

Electrophoretic separation of pressurized hot water extracts of *Hibiscus sabdariffa* L. using vitamin C as background electrolyte and contactless conductivity detection¹

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Construction of a contactless conductivity detector with a separate small dimension detection cell and integration into an automated capillary electrophoresis device is described. An optimized separation procedure of the pressurized hot water extract of *Hibiscus sabdariffa* L. and implementation of L-ascorbic acid as a novel background electrolyte in capillary zone electrophoresis with contactless conductivity detection are described. Optimized conditions allowed a rapid separation in less than 7 minutes with complementary data of UV-VIS absorbance and contactless conductivity detectors recorded simultaneously. The pressurized hot water extraction showed superior recovery to classical cold water extraction with increased peak areas by 9.4 times \pm 9.7%, 2.8 times \pm 9.7% and 4.1 times \pm 7.2% for pigments, inorganic cations and total peak area, respectively.

Key words: capillary electrophoresis, contactless conductivity detector, *Hibiscus sabdariffa* L., L-ascorbic acid, pressurized hot water extraction

INTRODUCTION

The electrophoretic separation technique was introduced by Stellan Hjertén in 1967 as free zone electrophoresis in a horizontally positioned, rotating, 3 mm inner diameter (ID) electrophoresis tube [1]. Later, it was described by Jorgenson and Lukacs [2] in 1981, where smaller ID capillaries were used. Afterwards, it was upgraded and described as “high-performance capillary electrophoresis” by Hjertén [3] and, undoubtedly, it reached a high development level as a modern separation technique. It is one of the most efficient and time saving separation techniques which can be applied for analysis of complex mixtures and is compatible to various detections. Originally, it was described as a separation method with UV on-tube scanning capability or indirect UV detection [1]. Later, in most applications it was used with on-capillary UV detection although other detection modes such as fluorescence, conductivity with contacting electrodes and mass spectrometry detection were utilized as well.

The capacitively coupled contactless conductivity detection (C4D) described by A. J. Zemann et al. in 1998 [4] has gained huge interest in the field of separation, when the principles and schematics of this detector were revealed [4–9]. An important reason for this was the simplicity of construction, cost effectiveness of such detector and its compatibility with capillary format separations and current separation systems [10]. Such detector is applicable for determination of inorganic ions [11], amino acids [12, 13], peptides [14], proteins [14, 15], saccharides [16], caffeine [17], etc. Electrode geometry and frequency dependence on detection performance is considered to be clear [18]. In addition to this, a miniaturized detection cell with electrodes separated from electronic circuit is described in literature [19]. Such setups allow easier integration of this detector in separation systems. An important issue of C4D is addressed to lower performance when detection is carried out in high conductivity buffers (phosphate, borate, etc.). This can be solved using low conductivity background electrolytes. A wide variety of low conductivity buffers have been studied and reviewed by Šolinova and Kašička [20].

Vitamin C (L-ascorbic acid or L-hexuronic acid) has not found its application in CE separations as a background

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¹The research was presented at the 8th International Scientific Conference *The Vital Nature Sign*, May 15–16, 2014, Kaunas (Lithuania).

electrolyte (BGE) due to its light absorbance in the UV region. L-ascorbic acid provides very low conductivity and acidic pH in aqueous solutions due to low dissociation (pK_a values 4.10 and 11.6) and bulky anions formed. Therefore, advantageous implementation of higher voltages, higher concentrations of BGE can be expected leading to better efficiency, sensitivity of detection and determination. Additionally, L-ascorbic acid solution can be an interesting alternative BGE for separation of analytes with antioxidant properties (or reactive analyte protection from oxidation) due to its reducing characteristics. A similar concept was described in literature where BGE containing EDTA was used for reactive metallochromic ligand separation [21].

Pressurized hot water extraction (PHWE) [22] is an extraction technique where water is used as a solvent under increased pressure conditions and temperatures ranging between 100 °C and 374 °C (critical water temperature), therefore the boiling point of the solvent could be increased resulting in different dissolution properties addressed to lower solvent polarity, which is related to lower dielectric constant at higher temperatures [23]. These characteristics of water offer beneficial extraction properties, such as applicability to low polarity compounds extraction with greater recoveries from various raw materials, green solvent properties and a possibility to adjust extraction solvent polarity selecting different conditions. Wide applicability and high efficiency of the pressurized hot water extraction was reported, including extraction of flavonoids, phenolic compounds [24], essential oils [25] and other physiologically important substances from plant material [26–28], soil remediation from polycyclic hydrocarbons [29].

Hibiscus sabdariffa L. was selected as an extraction and separation object using CE-C4D due to its popularity and wide usage in food, pharmaceutical industry and ethnopharmacy. Natural colorants [30–32] and other phenolic compounds [33] which provide high antioxidant activity are present in the plant. Pharmacological effects such as reduction of serum cholesterol [34], antihypertensive effect [35], apoptosis regulation [36], and inhibition of development of atherosclerosis [37] have been described in the literature.

The aim of this work was to construct and integrate a C4D detector into a commercial automated CE system in order to provide complementary detection data to the existing UV-VIS detector and explore the possibility to utilize L-ascorbic acid as a background electrolyte for CE separation of ions in pressurized hot water extracts of *Hibiscus sabdariffa* L.

EXPERIMENTAL

Chemicals

L-Ascorbic acid (99.7%), boric acid (99.5%) and calcium chloride (99%) were purchased from Sigma-Aldrich (Germany). L-histidin (L-His) (99%) and methanol (MeOH)

(99.9%) were from Carl Roth GmbH (Germany). 4-Morpholine ethanesulfonic acid monohydrate (MES) (98%), Alfa Aesar GmbH (Germany); tris(hydroxymethyl)aminomethane (Tris) (99.8%), Merck (Germany); chlorogenic acid (98%), Fluka (Germany); citric acid (99.5%), Lach-Ner (Czech Republic); sodium hydroxide (NaOH) (99%), ReaChem (Slovakia) and potassium acetate (99%), Fisher Scientific (USA) were used in this study. Bidistilled water was used for preparation of buffers and for extraction. Dried raw material of *Hibiscus sabdariffa* L. was purchased from the local store.

Instrumentation

The BioRad3000 CE system with the BioFocus 3000 software, BioRad (USA), the HP3DCE system with the ChemStation software purchased from Hewlett Packard (Germany), an ultrasonic bath, Cole-Parmer (USA), an orbital shaker, TiterTek (Germany) were used in this work. Data acquisition module DAQ1211 for conductivity signal acquisition was purchased from Futek Instruments (USA), a 14bit arbitrary waveform generator DDS-3005 was from Hantek (China), IC parts were purchased from the local store. Integration and data analysis was performed using the open-source software Chromulan developed by PiKRON (Czech Republic). The software written in a lab using LabWindows CVI (National Instruments, USA) was used for automation and autosaving of collected data in *.txt file format. The C4D system was constructed in the laboratory.

Contactless conductivity system

The C4D system consisted of a detection cell with the electrical circuit – Module1, and the signal conditioning electrical circuit – Module2 (Fig. 1). In the detection cell 3 mm radial electrodes made from a stainless steel syringe needle were soldered on the printed circuit board of the detection cell leaving a 0.8 mm air gap between them. Similar electrode geometry was reported in literature [5, 18]. A function generator provided an operating signal with fixed frequency and voltage that was fed directly to the detection cell actuator electrode. A DIP package OP37G operational amplifier was used as a current-to-voltage converter. The OPA2604 chip and additional passive components were used in the full wave precision rectifier circuit. Mentioned electronic parts with the passive integrator circuit were integrated into a small aluminum housing (5 × 1.5 × 1 cm), which was used as a shield and formed a small dimension detection cell (Module1). The detection cell was filled with thermally conductive silicone in order to minimize undesirable thermal fluctuations. The detection cell was connected to the signal conditioning module. In the Module2 the baseline was compensated, the signal was amplified (×41), filtered and fed via AD8139 (a differential ADC driver) (Fig. 1) directly to a 24-bit analog-to-digital converter (ADC). The developed software provided automated data acquisition and automated file saving. The C4D system described above was integrated into the BioRad 3000 capillary electrophoresis system. The detection cell was attached to the BioRad 3000

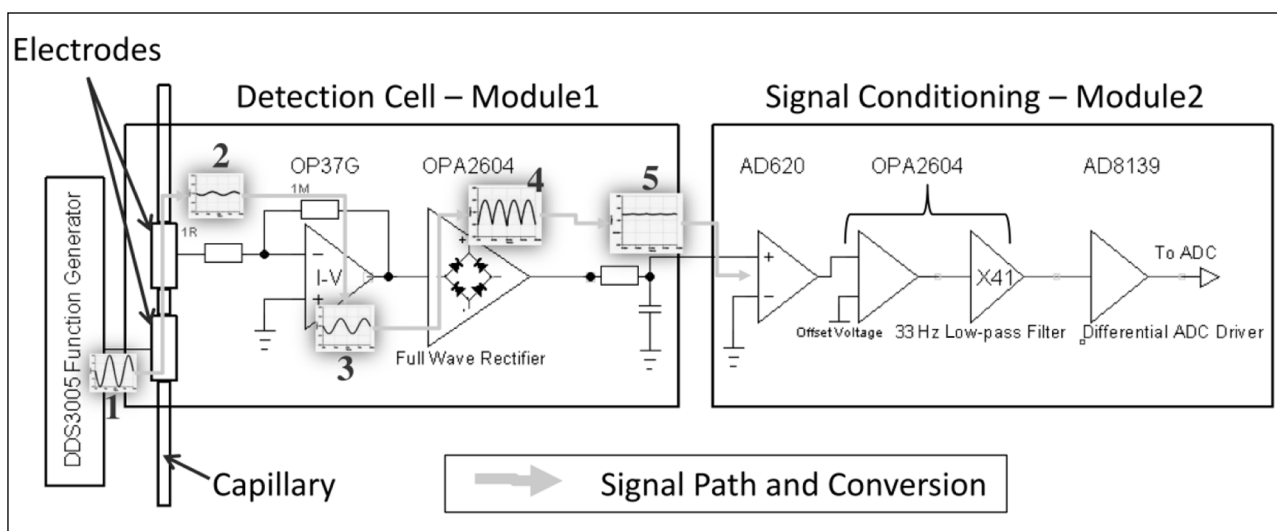


Fig. 1. A simplified circuit diagram of the C4D detector. A signal (1) is generated and fed to the actuator electrode of the detection cell; the attenuated signal (2) passes the detection gap and is amplified in the current-to-voltage converter (OP37G) (3), the amplified signal (4) is rectified in the full wave precision rectifier (OPA2604), high frequency filtered in the passive integrator circuit (5) and obtained DC is fed directly via the FTP cable to the signal conditioning Module2

cassette using an adhesive tape. A cooling tube was sealed with hot glue. Such set-up resulted in 6.5 cm distance between conductivity and UV-Vis detection windows in the BioRad 3000 CE apparatus, so that both detections with small and defined separation time and resolution difference could be carried out simultaneously. For comparative reasons the HP3DCE apparatus with the previously reported and integrated capacitance-to-digital (CDC) [38] detector was used.

Preparation of extracts

Two different extraction procedures were used for raw material of *Hibiscus sabdariffa* L. extraction, i. e. cold extraction using water and PHWE. A coffee-mill was used for raw material grinding to the particle fraction of 0.02–1.2 mm (measured by an optical microscope). For cold extraction the dry ground raw material was mixed with distilled water at the mass ratio 1/9 and left for extraction to proceed in an orbital shaker for 24 hours at 25 °C ambient temperature. For PHWE the espresso coffee apparatus Scarlett SC-037 (PRC) was used. The PHWE extraction principle is similar to the setup described in literature [23]. Water is heated and vapourized in a preheating coil then subjected to extraction material due to 4 bar pressure generated (technical specification), where a stainless steel filter basket serves as a back-pressure valve. The extraction procedure was performed according to the operation manual. A water reservoir was filled with bidistilled water. Raw material of *Hibiscus sabdariffa* L. was pressed into a filter basket and inserted into the apparatus. The same ratio of raw material and distilled water was maintained in both extraction modes.

Sample preparation

Crude extracts of *Hibiscus sabdariffa* L. raw material (both extraction modes) were diluted 10 times with bidistilled water and 5 times with BGE/MeOH 2/8 (V/V) mixture and

ultrasonicated for 20 seconds for gas removal and filtered through a 0.47 µm pore size disposable nylon filter.

RESULTS AND DISCUSSION

Optimization of separation

Separations were performed in fused silica capillary (L_{tot} 37.3 cm, C4D L_{eff} 26.5 cm, UV-VIS L_{eff} 33 cm, ID 50 µm) and different BGEs of alkaline, neutral and acidic pH. The capillary was washed with 0.5 M NaOH, bidistilled water and buffer solutions for 1 minute prior to each analysis. BGE of higher pH than 9 resulted in lower resolution. A similar separation profile to that described by Segura-Carretero et al. [30] was observed. The resolution of peaks registered using C4D was insufficient for identification of the compounds. 20 mM Tris/MES alkaline buffer, pH 9.0 (Fig. 2A) showed several unresolved peaks in pre- and post-electroosmotic time. The attempt to decrease pH and change the buffer composition, where 50 mM boric acid/Tris, pH 8.4, buffer (Fig. 2B) was implemented, resulted in a slightly better separation profile. More peaks were resolved, however, the separation was not satisfactory due to baseline fluctuations observed. Neutral conditions, where 30 mM L-His/MES, pH 6.7 buffer (Fig. 2C) was used, resulted in several separated peaks in pre- and post-electroosmotic time, however, an electroosmosis signal peak with tailing was observed (occurrence of undissociated analytes comigrating with electroosmotic flow (EOF)). The highest average efficiency ($N/m > 35\ 000$; RSD (%) < 6.3; $n = 10$), resolution, excellent migration time repeatability (intraday RSD (%) < 1.6; extra-day RSD (%) < 1.9, $n = 5$) and the best separation results were obtained in acidic conditions, using 75 mM L-ascorbic acid solution (pH 2.7) as BGE (Fig. 2D). Latter conditions allowed quantification of potassium ions down to 1 µM concentration (limit of determination, determined

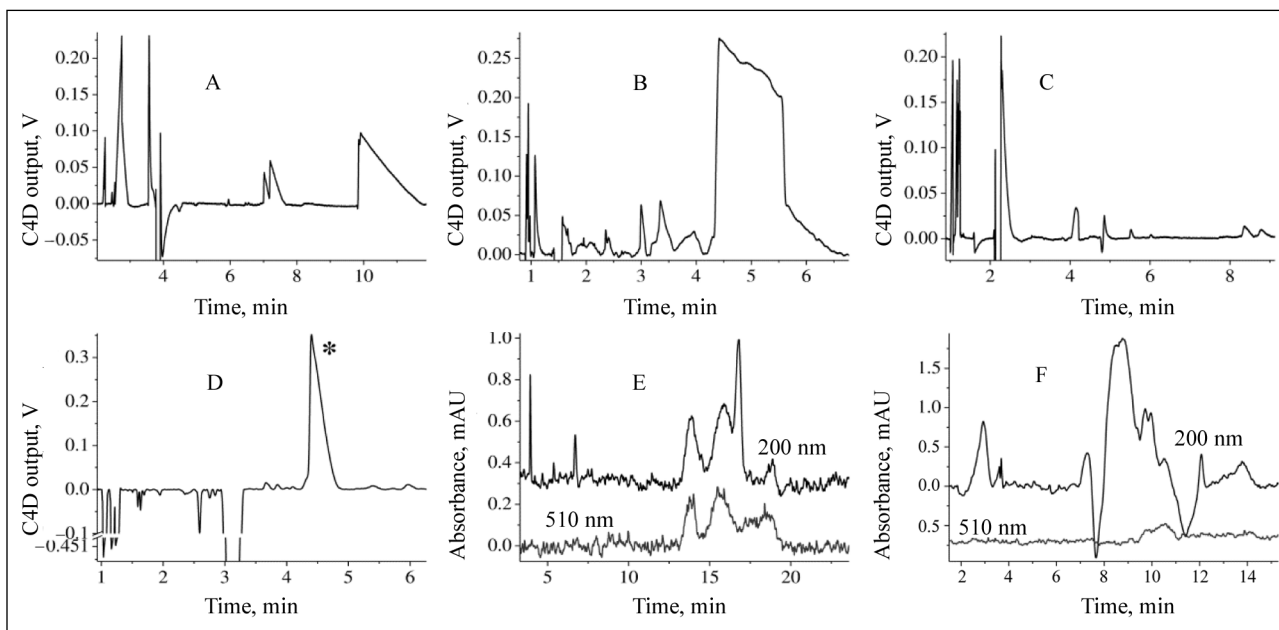


Fig. 2. Separation of *Hibiscus sabdariffa* L. extract using (A) 20 mM Tris/MES, pH 9.0, (B) 50 mM boric acid/Tris, pH 8.38, (C) 30 mM L-His/MES, pH 6.7 buffer, (D) 75 mM L-ascorbic acid BGE, pH 2.7, (E) 75 mM phosphate buffer, pH 2.7, (F) 75 mM citrate buffer, pH 2.7. Fused silica capillary, L_{tot} 37.3 cm, C4D L_{eff} 26.5 cm, UV-VIS L_{eff} 33 cm, ID 50 μm , O.D. 365 μm , 18 kV positive voltage, 45 °C cassette temperature, 30 °C carousel temperature. A, B, C and D C4D detection performed at 200 kHz, 4V amplitude sine wave; E, F UV absorbance at 200 nm and VIS absorbance at 510 nm detection. * Unidentified peak

in accordance with valid pharmaceutical method validation guidelines – ICHQ2R1). Most of the compounds present in *Hibiscus sabdariffa* L. have pK_a between 3 and 6 [39], therefore, small difference in pK_a value of the compounds allows to diversify the analytes electro-migration properties, using L-ascorbic acid (pH 2.7) as BGE. Compounds migrating before electroosmotic flow (EOF) were observed under the indirect mode and compounds migrating after EOF were observed under the direct mode of C4D detection. The direct or indirect detection mode is determined by the mobility difference of BGE and analyte cations. Similar detection properties for phenolic acids were reported by Kuban et al. [40] and indirect detection was found to be superior to the direct one.

The attempt to implement commonly used buffers and UV-VIS detection resulted in unsatisfactory separation of *Hibiscus sabdariffa* L. extract. 75 mM phosphate, pH 2.7, buffer (Fig. 2E) resulted in several unresolved peaks in 20 min. of analysis time for both UV and VIS absorbance modes. Higher level of noise was observed for common buffers in contrast to L-ascorbic acid as BGE. 75 mM citrate, pH 2.7, buffer (Fig. 2F) showed several peaks under indirect detection comigrating with several peaks which were observed under direct detection. Visible light (VIS) absorbance detection at 510 nm was used to reveal pigments peak profile of *Hibiscus sabdariffa* L. (similar spectral properties of colorants were reported by Ketmaro et al. [39]) (Fig. 3). Pigments peak was identified in the C4D detector recorded electropherograms calculating ion velocity from VIS absorbance detector data. 11.0 cm/min migration velocity was calculated for the pigments peak. The 1.5 times higher signal to noise ratio (S/N)

was observed for the C4D detector (36.7) than for the VIS detector (24.4). Chlorogenic and citric acids as well as inorganic cations were identified using standard solutions of those compounds. The chlorogenic acid peak was observed at 320 nm wavelength using UV detection and L-ascorbic acid as BGE. Interestingly, the UV absorbance detector showed inorganic cation peaks which were highest at 280 nm (Fig. 3). However, the C4D detector showed higher (1793.6) S/N ratio for the potassium peak than the UV detector (448.4) (S/N ratios for other peaks are presented in the Table). UV absorb-

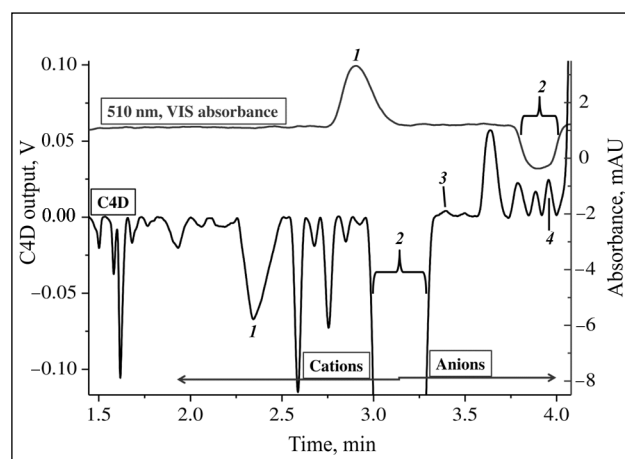


Fig. 3. PHWE extract *Hibiscus sabdariffa* L. separation and pigments peak identification using C4D (200 kHz, 4V) and VIS (510 nm) absorbance detections. Conditions: as described in Fig. 1D. Peaks: 1 – pigments, 2 – EOF, 3 – chlorogenic acid, 4 – citric acid

Table. Detection parameters using C4D detector

Parameter	Average*	Pigments	Inorganic cations (K^+ , Ca^{2+} , Na^+)	Chlorogenic acid	Citric acid	Unidentified peak**
S/N ratio for the peaks	–	36.6	1 793.6	17.3	29.0	856.1
Increase of peak corrected area*** (times)	4.1	9.4	2.8	7.4	7.7	3.6
RSD, % (n = 3) (different extractions)	7.2	9.7	9.7	14.0	13.3	8.1

* Average values for all peaks in electropherogram.

** Unidentified peak: see Fig. 4D.

*** Comparison of increase of analyte peak areas using PHWE instead of cold extractions.

ance spectra of 1000 times diluted BGE and 1 000 times diluted BGE with 20 μ M NaOH were recorded and compared. The results showed that the NaOH additive shifts the absorption maximum to higher wavelength by 2 nm and increases the absorbance maximum by 9% (Fig. 4). These observations can be explained by different absorbance of undissociated L-ascorbic acid, which is likely to undergo keto-enol tautomerism, and ascorbate anion, which can be found in different forms depending on α proton or β proton dissociation [41]. The pH of the solution is increased in the presence of inorganic cations and the equilibrium between L-ascorbic acid and ascorbate anions is shifted resulting in different absorption spectra. Spectral effects of absorption shifts for copper and mercury complexes with L-ascorbic acid have been described by E. Kleszczewska [42]. C4D detection resulted in higher resolution of inorganic cations than UV detection. Such effect was, probably, observed due to different width detection windows for both detectors. The UV detector has a constant width detection window. Contrary, the C4D detection window can be dynamic and it depends on high or low conductivity BGE is used [43]. In this case, the low conductivity L-ascorbic acid solution ensures a narrow detection window of C4D.

Peak area and concentration calculations

Figure 5 presents the electropherograms of cold water and PHWE extracts of *Hibiscus sabdariffa* L. PHWE provided

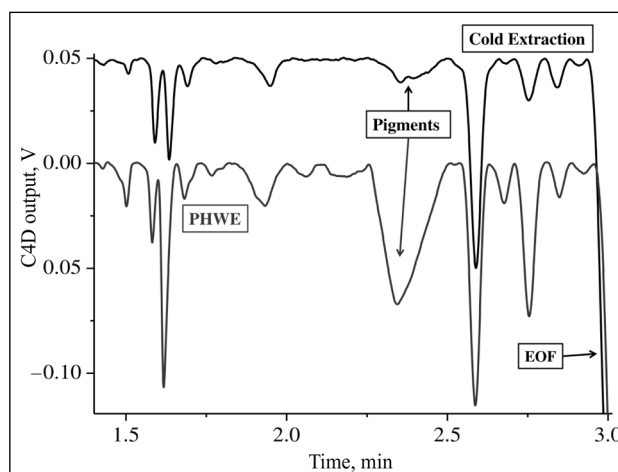


Fig. 5. *Hibiscus sabdariffa* L. extracts comparison of cold extraction and PHWE modes. C4D detection and separation conditions: as described in Fig. 2D

considerably higher recovery of the separated compounds than cold water extraction. PHWE took less than 15 minutes and cold water extraction took 24 hours. The calculated total and separated peak areas show different increase for different compounds (Table). This is due to the decreased polarity of water at higher temperature which affects extraction of different compounds. The lowest increase of the extracted amount (2.8 times \pm 9.7%) was observed for inorganic cations (K^+ , Ca^{2+} , Na^+) which are considered as highly polar. The

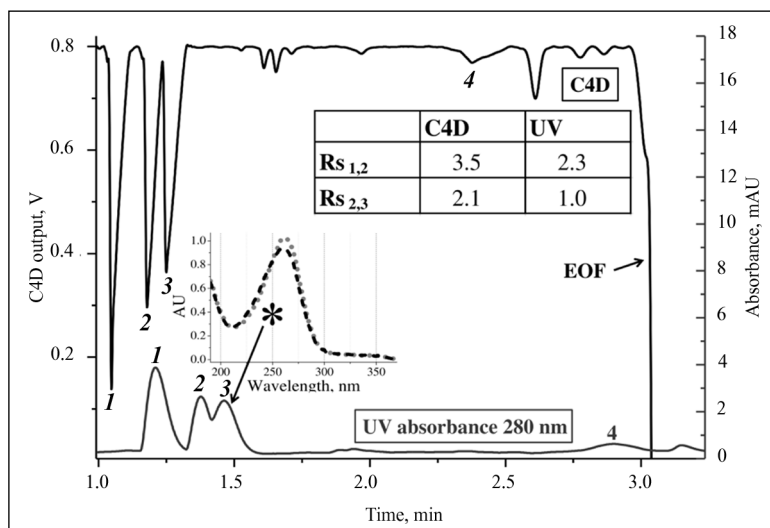


Fig. 4. Cold extraction of *Hibiscus sabdariffa* L. Comparison of C4D and UV absorbance detection modes. Peaks: 1 – K^+ , 2 – Ca^{2+} , 3 – Na^+ , 4 – pigments. R_s – resolution. * UV absorption spectra of 75 μ M L-ascorbic acid (grey dotted line) and 75 μ M L-ascorbic acid with 20 μ M NaOH (black dashed line) representing Na^+ peak spectra. Separation conditions: as described in Fig. 2D

highest increase of the amount of extracted compounds of interest was observed for pigments (9.4 times \pm 9.7%). Similar solubility tendencies for PHWE of heterocyclic compounds were reported by Karasek et al. [44]. Moderate increase was observed for chlorogenic and citric acid amounts. One of the major peaks (by integrated area) was not identified (* unidentified peak), however, the corrected area was calculated showing slightly higher increase than inorganic cations.

The optimized procedure was reproduced on the HP-3DCE system with the CDC detector implemented [38]. The obtained separation profile showed very similar electropherograms (Fig. 6) to Biorad3000-UV-C4D. Method reproducibility was evaluated showing less than 6.4% RSD for pigments and less than 7.7% for inorganic cations.

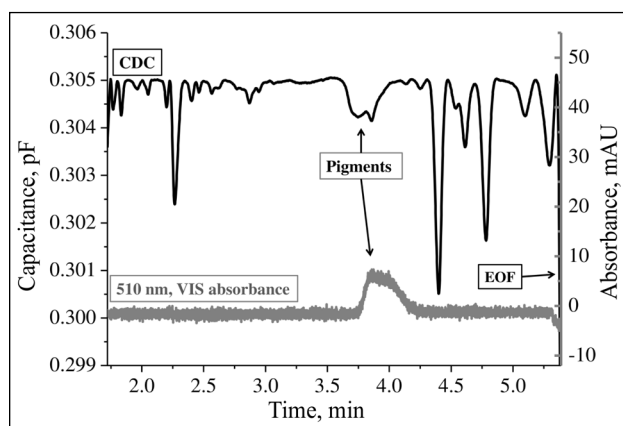


Fig. 6. Reproduced separation of *Hibiscus sabdariffa* L. PHWE extract using HP-3DCE-UV-CDC system. Conditions: 75 mM L-ascorbic acid BGE, pH 2.7, fused silica capillary, L_{tot} 62 cm, CDC L_{eff} 59.5 cm, UV-VIS L_{eff} 53.5 cm, ID 50 μm , O.D. 365 μm , 18 kV positive voltage, 25 $^{\circ}\text{C}$ cassette temperature, sample injection 50 mbar \times 10 s

CONCLUSIONS

The contactless conductivity detection system was constructed and integrated into the automated BioRad3000 capillary electrophoresis device. The proposed setup allowed simple and rapid implementation of the contactless conductivity detection system into the capillary electrophoresis device.

Complementary data of contactless conductivity and UV-VIS absorbance detectors were acquired and used for the identification and quantitation of the analytes. The defined capillary length between both detectors allowed simple calculation and evaluation of velocities of migrating analytes. L-ascorbic acid was demonstrated as a suitable background electrolyte in the CE-C4D separation of the pressurized hot water *Hibiscus sabdariffa* L. extract resulting in a rapid, efficient and reproducible analysis.

ACKNOWLEDGEMENTS

This research was supported by Vytautas Magnus University Grant No. BF-13-07.

Received 23 May 2014

Accepted 23 June 2014

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**KARŠTO VANDENS SLĖGINE EKSTRAKCIJA GAUTŲ
HIBISCUS SABDARIFFA L. EKSTRAKTŲ
ELEKTROFOREZINIS SKIRSTYMAS
NAUDOJANT DARBINĮ ELEKTROLITĄ – VITAMINĄ C
IR BEKONTAKTĘ LAIDUMO DETEKCIJĄ**

S a n t r a u k a

Darbe aprašyta bekontakčio laidumo detektoriaus su atskira mažo dydžio detekcijos cele gamyba ir integravimas į automatizuotą kapiliarinės elektroforezės įrenginį. Optimizuota skirstymo procedūra pritaikyta *Hibiscus sabdariffa* L. ekstraktams, gautiems karšto vandens slėgine ekstrakcija, skirstyti. Šiai procedūrai naudotas darbinis elektrolitas – vitaminas C yra visiškai suderinamas su bekontakčio laidumo detekcija. Optimizuotomis sąlygomis pavyko atlikti ekstraktų skirstymą per mažiau nei 7 min., taip pat tyrimui naudota sistema sudaro galimybę vienu metu gauti komplementarius bekontakčio laidumo ir ultravioletinės – matomos šviesos – absorbcijos detektorių duomenis. Karšto vandens slėginė ekstrakcija pasižymi didesne išgava nei įprasta ekstrakcija šaltu vandeniu. Nustatytų smailių plotas atskirtiems junginiams padidėjo 9,4 karto \pm 9,7 % (pigmentai), 2,8 karto \pm 9,7 % (neorganiniai katijonai) ir 4,1 karto \pm 7,2 % (bendras smailių plotas).