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Příloha 2.

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Research Article

Very fast electrophoretic determination of creatinine and uric acid in human urine using a combination of two capillaries with different internal diameters

A capillary system formed by combining 25 and 100 μm id capillaries was used in the short-end injection mode to determine creatinine and uric acid in human urine. The separation was performed at an electric field intensity of 2.3 kV/cm. Creatinine was determined in a BGE with a composition of 20 mM citric acid/NaOH (pH 3.0), and uric acid was determined in 20 mM MES/NaOH (pH 6.0). Under these conditions, migration times of 12.2 s for creatinine and 8.6 s for uric acid were achieved. The LOD value is 2.4 mg/L for creatinine and 0.9 mg/L for uric acid; the RSD for the migration time varies in the range 0.7–1.1% (intra day) to 1.0–7.5% (inter day); RSDs for the peak areas equalled 3.4–4.0% (intra day) and 4.3–4.7% (inter day). The determined creatinine values in seven urine samples vary in the range 221–1394 mg/L for creatinine and 87–615 mg/L for uric acid. *t*-Test did not reveal any statistically significant difference between the developed CE methodologies and reference methods – Jaffé reaction for creatinine and enzymatic uricase test for uric acid.

Keywords:
Capillary electrophoresis / Clinical analysis / Creatinine / High-speed analysis / Uric acid
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1 Introduction

Creatinine (Fig. 1A) is used in human medicine as an indicator of proper kidney function. Creatinine is a side product of energy metabolism in muscles and is excreted from the body through the kidneys [1]. As it is not resorbed in the kidneys, the creatinine levels in serum and urine are used to measure the blood flow through the kidneys [2]. The concentrations of the other substances present in the urine are recalculated to the urine creatinine level, eliminating the effect of variable diuresis. Consequently, the determination of creatinine in urine and blood is part of basic biochemical examination and is performed photometrically on the basis of the Jaffé reaction [3] or using enzymatic methods [4].

Uric acid (Fig. 1B) is the final product of purine metabolism in humans and is excreted from the body in urine in the form of urate salts, especially sodium and ammonium urate [1]. The limited solubility of uric acid and its salts is a problem and insoluble crystals can be formed in the tissues under pathological conditions. These diseases are known as gout, the Lesh–Nyhan syndrome and uric acid stone formation in the kidneys and are caused by hereditary defects, high intake of purines in foods or reduced excretion ability of the kidneys [5]. In clinical laboratories, uric acid is determined using the uricase enzyme [6] and the coloured products formed are measured photometrically.

Following alternative analytical techniques have been developed to determine these two substances in urine:

(i) for creatinine, HPLC with ultraviolet detection [7, 8], isotope dilution GC-MS [9], CE [10], methods of flow injection analysis [11] and electro-chemical biosensors [12] can be used;
(ii) uric acid has been determined using the methods of anion-exchange HPLC [13], RP HPLC with ultraviolet detection [14, 15], GC-MS [16, 17], the stopped-flow method for the fluorimetric determination [18], CE [19] and a wide range of other electro-chemical biosensors [20].

In the monitoring of creatinine and uric acid in extensive sets of biological samples, emphasis is placed on both reliability and accuracy as well as on the speed of the determination, treatment of the biological material, automation and, last but not least, the costs of the analyses. These goals can be attained using the capillary or chip electrophoresis methods [21, 22]. Electrophoretic determination is characterised by high separation efficiency, low demands on treatment and the amount

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Abbreviations: IS, internal standard; **PAS**, *p*-aminosalicylic acid

Colour Online: See the article online to view Figs. 3 and 4 in colour.

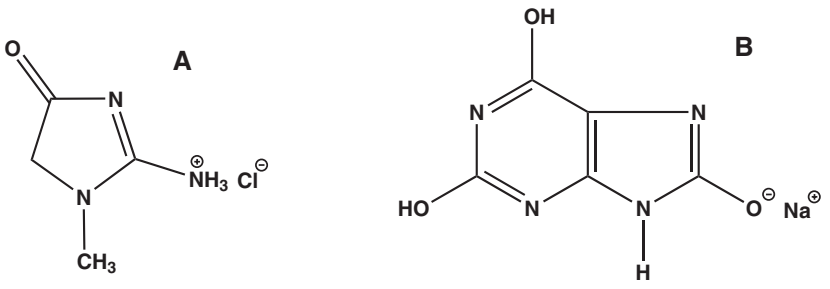

Figure 1. Structures of creatinine (A) and uric acid (B) in their protonated and dissociated forms, respectively.

of biological material and minimal operating costs. The speed of electrophoretic separation can be controlled by adjusting the length of the separation path and the electric field intensity. Very rapid separation can be achieved using microchips [23], laboratory-made electrophoretic instruments with a capillary length of 10 cm [24, 25] and commercial electrophoretic instruments using injection from the short end of the capillary [26]. A maximum electric field intensity of approximately 1 kV/cm can be employed when commercial electrophoretic instruments with a function for automatic analysis of sets of samples are used. It was demonstrated in a recent work that a combination of two capillaries of different internal diameters makes it possible to employ an intensity approaching 3 kV/cm when using commercial instruments [27]. Complete separation of a model mixture of adrenaline, noradrenaline and dopamine was achieved in 13 s using this procedure. This contribution describes the use of a combination of two capillaries for very fast determination of creatinine and uric acid in human urine.

2 Materials and methods

2.1 New procedure for joining two capillaries

Compared to the original work, the technique of joining two capillaries with different ids was simplified and improved [27]. The analytical capillary (id 25 μm, od 363 μm, length 9.7 cm) and auxiliary capillary (id 100 μm, od 363 μm, length 22.6 cm) with straight-cut ends were joined using a 9 mm-long PTFE capillary for HPLC (od 1.6 mm, id 0.25 mm, Watrex, Prague, Czech Republic). The tube was heated using a heat gun to a temperature above the melting point of PTFE −330°C (a temperature of 420°C was set on the heat gun). The heated softened PTFE tube was drawn over the ends of the two capillaries. After cooling (approx. 1 min), a hydrodynamically impermeable and mechanically strong join with minimum dead volume that was controlled under microscope was formed between the electrophoretic capillaries (Fig. 2). Finally, a detection window was created 8.4 cm from the injection end of the 25 μm capillary; the capillary was installed in the cassette of the CE instrument and is ready for analysis. This simple procedure for connecting two capillaries takes approximately 15 min.

2.2 CE conditions

All electrophoretic measurements were carried out using the HP^{3D}CE system (Agilent Technologies, Waldbronn, Germany) with an in-built diode-array detector (electropherograms were recorded at 214 nm for creatinine and at 292 nm for uric acid). The separations were performed in the short-end injection mode with an effective length of the coupled capillary of 8.4 cm (distance from the injection end to the detector) and overall length of 32.3 cm. The sample was injected hydrodynamically at a negative pressure of 50 mbar for 5 s in the inlet of analytical capillary, corresponding to an injection length of 4.1 mm (calculated from the Hagen–Poiseuille equation). CE separation was performed at a voltage of +25 kV, corresponding to an electric field intensity in the separation part of the capillary of 2.3 kV/cm. The pressure required for rinsing the capillary and for injection of the sample was applied to the free end of the auxiliary capillary (id 100 μm), which made it possible to successfully resolve the problems associated with elimination of air bubbles from the capillary, causing interruption of the CE separation. A separation capillary is placed in electrophoretic cassette and thermostatted at 25°C. A new capillary was gradually washed at a pressure 1000 mbar with 0.1 M NaOH (10 min), water (10 min) and BGE (10 min); 0.5 min washing with BGE was used between individual analyses.

2.3 Chemicals

All the chemicals used were of p.a. purity: citric acid (Penta, Czech Republic), creatinine (Fluka), imidazole (Fluka), MES (Sigma), NaOH (Fluka), *p*-aminosalicylic acid (PAS, Sigma), uric acid (Fluka). The stock solutions of the analytes (creatinine and uric acid) and internal standards (ISs; imidazole and PAS) were prepared at concentrations of 1 mg/mL; 1 M NaOH solution was added to dissolve uric acid and PAS. Deionized Milli-Q water (18.2 MΩ cm, Millipore, Molsheim, France) was used for preparation of the BGE and the stock

Figure 2. Detail of connection of the two capillaries: analytical capillary (1), auxiliary capillary (2) and PTFE tube (3).

solutions of the standards, which were stored in a refrigerator at 4°C.

2.4 Pre-treatment of urine samples and spectrophotometric determination

Samples of morning urine were obtained from seven healthy adult volunteers. The urine samples were stored in a refrigerator at 4°C and maintained at this temperature until the analysis, which was performed on the day of sampling or the next day. Prior to the measurement, the samples were filtered through a Durapore PVDF membrane (pore size 0.45 μm, centrifugal filter devices, Millipore, Bedford, MA, USA). CE analysis of creatinine: 20 μL of urine were mixed with 980 μL of 1 mM HCl with addition of 20 mg/L imidazole; CE analysis of uric acid: 20 μL of urine were mixed with 980 μL of 1 mM NaOH with addition of 20 mg/L PAS.

The control methods consisted of the bio-test (creatinine liquid 500, Lachema, Brno, Czech Republic) based on the Jaffé reaction and subsequent photometric determination at 505 nm and the bio-test (uric acid liquid 500, Lachema) based on the enzymatic reaction with uricase and subsequent photometric determination at 550 nm. All spectrophotometric measurements were performed in 1 cm cuvettes using a Boeco spectrophotometer (Model S-22, Hamburg, Germany).

2.5 Treatment and evaluation of the results

All the CE analyses of the model samples were carried out in five consecutive runs and the plots represent the average values ± SDs. The Origin 7.0 program (OriginLab, Northampton, MA, USA) was used to evaluate and statistically treat the experimental data. The number of theoretical plates was calculated from the formula, $N = 5.54\,(t_M/w_{1/2})^2$, where t_M is the migration time and $w_{1/2}$ is the peak width at half-height. The peak resolution, R, was computed from the relationship, $R = 2(t_{M2} - t_{M1})/(w_1 + w_2)$, where $w_1$and w_2 are the peak widths at the baseline.

3 Results and discussion

3.1 Optimisation of CE analysis of creatinine and uric acid in model samples

Creatinine is a weak base with a value of pK_A 4.8. Consequently, the creatinine determination was performed in an acidic BGE containing 20 mM of citric acid/NaOH at pH 3.0 and the separation was performed in the positive mode. At pH 3.0, creatinine migrates as a cation and EOF is relatively small (a water gap was not observed to 1 min). The IS was imidazole, which also exhibits cationic mobility and does not occur naturally in urine. Under these experimental conditions (see Fig. 3A), the creatinine migration time is 12.2 s

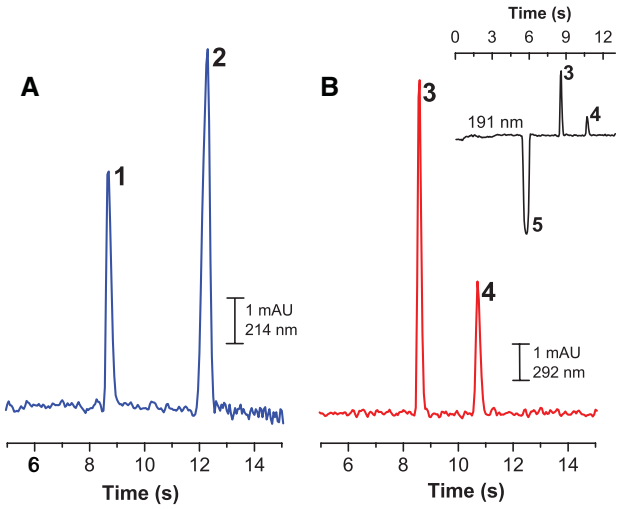

Figure 3. Electropherograms: (A) Separation of a model mixture of creatinine (40 mg/L) and imidazole (IS, 20 mg/L) and (B) separation of a model mixture of uric acid (40 mg/L) and PAS (IS, 20 mg/L) at an electric field intensity of 2.3 kV/cm. *Inset*: Recording of the separation of uric acid and PAS at 191 nm for identification of the water gap. Identification of peaks: imidazole (1), creatinine (2), uric acid (3), PAS (4) and the water gap (5).

Table 1. Migration time (t_M), number of theoretical plates (N), resolution (R) and mobility (u) for a model sample of creatinine and uric acid at concentrations of 40 mg/L

Parameters	Creatinine	Uric acid
t_M (s)	12.2 (0.0)	8.6 (0.1)
N (m^{-1})	118 000 (6 000)	354 000 (69 000)
N (s^{-1})	810 (40)	3450 (660)
R	8.9 (0.0)	9.4 (1.0)
u, 10^{-9} (m^2 s^{-1} V^{-1})	35.3 (0.1)	− 25.0 (0.5)

SDs are in parentheses.

and that of imidazole is 8.8 s, with a resolution value of 8.9 for both substances.

Uric acid is a weak acid with pK_A approx. 5.5, and its CE separation was performed in BGE with composition 20 mM MES/NaOH pH 6.0 in the positive mode (Fig. 3B). Under these conditions, uric acid migrates as an anion against the direction of EOF. The velocity of EOF at pH 6 is greater (water gap recorded at time 6 s) than the electrophoretic mobility of uric acid and EOF draws uric acid towards the detector; the uric acid peak (t_M 8.6 s) is recorded later than the position of EOF. The IS was PAS (pK_A 3.6), which exhibits higher electrophoretic mobility than uric acid and, moving against the direction of EOF, reaches the detector later (t_M for PAS is 10.7 s, the resolution of the two substances equals 9.4). PAS is also not a biogenic substance and does not occur naturally in urine.

It is apparent from the obtained electropherograms (Fig. 3) and calculation of the separation parameters (Table 1) that very short migration times of around 10 s can be obtained using an electric field intensity of 2.3 kV/cm and

Table 2. Parameters of the linear regression function for the peak area, the LOD and LOQ values

Parameters	Creatinine	Uric acid
Tested interval (mg/L)	5–70	10–70
Slope (mAU s mg^{-1} L)	0.0557 (0.0004)	0.0414 (0.0002)
Intercept (mAU s)	0.161 (0.017)	0.020 (0.007)
R	0.9998	0.99995
Noise (mAU)	0.2	0.1
LOD (mg/L, μM)	2.4 (21)	0.9 (5.4)
LOQ (mg/L)	7.9	3.0

effective separation path of 8.4 cm. On comparison with literature, the rapid CE determination of creatinine equals approx. 1 min in a capillary with an effective length of 10 cm [28] and 22 s, in a capillary with an effective length of 8.5 cm at 30 kV [29]; uric acid was determined on an 8.2 cm-long separation path at 20 kV in approx. 50 s [30].

The number of theoretical plates *N* related to 1 m capillary length for creatinine equals 118 000 m^{-1} and, for uric acid, equals 354 000 m^{-1}. The higher *N* value for uric acid is a consequence of the shorter t_M value and the smaller effect of electro-disperison on broadening of the peaks. For very fast separations, it is preferable to express the efficiency of the separation in the form N/t_M, which better corresponds to the need to separate a large number of substances in a short time. The attained N/t_M values are fully comparable with the values for separation of a mixture of basic neurotransmitters under similar experimental conditions [27]. The experimentally determined value of the mobility for creatinine under conditions with high intensity of the electric field and short-end injection corresponds to the theoretical values of the mobility calculated using the Peak-Master program (33.4×10^{-9} m^{2}s^{-1}V^{-1}) [31]. This is a further indication that, even when these extreme conditions are used, the conduction of the Joule heat away from the capillary is sufficient and the separation acts similarly as at low intensities of the electric field.

3.2 Method calibration

Calibration of creatinine and uric acid was performed in the concentration interval of 5–70 and 10–70 mg/L, respectively (Table 2). The tested concentration interval covers well the range of physiological levels of both substances in urine (creatinine 370–3000 mg/L, uric acid 80–1000 mg/L, http://metagene.de) using a 20- to 50-fold dilution of the urine prior to CE analysis. In this interval, the calibration dependences for both substances are linear with correlation coefficient values greater than 0.999. LODs were calculated from the relationship 3 × noise/slope and attain values of 2.4 mg/L for creatinine and 0.9 mg/L for uric acid; the higher LOD for creatinine is caused by the higher noise level of the detector in citrate/NaOH BGE, which partly absorbs radiation at 214 nm. The obtained LODs are somewhat worse compared with the LOD values for detection using other CE determinations of these analytes over a short separation path: (i) creatinine, 0.5 mg/L in a 50 μm capillary with an effective length of 8.5 cm [29], 0.7 mg/L in a 75 μm capillary with an effective length of 10 cm [28]; (ii) uric acid, 0.1 mg/L in a 75 μm capillary with an effective length of 8.5 cm [30]. Worse LODs when using a combination of two capillaries can be explained by the shorter absorption path in the 25 μm capillary and also the small injected sample amount. The sensitivity of the two developed methods is nonetheless sufficient for determination of creatinine and uric acid in 20- to 50-fold diluted urine samples.

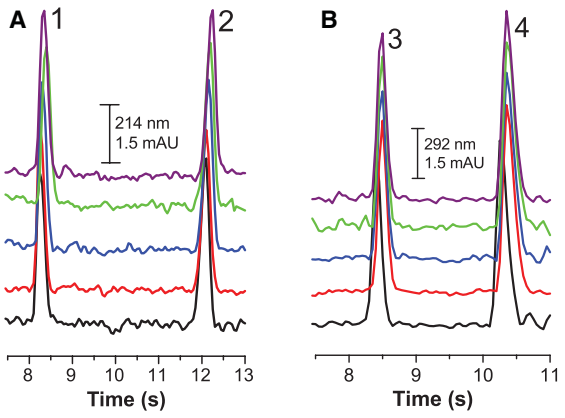

Figure 4. Five consecutive CE analyses of a 50-fold diluted sample of human urine containing creatinine 650 mg/L (A) and uric acid 615 mg/L (B). For the experimental conditions, see the caption in Fig. 3 and the text. Peak identification: imidazole (1), creatinine (2), uric acid (3) and PAS (4).

3.3 Analysis of creatinine and uric acid in urine samples

The peaks of creatinine and imidazole – IS are apparent in the electropherograms of 50-fold diluted urine in BGE 20 mM citrate/NaOH at pH 3.0 and 214 nm (Fig. 4); in BGE 20 mM

Table 3. Migration time, resolution, number of theoretical plates, slope and RSD values for determination of creatinine and uric acid in urine samples

Parameters	Creatinine	Uric acid
t_M (s)	12.1 (0.1)	8.6 (0.2)
R	11.9 (0.3)	6.2 (0.9)
N (m^{-1})	240 000 (27 000)	228 000 (30 000)
N (s^{-1})	1660 (170)	2 200 (20)
Slope (mAU s mg^{-1} L)	0.0556 (0.0015)	0.0400 (0.0015)
RSD – migration time (intra day) (%)	0.7	1.1
RSD – peak area (intra day) (%)	4.0	3.4
RSD – migration time (inter day) (%)	1.0	7.5
RSD – peak area (inter day) (%)	4.3	4.7

Table 4. Concentrations of creatinine and uric acid in urine determined by CE and comparison with the values determined by the Jaffé reaction (creatinine) and the enzymatic method (uric acid); SDs in parenthesis

Urine sample	Creatinine (mg/L)		Uric acid (mg/L)	
	CE	Jaffé reaction	CE	Enzymatic test – uricase
A	1320 (23)	1128 (23)	556 (33)	555 (24)
B	1394 (68)	1407 (94)	382 (23)	339 (20)
C	931 (44)	981 (119)	269 (11)	326 (24)
D	221 (26)	272 (10)	87 (8)	82 (7)
E	650 (23)	872 (51)	615 (29)	605 (18)
F	453 (31)	660 (31)	398 (14)	389 (9)
G	1023 (36)	1166 (92)	378 (11)	375 (9)

MES/NaOH at pH 6.0 and 292 nm the peaks of uric acid and PAS – IS are detected. The other substances present in the urine are not recorded in the electropherograms because of their low concentrations in the urine or because they do not absorb radiation at these wavelengths. The values of t_M (12.1 s for creatinine and 8.6 s for uric acid) are identical with t_M in the model samples (Table 3). To increase the precision of the quantification in urine and to eliminate fluctuations in injecting the sample, the peak areas were normalised to the peak area of IS (the normalised peak areas were used for a calculation of calibration parameters, Table 3, and determination of creatinine and uric acid in urine, Table 4). The obtained slopes of calibration dependencies for the peak areas measured for spiking the urine with additions of standards in the concentration range 0–70 mg/L also fully correspond to the values for real samples. Fiftyfold dilution of the urine is sufficient for eliminating the undesirable effect of the matrix on the CE separation and does not cause dilution of the urine below the LOD of the method. The RSD value for ten consecutive analyses of a single urine sample for t_M has values of 0.7% (creatinine) and 1.1% (uric acid) and, for the peak areas, 4.1 and 3.4%, respectively. The RSD values for ten analyses of one urine sample performed on three consecutive days for t_M equal 1.0 and 7.5% and, for the peak areas, 4.3 and 4.7%, respectively. The RSD values fully correspond to the values for normal electrophoretic determination of real biological samples.

The determined creatinine values in seven urine samples vary in the range 221–1394 mg/L for creatinine and 87–615 mg/L for uric acid (Table 4). Creatinine and uric acid were simultaneously determined using standard methods employed in clinical laboratories: the Jaffé reaction for creatinine and uricase enzymatic test for uric acid. The CE determination yields similar values for creatinine and uric acid as the standard clinical method and the performed paired sample *t*-test did not reveal any statistically significant difference between the two sets of data; the calculated value was $t = 1.22$ for creatinine ($p = 0.235$), $t = 0.17$ for uric acid ($p = 0.864$), critical value $t_{0.05} = 2.447$.

The total time of CE analysis is composed of (i) sample pre-treatment – urine filtration and dilution (0.5 min), (ii) CE analysis – capillary washing (0.5 min), sample injection and CE separation (20 s); in total 1.3 min. The total time of enzymatic (or colourimetric) method is composed of (i) mixing of buffer (or sodium hydroxide) and sample + 1–5 min incubation; (ii) then adding an enzyme (or picric acid) + 1–2 min incubation and (iii) absorbance measurement, in total 3–8 min.

4 Concluding remarks

The use of a combination of two capillaries with different inner diameters makes it possible to use commercial CE instruments at electric field intensities greater than 2 kV/cm without excessive release of Joule heat and destruction of the electrophoretic separation. The developed CE method for determining creatinine and uric acid is characterised by a separation time of approx. 10 s. The urine is only filtered and diluted prior to the analysis. This newly developed electrophoretic method is a suitable alternative to the standard colourimetric and enzymatic determinations that are routinely performed in clinical laboratories and permits rapid screening of creatinine and uric acid in extensive sets of samples.

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The authors have declared no conflict of interest.

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Analytical Methods

The use of capillary electrophoresis with contactless conductivity detection for sensitive determination of stevioside and rebaudioside A in foods and beverages

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We would like dedicate this paper to our teacher Professor František Opekar on the occasion of his 70th birthday.

Keywords:
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High-speed analysis
Rebaudioside A
Stevioside

ABSTRACT

Two electrophoretic methods with contactless conductivity detection have been developed for determination of the content of rebaudioside A and stevioside in samples of sweeteners and beverages prepared from extracts of the plant Stevia rebaudiana Bertoni. The total content of rebaudioside A and stevioside can be determined in a fused silica capillary with an inner diameter of 10 μm and total length of 31.5 cm in optimised background electrolyte with the composition 170 mM H_3BO_3/LiOH (pH 9.0). The combined peak of the two glucosides is characterised by a migration time of 54 s, which completely separates it from EOF. INST coating solution in an amount of 0.5% *v*/*v*, which effectively suppresses the electroosmotic flow, was added to the background electrolyte for mutual separation of rebaudioside A and stevioside. The CE method with suppression of EOF is characterised by complete separation of rebaudioside A and stevioside, LOD is 0.3 mg/L (0.1 μM).

1. Introduction

Extracts from tropical plants of the family Stevia rebaudiana Bertoni are currently used as one of the most widespread natural substitutes for saccharose (Lemus-Mondaca, Vega-Galvez, Zura-Bravo, & Ah-Hen, 2012; Yadav, Singh, Dhyani, & Ahuja, 2011). The sweetness of Stevia is a result of the presence of an extensive group of glycoside substances, which include rebaudiosides, stevioside, steviolbioside and dulcosides. The chemical structure of these substances is based on cyclic diterpene steviol, to which are bonded various numbers of glucopyranose, rhamnopyranose and xylopyranose units; a detailed survey of the chemical composition of steviol glucosides can be found in the articles (Jackson et al., 2009; Morlock, Meyer, Zimmermann, & Roussel, 2014; Zimmermann, 2011). These substances have a sweetening ability that greatly exceeds that of saccharose; for example, rebaudioside A and D have a purely sweet taste without any indication of bitterness and a sweetening ability that is 300–450 times greater than that of saccharose; on the other hand, the sweetening ability of dulcosides is only 100 × greater and they have a markedly bitter to metallic taste (Kinghorn, 2002).

From a practical point of view, stevioside and rebaudioside A (Fig. 1) are the most important of the wide range of various steviol glycosides. These two substances should make an approx. 95% contribution to the overall content of steviol glycosides used in foods and food supplements in order to achieve a purely sweet taste and sensorial feeling comparable to saccharose. The importance of steviol glycosides in the food industry is connected with their almost zero energy value (Lemus-Mondaca et al., 2012). Consequently, they are preferentially used to sweeten beverages for individuals suffering from overweight who are not capable of meeting their daily liquid needs with pure water. In this way, steviol glycosides can be effectively used in regulation of food intake and achieving a feeling of sweetness satisfaction (Carakostas, Curry, Boilea, & Brusick, 2008). Compared to artificial sweeteners of the aspartame type, steviol glycosides do not cause secretion of insulin and, in addition, can be used for individuals suffering from phenylketonuria (Kroger, Meister, & Kava, 2006). For all these reasons, the use of steviol glycosides is becoming increasingly widespread, leading to the requirement of controlling the content of steviol glycosides in foodstuffs to monitor possible falsification.

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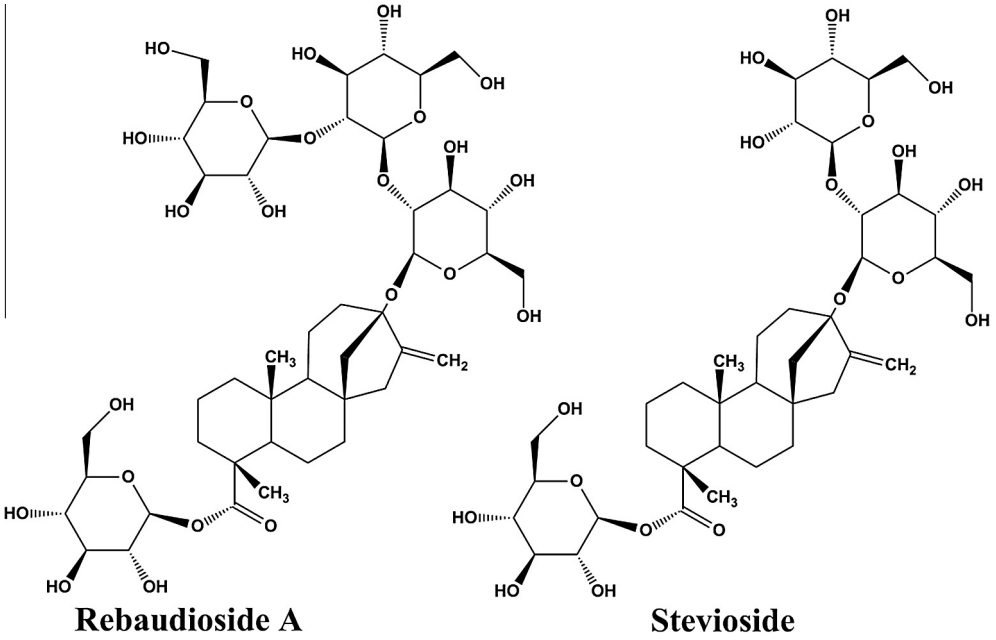

Fig. 1. Structure of the most important steviol glycosides used in the food industry.

Of the analytical methods known to date, steviol glycosides in Stevia leaves have been determined by desorption electrospray ionization mass spectrometry (Jackson et al., 2009), near infrared reflectance spectroscopy (Hearn & Subedi, 2009), high-performance thin-layer chromatography with densitometric quantification (Jaitak, Gupta, Kaul, & Ahuja, 2008) and electrospray ionization mass spectrometry (Morlock et al., 2014). However, HPLC techniques using an NH_2 column (Kitada, Sasaki, Yamazoe, & Nakazawa, 1989; Pól et al., 2007), RP18C column (Bililign, Moore, Tan, & Leeks, 2014; Bovanova, Brandsteterova, & Baxa, 1998) and newly also an HILIC column (Ahmed & Dobberstein, 1982; Zimmermann, Woelwer-Rieck, & Papagiannopoulos, 2012) hold a very dominant position in the analysis of foodstuffs. HPLC was also recommended in 2010 by the World Health Organisation as a reference method for determining steviol glycosides in foodstuffs (Tada et al., 2013). A difficulty encountered when using HPLC analysis of weakly absorbing glycosides lies in finding sensitive detection techniques. When using UV detection, measurements are carried out at short wavelengths around 190 nm (Makapugay, Nanayakkara, & Kinghorn, 1984; Wolwer-Rieck, Tomberg, & Wawrzun, 2010) or the sensitivity is increased by derivatisation of glycosides using p-bromophenacyl bromide (Kitada et al., 1989). Consequently, HPLC is combined with all types of mass analysers, which simultaneously ensure the selectivity of the determination (Gardana, Scaglianti, & Simonetti, 2010; Pól, Hohnová, & Hyotylainen, 2007; Wolwer-Rieck et al., 2010; Zimmermann et al., 2012).

The first experiments with analysis of steviol glycosides were performed using capillary electrophoresis (CE) (Liu & Li, 1995). The separation electrolyte was based on sodium tetraborate with addition of organic solvent and the capillary zone electrophoresis (CZE) technique was employed (Dacome et al., 2005; Liu & Li, 1995; Liu, Ong, & Li, 1997); in one case sodium dodecyl sulphate (SDS) was added to the sodium tetraborate and separation was performed by the micellar electrokinetic chromatography (MECK) technique (Mauri, Catalano, Gardana, & Pietta, 1996). In the newest CE study, rebaudioside A and stevioside were separated using addition of modified beta-cyclodextrin to phosphate buffer (Ayyappa et al., 2015). Detection was performed using low-sensitivity UV detection without derivatisation of steviol glycosides (Ayyappa et al., 2015; Dacome et al., 2005; Liu & Li, 1995); CZE analysis of extract from stevia leaves employed sensitive MS detection (Mauri et al., 1996). The sensitivity of the CE determinations described to date is low and this technique can be used only for determination of steviol glycoside in concentrated stevia extracts; as far as we have been able to determine, foodstuffs have not yet analysed. This communication describes the development and use of highly effective electrophoretic separation combined with sensitive contactless conductivity detection (C^4D) (Kubáň & Hauser, 2009, 2013) for the determination of rebaudioside A and stevioside in sweeteners and beverages commonly available in the commercial network. Compared with HPLC, CE has a number of advantages such as short analysis time, simple sample preparation based on only dilution and, last but not least, miniaturisation of the whole analytical process including minimal reagent consumption (Bergamo, da Silva, & de Jesus, 2011; Tůma, Málková, Samcová, & Štulík, 2011), which is currently in accord with the concept of green chemistry.

2. Materials and methods

2.1. Chemicals and BGE preparation

All the chemicals employed were of analytical purity: stevioside (Sigma), rebaudioside A (Sigma), rebaudioside B (Sigma), lithium hydroxide (Fluka), boric acid (Sigma), polyethylene glycol (PEG M_r 8000, Fluka), polyvinyl alcohol (PVA, Fluka), acetonitrile (ACN, Sigma), INST coating solution (Biotaq, U.S.A.). Deionized Milli-Q water (18.2 MΩ cm, Millipore) was used to prepare the background electrolytes (BGE) and 1 mg/mL stock solutions of rebaudioside A, rebaudioside B and stevioside, which were stored in a refrigerator at 4 °C until the analysis. Stock solutions of 20% *m*/*v* PEG and 5% *m*/*v* PVA were prepared by dissolving the solid substances in water (PEG 8000 in cold water) and then used at room temperature as an additive for BGE preparation. In preparing the BGE, the appropriate amount of H_3BO_3 was dissolved in deionized water and then solid LiOH was added to the solution until the required pH was attained. Titration with solid LiOH is important

for preventing undesirable dissolution of CO_2 in the BGE, which would form a system peak in the CE separation. The pH was measured using a pMX 3000 WTW laboratory pH meter (Germany).

2.2. CE apparatus and experimental conditions

The electrophoretic separations were performed using the HP^{3D} CE system (Agilent Technologies, Waldbronn, Germany) equipped with C^{4}D. The C^{4}D had tubular electrodes 2.5 mm long with a 1.0 mm long detection gap between the electrodes and operated with a sine-wave signal with a frequency of 1.0 MHz and an effective voltage of 50 V (Gaš, Zuska, Coufal, & van de Goor, 2002). Separation was performed in a fused silica capillary (Composite Metal Services, UK), 10 μm inner diameter, 363 μm outer diameter, with total length 31.5 cm (18.0 cm to C^{4}D), placed in an electrophoretic cassette and thermostated at 25 °C. The optimised BGE was i) 170 mM boric acid/LiOH, pH 9.0; ii) the same BGE with addition of 0.5% *v*/*v* INST coating solution. Separations were performed by applying a high voltage of +20 kV; the electric current under these conditions is 2.3 μA. Samples were injected into the capillary hydrodynamically by a pressure of 50 mbar for 100 s. The model samples and mixtures were obtained by diluting stock solutions with deionized water and ACN; the content of ACN in the samples equalled 80% *v*/*v*. A new capillary was washed stepwise with 0.1 M NaOH (10 min), water (10 min) and BGE (10 min); 3 min washing with BGE was used between the individual analyses.

2.3. Preparation of food and beverage samples

The sweetener and beverage samples were obtained from products commonly available on the Czech market. These were a liquid table sweetener based on steviol glycosides called Stevia (Supplied by F&N, ČR content of steviol glycosides 2.5%), free-flowing sweetener from natural sources called Stevia (Supplied by F&N, ČR, fructose, content of steviol glycosides not given), STEVIA sweetener in tablets (ARIKA s.r.o., SR, content of steviol glycosides 16.8 mg/tablet), Jupík Funny Fruit Cherry Cola fruit beverage (Kofola a.s., CR, content of steviol glycosides not given), Staropramen Cool Lemon fruit beer (Pivovary Staropramen, ČR, content of steviol glycosides not given). The solid samples were first dissolved in a ratio of 1 mg of solid sample in 1 mL of deionized water using ultrasound. Then all the samples were filtered through a Durapore membrane (pore size 0.45 μm, centrifugal filter devices, Millipore) for 2 min at 14.100 rpm in a centrifuge. Finally, the samples were diluted prior to the actual CE analysis 5–100 fold as required with an ACN/water mixture so that the final ACN content in the sample equalled 80% *v*/*v*.

2.4. Treatment and evaluation of the results

All the CE analyses of the model samples were carried out in five consecutive runs and the plots represent the average values ± the standard deviations. The Origin 8.0 program (OriginLab Corporation, Northampton, MA, USA) was used to evaluate and statistically treat the experimental data. The peak resolution, *R*, was computed from the relationship, $R = 2(t_{M2} - t_{M1})/(w_1 + w_2)$, where t_M is the migration time of the tested analyte and *w* is the peak width at the baseline; the electrophoretic mobility (u) was obtained from the formula, $u = L_T \cdot L_D / U \cdot (1/t_{EOF} - 1/t_M)$, where L_T is the total length of the capillary, L_D is the length to the detector, U is the separation voltage and t_{EOF} is the migration time of electroosmotic flow. The LOD and LOQ values were determined as the concentrations corresponding to peak heights, respectively, 3 and 10 times greater than the signal-to-noise ratio of the baseline. The C^{4}D background noise was 1.5 μV.

3. Results and discussion

3.1. BGE composition for determination of the total content of rebaudioside A and stevioside in sweeteners

The CE separation of saccharides based on deprotonation of the saccharides in strongly basic BGE can be used only for mono- and disaccharides which dissociate at pH around 12 (El Rassi & Mechref, 1996; Honda, Yamamoto, Suzuki, Ueda, & Kakehi, 1991; Tůma et al., 2011). Steviol glycosides are substances with a more complex nature with higher molecular weight and it was necessary to separate them at a higher pH of >13. These extreme conditions are not suitable for performance of normal electrophoretic analysis. From a practical point of view, it is far more advantageous to utilise the ability of boric acid to form stable complexes with molecules with vicinal 1,2 or sometimes 1,3 diol groups. Complexes of boric acid with saccharides normally deprotonate at pH 8–9 and exhibit anionic mobility.

The CE separation of steviol glycosides was performed in solutions of H_3BO_3/LiOH in the concentration range 25–200 mM at pH 9.0 (Fig. 2); LiOH was intentionally chosen because of the low conductivity of lithium ions. Under these conditions the fast cationic EOF flows through the capillary and after it can be observed the combined peak of rebaudioside A and stevioside (Fig. 3A). The experiments clearly demonstrate that the combined peak of steviol glycosides begins to separate from EOF at a H_3BO_3/LiOH concentration of 75 mM; the resolution value (*R*) related the tail of the water gap peak gradually increases over the whole tested range of BGE concentrations and attains a value of 7.0 at 200 mM H_3BO_3/LiOH, Fig. 2. With growing H_3BO_3 concentration, the fraction of glycosides bound to the charged complex increases, increasing its electrophoretic mobility. The sensitivity of the determination measured as the peak height attains a maximum around 170 mM H_3BO_3/LiOH. Performance of the separation in such concentrated solutions is necessarily connected with the use of a capillary of small inner diameter, in which effective dispersion of the Joule heat is ensured. Consequently, the separation was performed in a 10 μm capillary with a short separation length of approx. 31.5 cm (minimum capillary length for the Agilent instrument). Short capillaries permit the use of high electric field intensities (Tůma, Opekar, & Samcová, 2013), which are required for separation of the steviol glycoside/H_3BO_3 complex with low electrophoretic mobility of approx. $4 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} from the fast EOF with mobility of approx. $54 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} (Fig. 3).

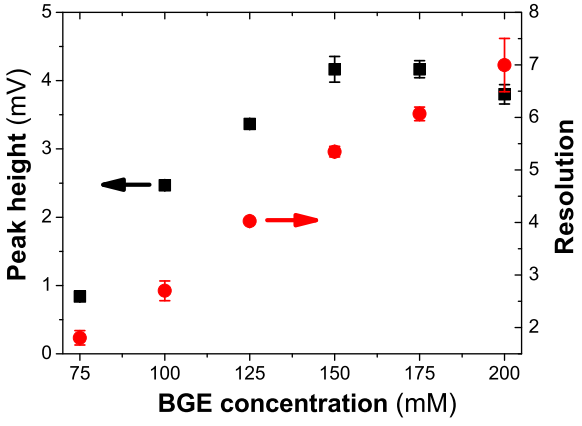

Fig. 2. The dependence of the peak height of rebaudioside A and its resolution from the zone of electroneutral substances on the concentration of BGE. The tested sample consisted of 50 mg/L rebaudioside A.

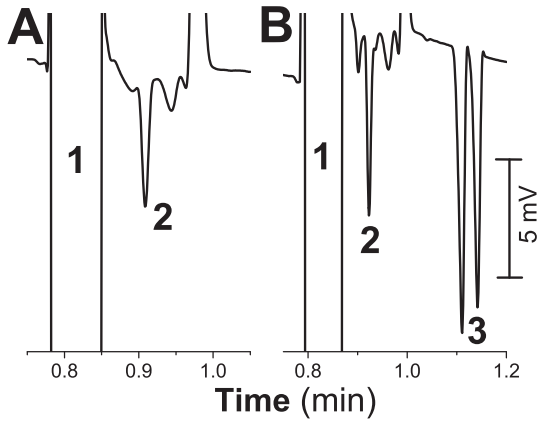

Fig. 3. CE separation of steviol glycosides in optimised BGE with a composition of 170 mM H_3BO_3/LiOH, pH 9.0. (A) Model mixture of 50 mg/L of rebaudioside A and stevioside, (B) STEVIA sweetener in tablets. Peak identification, EOF (1), combined peak rebaudioside A and stevioside (2), saccharides (3).

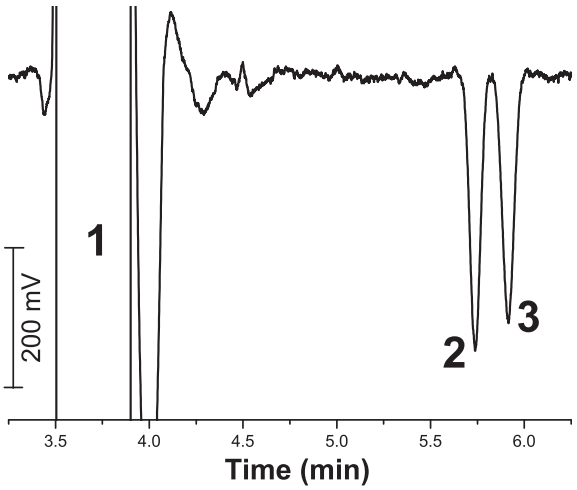

Fig. 4. CE separation of a mixture of stevioside and rebaudioside A dissolved in water at a concentration of 100 mg/L. Experimental conditions: BGE, 170 mM H_3BO_3/LiOH, +0.5% *v*/*v* INST, pH 9.0; separation voltage/current, +20 kV/2.3 μA; hydrodynamic sample injection by pressure 50 mbar for 100 s. Peak identification: 1 EOF, 2 rebaudioside A, 3 stevioside.

Under optimal conditions (170 mM H_3BO_3/LiOH, pH 9.0), the electropherogram of real samples at time 54.3 ± 0.7 s contains a combined peak of rebaudioside A and stevioside, which is separated from the zone of electrophoretic substances and also from other possible interfering saccharides such as saccharose, fructose and glucose, Fig. 3B. The calibration dependence for the area of the rebaudioside A peak in the concentration range 10–100 mg/L (each concentration was measured in five replicates) can be described by a linear regression equation, y[mV.s] = 0.035 (0.002) c[mg/L] + 0.026 (0.095), R^2 0.999; and for stevioside, y[mV.s] = 0.0342 (0.0004) c[mg/L] + 0.043 (0.022), R^2 0.999; SD values in parenthesis; the accuracy at low and high level is 10.0 ± 0.5 mg/L and 100.0 ± 4.0 mg/L for rebaudioside A; 10.0 ± 1.0 mg/L and 100.0 ± 5.1 mg/L for stevioside. LOD calculated from the dependence of the peak height on the concentration attains a value of 0.04 mg/L for both substances. The determined total content of rebaudioside A and stevioside in the Stevia sweetener equals 10.1 mg/tablet. The repeatability of the migration time for 20 consecutive analyses of a combined sample of rebaudioside A and stevioside with a concentration of 50 mg/L is 0.6% (RSD) and, for the peak area, 1.9%; the inter-day reproducibility measured for three consecutive days (10 analyses each day) equalled 1.3% for the migration time and 3.5% for the peak area.

3.2. Suppression of EOF using additive in the BGE to separate rebaudioside A and stevioside

For significant separation of slowly migrating rebaudioside A and stevioside, it is necessary to decrease the speed of EOF so that there is sufficient time for separation of substances with similar electrophoretic mobility values. For this purpose a number of experiments were performed, beginning with separation in a capillary of polyether ether ketone (PEEK) material – yields low separation efficiency; further a hydrophilic polymer was added to the BGE, binding to the inner walls of the capillary and suppressing EOF. Addition of PVA, PEG 8000 and INST at various concentrations was tested. These polymers are able to effectively reduce the speed of EOF to values of 10–20·10^{-9} m^2 V^{-1} s^{-1} leading to separation of rebaudioside A and stevioside down to the baseline. The best results were attained with addition of 0.5% *v*/*v* INST directly to 170 mM H_3BO_3/LiOH pH 9.0, Fig. 4. This neutral water soluble polymer is bonded most firmly to the inner capillary wall and its gradual washing out manifested in increasing speed of EOF is much slower than for 2% *m*/*v* PEG and 0.5% *m*/*v* PVA (Tůma, 2014). In optimised BGE with a final composition of 170 mM H_3BO_3/LiOH + 0.5% *v*/*v* INST, pH 9.0, the migration time for rebaudioside A is approx. 5.7 min and, for stevioside, approx. 5.9 min, with a resolution value of 2.0. Depending on the means of covering the capillary and its time of use, the migration times of the two steviol glycosides can be shifted in a wider interval of approx. 3–6 min, but remain stable throughout a single day, see Section 3.2.3.

3.2.1. Contactless conductivity detection and sensitivity of CE method

Steviol glycosides do not intensively absorb in the UV region of the electromagnetic spectrum and the use of indirect UV detection connected with the addition of an absorbing substance to BGE would yield very low sensitivity in solutions with a high concentration of H_3BO_3/LiOH (Johns, Macka, & Haddad, 2003). In addition, the separations are performed in narrow 10 μm capillaries with a short absorption path. These separation conditions are suitable for use in contactless conductivity detection, which is a universal detection method in CE and can be used to detect analytes independent of their absorption properties (Mai & Hauser, 2013; Tůma, Samcová, & Štulík, 2011; Zhang, Stamos, Amornthammarong, & Dasgupta, 2014). Transfer of the signal between the transmitting and receiving electrodes in C^4D is controlled primarily by the resistance of the solution in the detection cell. This means that the same sensitivity can be achieved in capillaries with large inner diameter and low specific BGE conductivity and in capillaries with small inner diameter and high specific BGE conductivity. This is the case here, where the separations are performed in highly conductive BGE using a 10 μm capillary, yielding LOD at the submicromolar level.

The use of ACN when preparing the solution makes a further contribution towards increasing the sensitivity of the CE/C^4D technique. 80% *v*/*v* content of ACN in the sample reduces the conductivity of the sample zone and, after turning on the separation voltage, leads to concentration of the glycosides at the ACN/BGE boundary, termed stacking (Tůma, Šustková-Fišerová, Opekar, Pavlíček, & Málková, 2013). This kind of stacking does not work for all kinds of analytes and was observed here for glycoside complexes with H_3BO_3.

3.2.2. Calibration dependence and LOD

The dependence of the peak area on the steviol glycoside concentration in the range 10–200 mg/L can be described by linear

regression equations in the form: rebaudioside A – y[mV.s] = 0.143 (0.003) c[mg/L] – 0.183 (0.341), R^2 0.999; stevioside – y[mV.s] = 0.145 (0.011) c[mg/L] – 0.120 (0.687), R^2 0.989. LOD calculated as 3 × *baseline noise/slope* (slope of the dependence of the peak height on the concentration) for both glycosides attains a value of 0.1 mg/L and LOQ – 10 × *baseline noise/slope* of peak height, value 0.3 mg/L. Following recalculation to molarity, LOD for rebaudioside A (M_r 967) is 0.1 μM and for stevioside (M_r 805) equals 0.1 μM. Such low LOD values permit the use of 10 to 100-fold solution dilution of beverage and food samples, suppressing the effect of the matrix.

3.2.3. Repeatability and reproducibility of method

The repeatability of the migration time for twenty consecutive analyses of 100 mg/L of a mixture of the two substances equals RSD 1.8% for both glycosides (without correction for changes in EOF); the repeatability of the peak area is 4.4% for rebaudioside A and 4.5% for stevioside (without correction for changes in the migration time). The repeatability of the migration time for three consecutive days (10 analyses each day) equals RSD 4.6% for rebaudioside A and 4.7% for stevioside; the RSD values for the peak area for the same conditions are 4.8% for rebaudioside A and 3.4% for stevioside. Frequent replacement of the separation capillary is a critical factor for attaining good reproducibility. After conditioning a new capillary, which was performed in the morning, the capillary was used for the whole day and the decrease in the EOF migration time equalled approx. 2%. The gradual decrease in the migration time is caused by the desorption of the surface active substance INST from the inner surface of the capillary by exchange for Li^+. Reactivation of a used capillary was not successful in slowing EOF so that it reattained its original value of a new capillary. Consequently the capillary was replaced after three days. Frequent replacement of the capillary does not substantially increase the cost of the analysis as a 31.5 cm capillary costs approx. USD 4 (50 m of capillary costs 600 USD, www.cmscientific.com).

3.2.4. Analysis of real samples

The high sensitivity of C^4D combined with on-line preconcentration of the sample ensures high sensitivity of the CE method. Consequently, sweetener samples and beverages can be sufficiently diluted, thus eliminating the effect of the matrix on the CE separation. Sample treatment is based on dissolution in a small amount of water, subsequent 10 to 100-fold dilution with 80% ACN and final filtration. This simple sample treatment was used for all the solid and liquid sweeteners and beverages. The determined values of the contents of rebaudioside A and stevioside are summarised in Table 1 and the corresponding electropherograms are given in Fig. 5. The contents of steviol glycosides in the individual samples vary in a very wide range and mutual separation of rebaudioside A and stevioside in such different mixtures can be achieved by suitable sample dilution. Rebaudioside B, which is separated well from the two monitored glycosides under these conditions, was not found in any of the tested samples. On the other hand, the peaks of simple sugars, such as saccharose in Jupík fruit beverage (Fig. 5D), are clearly visible in the electropherograms of the samples.

Table 1
Determination of the content of rebaudioside A and stevioside in samples of foods and beverages in the commercial network (the SD values are derived from the analysis of five replicates).

	Rebaudioside A, mg L^{-1}	Stevioside, mg L^{-1}
Free-flowing sweetener	1850 ± 30	620 ± 30
Sweetener tablets	6960 ± 310	2520 ± 90
Liquid sweetener	10,800 ± 400	3630 ± 120
Jupík fruit beverage	130 ± 5	40 ± 3
Staropramen fruit beer	29 ± 1	57 ± 2

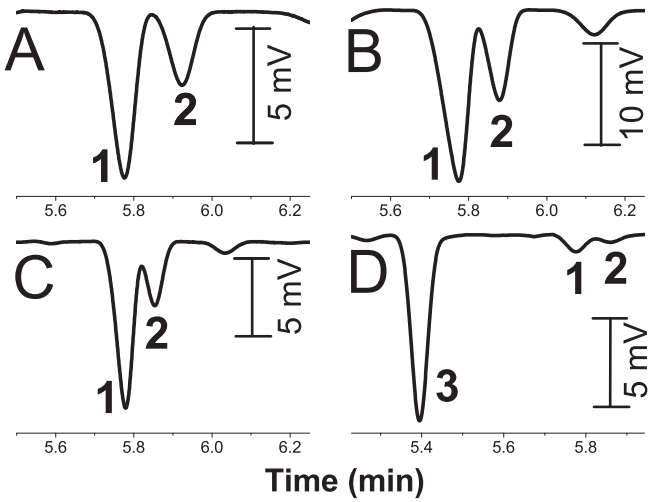

Fig. 5. CE/C^4D determination of rebaudioside A (1) and stevioside (2) in foodstuffs and beverages under optimised experimental conditions. (A) free-flowing table sweetener; (B) table sweetener in tablet form; (C) table sweetener in liquid form; (D) Jupík fruit beverage also with identification of saccharose (3).

The determined content of the steviol glycosides in the Stevia sweetener tablets corresponded to an amount of 7.0 mg rebaudioside A and 2.6 mg stevioside. The combined content of the two steviol glycosides determined by the CE method without EOF suppression was 10.1 mg. It is apparent from comparison of the obtained data that the two developed CE methods provide similar results and can be used for determination of rebaudioside A and stevioside in foodstuffs. It can be seen from comparison of the determined content of rebaudioside A and stevioside with the total amount of all steviol glycosides stated by the manufacturer (16.8 mg per tablet) that rebaudioside A and stevioside have predominant contents in natural products isolated from Stevia plants.

4. Conclusion

The contents of steviol glycosides in sweeteners in beverages can be simply determined using capillary electrophoresis combined with C^4D. CE separation in H_3BO_3 solutions must be performed at high concentrations, in which stable complexes are formed between steviol glycoside and H_3BO_3, which are subsequently separated from EOF. The performance of CE separation in highly concentrated BGE solutions is necessarily connected with the use of narrow capillaries with inner diameter of approx. 10 μm. Sensitive detection of steviol glycosides, which do not absorb in the UV region, in narrow capillaries can be achieved using C^4D, whose sensitivity is controlled by the conductivity of the solution in the capillary and not by the length of the optical path as in optical detectors. This simple instrumentation can be used to attain detection limits at submicromolar concentration levels even without requiring demanding sample derivatisation. This yields a simple and rapid treatment of sweetener and beverage samples for CE/C^4D, based on sample dilution and filtration.

Conflict of interest

The authors have declared no conflict of interest.

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