic voltammetry
DAD	diode array detector
DPV	differential pulse voltammetry
E	potential
E_p	peak potential
ED	electrochemical detection
EI	enzyme immunoassay
FA	ferulic acid
FIA	flow injection analysis
FS	flow system
FSDPV	fast scan differential pulse voltammetry
GA	gallic acid
GCE	glassy carbon electrode
GC	gas chromatography
HPLC	high performance liquid chromatography
HQ / Q	hydroquinone/quinone
I	current
I_p	peak current
L_D	limit of detection
L_Q	limit of quantification
MPA	multiple-pulse amperometry

MS	mass spectrometry
n	number of measurements
p-CA	*para*-coumaric acid
PG	propyl gallate
PDA	photo diode array
r	correlation coefficient
R^2	coefficient of determination
RSD	relative standard deviation
Ru	rutin
SGA	syringic acid
SOT	Scoville-Organoleptic test
SPA	sinapic acid
SPE	solid phase extraction
SV	stripping voltammetry
SWV	square wave voltammetry
Ty	tyrosol
*t*BHQ	*tert*-butylhydroquinone
UV-VIS	ultraviolet and visible spectroscopy

Abstract

In this Ph.D. thesis possibilities of using our proposed potential programs for a multiple-pulse amperometry and a fast scan differential pulse voltammetry in combination with flow systems are presented. The development of new sensitive amperometric and voltammetric methods for the determination of oxidisable biologically active organic compounds is another aim of this work.

In the first part of the work, the flow injection system and multiple-pulse amperometric detection were employed to develop and optimize a simple, low-cost, and rapid method for the simultaneous determination of natural and synthetic antioxidants. This technique involves the application of an appropriate potential waveform consisting of a suitable sequence of pulses on a single working electrode, thus allowing distinguish the analytes in a mixture with no need of separation. Conditions for the determination of antioxidants and modelling of the potential program were tested and studied, respectively.

Second part of the work describes and characterizes the application of the fast scan differential pulse voltammetry (FSDPV) in combination with the flow systems. FSDPV is the electroanalytical technique that use high scan rate to record voltammograms within several milliseconds and ensures high temporal resolution. This technique was characterized using the hydroquinone/quinone redox system.

During the characterization of the techniques in combination with the flow systems, the separability, the repeatability, and the concentration characteristics for the determination of commonly known antioxidants in standard solutions were performed. Successful testing has proved that these techniques have a good potential to be applied in routine analysis in substitution of expensive separation systems. Finally, the optimized procedures were applied for practical purposes to determine antioxidants contained in real matrices by applying a simple extraction procedure. The advantages and possibilities of the techniques are discussed, and the selectivity and sensitivity of the detectors are compared with other commonly used detection techniques, such as HPLC with DAD, ED or MS detection.

Abstrakt

Předložená disertační práce je zaměřena na možnosti využití navržených potenciálových programů pro multi-pulsní amperometrii a rychlou diferenční pulsní voltametrii, jakožto detekčních technik v kombinaci s průtokovými systémy. Dalším cílem této práce je vývoj nových citlivých amperometrických a voltametrických metod pro stanovení oxidovatelných biologicky aktivních organických sloučenin.

V první části studie byla průtoková injekční analýza a multi-pulsní amperometrická detekce použita pro vývoj a optimalizaci jednoduchého, levného a rychlého stanovení přírodních a syntetických antioxidantů. Zmíněna detekční technika zahrnuje aplikaci vhodného potenciálního průběhu, který se sestává z vhodné posloupnosti vkládaných pulsů na jednu pracovní elektrodu, což umožňuje selektivní stanovení analytů ve směsi, bez potřeby předchozí separace. Modelování použitých potenciálních programů bylo testováno a studováno, společně s ohledy na podmínky pro stanovení antioxidantů. Druhá část studie popisuje a charakterizuje použití rychlé diferenční pulsní voltametrie (FSDPV) v kombinaci s průtokovými systémy. FSDPV je elektroanalytická technika, která využívá vysokou rychlost skenování k záznamu velkého množství voltamogramů během několika málo sekund a zajišťuje vysoké rozlišení v čase analýzy. Tato technika byla charakterizována za použití redoxního systému hydrochinonu / chinonu.

Během charakterizace detekčních technik v kombinaci s průtokovými systémy byly provedeny separace, opakovatelnosti a koncentrační charakteristiky pro stanovení běžně známých přírodních a syntetických antioxidantů ve standardních roztocích. Po úspěšném testování, byly použité detekční techniky prakticky aplikovány v rutinní analýze, kde mohou nahradit drahé separační systémy. Nakonec, jako aplikace detekčních technik, byly optimalizovány postupy pro stanovení antioxidantů obsažených v reálných matricích za použití jednoduchého extrakčního postupu. V práci jsou diskutovány výhody a možnosti použitých detekčních technik; jejich selektivita a citlivost je porovnána s jinými běžně používanými detekčními technikami ve spojení s průtokovým systémem, jako je HPLC s detekcí DAD, ED nebo MS.

1. Aims of the Work

The main aim of the Ph.D. thesis was development and verification of electrochemical detection techniques in combination with flow systems based on a glassy carbon electrode adjusted in a wall-jet arrangement. For these purpose, different electrochemical techniques such as MPA and FSDPV were investigated for practical applications.

The combination of electrochemical detection techniques and the flow systems generates a lot of questions and problems, but it also offers an interesting field of possibilities, especially during the analysis of complicated samples of biological matrices, where the current information from different approaches can help to solve the difficulties in connection with the determination of different analytes. To meet the target it was necessary to realize the following steps:

⇒ To model the potential program for a multiple-pulse amperometry and develop a procedure for optimization of the electrochemical detection and the flow system a) basic measurement parameters, such as the flow rate, injected volume, and step height; b) advanced measurement parameters, such as the resulting peak potential, peak height, and their evaluation;

⇒ To model the potential program for a fast scan differential pulse voltammetry and develop a procedure for optimization of the electrochemical detection and the flow system a) basic measurement parameters, such as the scan rate and step height; b) advanced measurement parameters, such as the resulting peak potential, peak height and subtraction of background current; c) other parameter of the flow system, such as flow rate and injected volume;

⇒ The application of developed techniques for the determination of oxidisable biologically active organic compounds in model samples in flow systems (flow injection analysis and high performance liquid chromatography);

⇒ And lastly, to verify as an application of the techniques for the determination of oxidisable biologically active organic compounds in real matrices by applying a simple extraction procedure;

All results were published in following three papers and one paper is in press, the papers are attached as appendices:

1. **Bavol D.**, Dejmkova H., Scampicchio M., Zima J., Barek J., Combination of flow injection analysis and fast scan differential pulse voltammetry for the determination of antioxidants, *Electroanalysis 29*, 182–187 (**2017**).

2. **Bavol D.**, Economou A., Zima J., Barek J., Dejmkova H., Simultaneous determination of *tert*-butylhydroquinone, propyl gallate, and butylated hydroxyanisole by flow-injection analysis with multiple-pulse amperometric detection, *Talanta 178*, 231–236 (**2018**).

3. **Bavol D.**, Economou A., Zima J., Barek J., Dejmkova H., Simultaneous determination of sinapic acid and tyrosol by flow-injection analysis with multiple-pulse amperometric detection, *Monatshefte Für Chemie*, In Press (**2018**).

4. **Bavol D.**, Scampicchio M., Zima J., Barek J., Dejmkova H., Fast scanning voltammetric detector for high performance liquid chromatography, *Electrochimica Acta 281*, 534–539 (**2018**).

2. Introduction

In the last few decades, there has been a significant increase in demands for sensitivity, reliability, selectivity, and especially speed of measurement in analytical chemistry, with minimal demands on human labour and the cost of one analysis. Classical analytical procedures cannot guarantee this. Therefore, various types of automated analysers are used. Some of these requirements are satisfied by flow methods, namely flow injection analysis (FIA) and high performance liquid chromatography (HPLC). An important part of the proper analysis is also the used detection technique. Besides mass spectroscopy, spectrophotometry and fluorimetry, electrochemical detection techniques can also be advantageously used; they represent a useful, instrumentally unpretentious, and independent alternative to other commonly used detection techniques.

The usual methods of electrochemical detection, whether amperometric or coulometric, uses a single defined potential to monitor the course of the electrochemical reaction of the substance/s for which the current response is monitored. The disadvantage of this procedure is the fact that there is no information about the electrochemical behaviour of the monitored substance/s, which would significantly help in its/their characterization. This problem can be solved by using a set of electrodes (electrode arrays) each set to a different detection potential, but it is at the expense of higher prices and instrumental complexity (both technical and financial) in the form of a multichannel instrument and a complex spatial arrangement of the electrodes [1–4]. On the other hand, modern equipment, which works with potential programs, is now capable of sufficiently fast operation, in order to consider the application of varying potentials at one electrode and the use of acquired data.

The first question that needs to be solved before applying the variable electrical potential is the use of an appropriate potential program. The multiple-pulse amperometry (MPA) involves the application of an appropriate potential waveform consisting of a suitable sequence of pulses on a single working electrode,

thus allowing distinguishing the analytes in a mixture with no need of separation step, chemical pre-treatment of the sample or electrode modification – provided that the reaction potentials of the compound differ. MPA has been used for the simultaneous determination of different analytes [5–8]. This strategy was also used for simultaneous determination of sugars [9], drugs [10–13], antioxidants [14], synthetic colorants [15], as well as for the use of internal standard method in FIA [16]. This method has some important advantages: it is inexpensive, simple, has small sample and reagent consumption (with reduction of waste generation) and high sampling rates [17–19].

The extension of this approach is represented by differential pulse voltammetry or square wave voltammetry, when the whole potential scale is imposed on the electrode. Although DPV has been using extensively by physical electrochemists and electroanalytical chemists in static solutions, it has been used only rarely in flowing systems such as liquid chromatography and flow injection analysis. The use of this technique in the flow arrangement was verified using continuous flow mode [20]. This technique can provide a more complete identification of each peak: peak retention time and a characteristic shape of the voltammogram for each peak [21–23]. Measuring pulses may also be supplemented by pulses aimed at electrode cleaning and adjustment of its properties or on the pre-concentration of the analyte [21,24–26]. The voltammogram can facilitate identification or class identification of unknown compounds [27]. Further, the characteristic voltammograms of multicomponent samples are obtained with only one chromatogram in contrast to the time consuming generation of hydrodynamic voltammograms by electrochemical detection with the method of repeated injections at a series of applied potentials [28]. The technique also offers immediate identification of coeluting peaks if the coeluting components have different oxidation potentials and thus offers immediate selectivity, because the chromatogram can be plotted at the lower oxidation potential to remove the interference [29]. From an electrochemical point of view, the technique provides the capability of obtaining voltammograms of small volumes of sample.

Another approach is the use of cyclic voltammetry, where the potential is changed continuously between two boundary values. In this case, it is necessary to find a suitable compromise between the scan rate and the rise of the background current [30,31]. To increase the analyte signal and reduce noise during measurement, special computing numerical methods such as the Fourier Transformation [32,33] can be used for the treatment of the measured electrochemical signal.

The second measurement problem is the question of the used appropriate working electrode. With the current high demands placed on the electrode by the chromatographic and electrochemical components of the analysis, it is necessary to use the electrodes mechanically and electrochemically stable and durable, compatible with the mobile phases and with a sufficiently wide potential window and low noise. Very important is the possibility of an easy restoration of the electrode surface into the initial state, because in many cases electrochemical reactions results in the formation of polymeric products which are deposited on the surface of the electrode and thus passivate it [34]. In the case of flow techniques, the passivation is reduced due to a mobile phase, which continuously removes the products of electrochemical reactions from the surface of the electrode [35], but during the determination of complex samples, this process may not be sufficiently efficient. Preferences are given to the use of electrode materials that are minimally passivated, such as boron-doped diamond [36], but other possibilities, such as glassy carbon, platinum or a composite electrode, can also be considered. In addition, it may be necessary to insert cleaning pulses as mentioned earlier. Electrode modifications are also related to the possibility of using microelectrodes [30] and hence the utilization of their specific properties, such as low background current and related advantageous signal / noise ratio [37].

3. Analytes (antioxidants)

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation can produce free radicals, leading to chain reactions that may damage cells. Antioxidants (such as substances mentioned in this work) terminate these chain reactions. The term antioxidant is mainly used for two different groups of substances: industrial chemicals, which are added to products to prevent oxidation, and natural chemicals found in foods and body tissue, which are said to have beneficial health effects.

3.1. Natural antioxidants

Natural antioxidants can offer benefits to human health by reducing incidence of atherosclerosis and coronary heart diseases [38–41]. Many studies have confirmed that natural antioxidants including those mentioned in this work have multiple biological properties ranging from cardioprotective, antiinflammatory, antiallergenic and anticarcinogenic [42], to antiviral and antibacterial [43–45]; and are quite effective in combating analgesia [46], phlogosis [47], high cholesterol, and obesity [48]. These biological properties are attributed mainly to their powerful antioxidant and antiradical activity [49], which is related to their redox properties [50]. Some of them are used in pharmaceutical industry because of its pharmacological properties [51–53]. In recent years, lots of antioxidants have attracted the attention of scientists, as they are immensely beneficial for curing biological ailments and improving the overall health of human beings [54]. As the result of their healthy benefits [55,56], theirs determination is of special importance for food manufacturer that have to identify the content of such substances for their eventual declaration.

The first group of substances that were given attention were GA, FA, SRA, SPA, *p*-CA, Ru, and CA (Figure 3-1) that are commonly found in different products (e.g., food, agriculture, pharmaceuticals, and cosmetics) [57–59].

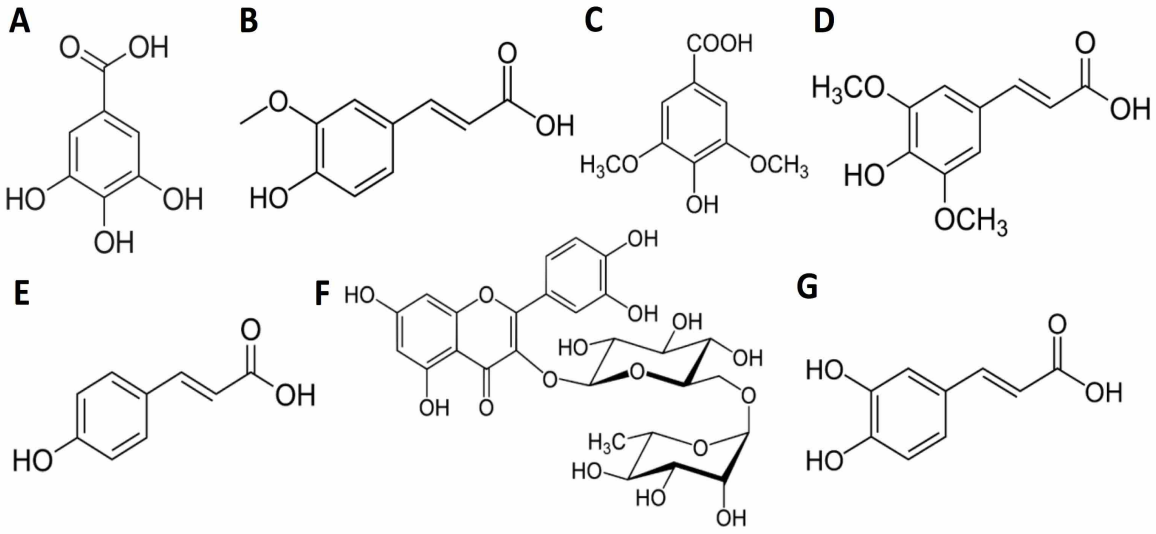

Fig. 3-1 Structure of gallic acid - **A**, ferulic acid - **B**, syringic acid - **C**, sinapic acid - **D**, *p*-coumaric acid - **E**, rutin - **F**, and caffeic acid - **G**.

Many different methods, including HPLC and CE in combination with different detectors, UV–Vis [60], PDA [61–63], DAD [64], and MS [65] have been used to monitor these compounds. CV was the first electrochemical method used for characterisation of natural antioxidants [66–68]. Also different electrochemical techniques such as SWV and DPV have been used [68–70].

Another group of natural antioxidants that was monitored were SA and Ty (Figure 3-2), which are common constituents of plants and fruits. These substances can be found for example in cranberry, wine and olive oil [71–74]. Ty is also one of the main natural phenols in argan oil [55].

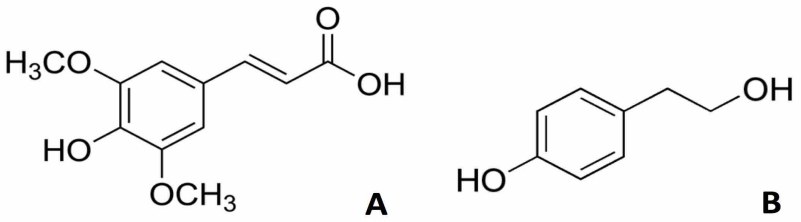

Fig. 3-2 Structure of sinapic acid (**A**) and tyrosol (**B**).

Several methods mainly based on voltammetry were reported for the individual determination of SA [75] or Ty [76]. However, to our knowledge, only few analytical methods have been reported for the simultaneous determination of SA and Ty including HPLC in combination with UV or MS detection [77–79].

Finally, the last compound that was given attention was capsaicin (Figure 3-3). Capsaicin is one of ten compounds of capsaicinoids, which are present in hot pepper. Hot pepper is one of oldest cultivated plants in the world. Its popularity is mainly due to its very pungent flavour. Capsaicin has the most pungent flavour [80] and it is a major constituent of capsaicinoids with representation nearly 70%.

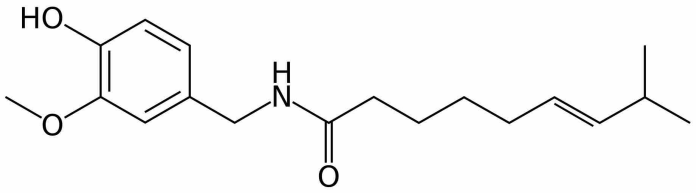

Fig. 3-3 Structure of capsaicin.

Some of the advanced analytical techniques have been used for the analysis of capsaicin such as SOT [81], EI [82], CE [83]. GC [84,85] and HPLC [86–89] are now widely and routinely used for the analysis of capsaicin.

3.2. Synthetic antioxidants

Synthetic phenolic antioxidants are extensively used in the food industry as additives to improve the stability of various products, especially for the prevention of lipid oxidation reactions, responsible for the production of volatile compounds with unpleasant flavours. Among the most commonly used additives are PG, *t*BHQ, and BHA (Figure 3-4), used alone or together. In many countries, the use of these antioxidants is controlled by official legislation, and consequently, it is important to be able to determine reliably the amounts of these substances in food products.

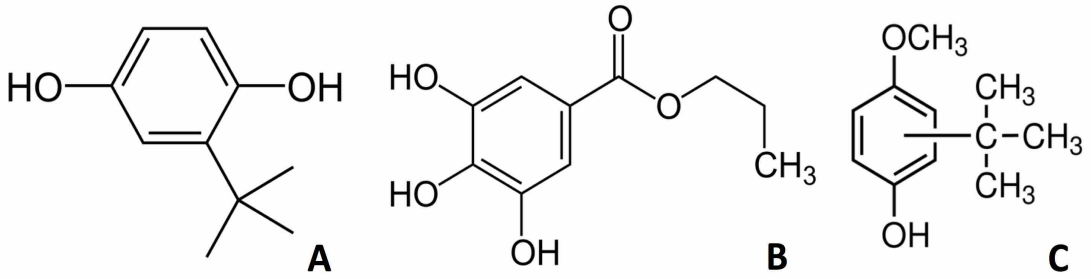

Fig. 3-4 Structure of *tert*-butylhydroquinone (**A**), propyl gallate (**B**), and butylated hydroxyanisole (**C**).

Many electrochemical methods, such as CV [90], DPV [90,91], SV [92], and SWV [90,93] have been used to determine these antioxidants. Also the adsorptive pre-concentration of synthetic antioxidant at a CPE has been described [94]. All these techniques generally have high sensitivity, and are widely used in many areas of analytical chemistry. However, their applicability for the determination of several components in mixtures is limited if the recorded voltammograms display significant partial overlapping. As a result, techniques preceded by a separation step, particularly HPLC with ED [95], DAD [96] or MS [97] detection are most frequently used for the determination of mixture of these antioxidants. However, application of such complex separation methods might not be necessary in many cases and FIA in combination with a selective detection technique might present a suitable alternative.

4. Results and Discussion

4.1. Multiple-pulse amperometry

The first detection technique that was used for determination of phenolic natural and synthetic antioxidants [98–100] in combination with the FIA system is a multiple-pulse amperometry (MPA) based on a glassy carbon electrode adjusted in a wall-jet arrangement [101]. It is well known in the literature that antioxidants provide the best performance for electrochemical oxidation in acidic media [102–104]. Due to the low solubility of these phenolic antioxidants in water, an aqueous-methanolic solution containing 10% (*v/v*) methanol in 0.040 M B-R buffer pH 2.0 was used both as a carrier solution for all experiments and for dilution of analysed solutions before injection. In the first step, the hydrodynamic voltammograms of all the targets were constructed, in order to find the potential values used for the construction of the pulse program. Next, the obtained peak heights were subtracted from each other within measurable concentration range and its verification was performed. Further, the basic FIA parameters and the other factors that could influence the determination itself, such as the effect of cleaning pulses or the effect of pulse width, was explored. And last but not least, as a practical application, the determination of these antioxidants contained in the real matrices was carried out, by applying a simple extraction procedure.

Determination of Peak Potential. In order to identify the potential of oxidation to perform the simultaneous determinations of all antioxidants, hydrodynamic voltammograms were first obtained separately for each compound. In the case of the determinations of *t*BHQ, PG, and BHA, eleven sequential potential pulses of 100 ms each from 0.20 to 0.70 V (step 0.05 V) were applied continuously. The current at each potential pulse was monitored continuously during three injections (injected volume 100 μL) of 0.1 mmol L^{-1} *t*BHQ.

The same procedure was performed during triplicate injections (injected volume 100 µL) of 0.1 mmol L^{-1} PG and BHA.

In the case of the determinations of SA and Ty (0.1 mmol L^{-1}), sequential potential pulses of 100 ms duration from +0.40 to +0.80 V for SA and from +0.70 to +1.10 V for Ty (step 0.05 V, in both cases) were applied continuously with injected volume 100 µL.

The average current peak (n = 3) at each potential pulse was monitored and used to construct a hydrodynamic voltammogram for the electrochemical oxidation of *t*BHQ, PG, and BHA; SA and Ty (Figure 4-1 and 4-2).

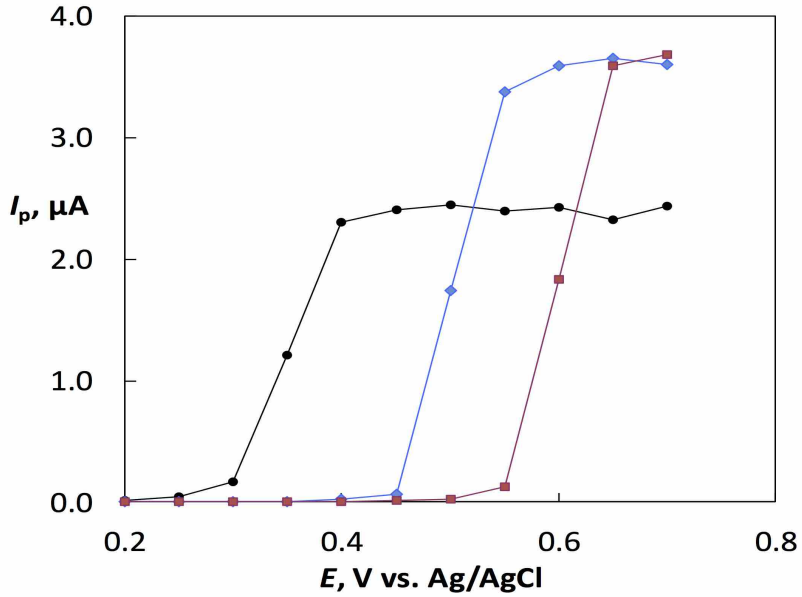

Fig. 4-1 Hydrodynamic voltammograms obtained by plotting peak current values as a function of the potential of applied potential pulses. The injected solutions contained *t*BHQ (•), PG (◆) or BHA (■) (all 0.1 mmol L^{-1}). Carrier solution: methanol − 0.040 M B-R buffer pH 2.0 (1:9, *v/v*); injected volume: 100 µL; flow rate: 1.0 mL min^{-1}.

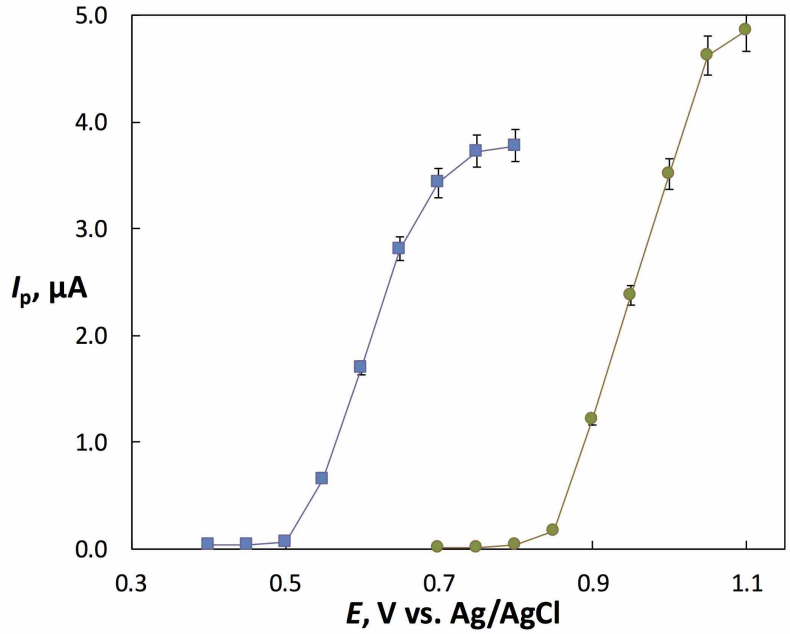

Fig. 4-2 Hydrodynamic voltammograms obtained by plotting peak current values as a function of the corresponding applied potential pulses. The injected solutions contained SA (■) or Ty (•) (both at 0.1 mmol L^{-1}). Carrier solution: methanol, 0.040 M B-R buffer pH 2.0 (1:9, *v/v*); injected volume: 100 µL; flow rate: 1.0 mL min^{-1}.

In the first case, according to the hydrodynamic voltammograms (Figure 4-1), the application of potentials lower than +0.45 V would lead to the detection of *t*BHQ without or with only insignificant interference of PG and BHA, and so +0.40 V (100 ms) was selected as the first potential pulse. As the second pulse, potential of +0.55 V (100 ms), was selected, which allows oxidation of both *t*BHQ and PG without the interference of BHA. PG can be quantified after the subtraction of the current from oxidation of *t*BHQ at +0.55 V. At +0.70 V (100 ms), all three target compounds were oxidized. BHA can be quantified if the currents from oxidation of *t*BHQ and PG at +0.70 V were previously subtracted. From the measured data of these antioxidants we can reach the conclusion, that the difference between peak potentials of 150 mV is sufficient enough to allow different analytes determinations.

In the second case the procedure for determination of the peak potential is very similar, according to the hydrodynamic voltammograms (Figure 4-2), it can be seen that the peak potentials differ enough to enable the selective determination of the analytes. Namely, potentials between +0.70 and +0.80 V would only cause the oxidation of SA without significant interference from Ty; therefore, +0.75 V (100 ms) was selected as the first potential pulse. Potential of +1.10 V (100 ms) was selected as the second potential pulse, where both target analytes are fully oxidized. Ty can be quantified if the current from the oxidation of sinapic acid at +1.10 V is previously subtracted.

Effect of Pulse Width. The influence of the width of the pulses inserted on the working electrode was tested as another measuring factor. The use of narrower pulses results in shorter pulse program. This means that higher flow rates can be used; and the total analysis time will be shorter; and it leads to lower consumption of the carrier solution. On the other hand, when wider pulses are inserted, it results in the opposite effect and it may result in an extension of the peaks, and thus reduce the signal. If we take into account these considerations, it is necessary to do some compromise between the width of the inserted pulses and the used flow rate. No significant influence of the pulse width (for measuring two or three substances) was observed between 70 and 150 ms and 100 ms pulse width was kept further. From the figures of repeatability of the mixtures of a standard solution, remarkable thing may be observed, namely difference between the values of currents of the baseline for each inserted potential [99]. Also, other publications mentioned earlier obtained the similar results [6] [19] [105]. This problem has a connection with the length of the individual pulses, the size of the inserted potential pulses, and the magnitude of the potential difference between the individual pulses, which are in very fast sequences during the measurement [106,107].

Current Subtraction. However, direct subtraction of the current response at +0.40 V (exclusive oxidation of *t*BHQ) from the current response at +0.55 V (oxidation of *t*BHQ and PG) would be equivalent to the current response of PG only if the current responses of *t*BHQ at both potentials pulses (+0.40 and +0.55 V) corresponded to the same value. Similar procedure should be used to obtain the current response of BHA only at +0.70 V. The current responses detected for *t*BHQ at +0.40 V and for PG at +0.55 V should be subtracted from the current response at +0.70 V (access to the current from the oxidation of BHA). In practice, the current responses detected for *t*BHQ at +0.40 V, +0.55 V, and +0.70 V and for PG at +0.55 V, and +0.70 V were not equal. Therefore, the direct subtraction of the currents detected at the applied potential pulses does not provide access to the oxidation currents from PG or BHA.

To overcome this problem, we have proposed to use simple correction factors (*CF*). For PG determination at +0.55 V without interference of *t*BHQ, the *CF* can be obtained by a simple injection of a standard solution containing only *t*BHQ and by using the following equation:

$$CF_1 = I_{t\text{BHQ }+0.55\text{ V}} / I_{t\text{BHQ }+0.40\text{ V}} \quad (1)$$

Then, if a standard or sample solution containing both *t*BHQ and PG is injected in the MPA-FIA system, the current originating from PG oxidation detected at +0.55 V can be calculated using the CF_1 value and the following equation:

$$I_{\text{PG}} = \text{I}_{+0.55\text{ V}} - (CF_1 \times \text{I}_{t\text{BHQ }+0.40\text{ V}}) \quad (2)$$

For BHA determination at +0.70 V without interference of *t*BHQ and PG, two *CF* values need to be used. The CF_2 corresponds to the difference between the current detected for *t*BHQ at +0.40 V and +0.70 V, and the CF_3 corresponds to the difference between the current detected for PG at +0.55 V and +0.70 V. The CF_2 value can be obtained by injection of a solution containing only *t*BHQ and the following equation:

$$CF_2 = I_{t\text{BHQ }+0.70\text{ V}} / I_{t\text{BHQ }+0.40\text{ V}} \quad (3)$$

The CF_3 value can be obtained by injection of a standard solution containing only PG and the following equation:

$$CF_3 = I_{\text{PG +0.70 V}} / I_{\text{PG +0.55 V}} \quad (4)$$

When a solution containing all three compounds was injected in the MPA-FIA system, the current originating from the oxidation of BHA at +0.70 V can be calculated using CF_2 and CF_3 values, the current obtained for PG through the equation 2 and the following equation:

$$I_{\text{BHA}} = I_{\text{+0.70 V}} - (CF_3 \times I_{\text{PG +0.55 V}}) - (CF_2 \times I_{t\text{BHQ +0.40 V}}) \quad (5)$$

In the development of a new proposed technique, an additional parameter should be considered: the *CF*s values must be relatively constant in the selected concentration interval of *t*BHQ (CF_1 and CF_2) and PG (CF_3). In the concentration interval between 10 to 100 μmol L^{-1} for *t*BHQ and PG, the following *CF* values were obtained (n = 3): $CF_1 = 1.04 \pm 0.03$, $CF_2 = 1.06 \pm 0.04$, and $CF_3 = 1.09 \pm 0.06$.

The same procedure of current subtraction was performed for the determination of the peak highs for SA and Ty. Results of additional parameter such as *CF* in the concentration interval between 10 to 100 μmol L^{-1} of SA was obtained (n = 3): the CF_{SA} value was calculated as 1.10 ± 0.06.

Optimization of FIA Parameters. Also other FIA parameters were optimized in order to obtain the highest signal for the specified substances; the effect of the injected sample volume on the MPA response, as well as the influence of the flow rate was investigated. The optimal injection volume was selected as the maximum volume above which the peak does not further increase, but only broadens. This point was reached for an injection volume of 100 μL of 0.1 mmol L^{-1} of *t*BHQ, PG, and BHA; the same injected volume was selected in the case of determination of SA and Ty (0.1 mmol L^{-1}) in the MPA-FIA system, this injected volume was thus selected for all further amperometric measurements. Another

investigated parameter was the flow rate. The flow rate was varied in the range from 1.0 to 3.0 mL min^{-1}, keeping the injection volume of 100 μL for 0.1 mmol L^{-1} of *t*BHQ, PG, and BHA; and from 1.0 to 5.0 mL min^{-1}, keeping the injection volume of 100 μL for 0.1 mmol L^{-1} of SA and Ty. An increase in the flow rate resulted in slight decrease in the peak area because of the shorter contact of the analytes with the electrode and in the rapid increase of the peak height due to its narrowing. On the other hand, excessive narrowing of the peak affects the resolution due to the 300 ms length of pulse program. As a compromise, flow rate of 2.0 mL min^{-1} for *t*BHQ, PG, and BHA was selected for all further amperometric measurements. In the case of SA and Ty determination the length of the pulse program was 200 ms, so a bit higher flow rate is possible. Therefore, flow rate of 3.0 mL min^{-1} was selected for all further amperometric measurements. Due to lower consumption of the carrier solution, higher flow rates over 3.0 mL min^{-1} were not selected.

Repeatability and calibration dependences. Under the optimized conditions, repeatability and calibration dependences were measured; ten successive injections of the mixture of a standard solution were carried out. The results demonstrate that the MPA-FIA system provides good repeatability (*RSD* <4.0%, in all cases) and high throughput (>140 injections h^{-1}). In the case of SA and Ty determination, the length of the pulse program is even shorter as mentioned earlier, therefor higher frequency of the injections is possible (>170 injections h^{-1}). Calibration curves for all targets were constructed using solutions containing varying concentrations of one antioxidant, while the concentration of the other remained constant. Linear regression of these series of experiments leads to excellent correlation coefficients ($r > 0.99$, in all cases) and the obtained L_Q calculated as the analyte concentration corresponding to a tenfold standard deviation of the lowest response, are at micromolar level for these antioxidants. However, lower L_Qs are not necessary for the analysis of these antioxidants in food samples, because their concentrations are usually relatively high; thus, the L_Qs obtained with this proposed method are more than adequate for the analysis of food samples (more information in Table 4-1).

Tab. 4-1 Parameters of calibration curves, limits of quantification and relative standard deviation of SA and Ty; *t*BHQ, PG, and BHA obtained by MPA-FIA.

Substance	Concentration range μmol L^{-1}	Slope nA mol^{-1} L	Intercept μA	Correlation coefficient	L_Q μmol L^{-1}	*RSD* (%) for 10 injections (100 μmol L^{-1})
SA	0.8–100	47.61	0.211	0.9956	0.86	2.48
Ty	1.0–100	89.78	0.076	0.9973	1.03	3.96
*t*BHQ	2.0–100	66.59	0.293	0.9955	2.51	0.84
PG	1.0–100	51.49	0.408	0.9901	1.45	1.53
BHA	0.8–100	28.14	0.114	0.9947	0.85	3.69

The factor that could have a significant effect on the measurement itself is the passivation of the electrode surface. After the data evaluation obtained from the repeatability of the measurement, it can be seen that the MPA-FIA system provides good repeatability (*RSD* <4.0%, in all cases; more details see in Table 4-1) and there was no significant effect of the passivation. In the case that the mentioned passivation occurs, the total potential program would have to be extended by the inserted pulse/s for the regeneration of the electrode surface. This setting changes the overall recording of the data received from the measuring device, and parameters such as the flow rate have to be set to an optimum value again.

Methods Aplication. As an application, the determination of *t*BHQ, PG, and BHA contained in real matrices was carried out, by applying a simple extraction procedure. As a real matrix, chewing gum was chosen because it contains all of mentioned antioxidants in high concentrations. It has been confirmed that the presence of any interfering substances do not significantly affect the course of the measurement and there is no need to use a separation technique. The trueness of the proposed method was first evaluated by determination in samples of the analytes spiked into extract

from chewing gum free from these compounds, in order to evaluate possible matrix effects. Recovery tests were carried out at 3 different concentration levels (5, 10, and 50 μmol L^{-1}); received values for *t*BHQ, PG, and BHA were in the range of 95-113%, 98-115%, and 104-116% ($n = 3$), respectively. HPLC with amperometric detection [102] provided recoveries between 82% and 102%, for the corresponding samples, underestimating the appropriate values probably due to the ineffective extraction step; MPA-FIA effectively compensated for this difference. The highest trueness for both techniques was observed for PG and the lowest for BHA. Finally, the proposed method was applied to the simultaneous determination of all three synthetic antioxidants in chewing gum samples. The samples were also analyzed by HPLC-ED for comparison [102]. The results are presented in graphical form in Figure 4-3. The presence of *t*BHQ was not detected in chewing gum by MPA-FIA, indicating its concentration below the limit of detection.

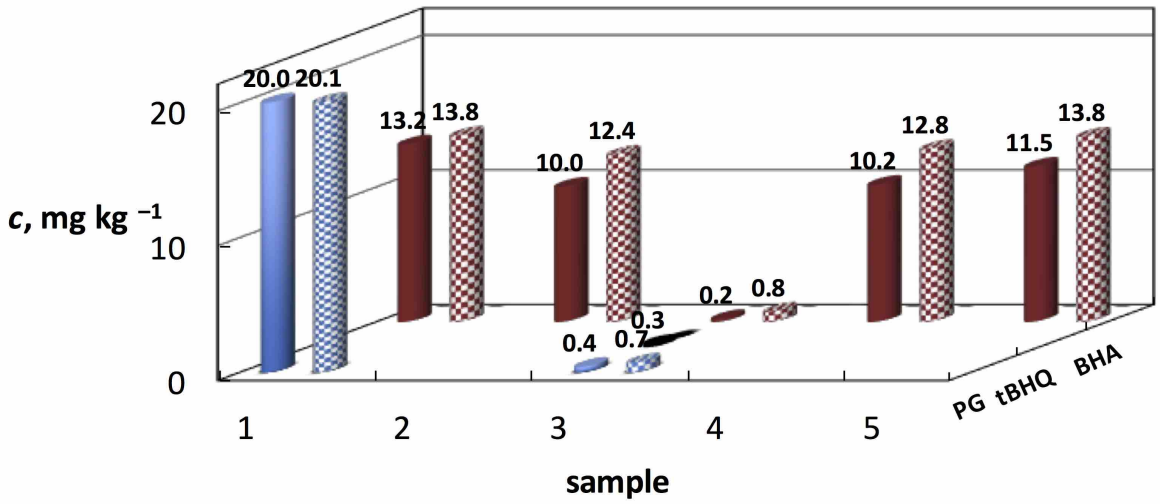

Fig. 4-3 Comparison of results from simultaneous determination of PG, *t*BHQ, and BHA content in analysed samples of chewing gums obtained by HPLC-ED (left) and MPA-FIA (right).

In the case of SA and Ty determination, method has a good potential to be applied in routine analysis in substitution of expensive chromatographic separation systems. But on the other hand, the course of determination of SA and Ty should be

without major complications in matrices mentioned earlier with a high proportion of these substances [71–74,77–79]. In the case of the rest of real samples, complications associated with the presence of other antioxidants naturally occurring in the real matrices can arise. The interference of other antioxidants depends highly on their properties, namely ascorbic acid and most other antioxidants oxidize earlier than the measured analytes using the given conditions [108,109]. This would change the procedure in the next step, namely recalculation of the peak heights of the determined substances by the correlation factor as explained earlier. A minor disadvantage may be that for each real sample a specific method for determination of the mentioned analytes would have to be developed.

4.2. Fast scan differential pulse voltammetry

4.2.1. FIA arrangement

The second detection technique that was used for the determination of antioxidants was a fast scan differential pulse voltammetry (FSDPV), when the whole potential scale in a short time is inserted on the electrode. Although DPV has been used extensively in static solutions, it has seen only limited use in flowing systems such as flow injection analysis and liquid chromatography. Preliminary experiments were aimed at characterizing the electrochemical detection system based on FSDPV in combination with flow injection analysis. This system was based on a glassy carbon electrode adjusted in a wall-jet arrangement using hydroquinone / quinone as model redox system [101]. Basic parameters, such as the scan rate and the resulting peak potential, peak height, and background current were evaluated together with other parameters of the flow system, such as flow rate and injected volume. However, these parameters are not the only factors that effect on the shape of the voltammetric signal and running of the entire analysis. Inserted reverse scan also plays important role in determining the current-potential response.

Effect of Flow Rate. The electrode configuration may be regarded as a cell in which the cell volume consists of a layer of solution between the electrode surface and the outlet of capillary. Under zero flow conditions, voltammograms are symmetrical peaks, as expected, but the introduction of convective flow modifies the electrochemical response. This can be seen in Figure 4-4, in which the recording of 10 μmol L^{-1} hydroquinone (injected volume 100 μL) is plotted at six different flow rates. The scan rate was held constant at 5.0 V s^{-1}. At potentials well below E_p, no oxidation occurs and the electrode surface region becomes filled with solute molecules. As the potential approaches E_p, the current increases causing the concentration of solute at the electrode surface to decrease and creating a flux to the electrode. When the surface concentration is essentially zero

flux (and thus current) reaches a maximum. Depletion begins at the electrode surface region and current decreases. At this point the effect of the restricted diffusion layer becomes evident. Under normal flowing conditions, flow delivers a constant flux to the electrode, causing the current to reach and maintain a "plateau" level greater than zero. As seen in Figure 4-4, the level at which the current plateau occurs is dependent upon the flow rate. As the flow rate increases, the waves become less peaked and steady state behaviour begins to prevail.

These results indicate that two models must be combined to describe this system. At the zero flow condition it is the thin layer model. It accounts for the depletion of the surface region and the peaked voltammetric wave. At the high flow extreme is the steady state hydrodynamic model. This describes the role of convective mass transfer and the resulting steady state voltammetric wave. At intermediate flow rates, a combination of the two models is observed, resulting in waves that show various degrees of peaking with the current reaching a steady state dependent on flow rate at high positive potentials. Flow rates from 0.2 to 1.5 ml min^{-1} were tested; based on the explanation in the previous paragraph, the value of flow rate of 0.8 ml min^{-1} (curve No. 4) was selected as optimal.

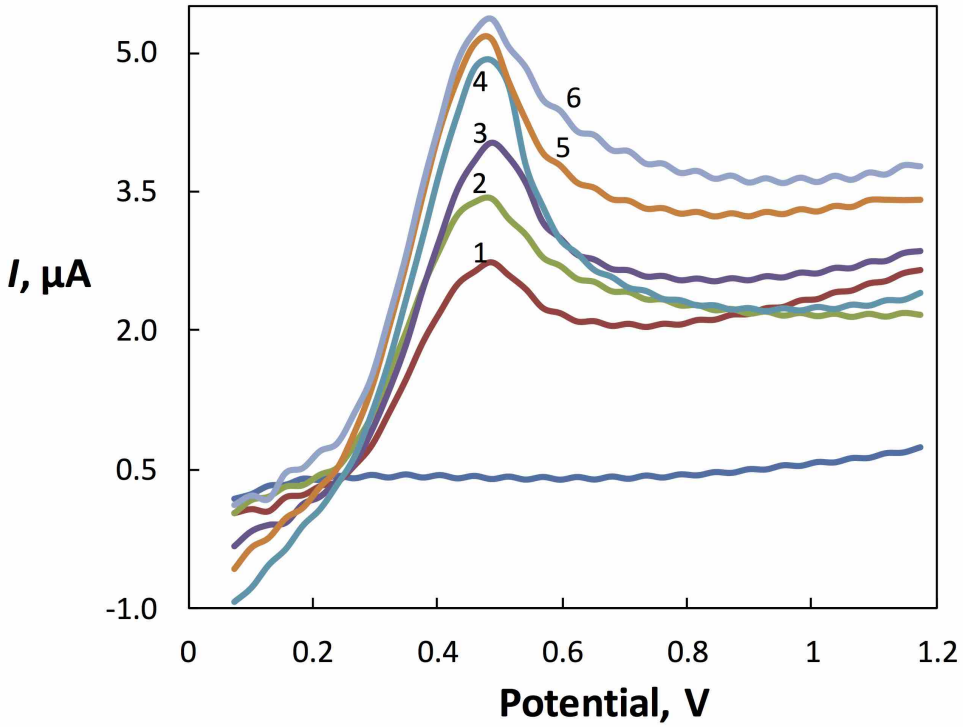

Fig. 4-4 Voltammograms of 10 µmol L^{-1} hydroquinone with addition of 0.10 mmol L^{-1} KCl as a function of flow rate (blank, 1 = 0.2, 2 = 0.4, 3 = 0.6, 4 = 0.8, 5 = 1.0, and 6 = 1.5 ml min^{-1}); scan rate: 5.0 V s^{-1}; injected volume: 100 µL. Detection at GCE in 0.04 M B-R buffer (pH 4).

Effect of Scan Rate. Figure 4-5 shows voltammograms of 10 µmol L^{-1} hydroquinone (injected volume 100 µL) at four different scan rates. The flow rate was kept constant at 0.8 ml min^{-1}. In a wall-jet cell, the peak current is directly proportional to scan rate. Under hydrodynamic conditions, current is independent of scan rate. Therefore, this system would exhibit some intermediate dependence on scan rate. The peak current shows the scan rate dependence, but it is not directly proportional as in a normally used wall-jet cell. At +1.2 V, where the current has reached a steady state, no scan rate dependence was observed. Furthermore, as the flow rate increases the peak current becomes less scan rate dependent. This is consistent with the combined thin layer/hydrodynamic model previously described. At low flow rates, the system approximates by a cell modified by convection. The peak current exhibits the scan rate

dependence, but that dependence is changed by the presence of the flowing stream. As the flow rate increases, conditions approach steady state and the peak current becomes less scan rate dependent. At high positive potentials, the system is under steady state conditions at all flow rates examined, and the current is not scan rate dependent. Scan rates from 1 to 5 V s^{-1} were tested and value of scan rate of 5 V s^{-1} (curve No. 4) was selected as optimal. In this case, the optimum scan rate could be even higher, but the device limitation did not allow us to do so.

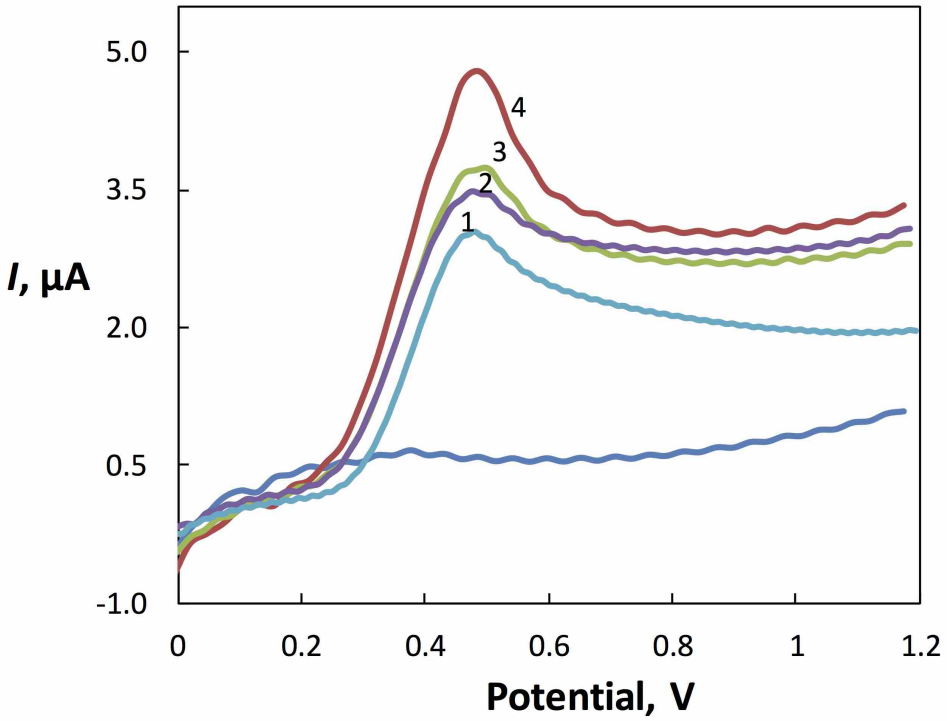

Fig. 4-5 Voltammograms of 10 µmol L^{-1} hydroquinone with addition of 0.10 mmol L^{-1} KCl as a function of scan rate (blank, 1 = 1.0, 2 = 2.0, 3 = 3.0, and 4 = 5.0 V s^{-1}); flow rate: 0.8 ml min^{-1}; injected volume: 100 µL. Detection at GCE in 0.04 M B-R buffer (pH 4).

Effect of *IR* Drop. One problem associated with wall-jet cells, and also encountered in this cell, is the occurrence of a potential gradient along the diameter of the electrode. The result of this is the shift of the voltammetric wave to more

positive potentials and lower peak currents. This can be seen in Figure 4-6, in which the concentration of KCl in the carrier solution was varied between 0.01 and 0.10 mmol L^{-1}. The higher KCl concentration resulted in a steeper wave at a lower potential and a higher peak current. Thus, the concentration of KCl of 0.10 mmol L^{-1} (curve No. 4) was selected as optimal.

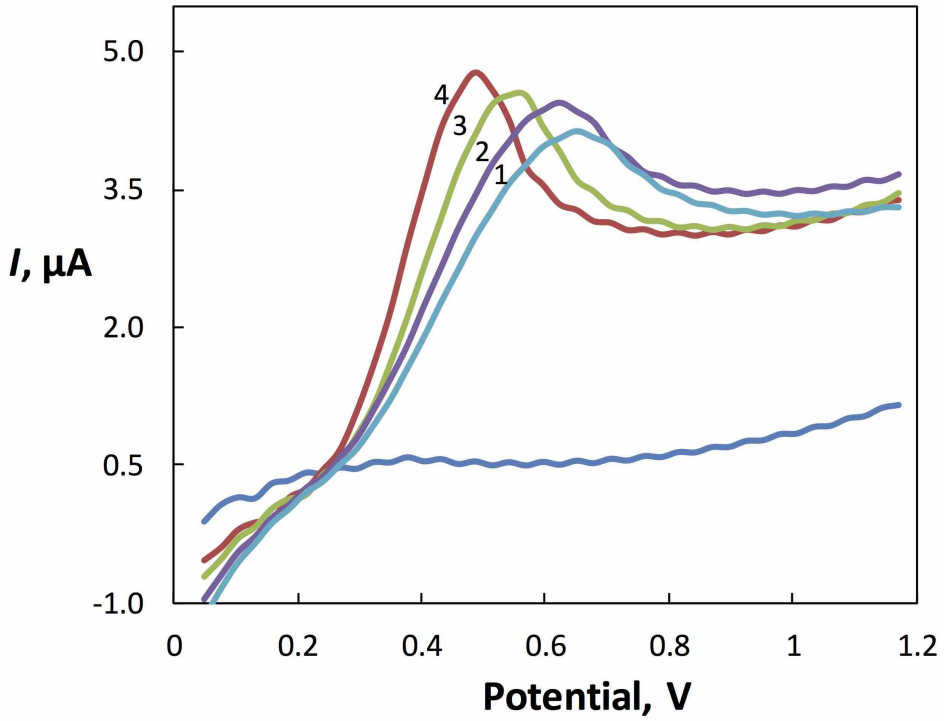

Fig. 4-6 Voltammograms of 10 µmol L^{-1} hydroquinone as a function of KCl basic electrolyte concentration (blank, 1 = 0.01, 2 = 0.02, 3 = 0.05, and 4 = 0.10 mmol L^{-1} KCl); scan rate: 5.0 V s^{-1}; flow rate: 0.8 ml min^{-1}; injected volume: 100 µL. Detection at GCE in 0.04 M B-R buffer (pH 4).

Effect of Injected Volume. The optimal injection volume was selected similarly as in the case of FIA with pulsed detection, as the maximum volume, above which the peak does not further increase its height, but only broadens (Figure 4-7). In this case, injection volumes from 20 to 150 µL were tested and the optimum value of

100 µL (curve No. 3) was selected as optimal. Under such optimized conditions, the injection of a blank sample provided negligible response.

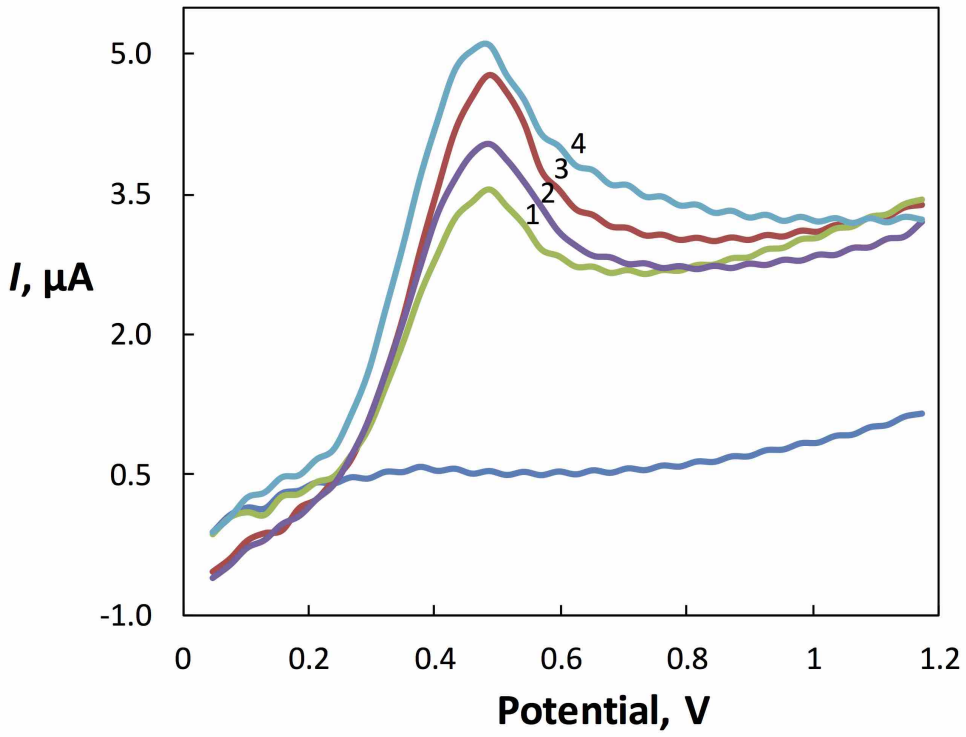

Fig. 4-7 Voltammograms of 10 µmol L^{-1} hydroquinone with addition of 0.10 mmol L^{-1} KCl as a function of injected volume (blank, 1 = 20, 2 = 50, 3 = 100, and 4 = 150 µL); scan rate: 5.0 V s^{-1}; flow rate: 0.8 ml min^{-1}. Detection at GCE in 0.04 M B-R buffer (pH 4).

Effect of Reverse Scan. A basic FSDPV measurement generally comprise one move, there is a potential ramp step when current measurements are repeatedly made. It should also be taken into account that in many cases electrochemical reactions result in the formation of polymeric products which are deposited on the surface of the electrode and thus passivates it [34,35]. If a reverse scan is being set up into a measurement program, it can partly remove the adsorbed products from the electrode surface. On the other hand, the rest of amount of adsorbed products of first

oxidation still remains on the electrode surface, leading to the red/ox inhibition of the electrode surface. Another, more important reason for insertion of the reverse scan is that with the gradual insertion of a large number of voltammetric scans, the increasing current at the beginning of the potential window degrades the readability and the evaluation processes of the measured substances during the analysis, so that the electrode is kept in the constantly cycling voltage state. Therefore, reverse scan with the same scan rate was also included, but only a forward scan was recorded. A predetermined number of scans was recorded, collected, and stored for each chromato-voltammograms; then processed using a computer program.

One obtained chromato-voltammogram is shown in Figure 4-8. This picture shows the resulting three-dimensional FIA-FSDPV recordings of model sample of hydroquinone oxidation measured under the optimized conditions; scan rate: 5.0 V s^{-1}; flow rate: 0.8 ml min^{-1}; electrolyte concentration 0.10 mmol L^{-1}; injected volume: 100 µL; the peaks are well defined and the relative standard deviation of their height is less than 3.5% (n = 10).

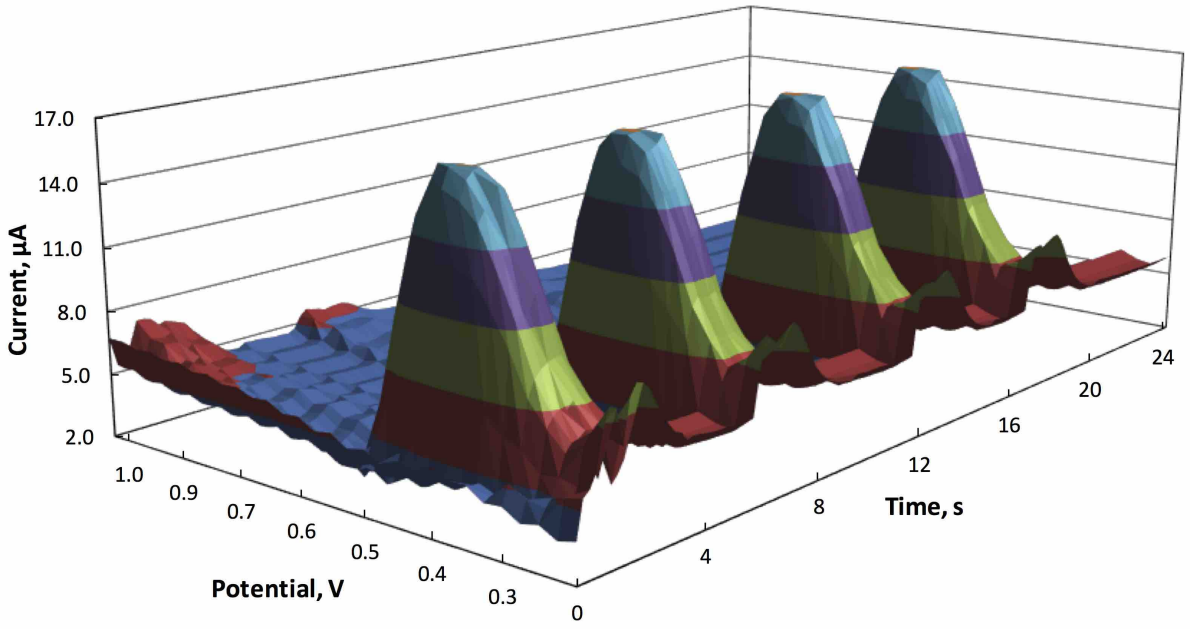

Fig. 4-8 Three-dimensional representation of FIA-FSDPV recordings of 100 µmol L^{-1} hydroquinone with addition of 0.1 mmol L^{-1} KCl; scan rate 5 V s^{-1}, flow rate 0.8 ml min^{-1}, four repeated injections of 100 µL. Detection at GCE in 0.04 M B-R buffer (pH 4).

Enhanced Resolution. After having defined the best conditions for the determination of a single redox species, we have paid attention to the enhanced spatial resolution offered by FSDPV for the simultaneous detection of more than one antioxidant. Caffeic acid and *p*-coumaric acid were selected as model analytes [110]. Their peak potentials differ of about 0.35 V, which is large enough to be resolved by batch voltammetric techniques. Therefore, this determination allows us to explore the real peak resolution on the potential axis. Measurements of a series of solutions of these individual compounds and in various concentration ratios were performed in non-aqueous medium of acetonitrile:ethanol mixture (1:1, *v/v*) containing 0.1 mmol L^{-1} of lithium perchlorate under the previously optimized conditions. High background current is attributed to the high scan rate, but also to the non-aqueous medium; nevertheless, the background magnitude is constant in time. The relative standard deviation of the peak heights was <4.0% ($n = 10$, in both cases), which confirms good stability

and repeatability of the measurements. The measured FIA-FSDPV peak current signal at constant time is linearly related to the concentration in the range from 0.01 to 1 mmol L^{-1} for caffeic acid and from 0.02 to 1 mmol L^{-1} for *p*-coumaric acid (r >0.98, in both cases). Calculated values prove the suitability of the proposed technique to monitor the redox species in real time. Furthermore, in comparison with classical FIA technique with amperometric detection, the use of FIA-FSDPV provides an enhanced selectivity, which enables to distinguish two compounds having different oxidation potentials without the need of previous separation step or the use of chromatographic columns. To obtained good peak resolution of measured substances on the potential axis, difference between peak potentials should exceed from 150 to 200 mV.

Methods Aplication. Last experiments aimed at verifying the suitability of the proposed procedure to quickly measure the total phenol content of olive oil extracts and then on the determination of a total capsaicin value in 9 samples of chilli extracts. Results obtained by this measurement, provides proof of the concept of the suitability of the proposed procedure for the rapid monitoring of complex sample extracts [110].

4.2.2. HPLC arrangement

Optimized FSDPV can also be combined with HPLC system, where preliminary separation of the analytes is expected [111]. One of many perspicuous advantages of this combination is its two-dimensional resolving power. One typical obtained chromato-voltammogram of standard solution of antioxidants (namely gallic acid - GA, caffeic acid - CA, syringic acid - SRA, and *p*-coumaric acid - *p*-CA) is shown in Figure 4-9. The use of HPLC-FSDPV provides an enhanced selectivity, which allows to separate substances using of chromatographic column and also to distinguish two compounds having different oxidation potentials at the same elution time, as in the case of CA and SRA (Figure 4-9). They are eluted from the column approximately at the same time, but the difference between oxidation potentials allows perfect separation of their electrochemical signals. On the other hand, *p*-CA provides two peaks during measurement, which may be caused by subsequent electrochemical reaction.

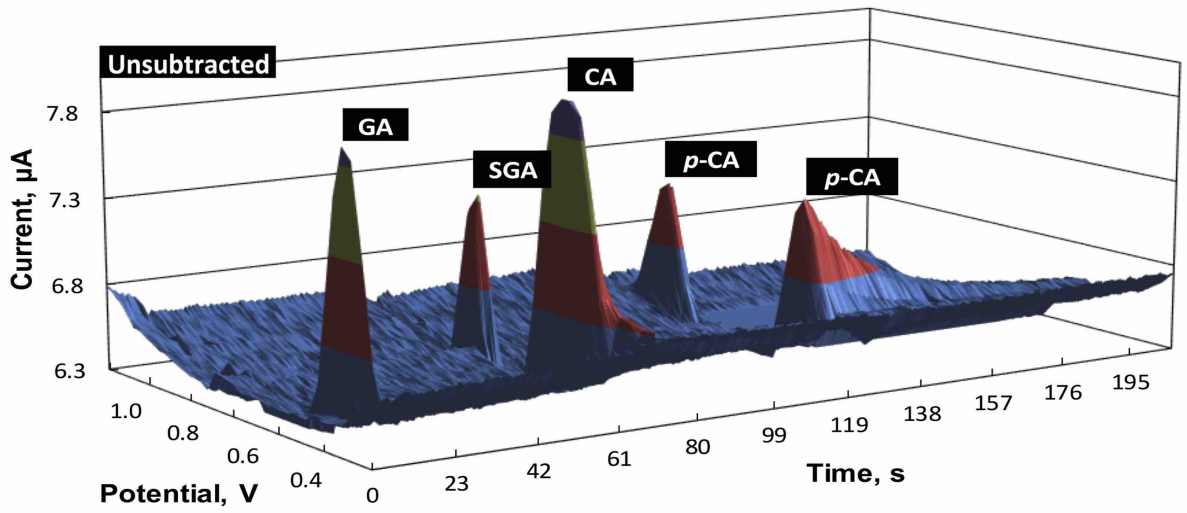

Fig. 4-9 Three-dimensional representation of unsubtracted HPLC-FSDPV recordings of one repeated injection (20 µL) of a mixture of antioxidant (GA, CA, SRA, and *p*-CA; $c = 100$ µmol L^{-1}) at GCE; mobile phase: acetonitrile:B-R buffer pH 4.0 (1:20, *v/v*); column: ATP 120 EC-C18 (3.0×0.5 cm *ID*, 2.7 µm); scan rate: 5 V s^{-1}; flow rate: 0.8 ml min^{-1}.

Effect of Background Subtraction. FSDP voltammograms obtained during a chromatographic run at low concentrations of analytes are significantly distorted by the relatively high background current upon which the faradaic current is superimposed. This can be seen in Figure 4-10A, where the FSDP voltammetric crossection of the mobile phase and that for the mixture of 10 μmol L^{-1} CA and SGA are plotted. This distortion could be avoided by the baseline subtraction. The shape of the FSDP voltammograms of the mobile phase is very reproducible, but its magnitude changes, particularly during the first 10 - 20 scans. To stabilize the FSDP voltammogram of the mobile phase prior the measurement, potential scanning was started about 20 s before injection of the sample. After that, the shape of the FSDP voltammogram of the mobile phase is reproducible and the magnitude changes are small; therefore, the FSDP voltammogram of the mobile phase can be subtracted from the total current to give a FSDP voltammogram corresponding to faradaic process as shown in Figure 4-10B; in a 3D dimension, the area consisting of voltammograms of the mobile phase is subtracted from the total current area of the sample. This subtraction technique assumes that the recorded voltammogram remains constant during the elution of all peaks. Otherwise, a FSDP voltammogram scan immediately prior to each peak can be chosen for the subtraction. The result of the subtraction of the model sample illustrated in Figure 4-9 can be seen in Figure 4-11. From the Table 4-2 it can be observed that background subtraction does not improve the repeatability and slope of the measurement (which was expected), but on the other hand, a pronounced decrease of L_Q for tested analytes was caused by the use of background subtraction.

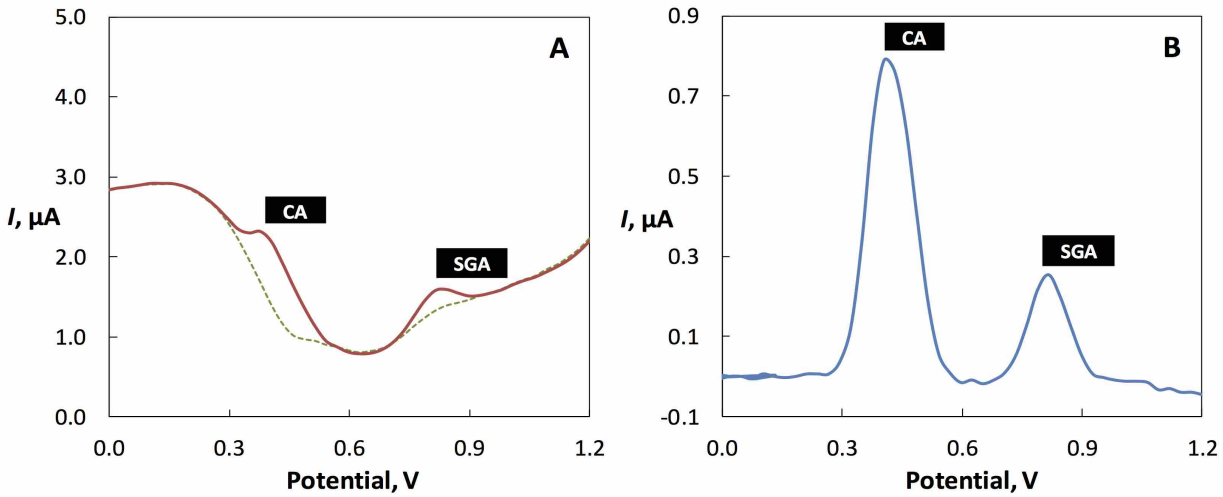

Fig. 4-10 (**A**) Unsubtracted FSDP voltammograms of the mobile phase (dotted line) and a mixture of 10 µmol L^{-1} CA and SGA (solid line) and (**B**) subtracted FSDP voltammogram; measured at GCE. For measuring conditions see Fig. 4-9.

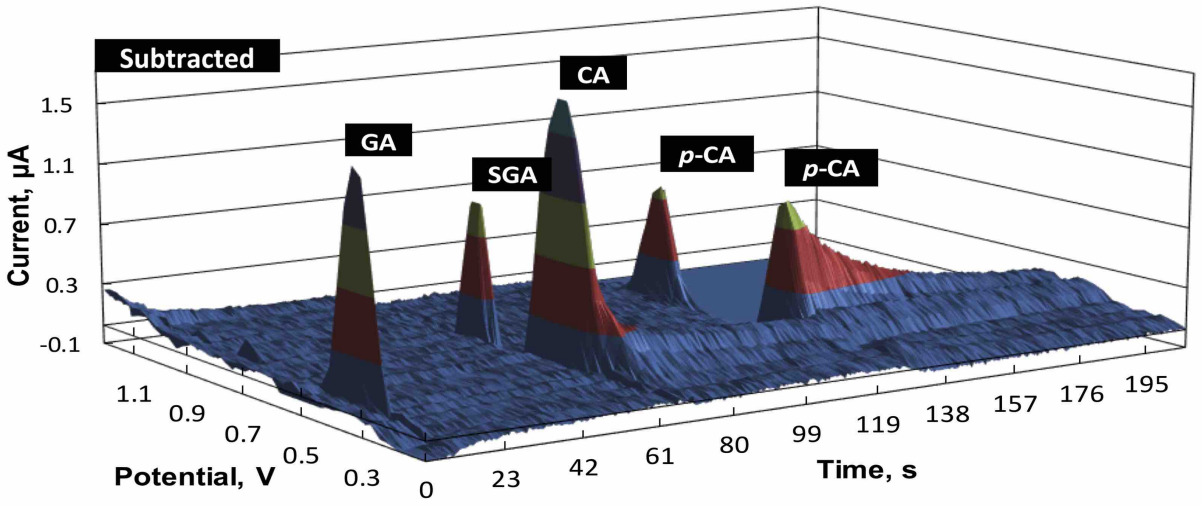

Fig. 4-11 Three-dimensional representation of subtracted HPLC-FSDPV recordings of one repeated injection (20 µL) of a mixture of antioxidant (GA, CA, SRA, and *p*-CA; c = 100 µmol L^{-1}) at GCE. For measuring conditions see Fig. 4-9.

Tab. 4-2 Parameters of calibration straight lines of the proposed HPLC-FSDPV method for the determination of tested antioxidants (unsubtracted vs. subtracted).

	Antioxidant	Concentration range μmol L^{-1}	Slope mA mol^{-1} L^{-1}	Intercept nA	Correlation coefficient	L_Q μmol L^{-1}	*RSD* (%) for 10 injections (100 μmol L^{-1})
Unsubtracted	GA	2–100	14.6	162	0.9913	16	2.48
	CA	2–100	16.1	387	0.9877	22	2.97
	SRA	2–100	11.8	98	0.9821	11	4.16
	p-CA	2–100	8.09	44	0.9859	14	3.81
Subtracted	GA	1–100	14.8	107	0.9879	1.2	2.85
	CA	1–100	17.8	197	0.9861	0.8	4.03
	SRA	1–100	12.3	66	0.9817	1.4	3.26
	p-CA	1–100	8.14	31	0.9836	1.2	3.23

The factor that could have a significant effect on the measurement itself is the passivation of the electrode surface. After the data evaluation obtained from the measurement of model sample, it can be seen, that there was no significant effect of the passivation. In the case that the above mentioned passivation occurs, the total potential program would have to be extended by the inserted pulse/s for the regeneration of the electrode surface. The efficiency of cleaning pulses increases with their insertion length [112], but in this case, there is a limitation that consists of a combination of scan rate and flow rate, which are the most important parameters for the measurement itself. The potential window and the number of current readings were limited by the 6 ms limit of the potentiostat used; the scan rate value determines the distribution of resolution between the respective axes. Increased scan rate results, besides the lower resolution of the potential axis, in the increased background current. Low scan rate, on the other hand, leads to the lower resolution of the time axis; this effect can be partly compensated by lower flow rate, i.e. slowing the measurement down to the level, where the diffusion causes unnecessary peak

broadening. From this consideration, it can be assumed that the efficiency of the cleaning pulses would be inconsistent with the sufficiently good elution force of the given system, and thus with the separation and dissolution of the substances themselves in the column. Therefore, measurement of real samples were tested without cleaning pulses to detect whether and how strong passivation of the electrode could occur during the analysis.

Methods Aplication. Finally, determination of antioxidants in 8 samples of tea extracts was selected as a suitable problem for testing the performance of the newly developed technique. Antioxidants such as gallic acid - GA, affeic acid - CA, rutin - Ru, sinapic acid - SPA, and ferulic acid - FA are well-known species present in various kinds of tea. Separation was performed according to the article [62] and the samples were also analyzed by HPLC-DAD [113] as a comparative method. Four obtained chromato-voltammograms of the selected samples of tea extracts are shown in Figure 4-12; some of the antioxidants (marked in Figure 4-12) have been identified based on their UV spectra. Other antioxidants or/and interfering substances presented in tea had no influence on the peak currents of the detected compound. We can observe the tailing of some peaks, caused probably by the deposition of the product of the electrode reaction on the surface of the electrode. In this case, inserted cleaning pulses could eventually help, but with all the disadvantages associated with it, which are discussed in the previous paragraph. As the passivation of the electrode surface was not too pronounced, it was concluded that all further measurements with real samples would be carried out without insertion of the cleaning pulses. Also, comparison of the potentials of the mentioned five peaks with potentials obtained from cyclic voltammetry and differential pulse voltammetry (under the same experimental conditions) in unstirred solutions was performed [75,114–118], and it was found that the peaks in the flow system were shifted by about 50 mV to a more positive potential.

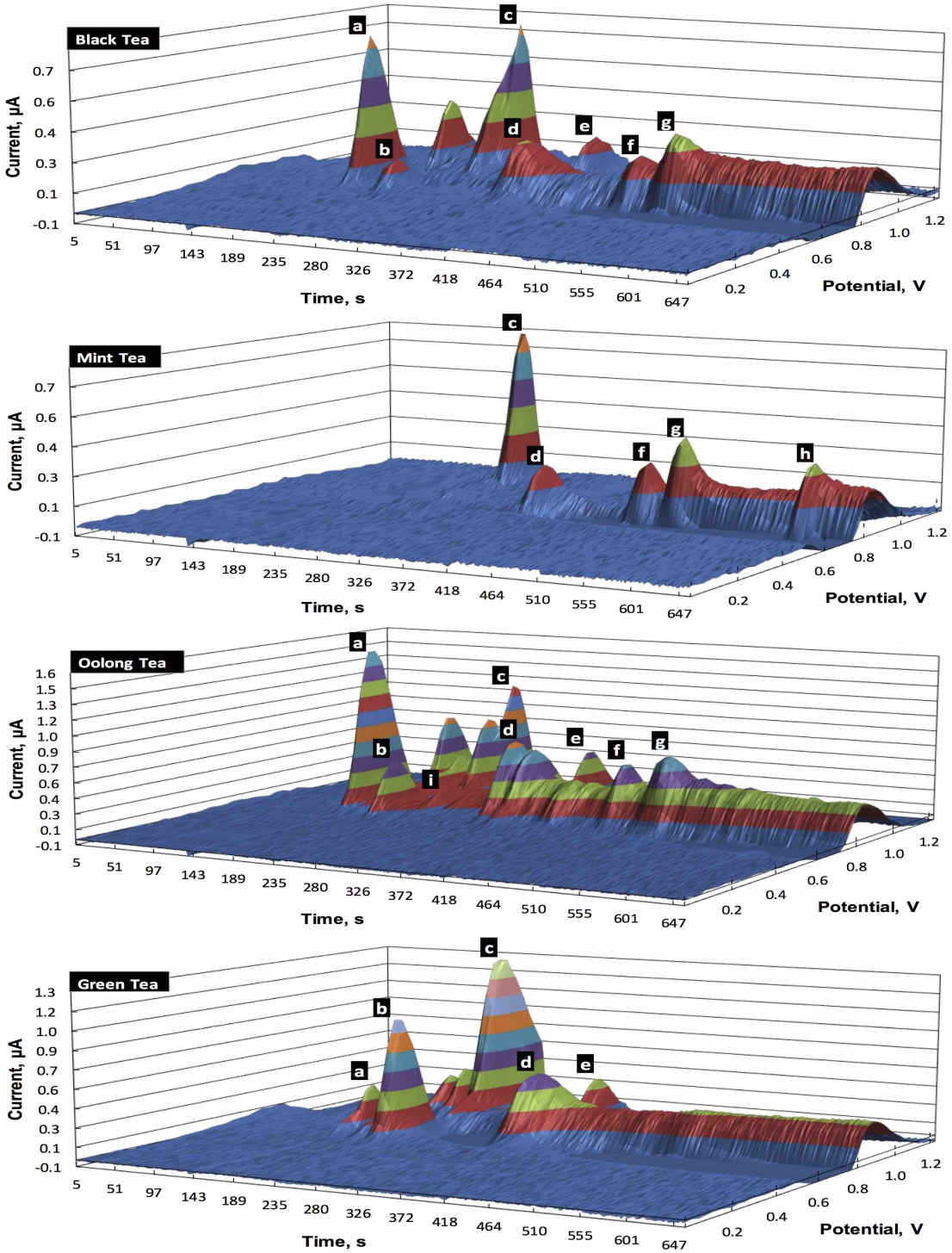

Fig. 4-12 Three-dimensional representation of subtracted HPLC-FSDPV recordings of one repeated injection (20 µL) of the selected samples of tea extracts (gallic acid - **a**, gallocatechin or epigallocatechin - **b**, catechin - **c**, caffeic acid - **d**, epicatechin or epicatechin gallate - **e**, rutin - **f**, sinapic acid - **g**, chlorogenic acid - **h**, 3-hydroxybenzoic acid - **i**; compounds **b**, **c**, **e**, **h,** and **i** are predicted from their UV spectra.) at GCE; mobile phase: acetonitrile:phosphate buffer pH 2.5 (0 min: 15:85; 11 min: 30:70, *v/v*); column: Purospher STAR RP-C18 (12.5×0.4 cm *ID*, 5.0 µm); scan rate: 5 V s^{-1}; flow rate: 0.8 ml min^{-1}.

5. Conclusion

In submitted Ph.D. thesis, two types of detection techniques in combination with flow systems were tested, characterized in many possible ways and obtained results were compared with others commonly used techniques, such as HPLC with DAD, ED or MS detection. Moreover, their practical applicability was verified by the development of the methods for the determination of natural and synthetic antioxidants in model samples and real matrices. Their detection is of special importance for food manufacturer that have to identify the content of such substances for their eventual declaration.

The obtained results can be summarized as follows:

FIA - MPA

- Potential program for an MPA was developed and optimum conditions for electrochemical detection of natural and synthetic antioxidants in combination with the flow system were found.

- MPA detection with GCE as a working electrode coupled to wall-jet configuration in FIA system was successfully applied for simultaneous determination of two (namely SA and Ty) and three antioxidants (namely *t*BHQ, PG, and BHA).

- The detection technique in combination with FIA system provides short analysis time, low consumption of reagents and samples; high precision (*RSD* <4.0%, for all measured substances) and linear calibration curves (r >0.99, in all cases) across two concentration ranges from 1.0 to 100 μmol L^{-1}. The limits of quantification were around 1 μmol L^{-1}. Furthermore, the method requires simpler instrumentation and provides lower investment and running cost in comparison with other commonly used techniques, e.g. HPLC with ED, DAD

or MS detection, typically applied for simultaneous determinations of more than one antioxidant.

- Lastly, this newly developed method was successfully applied for simultaneous determination of *t*BHQ, PG, and BHA in chewing gum samples by applying a simple extraction procedure.

FIA / HPLC - FSDPV

- Potential program for a FSDPV was developed together with optimization procedure for characterization of the electrochemical detection of natural antioxidants in combination with the flow systems.

- To find out the overall optimization parameters of the method in this arrangement, the well-known redox system, such as HQ / Q was used.

- The newly developed detection technique in combination with flow systems presented good correlation with traditional methods used for the determination of natural antioxidants in real matrices, such as HPLC with DAD.

- The main advantages of the FSDPV in combination with FIA system are small amount of the consumed sample and speed of measurement, enabling to obtain a complete voltammogram each 10 s, together with the selectivity provided by the electrochemical part of the measurement. Under optimal experimental conditions, the selectivity of the measurement is sufficient to distinguish several voltammetric responses in the potential window over time.

- The main advantage of proposed setting with HPLC system is detection of substances with similar retention times, but with a different potential of oxidation. Another important point of this work is the successful solving of the main difficulties encountered during the measurements. On the other hand, disadvantage can be the higher limit of quantification (around 1 μmol L^{-1}), which, nevertheless, does not negatively influence the applicability of the technique for the determination of antioxidants in various matrices.

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Publication I

Combination of Flow Injection Analysis and Fast Scan Differential Pulse Voltammetry for the Determination of Antioxidants

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Combination of Flow Injection Analysis and Fast Scan Differential Pulse Voltammetry for the Determination of Antioxidants

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Abstract: Fast-scan differential pulse voltammetry (FSDPV) is an electroanalytical technique that uses high scan rate to record voltammograms within several milliseconds and ensures high temporal resolution. Here, a FSDPV on a glassy carbon working electrode in combination with a flow injection analysis (FIA) system was developed and characterized using the hydroquinone/quinone redox system. Later, enhanced resolution of the technique was confirmed with the parallel determination of caffeic acid and *p*-coumaric acid. Finally, the optimized procedure has been applied for the first time to determine capsacinoids in chili pepper and total phenols in extra virgin olive oils. The proposed procedure is fast, simple, and enables the monitoring of complex samples in real-time.

Keywords: flow injection analysis • fast scan differential pulse voltammetry • glassy carbon electrode • antioxidants

1 Introduction

With the increasing pressure of industry to monitor and control manufacturing processes, there is a growing demand to establish reliable tools and systems capable of meeting this need. The challenge for research is to translate such needs into robust instrumentation capable of monitoring the critical process parameters in real time. In addition, due to the recent advances in signal processing, there is a growing interest in the development of analytical systems that can record in real-time a huge amount of multivariate signals and transform them into the relevant key-parameters of the process.

This approach is popular nowadays for the spectrometric detection techniques, but it is less frequent for the electrochemical detection. Occasionally, multiple electrodes are used in the so-called electronic tongue systems, where several sensors, made by different metals or polarized with different potentials, are applied on the same sample to achieve a multivariate electroanalytical signal and, thus, enhance the discriminatory capacity of the system. However, this setup has also the disadvantage because it increases the complexity of the instrumentation.

Alternatively, traditional single electrode systems can be also used to achieve multivariate signals in the so-called fast-scan voltammetry technique, where the electrode potential is quickly raised and lowered in a triangular wave fashion to allow the rapid acquisition of voltammograms within several milliseconds and ensures high temporal resolution. This approach was tested several times in the past, first on traditional glassy carbon electrodes and on microelectrode arrays [1], later particularly on microelectrodes due to the better suppression of the capacitance current [2–3]. Besides the combination with HPLC and flow injection analysis (FIA), the technique was also applied in capillary zone electrophoresis [4]. Selected potential programs were compared, including normal pulse voltammetry, staircase voltammetry [1], cyclic voltammetry [4], and square-wave voltammetry [5]. The studies were generally aimed to the determination of neurotransmitters, hormones, and metabolites in biological systems. Besides, this approach enables the determination of even electrochemically inactive species by observing their adsorption on the electrode [6–8].

Despite its long success, fast scan voltammetry techniques have a number of problems, namely the large background current, distorted ohmic drop, instrument low-pass filtering and distortion caused by the cell time constant [9]. Such problems are derived mainly because, under conditions of linear diffusion, the faradaic current (i_f) for a simple redox reaction increases with the square root of the scan rate, while the current arising from charging the double layer (i_c) increases with scan rate. This, in turn, decreases the i_f/i_c ratio as the scan rate is increased, resulting in lower analytical performance.

Although these drawbacks at fast sweep rates can be minimised by background subtraction, the subtracted signal remains distorted by the ohmic drop resulting from the background current. Furthermore, complications of the electron transfer at fast sweep rates can be encoun-

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tered when the technique is applied not only to simple redox reactions but to more complex real samples. Not surprisingly, to date, the application of fast scan voltammetry on the determination of bioactive food polyphenols has received very little attention in the research literature.

In this study, fast scan differential pulse voltammetry (FSDPV) in connection with flow injection analysis (FIA) has been investigated using hydroquinone, *p*-coumaric acid, and caffeic acid as model compounds and then applied for the first time for the determination of capsaicinoides, compounds responsible for the pungency of chili pepper [10] (structures of the used compounds are in Fig. 1). Furthermore, as a second application, FSDPV with FIA has been applied for the determination of total phenol content in extra virgin olive oils [11–12]. The potential advantages and limitations of the proposed procedure are assessed, giving particular emphasis on the easy sample handling, high throughput of the samples, and the selectivity for the determination in food analysis.

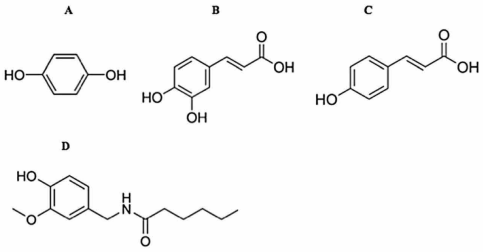

Fig. 1. Structure of hydroquinone (A), p-coumaric acid (B), caffeic acid (C), and capsaicin (D).

2 Experimental

2.1 Reagents

Hydroquinone (CAS Number: 123–31–9), gallic acid (CAS Number: 149–91–7), *p*-coumaric acid (CAS Number: 501–98–4), caffeic acid (CAS Number: 331–39–5), and capsaicin (CAS Number: 404–86–4) were supplied by Sigma-Aldrich. The stock solutions ($c = 1\ \mathrm{mmol\,L^{-1}}$) were prepared by dissolving the exact amount of the respective substance in deionized water with addition of B-R buffer solutions of appropriate pH, or in acetonitrile:ethanol mixture (both from Fluka) (1:1, *v/v*) containing 0.1 mM lithium perchlorate (Sigma-Aldrich), according to the medium in which the measurement was performed. All stock solutions were kept at low temperature in the dark. More diluted solutions were prepared by exact dilution of the stock solutions with above mentioned aqueous buffer or acetonitrile-ethanol mixture.

Voltammetric experiments in aqueous media were carried out in B-R buffer solutions prepared by mixing 0.2 M sodium hydroxide (Fluka) with acidic solution consisting of 0.04 M boric acid, 0.04 M phosphoric acid, and 0.04 M acetic acid (all by Sigma-Aldrich).

Voltammetric experiments in a non-aqueous medium were carried out in the mixture of acetonitrile:ethanol, (1:1, *v/v*) containing 0.1 mM lithium perchlorate.

Other used chemicals were sodium carbonate (Sigma-Aldrich), potassium chloride (Sigma-Aldrich), Folin-Ciocalteu reagent (Sigma-Aldrich) and deionized water (Millipore Q-plus System, Millipore, USA). All used chemicals were of analytical grade purity.

2.2 Electrode Preparation

The three-electrode wall-jet system was used for FSDPV. Glassy carbon electrode (GCE) (Metrohm, Switzerland, diameter of 2 mm and geometric area 3.1 $\mathrm{mm^2}$) was used as a working electrode. Before each run, the surface of GCE was polished for 1 min with alumina (particle size 1.0 μm). After polishing, the electrode was thoroughly washed with distilled water. Ag/AgCl (3 M KCl) reference electrode (Monokrystaly Turnov, Czech Republic) was used for all measurements in aqueous media; non-aqueous silver/silver ion reference electrode (0.01 M $AgNO_3$; 0.1 M TBAP in acetonitrile) (BASi, USA) was used in non-aqueous media. The auxiliary electrode was made of a Pt wire, 1 cm in length and 0.5 mm in diameter.

2.3 Apparatus

FIA measurements were performed using a high pressure pump Waters 515 HPLC pump (Waters Corporation, USA) and a six-way injection valve (Supelco Rheodyne Model 7725i) with a 100 μL sample injection loop. FSDPV detection was performed using a Twelve Channel Multi Autolab Potentiostat/Galvanostat, controlled by NOVA version 10.2 software (Metrohm, Switzerland) working under Windows 7 (Microsoft Corporation). A Basic 20+ pH meter (Crison Instruments, Spain) equipped with a combined glass pH electrode was used for pH measurements. The pH meter was calibrated with aqueous buffers at a laboratory temperature. The spectrophotometric measurements were performed using UV/Vis spectrophotometer G9820A (Agilent Technologies, USA) in 1 mm quartz cuvettes.

2.4 FIA Procedures

FSDPV was performed with the following parameters: pulse width 100 ms, pulse amplitude 50 mV, and scan rate $5\ \mathrm{V\,s^{-1}}$. Reverse LSV scan with the same scan rate was also included. The flow rate of the carrier solution during the experiments was set to 0.8 mL/min. All these parameters were set up after the optimization of detection system.

All the injections were repeated three times unless stated otherwise and the measurements were carried out at laboratory temperature.

The peak height was evaluated from DPV recordings. The limit of quantification (L_Q) was calculated as the analyte concentration corresponding to a tenfold standard deviation of the respective response from ten consecutive determinations at the lowest measurable concentration [13].

2.5 Sample Preparation

The olive oils used for measurements were purchased at local store in Bolzano, Italy. Fruits of the chili pepper varieties in ripe state were provided by Xundgarten; St. Jakob, Leifers.

Analyzed extracts of olive oil were prepared by shaking 10 ml of oil with 5 ml of 0.1 M $LiClO_4$ in acetonitrile:ethanol (1:1) mixture for five minutes. After the phase separation, upper part was collected and used directly.

The chili peppers were heat-dried at 65 °C and grounded by vibrational mill. The extraction of capsaicinoides was performed by stirring of 0.2 g of pepper powder with 10 mL of acetonitrile for 15 min under room temperature. The aliquots were then filtered using 0.45 µm membrane filters and diluted ten times by the carrier solution before injection.

2.6 Folin-Ciocalteau Micro Method for Total Phenol in Oil

Ten calibration stock solutions from 0 to 500 mg L^{-1} of gallic acid were prepared. Each calibration solution and blank were mixed with water, Folin-Ciocalteu reagent and sodium carbonate solutions, as described in [14]. After 2 h at 20 °C the absorbance of each solution was measured at 765 nm against the blank.

3 Results and Discussion

3.1 Fast Scan Differential Pulse Voltammetry

Preliminary experiments were aimed at characterizing the electrochemical detection system based on FSDPV using hydroquinone/quinone as model redox system. Basic parameters, such as the scan rate of the electrochemical detection and the resulting peak potential, peak height, and background current were evaluated together with other parameters of the flow system, such as flow rate and injected volume.

The most important parameter for the optimized procedure is the scan rate. The potential window and the number of current readings were limited by the 6 ms limit of the potentiostat used; the scan rate value determines the distribution of resolution between the respective axes. Increased scan rate results, besides the lower resolution of the potential axis, in the increased background current. Low scan rate, on the other hand, leads to the lower resolution of the time axis; this effect can be partly compensated by lower flow rate, i.e. slowing the measurement down to the level, where the diffusion causes unnecessary peak broadening. Scan rate from 1 to 10 V s^{-1} and flow rate from 0.4 to 2 ml min^{-1} were tested and values of scan rate of 5 V s^{-1} and flow rate of 0.8 ml min^{-1} were selected as optimal.

The optimization of the injected volume was done similarly to any FIA system, i.e. the optimal injection volume was selected as the maximum volume above which the peak does not further increase its height, but only broadens. In this case, injection volumes from 20 to 200 µL were tested and the optimum value of 100 µL was selected. Under such optimized conditions, the injection of a blank sample provided negligible response.

Fig. 2 shows the resulting three-dimensional FIA-FSDPV recordings of hydroquinone oxidation measured under the optimized conditions; the peaks are well defined and the relative standard deviation (RSD) of their height is 3.5 % (n = 10).

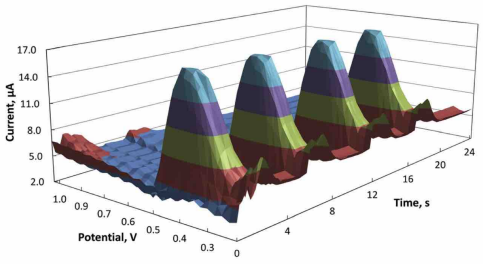

Fig. 2. Three-dimensional representation of FIA-FSDPV recordings of 0.1 mmol L^{-1} hydroquinone with addition of 0.1 mol L^{-1} KCl; scan rate 5 V s^{-1}, flow rate 0.8 ml min^{-1}, four repeated injections of 100 µL. Detection at GCE in BR buffer (pH 4).

3.2 Simultaneous Determination of Caffeic Acid and *p*-Coumaric Acid

After having defined the best conditions for the determination of a single redox species, we have paid attention to the enhanced spatial resolution offered by FSDPV for the simultaneous detection of caffeic acid and *p*-coumaric acid. These two redox species are common antioxidants found in many vegetable products, including wine. Also, their peak potentials differ of about 0.35 V, which is large enough to be resolved by batch voltammetric techniques. Therefore, this determination allows us to explore the real peak resolution on the potential axis.

FIA-FSDPV recordings of a series of solutions of these individual compounds and in various concentration ratios were measured in non-aqueous medium of acetonitrile:ethanol mixture (1:1, *v/v*) containing 0.1 mmol L^{-1} of lithium perchlorate under the previously optimized conditions. Selected record is shown in Fig. 3. High background current is attributed to the high scan rate, but also to the non-aqueous supporting electrolyte; nevertheless, the background magnitude is constant. The relative standard

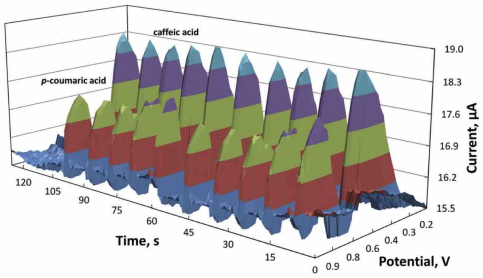

Fig. 3. Three-dimensional representation of FIA-FSDPV recordings of ten times repeated injection of the mixture of caffeic acid (A) and *p*-coumaric acid (B), $c = 1 \times 10^{-3}$ mol L^{-1}. Measured at GCE in a mixture of acetonitrile:ethanol, (1:1, *v/v*) containing 0.1 mmol L^{-1} of lithium perchlorate.

deviation of the peak heights was 3.3% for caffeic acid and 3.8% for *p*-coumaric acid (n=10), which confirms good stability and repeatability of the measurements.

The measured FIA-FSDPV peak current signal at constant time is linearly related to the concentration in the range from 0.01 to 1 mmol L^{-1} for caffeic acid and from 0.02 to 1 mmol L^{-1} for *p*-coumaric acid (Fig. 4). The correlation coefficients close to one and the limit of quantification 15 µmol L^{-1} and 26 µmol L^{-1} for caffeic acid and *p*-coumaric acid, respectively (Table 1), prove the suitability of the proposed technique to monitor the redox species in real time. Furthermore, in comparison with classical FIA technique with amperometric detection, the use of FIA-FSDPV provides an enhanced selectivity, which enables to distinguish two compounds having different oxidation potentials without the need of previous separation step or the use of chromatographic columns.

3.3 Determination of Capsaicin in Chili Pepper Extracts

Determination of capsaicinoides in chili peppers was selected as a suitable problem for testing the performance of the technique. Capsacinoids such as capsaicin are well-known redox species presenting a pH dependent anodic peak corresponding to one electron/one proton reaction mechanism [15]. Furthermore, their fast detection is of special importance for food manufacturers and pharmaceutical industries that have to dose the potency of such ingredient in their formulations or premixes.

The response of standard solutions of capsaicin (from 0.5 to 0.01 mmol L^{-1}) is shown in Fig. 6A. The position of the peak, i.e. 0.6 V, is the same as observed during the measurement by classical batch cyclic voltammetry. As

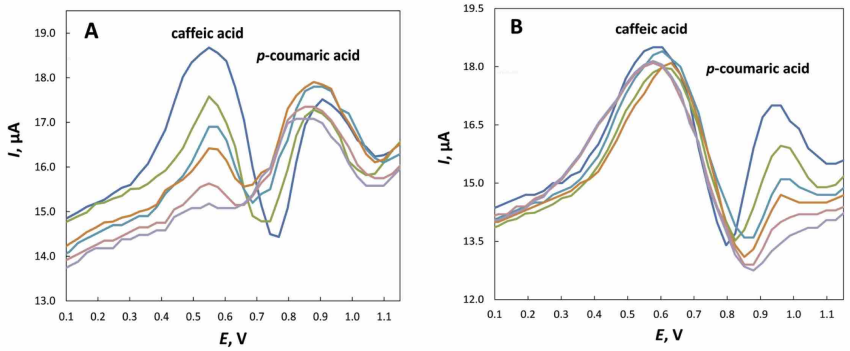

Fig. 4. FIA-FSDP voltammograms of caffeic acid (0.02, 0.06, 0.1, 0.2, 0.6, and 1 mmol L^{-1}) in the presence of 1 mmol L^{-1} p-coumaric acid (A) *p*-coumaric acid (0.02, 0.06, 0.1, 0.2, 0.6, and 1 mmol L^{-1}) in the presence of 1 mmol L^{-1} caffeic acid (B) at GCE in a mixture of acetonitrile:ethanol, (1:1, *v/v*) containing 0.1 mmol L^{-1} of lithium perchlorate.

Table 1. Parameters of calibration curves and limits of quantification of caffeic acid, *p*-coumaric acid, and capsaicin measured in a mixture of acetonitrile:ethanol, (1:1, *v/v*) containing 0.1 mmol L^{-1} of lithium perchlorate; obtained by FIA-FSDPV at GCE, variability expressed as standard deviation.

Substance	Concentration range mmol L^{-1}	Slope mA mol^{-1} L	Intercept nA	Correlation coefficient	L_Q mmol L^{-1}	RSD (%), n=10
caffeic acid	0.01–1	3.78±0.09	422±13	0.9823	15±0.5	3.3 (1 mmol L^{-1})
p-coumaric acid	0.02–1	2.36±0.07	521±15	0.9844	26±0.6	3.8 (1 mmol L^{-1})
capsaicin	0.01–0.5	6.32±0.21	462±11	0.9829	16±0.5	4.0 (0.5 mmol L^{-1})

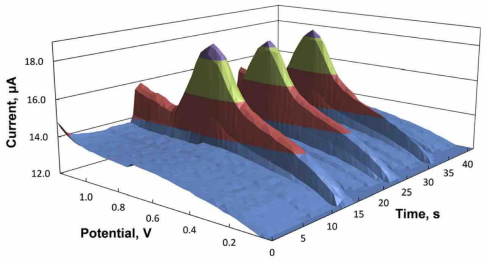

Fig. 5. Three-dimensional representation of FIA-FSDPV recordings of three repeated injection of the selected sample of chili pepper extract. Measured at GCE in a mixture of acetonitrile:ethanol, (1:1, *v/v*) containing 0.1 mmol L^{-1} of lithium perchlorate.

observed before, the variability of the peak heights for the same concentration of capsaicin standard (0.5 mmol L^{-1}) is below 4% (n=10). The figures of merit of the applied procedure for the detection of capsaicin are summarized in Table 1, confirming the linear dependence of the peak current on increasing concentration of the standard and L_Q of 16 µmol L^{-1}.

The procedure was then applied on the determination of a total capsaicin value in 9 samples of chili pepper extracts (Fig. 5). The graphical comparison of the results obtained with the FIA-FSDPV and with HPLC with spectrophotometric detection is shown in Figure 6B. Parameters of the dependence are *c*(electrochemical)= 0.9549.*c*(HPLC) - 82.585 (R^2=0.9451), confirming the high correlation between the two methods. Also, this result provides proof of the concept of the suitability of the proposed procedure for the rapid monitoring of complex sample extracts.

3.4 Determination of Total Phenol in Olive Oils

Last experiments aimed at verifying the suitability of the proposed procedure to quickly measure the total phenol content of olive oil extracts. Fig. 7A shows the resulting FIA-FSDPV recordings of olive oil extracts. In contrary to the expectations, only single peak with the potential of approx. 0.75 V was observed in the voltammogram; for that reason, suitable potential range for the charge calculation was not sought, but the peak area was evaluated instead. For the calculation of phenol content, concentration dependence of gallic acid measured under the same conditions was used. For comparison, the total phenol values obtained by the Folin-Ciocalteu (FC) assay, a popular spectrometric method used in quality control, are also reported (Fig. 7B). The correlation between the two methods is not too good, showing that the results obtained by FSDPV are always lower than those obtained by the FC. This can be explained by the presence of redox compounds in the test solution that react positively to the Folin-Ciocalteu test, but have oxidation potential higher than the potential window used in our procedure.

4 Conclusions

This study presents a newly developed method of capsaicin determination and determination of total polyphenols content in olive oils performed by FSDPV in FIA system, using GCE as a working electrode. To find out the overall optimization parameters of the method in this arrangement, the well-known redox system hydroquinone/quinone was used. The best measurement conditions with scan rate 5 Vs^{-1}; flow rate 0.8 ml min^{-1}; injection volume 100 µL were found. Under these conditions, the selectivity of the measurement is sufficient to distinguish several voltammetric responses in the potential window.

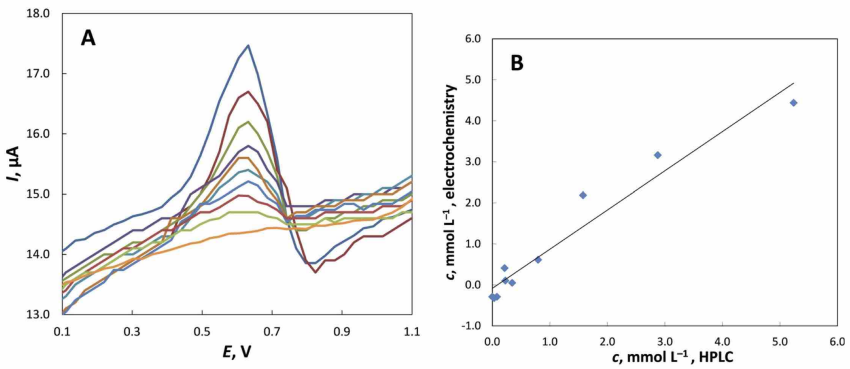

Fig. 6. (A) FIA-FSDP voltammograms of concentration dependence of capsaicin at GCE in a mixture of acetonitrile:ethanol, (1:1, v/v) containing 0.1 mmol L^{-1} of lithium perchlorate (concentrations of analyte 0, 0.01, 0.02, 0.04, 0.06, 0,08, 0.1, 0.2, 0.4 and 0.5 mmol L^{-1}); (B) correlation of capsaicin concentration in analyzed samples of chili pepper obtained by FIA-FSDPV and by HPLC with diode array detection.

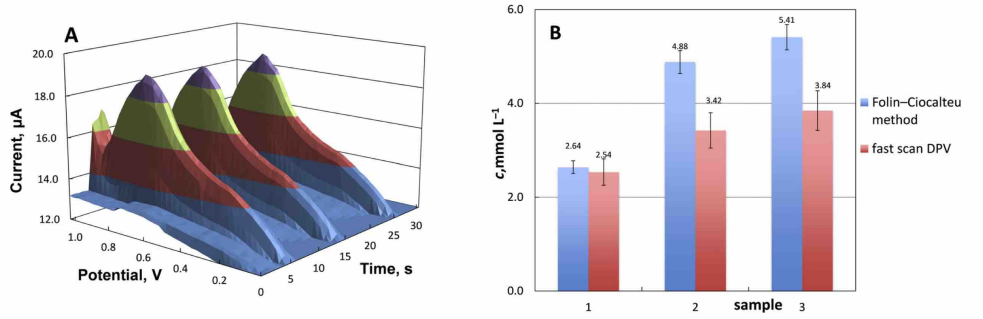

Fig. 7. (A) Three-dimensional representation of FIA-FSDPV recordings of three repeated injection of the selected sample of olive oil extract. Measured at GCE in a mixture of acetonitrile:ethanol, (1:1, *v/v*) containing 0.1 mmol L^{-1} of lithium perchlorate. (B) Comparison of total phenol content in analyzed samples of olive oils obtained by FIA-FSDPV and by Folin-Ciocolteu micro method.

The newly developed method presented good correlation with traditional methods used for the determination of capsaicin, HPLC with diode array detection; the correlation of the total polyphenols content in olive oils is less tight, probably due to the presence of compounds with detection potential outside of the applied range.

The main advantages of the FSDPV are small amount of the consumed sample and speed of measurement, enabling to obtain a complete voltammogram each 10 s, together with the selectivity provided by the electrochemical part of the measurement. On the other hand, disadvantage of the technique can be the higher limit of quantification (20 µmol L^{-1}), which, nevertheless, does not negatively influence the applicability of the methods for the determination of total polyphenols in olive oils and capsaicin in chili peppers.

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Publication II

Simultaneous determination of tert-butylhydroquinone, propyl gallate, and butylated hydroxyanisole by flow-injection analysis with multiple-pulse amperometric detection

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Simultaneous determination of *tert*-butylhydroquinone, propyl gallate, and butylated hydroxyanisole by flow-injection analysis with multiple-pulse amperometric detection

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ABSTRACT

We report the first amperometric method for the simultaneous determination of *tert*-butylhydroquinone (tBHQ), propyl gallate (PG), and butylated hydroxyanisole (BHA) using flow injection analysis coupled to multiple-pulse amperometry. A sequence of potential pulses was selected in order to detect tBHQ, PG, and BHA separately in a single injection step at a glassy carbon electrode without the need of a preliminary separation. A mixture of methanol and 0.040 M Britton-Robinson buffer was used both as a carrier solution and for dilution of analyzed solutions before injection. The method is precise ($RSD < 5\%$, $n = 10$), fast (a frequency of 140 injections h^{-1}), provides sufficiently low quantification limits (2.51, 1.45, and 0.85 μmol L^{-1} for tBHQ, PG, and BHA, respectively) and can be easily applied without high demands on instrumentation. As a practical application, the determination of these antioxidants contained in commercial chewing gum samples was carried out by applying a simple extraction procedure.

1. Introduction

Synthetic phenolic antioxidants are extensively used in the food industry as additives to improve the stability of various products, especially for the prevention of lipid oxidation reactions, responsible for the production of volatile compounds with unpleasant flavours. Among the most commonly used additives are propyl gallate (PG), *tert*-butylhydroquinone (tBHQ), and butylated hydroxyanisole (BHA), used alone or together (Fig. 1). In many countries, the use of these antioxidants is controlled by official legislation, and consequently, it is important to be able to determine reliably the amounts of these substances in food products. Last but not least, determination of antioxidants, and eventually, mixtures of antioxidants (PG, tBHQ, and BHA) can provide important information on the quality of food products, because the concentration of antioxidants may be related to their oxidation stability.

Many electrochemical methods, such as cyclic voltammetry [1], differential pulse voltammetry [1,2], stripping voltammetry [3], and square-wave voltammetry [1,4] have been used to determine phenolic antioxidants. Also the adsorptive preconcentration of synthetic antioxidant at a carbon paste electrode has been described [5]. All these techniques generally have high sensitivity, and are widely used in many areas of analytical chemistry. However, their applicability for the determination of several components in mixtures is limited when the recorded voltammograms display significant partial overlapping.

As a result, techniques preceded by a separation step, particularly HPLC with electrochemical [6], DAD [7] or MS [8] detection are most frequently used for the determination of the mixture of antioxidants. Usually, HPLC may be employed for the separation of the analytes previously to their quantification, or two or more sensors are used with the application of a different constant potential at each sensor, whose resulting signals are analyzed with a multivariate calibration method. However, application of such complex separation methods might not be necessary in many cases and flow injection analysis (FIA) in combination with a selective detection method might present a suitable alternative. Multiple-pulse amperometry (MPA) has been used for the simultaneous determination of different analytes [9–11]. It involves the application of an appropriate potential waveform consisting of a suitable succession of pulses on a single working electrode, thus allowing to distinguish the analytes in a mixture with no need of separation, chemical pretreatment of the sample or electrode modification, or the application of mathematical techniques for data analysis. This strategy was used for simultaneous determination of sugars [12], drugs [13–15], antioxidants [16], synthetic colorants [17], as well as for the use of

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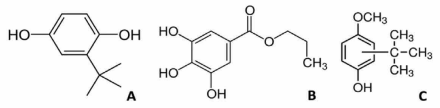

Fig. 1. Structure of *tert*-butylhydroquinone (**A**), propyl gallate (**B**), and butylated hydroxyanisole (**C**).

internal standard method in FIA [18]. This method has some important advantages: it is inexpensive, simple, has small sample and reagent consumption (with reduction of waste generation) and high sampling rates. Considering the number of injections that can be done in one hour, less than 1 mL of carrier solution (i.e. 100 μL of methanol) is consumed per one sample. Moreover, the use of MPA detection can prevent contamination of the working electrode surface by inserting cleaning pulses in the potential program.

The purpose of this paper is to report a MPA-FIA method using a single working electrode for simultaneous determination of three compounds (tBHQ, PG, and BHA). The novelty of the newly developed method rests in the continuous application of three potential pulses (with simultaneous acquisition of three separate amperograms) at a single-injection step. It was confirmed that this approach enables the determination of the tested antioxidants contained in chewing gum as an example of practical application of the new method.

2. Experimental

2.1. Reagents

Propyl gallate (CAS Number: 121-79-9), *tert*-butylhydroquinone (CAS Number: 1948-33-0), and butylated hydroxyanisole (CAS Number: 25013-16-5) were supplied by Sigma-Aldrich. Their individual stock solutions (c = 1.00 mmol L^{-1}) were prepared by dissolving the exact amount of the respective substance in methanol (Merck Millipore, Germany) and were kept at 4 °C. More diluted solutions were prepared by exact dilution of the stock solutions with mixture of methanol and 0.040 M Britton-Robinson (B-R) buffer (1:9, *v/v*). All electrochemical measurements were carried out in the same solution. The B-R buffer was prepared by mixing 0.20 M sodium hydroxide (Lach-Ner Neratovice, Czech Republic) with acidic solution consisting of 0.040 M boric acid (Lach-Ner Neratovice, Czech Republic), 0.040 M phosphoric acid (Merck Millipore, Germany) and 0.040 M acetic acid (Merck Millipore, Germany). All chemicals used for buffer preparation were of analytical grade purity. Distilled water was provided from a Mega-Pure 3A Liter Automatic Distillation System, USA.

2.2. Instrumentation and apparatus

All electrochemical recordings were performed using an Autolab PGSTAT12 potentiostat/galvanostat, controlled by NOVA version 1.11.2 software (Metrohm, Switzerland) working under Windows 7 (Microsoft Corporation). The three-electrode wall-jet configuration described in our previous paper [19] included a glassy carbon working electrode (GCE) (Metrohm, Switzerland, diameter of 2 mm and geometric area 3.1 mm^2), a platinum wire, 1 cm in length and 0.5 mm in diameter, as a counter electrode, and an Ag/AgCl (3 M KCl) electrode as a reference electrode (Monokrystaly Turnov, Czech Republic). Flow of the carrier solution was provided by peristaltic pump MINIPULS Evolution (Gilson, USA) and injection of the sample was performed with a six-way injection valve (VICI Valco Instruments, Canada) equipped with a 100 μL sample injection loop. An ultrasonic bath (Ultrasonic PS 02000A, DANAE VISION, Czech Republic) was used during the sample preparation and an Orion 266S pH meter (Thermo Fisher Scientific, USA) equipped with a combined glass pH electrode was used for pH measurements. The pH meter was calibrated with aqueous standard buffer solutions at room temperature.

2.3. Procedures

Pre-treatment of the GCE was done by polishing with alumina powder suspension (0.1 μm) on a damp polishing cloth (Metrohm, Switzerland) before fixing to the flow cell. This procedure was performed at the beginning of the working day.

Hydrodynamic voltammograms of tBHQ, PG, and BHA were obtained separately by application of eleven sequential potential pulses (from +0.20 to +0.70 V; pulse width: 100 ms) in triplicate injections of standard solutions through the FIA system using the MPA technique. The same technique was used for simultaneous amperometric detection of tBHQ, PG, and BHA, applying pulses of +0.40 V for 100 ms, +0.55 V for 100 ms, and +0.70 V for 100 ms continuously, and sampling the current once during each potential pulse. Thus, the current was sampled in each amperogram every 300 ms (total time of the potential waveform). The peak width under the proposed conditions is approximately 12 s, the current was therefore sampled around 40 times during each peak.

The samples analyzed were chewing gums purchased in a local supermarket; the quantity of synthetic antioxidants was not declared. About 1.5 g of finely cut sample was extracted with 5.0 mL of methanol in the ultrasonic bath for 15 min. Afterwards, the mixture was placed at 4 °C for 2 h in order to precipitate the gum-base polymer components. Then, 1.0 mL aliquot of the supernatant solution was mixed with B-R buffer pH 2.0 (1:1 *v/v*), filtered through a syringe filter (Nylon (PA), 0.45 μm) and injected into the system [20].

The peak height (I_p) was evaluated from the amperometric FIA recording. The limit of quantification (L_Q) was calculated as the analyte concentration corresponding to a tenfold standard deviation of the respective response from ten consecutive determinations in the lowest measurable concentration range [21].

3. Results and discussion

3.1. Determination of the peak potential

Previous electrochemical investigations of phenolic antioxidants (which includes also tBHQ, PG, and BHA) using GCEs have demonstrated that acidic media provide the best performance for electrochemical oxidation [20,22,23]. These results of detailed voltammetric study of the tested antioxidants were taken into account in searching for optimum conditions for their amperometric detection. Due to low solubility of these phenolic antioxidants in water, an aqueous-methanolic solution containing 10% (*v/v*) methanol in 0.040 M B-R buffer pH 2.0 was used as a carrier solution in this work.

In order to identify the potential of oxidation to perform simultaneous determinations of tBHQ, PG, and BHA, hydrodynamic voltammograms were first obtained separately for each compound using MPA-FIA. The current at each potential pulse (from +0.20 to +0.70 V; pulse width: 100 ms) was monitored continuously during three injections of 0.1 mmol L^{-1} solution of the target analyte. The average value of peak current (n = 3) at each potential pulse was used to construct the hydrodynamic voltammogram for the electrochemical oxidation of tBHQ, PG, and BHA (Fig. 2).

It can be seen that the peak potentials of the obtained curves differ enough to enable the selective determination of the analytes. Namely, a potential of +0.40 V can be used for determination of tBHQ alone. Under potential of +0.55 V, both tBHQ and PG provide response; the current response of PG can be calculated by subtraction. The current response of BHA is two orders of magnitude lower under this potential and therefore can be considered negligible. At +0.70 V, all three target compounds are oxidized and the current response of BHA can be again obtained by subtraction. The direct subtraction of the current obtained by lower-potential pulse is generally impossible due to the difference

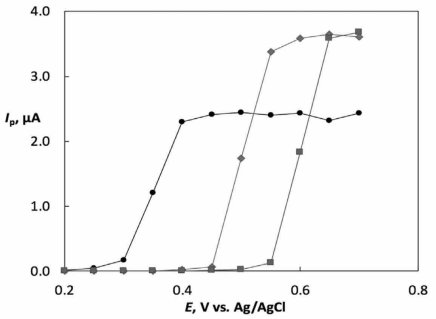

Fig. 2. Hydrodynamic voltammograms obtained by plotting peak current values as a function of the potential of applied potential pulses. The solutions contained tBHQ (•), PG (◆) or BHA (■) (all 0.1 mmol L^{-1}). Carrier solution: methanol – 0.040 M B-R buffer pH 2.0 (1:9, *v/v*); injected volume: 100 µL; flow rate: 1.0 mL min^{-1}.

between the heights of the peaks at different potentials. In our case, potential differences are small, so one would assume that the differences between the heights of the peaks would be minimal and can be neglected. Actually that is possible only in the case of mutually similar concentrations. For subtraction even in the case of varying concentrations, we have used correction factors (*CFs*). A *CF* is obtained as the proportion of the current response of the particular compound obtained under the higher and lower potential; knowledge of its value enables us to calculate the current response at higher potential from the known response at lower potential, thus offering the value suitable for subtraction. The values of *CF* must be independent of the concentration of determined compounds, thus the concentration interval between 10 and 100 µmol L^{-1} for tBHQ and PG measurement was used for its assessment. For example, $CF_{\text{tBHQ }+0.55\text{ V}}$ was obtained from the injection of a standard solution containing only tBHQ by the equation:

$$CF_{\text{tBHQ }+0.55\ V} = I_{\text{tBHQ }+0.55\text{ V}}/I_{\text{tBHQ }+0.40\text{ V}} \quad (1)$$

Then, the current originating from PG oxidation at +0.55 V during the analysis of solution containing both tBHQ and PG can be calculated using the equation:

$$I_{\text{PG}} = I_{+0.55\text{ V}} - (CF_{\text{tBHQ }+0.55\text{ V}} \times I_{\text{tBHQ }+0.40\text{ V}}) \quad (2)$$

CFs necessary for the determination of BHA were calculated similarly. The obtained *CF* values for tBHQ and PG were: $CF_{\text{tBHQ }+0.55\text{ V}}$ = 1.04 ± 0.03, $CF_{\text{tBHQ }+0.70\text{ V}}$ = 1.06 ± 0.04, and $CF_{\text{PG }+0.70\text{ V}}$ = 1.09 ± 0.06.

3.2. Optimization of FIA parameters

The FIA parameters were optimized in order to obtain the highest signal for tBHQ, PG, and BHA. Fig. 3 presents the dependence of the peak current of the analytes on the injected volume (Fig. 3A) and flow rate (Fig. 3B). The optimization of the injected volume was done according to usual FIA approach, i.e. the optimal injection volume was selected as the maximum volume above which the peak does not further increase its height, but only its width. This point was reached for an injection volume of 100 µL of 0.1 mmol L^{-1} of tBHQ, PG, and BHA in the MPA-FIA system (Fig. 3A), which was thus selected for further measurements. Another investigated parameter was the flow rate. The flow rate (Fig. 3B) was varied in the range from 1.0 to 3.0 mL min^{-1}, keeping the injection volume of 100 µL for 0.1 mmol L^{-1} of tBHQ, PG, and BHA. An increase in the flow rate resulted in the slight decrease in the peak area because of the shorter contact of the analytes with the electrode and in the rapid increase of the peak height due to its narrowing. On the other hand, excessive narrowing of the peak affects the resolution due to the 300 ms length of pulse program. As a compromise, flow rate of 2.0 mL min^{-1} was selected for further amperometric measurements, due to lower consumption of the carrier solution. No influence of the pulse width was observed between 70 and 150 ms and thus 100 ms pulse width was used further.

To examine the stability of the analytical signal, a repeatability study (Fig. 4) was conducted; under the optimized conditions, ten successive injections of the mixture of a standard solution (all compounds at 0.1 mmol L^{-1}) were carried out. The results demonstrate that the MPA-FIA system provides good repeatability (*RSD* < 5%, n = 10). Besides, this arrangement demonstrates the high throughput (140 injections h^{-1}).

3.3. Concentration dependences

Using the optimized conditions, calibration curves for tBHQ, PG, and BHA were constructed using solutions containing varying concentrations of one antioxidant while the concentration of the other two remained constant. Fig. 5 shows the amperometric responses of this measurement at one concentration level (100–10 µmol L^{-1}) and the calibration plots proving the proportionality between the amperometric current and the concentrations of the analytes. Linear regression of these three series of experiments leads to excellent correlation coefficients ($r > 0.99$ in all cases) and the obtained limits of quantification (L_Q) calculated as the analyte concentration corresponding to a tenfold

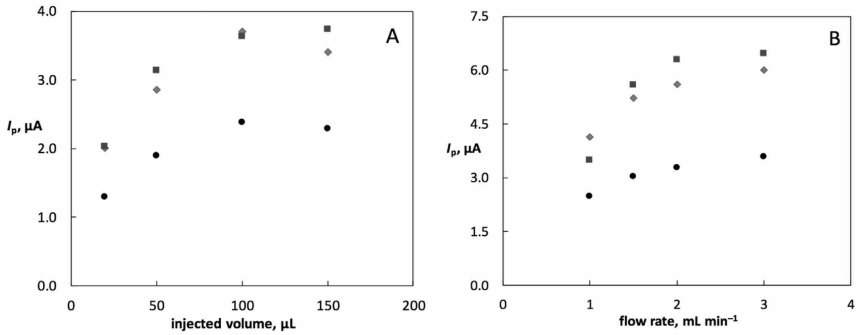

Fig. 3. Optimization of FIA parameters: dependence of the peak height on (**A**) injected volume (20, 50, 100, and 150 µL) and (**B**) flow rate (1.0, 1.5, 2.0, and 3.0 mL min^{-1}) based on triplicate injections of tBHQ (•), PG (◆) or BHA (■) (all 0.1 mmol L^{-1}). Potential pulse: +0.40, +0.55, and +0.70 V for 100 ms each; carrier solution: methanol – 0.040 M B-R buffer pH 2.0 (1:9, *v/v*).

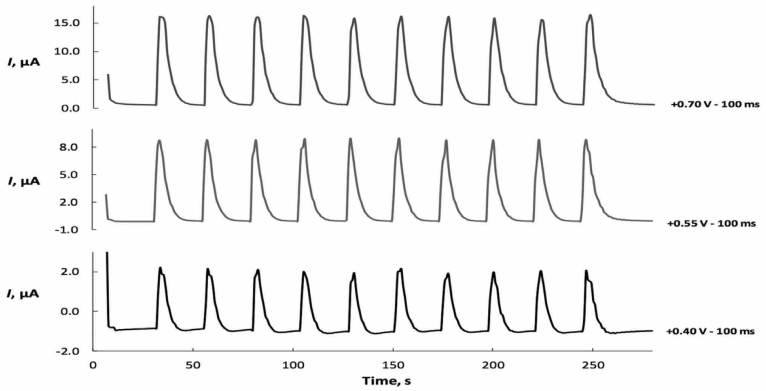

Fig. 4. Repeatability data obtained from successive injections of a solution containing tBHQ, PG, and BHA (all 0.1 mmol L^{-1}) (n = 10). Potential pulse: +0.40, +0.55, and +0.70 V for 100 ms each; carrier solution: methanol – 0.040 M B-R buffer pH 2.0 (1:9, *v/v*); injected volume: 100 µL; flow rate: 2.0 mL min^{-1}.

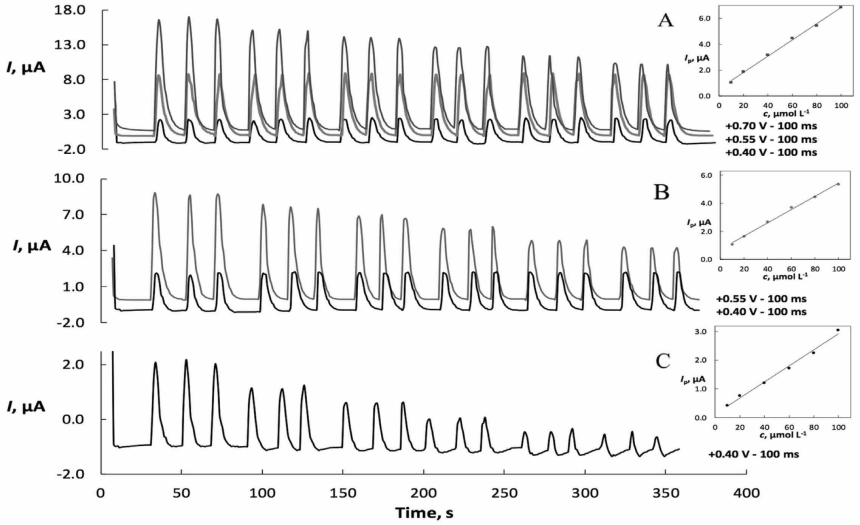

Fig. 5. MPA-FIA recordings obtained after injections of BHA (**A**), six standard solutions (100–10 µmol L^{-1}) + tBHQ and PG (both 0.1 mmol L^{-1}); PG (**B**), six standard solutions (100–10 µmol L^{-1}) + tBHQ and BHA (both 0.1 mmol L^{-1}) and tBHQ (**C**), six standard solutions (100–10 µmol L^{-1}) + PG and BHA (both 0.1 mmol L^{-1}). Calibration curves for tBHQ (•), PG (◆) or BHA (■) are presented on the right hand side. For measuring conditions see Fig. 4.

Table 1
Parameters of calibration curves, limits of quantification, and relative standard deviation of tBHQ, PG, and BHA obtained by MPA-FIA.

Substance	Studied concentration range (µmol $L^{-1)}$	Slope (nA mol $^{-1}$ L)	Intercept (nA)	Correlation coefficient	L_Q (µmol L^{-1})	*RSD* (%) for 10 injections (100 µmol L^{-1})
tBHQ	2–100	66.59	293	0.9955	2.51	0.84
PG	1–100	51.49	408	0.9901	1.45	1.53
BHA	0.8–100	28.14	114	0.9947	0.85	3.69

standard deviation of the lowest response, are at micromolar level for these antioxidants. However, lower L_Qs are not necessary for the analysis of these antioxidants in food samples, because their concentrations are usually relatively high; thus, the L_Qs obtained with this proposed method are more than adequate for the analysis of food samples (for more details see Table 1). Similar experiments with simultaneously increasing concentration of all three analytes (results not depicted) confirmed these results and the fact that under the given conditions the calculated signal of one analyte is practically not influenced by changing concentration of other analytes.

Table 2
Recovery of tBHQ, PG, and BHA from spiked chewing gum samples at various concentrations.

Number	Added concentration (μmol L^{-1})			Recovery measured by HPLC-ED			Recovery measured by MPA-FIA		
	PG	tBHQ	BHA	PG	tBHQ	BHA	PG	tBHQ	BHA
1	50	50	50	91%	86%	87%	112%	101%	112%
2	10	10	10	99%	87%	82%	115%	105%	104%
3	5	5	5	99%	99%	94%	103%	95%	116%
4	50	10	5	80%	86%	87%	109%	113%	113%
5	10	5	50	95%	95%	85%	98%	110%	115%
6	5	50	10	102%	85%	85%	101%	106%	113%

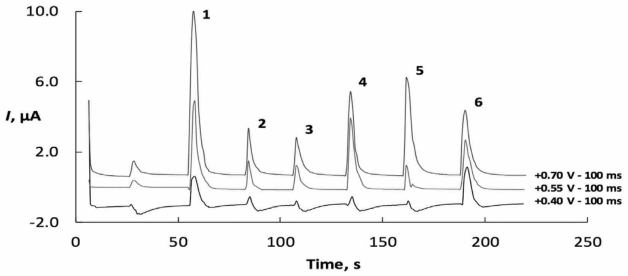

Fig. 6. Amperograms obtained after injections of unspiked sample solution prepared from chewing gum + six samples (1–6) of sample solution spiked with 3 different levels of concentration of tBHQ, PG, and BHA as indicated in the Table 2. For measuring conditions see Fig. 4.

Table 3
Content of tBHQ, PG, and BHA in chewing gum samples found by HPLC-ED and the proposed method MPA-FIA, expressed as (average ± *SD*).

Sample	Samples measured by HPLC-ED (mg kg^{-1})			Samples measured by MPA-FIA (mg kg^{-1})		
	PG	tBHQ	BHA	PG	tBHQ	BHA
1	20.0 ± 0.3	–[a]	13.2 ± 0.4	20.1 ± 0.5	–[a]	13.8 ± 0.5
2	–[a]	–[a]	10.0 ± 0.3	–[a]	–[a]	12.4 ± 0.5
3	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.7 ± 0.1	–[a]	0.8 ± 0.1
4	–[a]	–[a]	10.2 ± 0.3	–[a]	–[a]	12.8 ± 0.4
5	–[a]	–[a]	11.5 ± 0.4	–[a]	–[a]	13.8 ± 0.4

[a] Below detection limit.

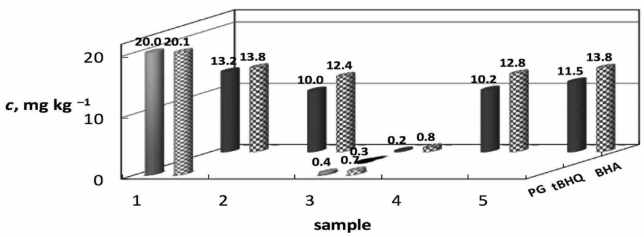

Fig. 7. Comparison of results from simultaneous determination of PG, tBHQ, and BHA content in analyzed samples of chewing gums obtained by HPLC-ED (left) and MPA-FIA (right).

3.4. Recovery study

From available reports (e.g. Ref. [20]), only few electroactive substances could potentially occur in chewing gums and affect the determination of the tested antioxidants. The trueness of the proposed method was first evaluated by recovery studies using unspiked sample solution prepared from chewing gum and six samples of solution spiked with different levels of concentration of tBHQ, PG, and BHA, to evaluate matrix effects after addition of standard solutions. For this purpose, a sample of chewing gum free of the mentioned antioxidants was selected. Recovery tests were carried out at 3 different levels of concentration, as indicated in the Table 2. Recovery values for tBHQ, PG, and BHA were in the range of 95–113%, 98–115%, and 104–116% (n = 3), respectively. HPLC with amperometric detection [20] provided recoveries between 82% and 102%, for the corresponding samples, underestimating the appropriate values probably due to the ineffective extraction step; MPA-FIA effectively compensated for this difference. The highest trueness for both techniques was observed for PG and the lowest for BHA. Example of amperograms obtained after injections of spiked samples of chewing gum with tBHQ, PG, and BHA is shown in Fig. 6.

3.5. Determination in chewing gum samples

Finally, the proposed method was applied to the simultaneous determination of all three synthetic antioxidants in chewing gum samples.

The samples were also analyzed by HPLC-ED for comparison [20]. The results are presented in Table 3 and shown in graphical form in Fig. 7. The obtained values of PG determination from MPA-FIA are in agreement with those obtained using the reference HPLC-ED method according to the paired Student *t*-test at a confidence level of 95%. In the case of BHA determination, the difference between the methods follows the difference observed in the recovery measurements, suggesting that the results obtained by the proposed method compared with HPLC-ED are satisfactory enough for the determination of antioxidants in this kind of matrix. The presence of tBHQ was not detected in chewing gum by MPA-FIA, indicating that its concentration is below the limit of detection. No observed signals in the matrix of chewing gum interfered with the proper determination of tested antioxidants.

4. Conclusions

We have demonstrated the applicability of multiple-pulse amperometric detection with GCE as a working electrode coupled to wall-jet configuration in FIA system for simultaneous determination of three antioxidants, namely tBHQ, PG, and BHA. The technique provides short analysis time (a frequency of 140 injections h^{-1}), low consumption of reagents and samples, high precision ($RSD < 5.0\%$; $n = 10$) and linear calibration curves ($r > 0.99$ in all cases). The limits of quantification were 2.51, 1.45, and 0.85 μmol L^{-1} for tBHQ, PG, and BHA, respectively. Furthermore, the method requires simpler instrumentation and lower investment and running cost in comparison with others more expensive techniques, e.g. HPLC with ED, DAD or MS typically applied for simultaneous determinations of more than one antioxidant. The newly developed method was successfully applied for simultaneous determination of tBHQ, PG, and BHA in chewing gum samples by applying a simple extraction procedure.

Acknowledgment

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Publication III

Simultaneous determination of sinapic acid and tyrosol by flow-injection analysis with multiple-pulse amperometric detection

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ORIGINAL PAPER

Simultaneous determination of sinapic acid and tyrosol by flow-injection analysis with multiple-pulse amperometric detection

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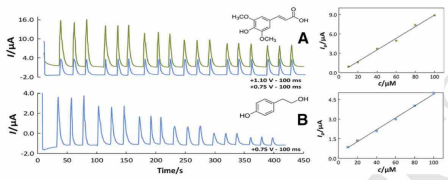
Abstract

This work describes a simple, fast (frequency of 170 injections h^{-1}), and low-cost method for the simultaneous determination of two antioxidants, sinapic acid and tyrosol, using multiple-pulse amperometric detection at a glassy carbon electrode incorporated in a flow-injection analysis cell. A sequence of potential pulses was selected to detect sinapic acid and tyrosol separately in the course of a single injection step. During the characterization of electrochemical detection, conditions for the determination of the two antioxidants (such as the injected volume and the flow rate) were studied and the analytical figures of merit were calculated. The repeatability (expressed as %) RSD was < 4.0% (n = 10) and excellent linearity was obtained across two concentration ranges from 1.0 to 100 μM; the limits of detection of sinapic acid and tyrosol were around 1.0 μM.

Graphical abstract



Keywords Electrochemistry · Flow-injection analysis · Glassy carbon electrode · Oxidations · Voltammetry

Introduction

Sinapic acid and tyrosol (Fig. 1) are common constituents of plants and fruits. These substances can be found for example in cranberry, wine, mustard seeds, and selected types of oils [1–3]. Tyrosol is also one of the main natural phenols in argan oil [4]. As antioxidants, they can protect cells against oxidation [4, 5]. Even though they are not as potent as other antioxidants, their higher concentration and good bioavailability indicate that they may have an important overall effect in the antioxidant properties of natural products. This effect may contribute significantly to the health benefits for example of olive oil and, more generally, the Mediterranean diet [5].

Several methods, mainly based on cyclic voltammetry and differential pulse voltammetry, have been reported for the determination of sinapic acid [6] or tyrosol [7]. However, to the best of our knowledge, only a few analytical methods have been reported for the simultaneous

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Journal : Large 706 | Dispatch : 21-3-2018 | Pages : 6
Article No. : 2189 | ☐ LE | ☐ TYPESET
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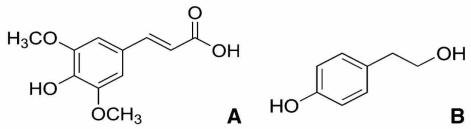

Fig. 1 Structure of sinapic acid (**a**) and tyrosol (**b**)

determination of sinapic acid and tyrosol including HPLC with UV or MS detection [8–10].

Recent publications have demonstrated that flow-injection analysis (FIA) with multiple-pulse amperometric (MPA) detection could be used for simultaneous measurement of two or more electroactive species [11–13]. An aliquot of sample solution is directly injected into a FIA system and the compounds are selectively detected at a single working electrode by applying two sequential potential pulses. A simple correction factor must be used for the calculation. This approach has some important advantages: it is inexpensive and simple, and has small sample and reagent consumption (with reduction in waste generation) and high sampling rates [14]. This strategy was used for simultaneous amperometric detection of sugars [15], drugs [16–18], antioxidants [19], synthetic colorants [20], as well as for the use of internal standard method in FIA [21].

This paper demonstrates that MPA detection in combination with FIA on a glassy carbon electrode (GCE) can be used for the simultaneous determination of sinapic acid and tyrosol. Results obtained with this method were evaluated with respect to recovery, repeatability, linearity, and detection limits.

Results and discussion

The influence of pH on the cyclic voltammograms of oxidizable sinapic acid and tyrosol (both at 0.1 mM) was investigated in a mixed methanol and 0.040 M B-R buffer (1:9, v/v) medium. Both sinapic acid and tyrosol have in this medium single peak, whose position and height depend on the pH. The dependence of E_p on pH for sinapic acid can be described using linear regression as E_p (-mV) = − 47.1 pH + 773.2 ($r > 0.98$). In the case of tyrosol, the dependence can be described as E_p (mV) = − 58.5 pH + 1098.2 ($r > 0.99$). In both cases, with rising pH, there is a rapid drop in the peak heights. The carrier solution of pH 2.0 was selected for further experiments, because in this medium, the oxidation peaks of sinapic acid and tyrosol were well separated (> 350 mV) and the peaks were highest in the CV experiments; addition of methanol was necessary due to the low solubility of these phenolic antioxidants in water. To identify the optimal oxidation potentials to perform simultaneous determination of sinapic acid and tyrosol, hydrodynamic voltammograms were first obtained separately for each compound using MPA-FIA (Fig. 2). In this case, standard solutions containing sinapic acid or tyrosol (0.1 mM) were injected into the system. Sequential potential pulses of 100 ms duration from + 0.40 to + 0.80 V for sinapic acid and from + 0.70 to + 1.10 V for tyrosol were applied continuously; the current at each potential pulse was monitored and used to construct the hydrodynamic voltammogram for the electrochemical oxidation of both compounds.

It can be seen that the peak potentials of the obtained curves differ enough to enable the selective determination of the analytes. Namely, potentials between + 0.70 and + 0.80 V would only cause the oxidation of sinapic acid without significant interference from tyrosol; therefore, + 0.75 V (100 ms) was selected as the first potential pulse. Potential of + 1.10 V (100 ms) was selected as the second potential pulse, where both target analytes are fully oxidized. Tyrosol can be quantified if the current from the oxidation of sinapic acid at + 1.10 V is previously subtracted. However, direct subtraction of the current response at + 0.75 V (exclusive oxidation of sinapic acid) from the current response at + 1.10 V (oxidation of both target analytes) is not possible, as the current responses detected for sinapic acid at + 0.75 and + 1.10 V are not equal, and a correction factor (*CF*) must be used. For tyrosol determination at + 1.10 V without interference from sinapic acid, the *CF* can be obtained by a simple injection of

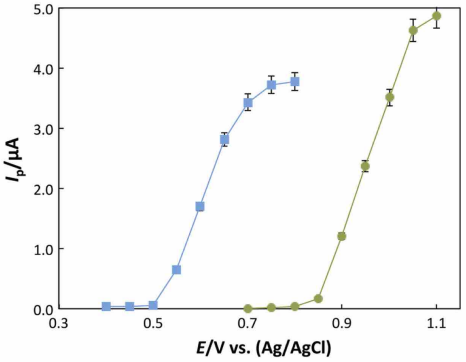

Fig. 2 Hydrodynamic voltammograms obtained by plotting peak current values as a function of the corresponding applied potential pulses. The solutions contained sinapic acid (filled squares) or tyrosol (filled circles) (both at 0.1 mM). Carrier solution: methanol, 0.040 M B-R buffer pH 2.0 (1:9, v/v); injected volume: 100 mm^3; flow rate: 1.0 cm^3 min^{-1}

Journal : Large 706 | Dispatch : 21-3-2018 | Pages : 6
Article No. : 2189 | LE | TYPESET
MS Code : MCCM-D-18-00020 | CP | DISK

a standard solution containing only sinapic acid and by the application of the following equation:

$$CF = I_{\text{sinapicacid}+1.10\ \text{V}} / I_{\text{sinapicacid}+0.75\ \text{V}}. \quad (1)$$

Then, the current originating from tyrosol oxidation at + 1.10 V during the analysis of solution containing both sinapic acid and tyrosol can be calculated using the equation:

$$I_{\text{tyrosol}} = I_{+1.10\ \text{V}} - (CF \times I_{\text{sinapicacid}+0.75\ \text{V}}). \quad (2)$$

In the development of the proposed method, an additional parameter should be considered: the *CF* value must be constant in the selected concentration interval. In the concentration interval between 10 to 100 μM of sinapic acid, this requirement was fulfilled and the *CF* value was calculated as 1.10 ± 0.06 (n = 3).

Other FIA parameters were optimized to obtain the highest signal for sinapic acid and tyrosol. Figure 3 illustrates the dependence of the peak current of the analytes on the injected volume (Fig. 3a) and flow rate (Fig. 3b). The optimization of the injected volume was done similar to any FIA system, i.e., the optimal injection volume was selected as the maximum volume above which the peak height does not further increase. The effect of the injected sample volume (Fig. 3a) on the MPA response was investigated in the range from 20 to 150 mm^3, using solutions of each antioxidant and applied electrode potentials of + 0.75 V for sinapic acid and + 1.10 V for tyrosol. The amperometric signal increased with the injected sample volume up to 100 mm^3 and then remained almost constant for higher injected volumes; this value was thus selected for a subsequent measurements. The effect of the flow rate (Fig. 3b) was evaluated by varying its values from 1.0 to 5.0 $cm^3\ min^{-1}$, using injected volume 100 mm^3 and applying electrode potentials of + 0.75 V for sinapic acid and + 1.10 V for tyrosol. The electrode response increased with flow rate up to 3.0 $cm^3\ min^{-1}$ and then remained almost constant for higher flow rates; thus, this value of flow rate was selected for further amperometric measurements, due to lower consumption of the carrier solution and higher peaks.

To examine the stability of the analytical signal, a repeatability study was conducted (Fig. 4); under the optimized conditions, ten successive injections of a standard solution containing sinapic acid and tyrosol (both 0.1 mM) were carried out. The results demonstrate that the MPA-FIA system provides good repeatability (RSD < 4.0%, n = 10) and a high sampling rate (around 170 determinations h^{-1}). From the same figure, difference between the values of the baseline for each inserted potential may be observed. In addition, other publications mentioned earlier obtained similar results [12–14]. This problem has a great connection with the length of the individual pulses, the size of the inserted potential pulses, and the magnitude of the potential difference between the individual pulses, which are in very fast sequences during the measurement only the carrier solution or carrier solution and analytes [22, 23].

Using the optimized experimental conditions selected for the determination of sinapic acid and tyrosol, analytical figures of merit were obtained using solutions containing varying concentrations of one antioxidant, while the concentration of the other antioxidant remained constant. Figure 5 illustrates the amperometric responses of this measurement at one concentration level (100–10 μM) and the calibration plots proving the proportionality between the amperometric current and the concentrations of the analytes. Linear regression of these two series of

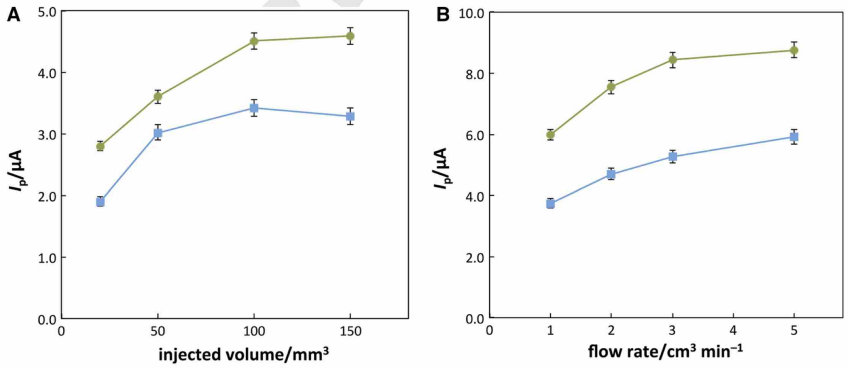

Fig. 3 Optimization of FIA parameters: dependence of current response on **a** injected volume and **b** flow rate of sinapic acid (filled squares) or tyrosol (filled circles) (both 0.1 mM). Potential pulses: + 0.75 (sinapic acid) and + 1.10 V (tyrosol) of 100 ms duration; carrier solution: methanol − 0.040 M B-R buffer pH 2.0 (1:9, v/v)

Journal : Large 706 | Dispatch : 21-3-2018 | Pages : 6
Article No. : 2189 | LE | TYPESET
MS Code : MCCM-D-18-00020 | CP | DISK

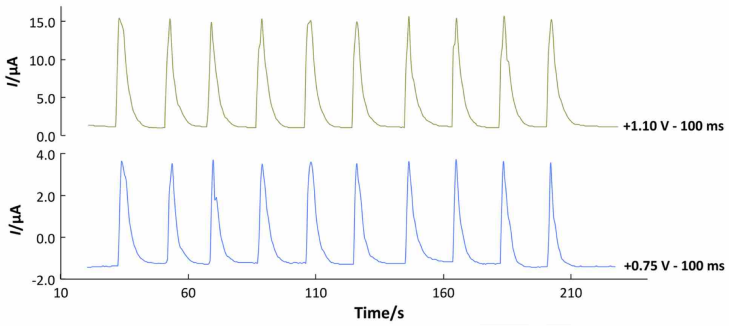

Fig. 4 Repeatability data obtained from successive injections of a solution containing sinapic acid and tyrosol (both 0.1 mM) (n = 10). Potential pulses: + 0.75 (sinapic acid) and + 1.10 V (tyrosol) for 100 ms each; carrier solution: methanol – 0.040 M B-R buffer pH 2.0 (1:9, v/v); injected volume: 100 mm^3; flow rate: 3.0 cm^3 min^{-1}

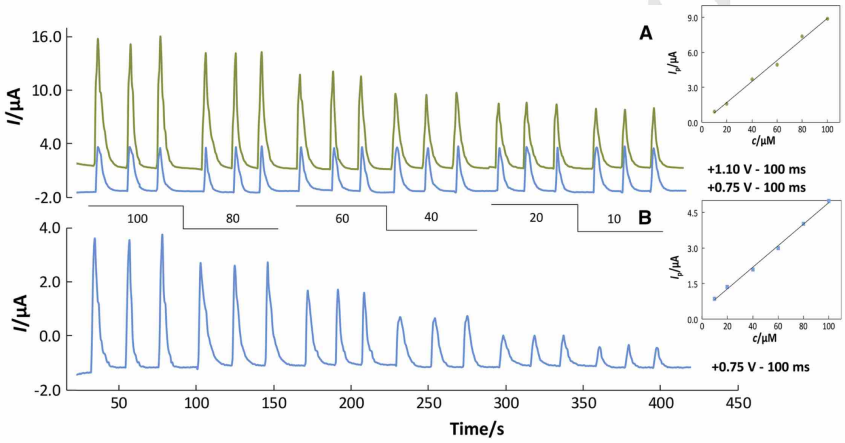

Fig. 5 MPA-FIA recordings obtained after injections of **a** six standard solutions (100–10 μM) of tyrosol + sinapic acid (0.1 mM) and **b** six standard solutions (100–10 μM) of sinapic acid + tyrosol (0.1 mM). Inset shows calibration curves for tyrosol (filled circles) and sinapic acid (filled squares). For measurement conditions, see Fig. 4

Table 1 Figures of merit of the proposed method for the simultaneous MPA-FIA determination of sinapic acid and tyrosol

Substance	Concentration range/ μM	Slope/ nA mol^{-1} dm^3	Intercept/ nA	Correlation coefficient	L_Q/ μM	RSD/% for 10 injections (100 μM)
Sinapic acid	0.8–100	47.61	211	0.9956	0.86	2.48
Tyrosol	1.0–100	89.78	76	0.9973	1.03	3.96

experiments leads to excellent correlation coefficients ($r > 0.99$, in both cases) and the L_Q values obtained for these antioxidants are at micromolar level (see Table 1). The course of determination should be without major complications in the case of the measurement of sinapic acid and tyrosol in matrices mentioned earlier with a high proportion of these substances. In the case of the rest of real samples, complications associated with the presence of other antioxidants, which naturally occurring in the real matrices can arise. The interference of other antioxidants depends highly on their properties, namely ascorbic acid, and most other antioxidants oxidize earlier than the

Journal : Large 706 | Dispatch : 21-3-2018 | Pages : 6
Article No. : 2189 | LE | TYPESET
MS Code : MCCM-D-18-00020 | CP | DISK

measured analytes using the given conditions. This would change the procedure in the next step, namely recalculation of the peak heights of the determined substances by the correlation factor as explained earlier. A minor disadvantage may be that, for each real sample, a specific method for determination of the mentioned analytes would have to be developed.

Conclusion

The present work demonstrates the possibility of simultaneous determination of sinapic acid and tyrosol using a flow-injection system with multiple-pulse amperometric detection. The advantages of the technique are short time of analysis (170 injections h^{-1}), low consumption of samples and reagents, high precision (RSD < 4.0%; $n = 10$), and linear calibration curves ($r > 0.99$). The limits of quantification were 0.86 and 1.03 μM for sinapic acid and tyrosol, respectively. This method has a good potential to be applied in routine analysis in substitution of expensive chromatographic separation systems.

Experimental

Sinapic acid (CAS number 530-59-6) and tyrosol (CAS number 501-94-0) were supplied by Sigma-Aldrich. Their individual stock solutions ($c = 1.00$ mM) were prepared by dissolving the exact amount of the respective substance in methanol (Merck Millipore, Germany) and they were kept at 4 °C. More diluted solutions were prepared by exact dilution of the stock solutions with mixture of methanol and 0.040 M Britton-Robinson (B-R) buffer (1:9, v/v). All electrochemical measurements were carried out in the same solution. The B-R buffer was prepared by mixing 0.20 M sodium hydroxide (Lach-Ner Neratovice, Czech Republic) with acidic solution consisting of 0.040 M boric acid (Lach-Ner Neratovice, Czech Republic), 0.040 M phosphoric acid (Merck Millipore, Germany), and 0.040 M acetic acid (Merck Millipore, Germany). All chemicals used for buffer preparation were of analytical grade purity. Distilled water was provided from a Mega-Pure 3A Liter Automatic Distillation System, USA.

Instrumentation and apparatus

All electrochemical recordings were performed using an Autolab PGSTAT12 potentiostat/galvanostat, controlled by NOVA version 1.11.2 software (Metrohm, Switzerland) working under Windows 7 (Microsoft Corporation). The three-electrode wall-jet configuration included a glassy carbon working electrode (GCE) (Metrohm, Switzerland, diameter of 2 mm and geometric area 3.1 mm^2), a platinum wire, 1 cm in length and 0.5 mm in diameter, as a counter electrode, and an Ag/AgCl (3 M KCl) electrode as a reference electrode (MonokrystalyTurnov, Czech Republic) [24]. Flow of the carrier solution was provided by peristaltic pump MINIPULS Evolution (Gilson, USA) and injection of the sample was performed with a six-way injection valve (VICI Valco Instruments, Canada) equipped with a 100 mm^3 sample injection loop. An Orion 266S pH meter (Thermo Fisher Scientific, USA) equipped with a combined glass pH electrode was used for pH measurements. The pH meter was calibrated with aqueous standard buffer solutions at ambient temperature.

Procedures

Pre-treatment of the GCE was done by polishing with alumina powder suspension (0.1 μm) on a damp polishing cloth (Metrohm, Switzerland) before fixing to the flow cell. This procedure was performed at the beginning of the working day.

Hydrodynamic voltammograms of sinapic acid and tyrosol were obtained separately by application of nine sequential potential pulses (from + 0.40 to + 0.80 V for sinapic acid and from + 0.70 to + 1.10 V for tyrosol, pulse width 100 ms) in triplicate injections of standard solutions through the FIA system using the MPA technique. The same technique was used for simultaneous amperometric detection of sinapic acid and tyrosol, applying pulses + 0.75 V for 100 ms (sinapic acid) and + 1.10 V for 100 ms (tyrosol) continuously (total time of the potential waveform was 200 ms).

The peak height (I_p) was evaluated from the amperometric FIA recording. The limit of quantification (L_Q) was calculated as the analyte concentration corresponding to a tenfold standard deviation of the respective response from ten consecutive determinations at the lowest measurable concentration [25].

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Publication IV

Fast Scanning Voltammetric Detector for High Performance Liquid Chromatography

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Fast scanning voltammetric detector for high performance liquid chromatography

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ABSTRACT

This article describes the application of fast scan differential pulse voltammetry on a glassy carbon electrode as a working electrode in a wall-jet arrangement in combination with a high performance liquid chromatography. During the characterization of electrochemical detection, the separability, the repeatability, and the concentration characteristics for determination of common antioxidants in standard solutions were found. Finally, as an application of the technique, the optimized procedure has been used for the first time to determine antioxidants contained in tea samples by applying a simple extraction procedure.

1. Introduction

Electrochemical detectors for high performance liquid chromatography (HPLC) are widely used for detection of electrochemically active organic compounds. It is particularly valid for the most ordinary amperometric detector, because it requires relatively simple instrumentation, offers high sensitivity, and provides considerable selectivity for many electroactive compounds through reasonable selection of the applied potential [1,2]. Improved potential selectivity can be obtained with the use of dual electrodes (in parallel, in series or geometrically opposed) operated at different potentials [3]. Another mode of detection, which has been investigated, involves potential pulse techniques, because pulse techniques can also be used for increased selectivity and for electrode cleaning [4–7].

Despite the advantages of all of the mentioned detection techniques, none of them provides complete electrochemical information during the flow measurement. This can only be provided by voltammetric technique, in our case fast-scan differential pulse voltammetry (FSDPV). Although DPV has been used extensively by physical electrochemists and electroanalytical chemists in static solutions, it has seen only limited number of applications in flowing systems such as liquid chromatography and flow injection analysis. FSDPV technique is interesting for electrochemical measurements, because the detector response is relatively insensitive to the stability of the flow rate with time [8–11]. This technique in combination with flowing systems can provide more complete information about each peak: a characteristic shape of the voltammogram for each peak and peak retention time [12–14]. The obtained voltammogram can relieve difficulty identification or class identification of unknown compounds [8]. Furthermore, the characteristic voltammograms of multicomponent samples can be evaluated from only one obtained chromatogram in comparison with the time consuming generation of hydrodynamic voltammograms by electrochemical detection with the method of repeated injections at a series of gradually changed applied potentials [15]. The FSDPV technique offers immediate identification or class identification of coeluting peaks if the coeluting compounds have different oxidation potentials. If the peak of the coeluting compound has a higher oxidation potential, it can be removed as the interference, because the chromatogram can be plotted at the lower potential [16].

The present study was focused on FSDPV technique in combination with HPLC and its performance was tested on the detection

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of selected antioxidants (Fig. 1) contained in tea samples. The advantages and possibilities of the technique are discussed, and the selectivity and sensitivity of the detector are compared with HPLC-DAD.

2. Experimental

2.1. Reagents

Gallic acid – GA (CAS Number: 149-91-7), ferulic acid – FA (CAS Number: 1135-24-6), syringic acid – SGA (CAS Number: 530-57-4), sinapic acid – SPA (CAS Number: 530-59-6), *p*-coumaric acid – *p*-CA (CAS Number: 501-98-4), rutin – R (CAS Number: 207671-50-9), and caffeic acid – CA (CAS Number: 331-39-5) were supplied from Sigma-Aldrich. Their individual stock solutions ($c = 1.00$ mmol L^{-1}) were prepared by dissolving the exact amount of the respective substance in mixture of acetonitrile (Fluka) and deionized water (Millipore Q-plus System, Millipore, USA) (30:70, *v/v*) and were kept in the refrigerator. For their dilution, mixture of acetonitrile:B-R buffer or phosphate buffer was used according to the mobile phase composition. The B-R buffer solutions and 0.05 M phosphate buffer solution were prepared of sodium hydroxide (Fluka) and boric acid, phosphoric acid, and acetic acid (all Sigma-Aldrich). All chemicals used for measurement were of analytical grade purity.

2.2. Detection cell

The three-electrode wall-jet system with all electrodes placed in an overflow vessel [17] was used for all voltametric measurements. A glassy carbon working electrode (GCE; Metrohm, Switzerland, diameter of 2 mm and geometric area 3.1 mm^2) was cleaned by polishing for 1 min with alumina powder suspension (particle size 0.1 μm) and washing with distilled water daily on the beginning of the measurement. An Ag/AgCl (3 M KCl) reference electrode (Monokrystaly Turnov, Czech Republic) was used. An auxiliary electrode was made of a Pt wire, 1 cm in length and 0.5 mm in diameter.

2.3. Apparatus

HPLC apparatus consisted of a high pressure pump Waters 515 HPLC (Waters Corporation, USA), a six-way injection valve (Supelco Rheodyne Model 7725i) with a 20 μL loop and Twelve Channel Multi Autolab potentiostat/galvanostat, controlled by NOVA version 10.2 software (Metrohm, Switzerland). Two types of columns were used for separation; Agilent Technologies Poroshell 120 EC-C18 (3.0×0.5 cm *ID*) with 2.7 μm packing was used for preliminary experiments with GA, CA, SGA, and *p*-CA and Purospher STAR RP-C18 (12.5×0.4 cm *ID*) with 5.0 μm packing was used for determination of tea samples including the standards of GA, CA, R, SPA, and FA. Solution pH was measured by pH meter Basic 20+ (Crison Instruments, Spain) equipped with a combined glass pH electrode; aqueous standard buffer solutions at ambient temperature were used for its calibration.

2.4. HPLC-FSDPV procedures

Parameters of FSDPV were set as follows: scan rate 5 V s^{-1}, pulse amplitude 50 mV, pulse width 100 ms, E_{start} 0.0 V, E_{end} 1.2 V, and step height 15 mV. Reverse scan with the same scan rate was included in the potential program, but the response was not recorded. A predetermined number of scans was recorded, collected, and stored for each chromato-voltammograms. During the data processing, the subtraction of the background current and noise filtering was performed, and the peak position and the peak height for each antioxidant was calculated [10]. The peak height was evaluated from the voltammetric dimension of the recordings. The limit of detection (L_D) was calculated as the analyte concentration corresponding to a threefold standard deviation of the respective response from ten consecutive determinations at the lowest measurable concentration [18]. The flow rate of the mobile phase during the experiments was set to 0.8 ml min^{-1} [10]. All the injections were repeated three times unless stated otherwise and the measurements were carried out at ambient temperature.

2.5. Sample preparation

Samples of four kinds of teas (oolong, black, green, and mint tea) were purchased from a local supermarket. Dried tea sample of 2.0 g was crushed with a mortar and pestle. The tea infusion was prepared by an extraction of tea with 100 mL water at 80 °C, under stirring with a magnetic stirrer for 10 min. After cooling to ambient temperature, tea extract was filtered through paper filter, then through 0.45 μm syringe filter and injected into the system. Tea infusions were prepared daily [19].

3. Results and discussion

3.1. Model sample of antioxidants measured by HPLC-FSDPV

Principal determination conditions, such as scan rate 5 V s^{-1}, step height 15 mV, flow rate 0.8 ml min^{-1}, and injected volume 20 μL, were optimized previously [10]. Under these optimized

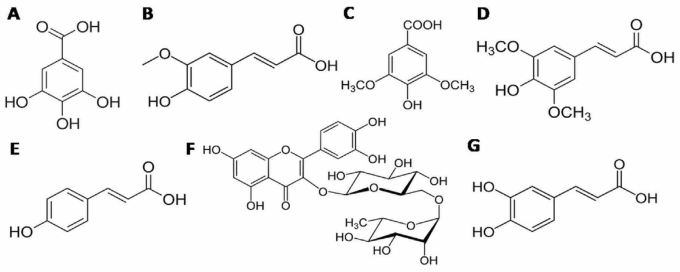

Fig. 1. Structure of tested antioxidants. Gallic acid (GA) - **A**, ferulic acid (FA) - **B**, syringic acid (SGA) - **C**, sinapic acid (SPA) - **D**, *p*-coumaric acid (*p*-CA) - **E**, rutin (R) - **F**, and caffeic acid (CA) - **G**.

conditions, model sample consisting of four antioxidants, namely gallic acid, caffeic acid, syringic acid, and *p*-coumaric acid was analyzed. The use of HPLC-FSDPV provides an enhanced selectivity, which allows to separate substances using a chromatographic column and also to distinguish two compounds having different oxidation potentials by FSDPV at the same time. This illustrative example is demonstrated in Fig. 2, where caffeic acid and syringic acid are eluted from the column approximately at the same time, but the difference between oxidation potentials allows perfect separation of corresponding signals. Two FSDPV peaks of *p*-CA suggest two-step oxidation of the compound. After successful separation of chosen model substances, the repeatability of peak heights was investigated. Relative standard deviation (*RSD*) of the peak heights ($c = 100\ \mu mol\ L^{-1}$) for GA is 2.84%, CA 2.97%, SGA 4.16%, and 3.81% for *p*-CA, which confirms good stability of the response signals and repeatability of FSDPV peak heights. As a part of the validation method the intra-day reproducibility was also evaluated by calculating the *RSD* of the peak heights ($c = 100\ \mu mol\ L^{-1}$) for the analysis of this group of antioxidants in three replicates within a six-hour range, namely GA 4.28%, CA 4.78%, SGA 6.17%, and 5.72% for *p*-CA. The measured peak current is linearly related to the concentration from 2 to 100 $\mu mol\ L^{-1}$ for all compounds. The correlation coefficients are close to one specifically higher than 0.98 (in all cases) and the limits of detection (L_D) are 4.8, 6.6, 3.3, and 4.2 $\mu mol\ L^{-1}$ for gallic acid, caffeic acid, syringic acid, and *p*-coumaric acid, respectively (Table 1), which proves the suitability of the proposed technique for monitoring of the target analytes.

3.2. Background adjustment

FSDP voltammograms obtained during a chromatographic run at low concentrations of analytes are significantly distorted by the relatively high background current, upon which the faradaic current is superimposed. This can be seen in Fig. 3A, where the FSDP voltammogram of the mobile phase and that for the mixture of 10 $\mu mol\ L^{-1}$ CA and SGA are plotted. This distortion could be avoided by subtracting the FSDP voltamgram of the mobile phase from that FSDP voltamgram of the mixture of the analytes. The shape of the FSDP voltammograms of the mobile phase is very reproducible, but its magnitude changes, particularly during the first 10–20 scans. To stabilize the FSDP voltammogram of the mobile phase prior the measurement, potential scanning was started about 20 s before injection of the sample. After that, the shape of the FSDP voltammogram of the mobile phase is reproducible and the magnitude changes are small; therefore, the FSDP voltammogram of the mobile phase can be subtracted from the total current to give a FSDP voltammogram corresponding to faradaic process as shown in Fig. 3B. Thus, in a 3D dimension, the area consisting of voltammograms of the mobile phase is subtracted from the total current area of the sample. The explained subtraction technique assumes that the recorded voltammogram stays constant during the elution of all peaks. Otherwise, a FSDP voltammogram scan immediately prior to each eluted peak is chosen for the subtraction. Subtraction of the background was successfully tested on the same model sample (Fig. 2) and then subsequently on real samples. From Table 1 can be observed, that a pronounced decrease of L_Q (L_D) for tested analytes was caused by the use of background subtraction. Also a minor improvement of the parameter, such as a smaller intercept was observed; slope and correlation coefficient remained almost unchanged.

3.3. Measurement of standard antioxidants occurring in various kinds of tea

Determination of antioxidants in tea sample was selected as a suitable issue for testing the performance of the technique. Antioxidants such as gallic acid, caffeic acid, rutin, sinapic acid, and ferulic acid are well-known species present in various kinds of tea. Separation of chosen standards was performed according to the

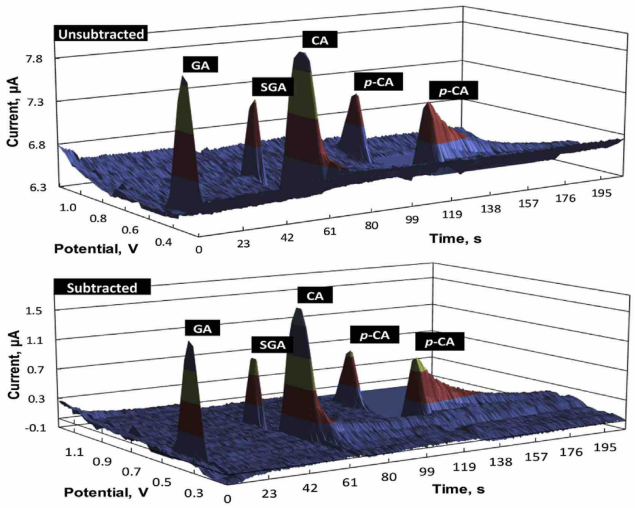

Fig. 2. Three-dimensional representation of HPLC-FSDPV recordings (unsubtracted vs. subtracted) of one repeated injection (20 µL) of a mixture of antioxidants (GA, CA, SGA, and *p*-CA; $c = 100\ \mu mol\ L^{-1}$) at GCE. Mobile phase: acetonitrile:Britton-Robinson buffer pH 4.0 (1:20, *v*/*v*); column: ATP 120 EC-C18 (3.0 × 0.5 cm *ID*, 2.7 µm); scan rate: 5 V s^{-1}; flow rate: 0.8 ml min^{-1}.

Table 1
Parameters of calibration straight lines of the proposed HPLC-FSDPV method for the determination of tested antioxidants (unsubtracted vs. subtracted).

Antioxidant		Concentration range μmol L^{-1}	Slope mA mol^{-1} L^{-1}	Intercept nA	Correlation coefficient	L_Q μmol L^{-1}	L_D μmol L^{-1}
Unsubtracted	gallic acid	2–100	14.56 ± 0.19	162	0.9913	16	4.8
	caffeic acid	2–100	16.12 ± 0.11	387	0.9877	22	6.6
	syringic acid	2–100	11.82 ± 0.16	98	0.9821	11	3.3
	p-coumaric acid	2–100	8.07 ± 0.10	44	0.9859	14	4.2
Subtracted	gallic acid	1–100	14.78 ± 0.21	107	0.9879	1.2	0.36
	caffeic acid	1–100	17.82 ± 0.14	197	0.9861	0.8	0.24
	syringic acid	1–100	12.33 ± 0.11	66	0.9817	1.4	0.42
	p-coumaric acid	1–100	8.14 ± 0.13	31	0.9836	1.2	0.36

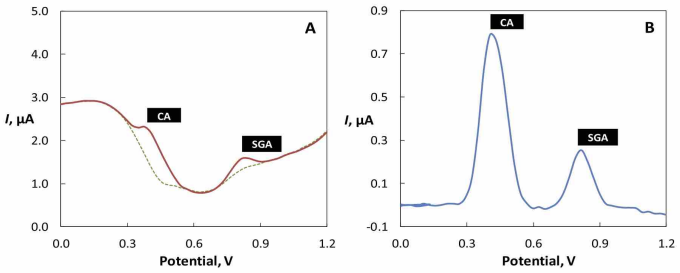

Fig. 3. (**A**) Unsubtracted FSDP voltammograms of the mobile phase (dotted line) and a mixture of 10 μmol L^{-1} CA and SGA (solid line) and (**B**) subtracted FSDP voltammogram; measured at GCE; mobile phase: acetonitrile:Britton-Robinson buffer pH 4.0 (1:20, *v*/*v*); column: ATP 120 EC-C18 (3.0 × 0.5 cm *ID*, 2.7 μm); scan rate: 5 V s^{-1}; flow rate: 0.8 ml min^{-1}; injected volume: 20 μL.

article [19]; one obtained chromato-voltammogram of standard solution of antioxidants is shown in Fig. 4. The repeatability, the reproducibility of peak heights and the concentration dependences of tested antioxidants were verified. The variability of the peak heights of antioxidants standard is below 5% ($n = 10$). A *RSD* is 3.87% for GA (17 mg kg^{-1}), 4.34% for CA (18 mg kg^{-1}), 4.97% for R (60 mg kg^{-1}), 4.41% for SPA (22 mg kg^{-1}), and 4.45% for FA (20 mg kg^{-1}). Also the intra-day reproducibility within the same concentration range was calculated for the analysis of this group of antioxidants in three replicates within a six-hour range, namely GA 5.80%, CA 7.33%, R 6.72%, SPA 5.91%, and 5.83% for FA. These values confirm good stability of the response signals, repeatability, and reproducibility of the measurements. Parameters of calibration straight line of the applied procedure for the determination of antioxidants are summarized in Table 2, confirming the linear dependence of the peak current on the concentration of tested antioxidants and relatively good L_D around 0.1 mg kg^{-1}. A pronounced decrease of L_D for tested analytes was caused by the use of background subtraction. In Fig. 4, we can also observe the tailing of some peaks, caused probably by the deposition of the product of the electrode reaction on the surface of the electrode. Comparison of the potentials of the mentioned five peaks with potentials obtained from fast scan differential pulse voltammetry [20–25] under the flow and stop flow conditions was performed, keeping the same experimental conditions, such as concentration or scan rate, and it was found that the main difference was the shift of the peaks in the flow system by about 50–70 mV to a more positive potential. Five obtained voltammograms (unsubtracted vs. subtracted) of standard solutions of antioxidants measured by FSDPV in combination with stop flow condition are enclosed in the Supporting Information.

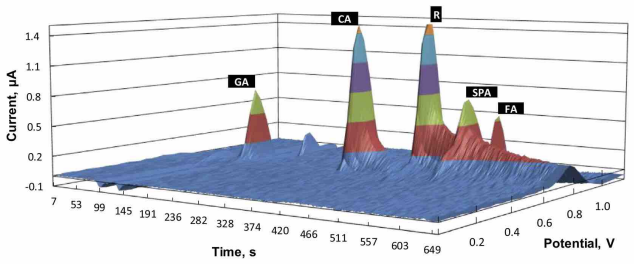

Fig. 4. Three-dimensional representation of HPLC-FSDPV recordings of one repeated injection (20 μL) of a model mixture of standard antioxidants (GA, CA, R, SPA, and FA; $c = 17$, 18, 60, 22, and 20 mg kg^{-1}) at GCE. Mobile phase acetonitrile:phosphate buffer pH 2.5 (0 min: 15:85; 11 min: 30:70, *v*/*v*); column: Purospher STAR RP-C18 (12.5 × 0.4 cm *ID*, 5.0 μm); scan rate: 5 V s^{-1}; flow rate: 0.8 ml min^{-1}.

Table 2
Parameters of calibration straight lines of the proposed HPLC-FSDPV method for the determination of five tested antioxidants.

Substance	Concentration range mg kg^{-1}	Slope nA mg^{-1} kg^{-1}	Intercept nA	Correlation coefficient	L_Q mg kg^{-1}	L_D mg kg^{-1}
Gallic acid	0.17–34	49.29 ± 0.98	58.3	0.9863	0.2	0.06
Caffeic acid	0.18–18	93.78 ± 1.20	20.3	0.9948	0.1	0.04
Rutin	0.60–60	38.54 ± 0.76	93.1	0.9908	0.8	0.24
Sinapic acid	0.22–22	34.51 ± 0.69	36.2	0.9961	0.3	0.08
Ferulic acid	0.20–20	40.87 ± 0.67	43.2	0.9853	0.4	0.12

Table 3
Determination of the contents (mg/kg) of 5 tested antioxidants in tea samples using the proposed HPLC-FSDPV method. Values in the brackets were obtained by HPLC-DAD comparative method.

Sample	Gallic acid	Caffeic acid	Rutin	Sinapic acid	Ferulic acid
Oolong tea 1	30.39 ± 2.43	7.52 ± 0.61	3.08 ± 0.44	6.62 ± 1.19	–
	(29.09 ± 0.83)	(6.70 ± 0.31)	(2.63 ± 0.12)	(5.58 ± 0.26)	–
Oolong tea 2	29.47 ± 1.57	–	1.53 ± 0.31	1.66 ± 0.38	–
	(31.21 ± 0.41)	–	(1.39 ± 0.06)	(1.89 ± 0.07)	–
Black tea 1	22.54 ± 1.58	0.46 ± 0.11	2.14 ± 0.43	4.39 ± 0.83	–
	(24.03 ± 0.69)	(0.60 ± 0.04)	(1.81 ± 0.09)	(3.52 ± 0.18)	–
Black tea 2	13.13 ± 0.92	1.93 ± 0.19	1.97 ± 0.45	4.05 ± 0.82	–
	(11.89 ± 0.43)	(1.79 ± 0.10)	(2.52 ± 0.09)	(5.01 ± 0.16)	–
Green tea 1	2.06 ± 0.49	4.58 ± 0.47	–	–	–
	(1.49 ± 0.06)	(5.11 ± 0.18)	–	–	–
Green tea 2	6.65 ± 0.53	6.78 ± 0.72	–	0.85 ± 0.25	–
	(5.77 ± 0.27)	(7.76 ± 0.34)	–	(1.07 ± 0.05)	–
Mint tea 1	–	1.84 ± 0.33	4.41 ± 0.64	8.07 ± 0.71	–
	–	(2.28 ± 0.12)	(5.09 ± 0.25)	(9.28 ± 0.48)	–
Mint tea 2	–	0.98 ± 0.23	15.17 ± 1.33	8.66 ± 0.78	–
	–	(1.21 ± 0.06)	(16.65 ± 0.91)	(9.87 ± 0.52)	–

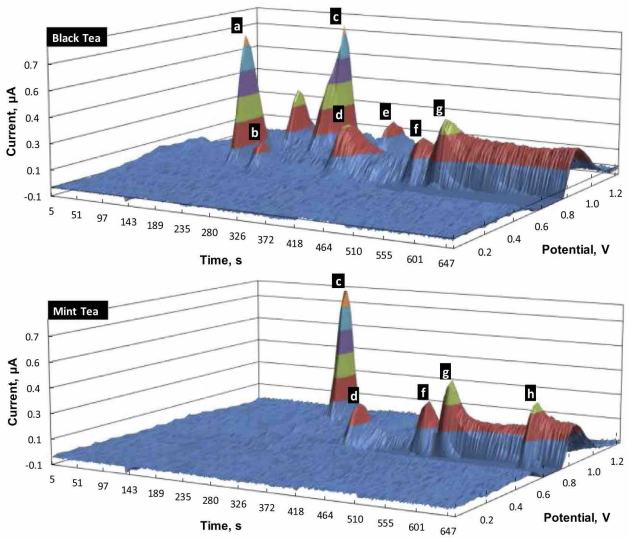

Fig. 5. Three-dimensional representation of HPLC-FSDPV recordings of one repeated injection of the selected samples of tea extracts (black tea above and mint tea below); (gallic acid - **a**, gallocatechin or epigallocatechin - **b**, catechin - **c**, caffeic acid - **d**, epicatechin or epicatechin gallate - **e**, rutin - **f**, sinapic acid - **g**, chlorogenic acid - **h**). Compounds **b**, **c**, **e**, and **h** are predicted from their UV spectra. For experimental conditions see Fig. 4.

3.4. Determination of antioxidants in tea extracts

Finally, the optimized procedure was used on the determination of tested antioxidants in 8 samples of tea extracts. The samples were also analyzed by HPLC-DAD [26] as a comparative method. The results of analysis of tea samples are shown in Table 3 and two obtained chromato-voltammograms of the selected samples of tea extracts are shown in Fig. 5. Other antioxidants or/and interfering

substances presented in tea had no influence on the peak currents of the detected compound. Also, some of these antioxidants (marked in Fig. 5) have been identified based on their UV spectra. The obtained values of four out of five antioxidants determination in tea samples from HPLC-FSDPV are in agreement with those obtained using the reference HPLC-DAD method. The paired Student *t*-test indicates that there is no significant difference between the results obtained by both methods, at a confidence level of 95%. The presence of a fifth antioxidant (ferulic acid) was not detected in any tea samples by HPLC-FSDPV, suggesting its concentration below the limit of detection. Also, these results provide proof of the concept of the suitability of the proposed procedure for the monitoring of complex sample extracts.

4. Conclusion

This study presents a newly proposed method, which may be used for determination of multicomponent samples of antioxidants. Under optimal conditions, this method provides an enhanced selectivity, which allows to separate substances using a chromatographic column and also to distinguish compounds having different oxidation potentials at the same time. Another important point of this work is the successful solving of the main problems accompanying measurements, namely subtracting of the background current of FSDP voltammograms of the analytes and relating decrease of L_Ds for all targets. Last but not least, combining of proposed technique with the flow systems could help with the identification and quantification of unknown analytes in the real matrices. Results of the proposed method gave good correlation with traditionally used methods for the determination of antioxidants, such as HPLC with DAD. The analysis time and sample preparation itself is comparable between these two techniques. The difference between them is given mostly by the general selectivity difference between spectrophotometric and electrochemical technique: higher selectivity of the electrochemical detection makes it suitable only for the determination of specific classes of compounds, but in the same time it produces less populated chromatograms, with higher peak resolution. This is advantageous in the case of the determination of antioxidants, which are naturally electrochemically active. On the other hand, disadvantage compared to HPLC with DAD can be the higher limit of detection (around 0.1 mg kg^{-1}), which, nevertheless, does not negatively influence the applicability of the technique for the determination of antioxidants in various matrices.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.electacta.2018.05.199.

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Confirmation of participation

[1] **Bavol D.**, Dejmkova H., Scampicchio M., Zima J., Barek J., Combination of flow injection analysis and fast scan differential pulse voltammetry for the determination of antioxidants, *Electroanalysis 29*, 182–187 (**2017**).
Impact factor: **2.851**; Percentage of participation of Mgr. Dmytro Bavol ~ **50 %**.

[2] **Bavol D.**, Economou A., Zima J., Barek J., Dejmkova H., Simultaneous determination of *tert*-butylhydroquinone, propyl gallate, and butylated hydroxyanisole by flow-injection analysis with multiple-pulse amperometric detection, *Talanta 178*, 231–236 (**2018**).
Impact factor: **4.162**; Percentage of participation of Mgr. Dmytro Bavol ~ **70 %**.

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Impact factor: **1.282**; Percentage of participation of Mgr. Dmytro Bavol ~ **80 %**.

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Impact factor: **4.798**; Percentage of participation of Mgr. Dmytro Bavol ~ **70 %**.

I declare that the percentage of participation of Mgr. Dmytro Bavol at the above given papers corresponds to above given numbers.

Prague, 02. 07. 2018 RNDr. Hana Dejmková, Ph.D.

List of publications

1. **Bavol D.**, Zima J., Barek J., Dejmkova H., Voltammetric determination of cymoxanil and famoxadone at different types of carbon electrodes, *Electroanalysis 28*, 1029–1034 (**2016**).

2. **Bavol D.**, Dejmkova H., Scampicchio M., Zima J., Barek J., Combination of flow injection analysis and fast scan differential pulse voltammetry for the determination of antioxidants, *Electroanalysis 29*, 182–187 (**2017**).

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1. **Bavol D.**, Zima J., Barek J., Dejmkova H.: Voltammetric determination of cymoxanil on carbon-based electrodes, *XXXIV. Modern Electrochemical Methods* – Jetřichovice u Děčína, Czech Republic (19. – 23. 05. 2014).

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Internships

1. Foreign internship in Erasmus+ Intensive Programme, Environmental Remediation and Energy Production Technologies, ESTG-IPPortalegre – Portalegre, Portugal (22. 06. – 05. 07. 2014).

2. Long-term foreign internship in laboratory of prof. Matteo Mario Scampicchio, Faculty of Science and Technology, Free University of Bozen-Bolzano – Bolzano, Italy (15. 07. – 30. 10. 2014).

3. Long-term foreign internship in laboratory of prof. Anastasios Economou, Laboratory of Analytical Chemistry, Department of Chemistry, School of Sciences, National and Kapodistrian University of Athens – Athens, Greece (01. 10. 2015 – 04. 07. 2016).

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