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Capillary electrophoresis for the simultaneous separation of selected carboxylated carbohydrates and their related 1,4-lactones

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Abstract

A capillary electrophoresis method has been developed for the separation of acidic monosaccharides and their corresponding 1,4-lactones in a single run without derivatisation. The controlled changes of the concentration in borate and the pH of the buffers allowed good selectivity in the separation of the 18 studied compounds and of two isosaccharino-1,4-lactone isomers. Best separations were at neutral pH to avoid the saponification of the 1,4-lactones to the acidic carbohydrates, and with high borate concentration to form the negatively charged bidentate borate esters (BL^-, 1:1 complexes). The BL^- complex formation could be modeled and the corresponding measured association constants could be calculated; the selective controlled association of borate ions to specific 1,2- and 1,3-diol structures can be a useful tool in the selectivity of the separation of known carbohydrate-type mixtures.

Keywords: Carbohydrates; Lactones; Sugar acids; Isosaccharinic acid

1. Introduction

In the field of environmental engineering, natural chelating agents are needed for the removal of heavy metals from polluted materials by leaching techniques. One class of compounds which meets this requirement, are sugar acids. Sugar acids form heavy metal complexes with high stabilities at alkaline conditions. Practically they are applied in mixtures with other complexants [1,2]. They are generally obtained from oxidations of carbohydrate-rich residues or generated from the pulp industry in kraft pulping. Wood pulp fibers are mainly constituted of cellulose [homopolymer of β-(1,4)glycoside] and different hemicelluloses (heteroglycans constituted of neutral and acidic monosaccharides); the presence of acidic carbohydrates is dependant on wood origin and industrial processing and contributes significantly to the total amount of charged groups and to the fiber physicochemical properties of chemical pulps. Besides the need of analytical techniques for the analysis and control of sugar acids in these applications, analytical techniques are developed for sugar acids in the field of phytochemistry, phytopathology, clinical chemistry, microbiology as well as food and beverage engineering [3].

As most carbohydrate compounds do not possess strong chromophores, the UV detection possibilities of classical liquid separation techniques set the limit. With ion-exclusion chromatography these separations allow a detection limit in the $\mu mol\,l^{-1}$ range [4]. In liquid chromatography as well as in capillary electrophoresis (CE), derivatisation techniques with strong UV absorbing or fluorescing tags have been developed to overcome these problems [5,6]. These selective techniques, like for example reductive

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aminations, allow good separations and low detection limits of carbohydrates [7,8], but generally involve the carboxyl group of the analyte so that the quantification of the lactones together with the acid species in a single run is difficult. In capillary zone electrophoresis (CZE), the appropriate buffer systems (undergoing specific reactions with the analytes) can be used to overcome these problems. CE methods with borate buffers, for example, were successfully used for the analysis of acidic mono- and oligosaccharides from kraft pulps [9–11].

The aim of this study was to develop a simple and efficient method to separate two isosaccharino-1,4-lactone isomers obtained from the alkaline hydrolysis of xylan [12] in the presence of other 1,4-lactones and the corresponding sugar acids. One of the isomers was identified as the α-isomer, the other compounds estimated to be the β-isomer. Due to the fast saponification kinetic of the lactones to their corresponding acids, the method must allow the possibility of rapidly detecting the two species in a single run without altering the sample composition.

2. Experimental

2.1. Instrumentation

CE instrumentation consisted of a Beckman P/ACE 2050 Series HPCE with Beckman System Gold Chromatography Software and a BioFocus 3000 CE system from Bio-Rad. Uncoated fused-silica CE columns [57 cm (50 cm to detectors)×75 μm I.D.×375 μm O.D., 100 cm (94 cm to detectors)×75 μm I.D.×375 μm O.D. for the two systems, respectively) were obtained from Polymicro (Laser 2000 Wessling, Germany).

2.2. Separation

CZE conditions for the separation of the analytes were: temperature, 30°C; voltage, 20 kV; detector wavelength, 200 nm. The injection was in the hydrodynamic injection modus for 5 or 10 s (Beckman) or 30 s at 0.5 p.s.i. (Bio-Rad) (1 p.s.i.= 6894.76 Pa).

2.3. Buffers

Buffer were prepared by adjusting the pH of the boric acid solution at desired molarities with 0.1 *M* NaOH.

2.4. Sample preparation

D-Erythrono-1,4-lactone, D-ribono-1,4-lactone, D-galactono-1,4-lactone, 5-keto-D-gluconic acid were purchased from Fluka (Buchs, Switzerland); D-gluconic acid was obtained from Merck (Darmstadt, Germany); 2-keto-D-gluconic acid, D-mannono-1,4-lactone from Sigma (St. Louis, MO, USA); glucuronic acid from Roth (Karlsruhe, Germany). Lactones were dissolved in distilled water and the corresponding sugars were obtained by saponification of the lactones overnight with 0.1 *M* NaOH. All compounds were dissolved in tridistilled water at a final concentration of 50 ppm. The structures of the interesting compounds are given in Fig. 1a–c.

3. Results and discussion

3.1. Sample stability

Our first approach in the separation of isosaccharinic acid (ISA) and the two isosaccharino-1,4-lactone isomers was to use a classical indirect detection method [13] as adapted by the authors for the analysis of aliphatic and phenolic acids at pH 11.0 using 2,6-naphthalenedicarboxylate as UV-absorbing background electrolyte (unpublished data). ISA as well as the other sugar acids showed very good separation under these conditions but the corresponding 1,4-lactones could not be detected. When the pH was increased to pH 12 and 12.5 to ionize partially the hydroxyl groups of the sugars, ISA was measured in high concentrations in both pure lactone samples. Because the lactone samples were known to be of good purity, the measured ISA signals were supposed to originate from the saponification of the lactones to ISA in the alkaline buffer pH during the separation run. The high buffer pH, needed to get theoretically CZE separation of these compounds (dissociation constants of the side chain hydroxyl groups over 12), affected the samples

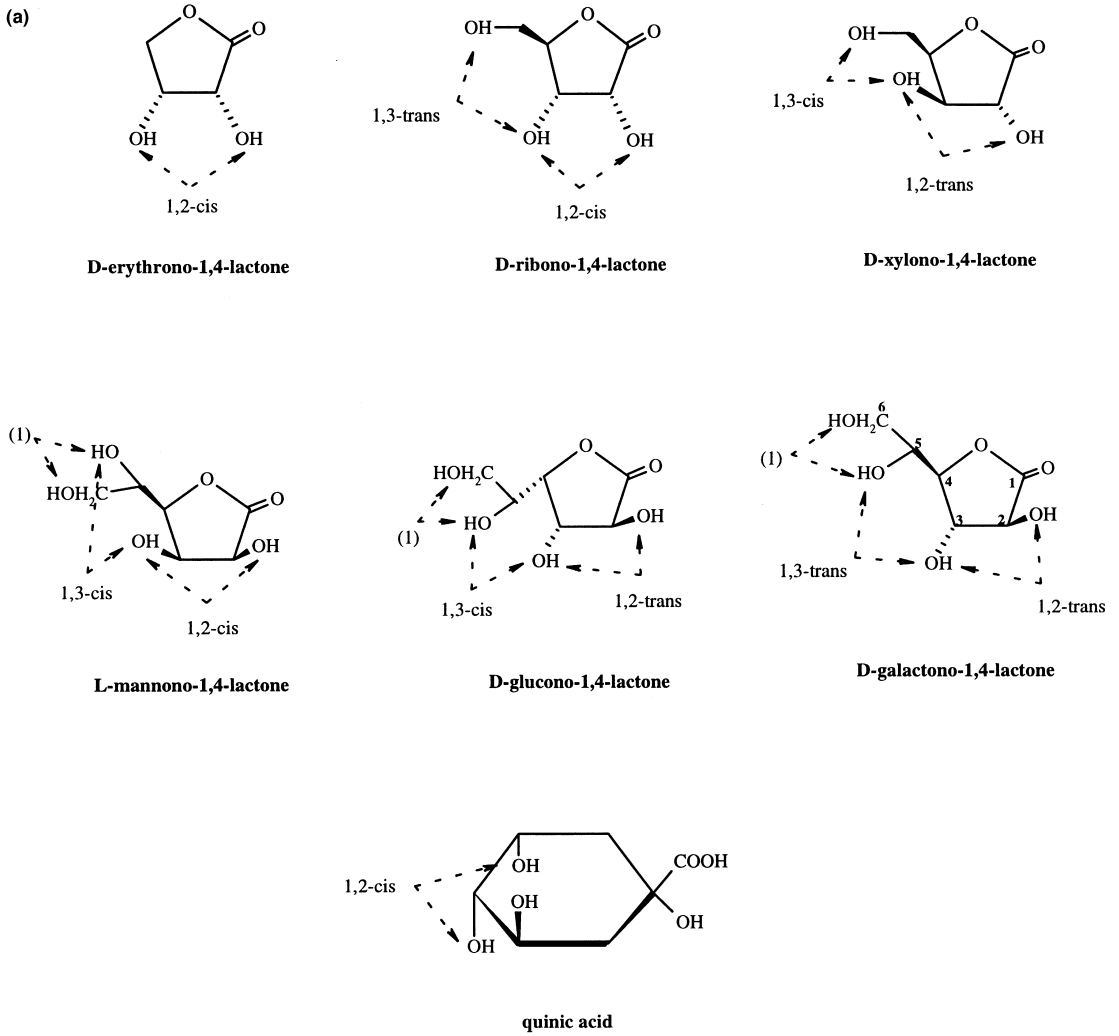


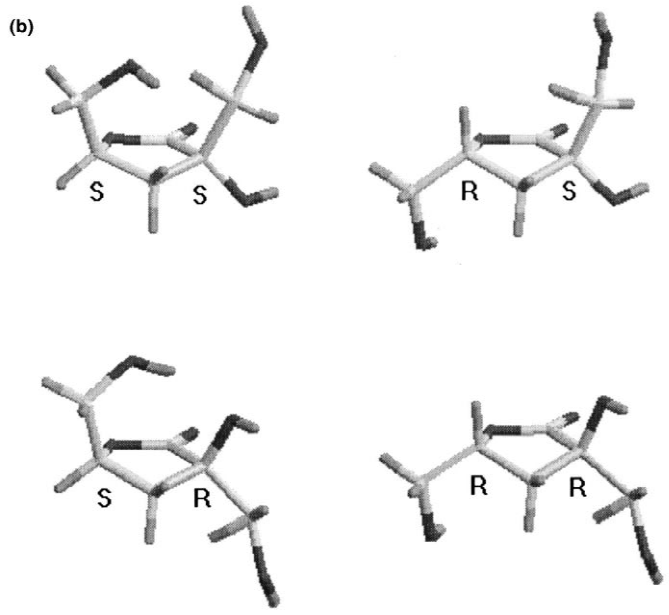
Fig. 1. (a) Structures of the studied 1,4-lactones; the arrows show the possible borate complexation sites. (b) Possible isomers of isosaccharino-1,4-lactone; structure of α-isosaccharino-1,4-lactone and proposed structure for β-isosaccharino-1,4-lactone. (c) Structures of studied sugar acids; the arrows show the possible borate complexation sites.

significantly. This rapid hydrolysis of the sample during the separation run was verified by using a carbonate buffer (50 m*M*, pH 9.0): the injection of the two pure isosaccharino-1,4-lactone isomers resulted in the detection of ISA in the electropherograms in significant amounts. A neutral separation buffer pH is thus a necessity to avoid changes in the sample composition.

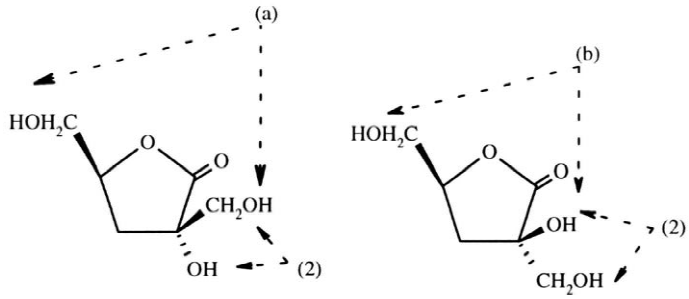
The hydrolytic stability of the sample is thus an important factor that should always be controlled when developing methods with buffers at extreme pH values.

3.2. Separation of 1,4-lactones

Boric acid and borate are well known to complex with diols in 1,2- and 1,3-position to form stable borate complexes. This property was used to separate



possible isomers of isosaccharino-1,4-lactone

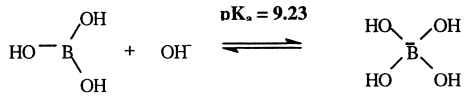


α-isosaccharino-1,4-lactone **β-isosaccharino-1,4-lactone**

Fig. 1. (*continued*)

the two isosaccharinolactone isomers, the other 1,4-lactones and their related sugar acids at around neutral pH values.

Equilibrium (1) corresponds to the ionization of boric acid to borate (tetrahydroxy borate) ions at a pH around the pK_a.

(1) 

1 **2**

Both **1** and borate anions are known to complex

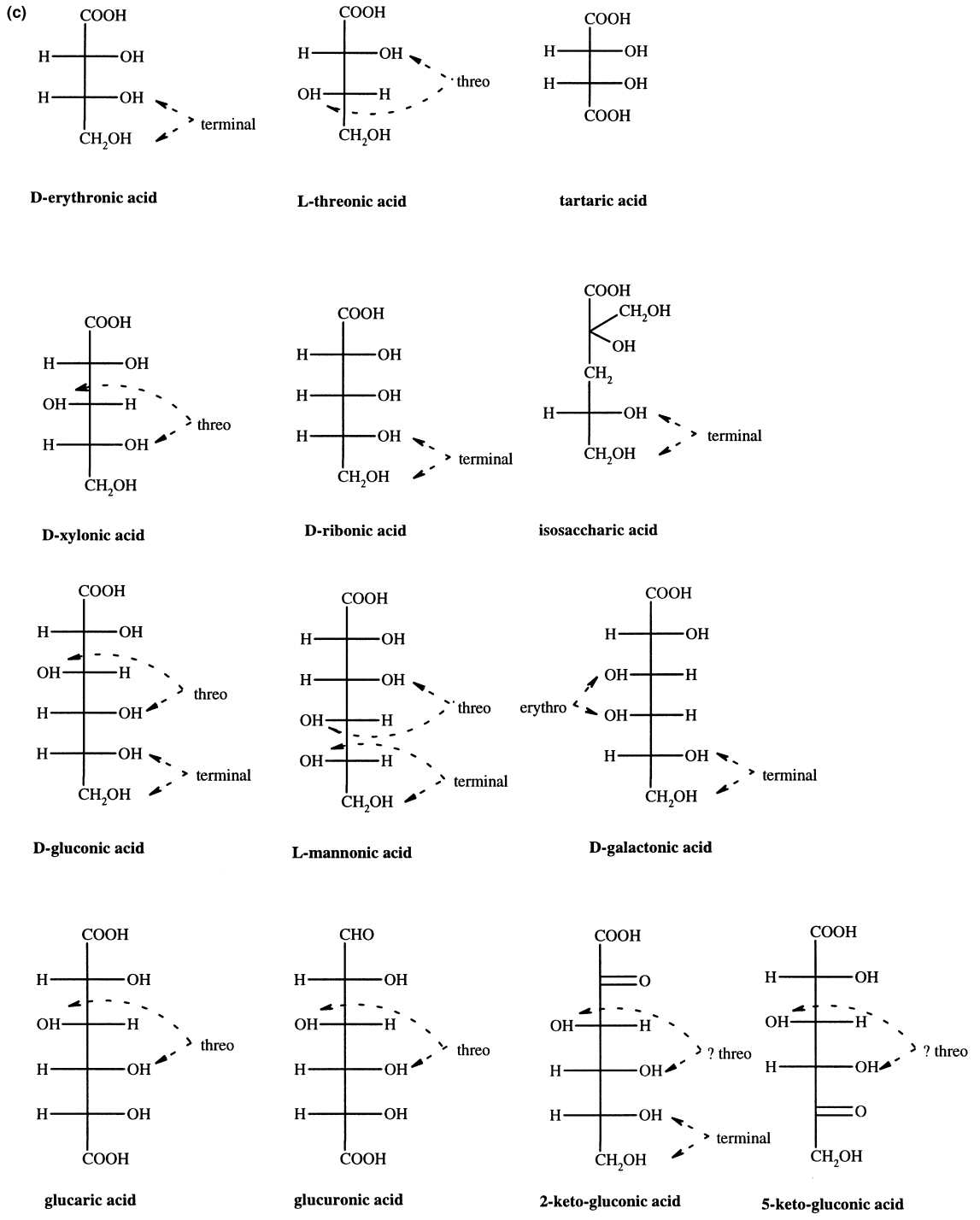
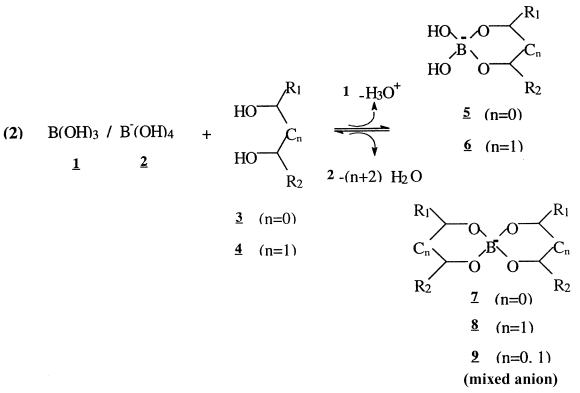


Fig. 1. (*continued*)

diol groups of the proper geometry in cyclic and noncyclic polyhydroxy compounds [14]. Five-membered ring complexes with 1,2-diols ($n=0$:**3**) or six-membered rings with 1,3-diols ($n=1$:**4**) as shown in equilibrium (2) giving bidentate esters (**5**, **6**), also called the 1:1 monocomplex (BL^-), as well as the spiranes (**7**–**9**) or tetradentate esters (1:2 dicomplex BL_2^-).



Borate complexation induces changes in the charge-to-mass ratios of the ligands; this property was already used in the early 1950s to determine the configuration of carbohydrates [14–16] by means of electrophoretic techniques (zone and gel electrophoresis). The routine analysis of carbohydrates and oligosaccharides with CZE is based on these properties; borate not only induces the formation of charged and mobile complexes from uncharged carbohydrates thus increasing the selectivity of their separation [17], but also significantly enhances their UV-absorbance, facilitating their on-line UV detection [18] and their spectrophotometric quantification [19].

For the separation of D-galactono-1,4-lactone, the isosaccharino-1,4-lactone isomers, D-erythrono-1,4-lactone, L-mannono-1,4-lactone and their corresponding acids, the optimal borate concentration was evaluated by changing the molarity of borate in the buffer at pH 7.1 from 25 m*M* to 400 m*M* (electropherograms in Fig. 2). One can clearly recognize that the presence of borate ions in the separation buffer increases both the absorbance and the mobility of the lactones, leading to high selectivity and sharp peaks. The isosaccharino-1,4-lactone isomers are well separated with a molarity of 400 m*M* borate. The changes in mobility of these compounds are resumed in Fig. 3.

The bidentate-type of association (BL^-) between borate and the sugar ligands to form a 1:1 complex can be described as: $L + B \underset{}{\overset{K}{\rightleftharpoons}} L\text{-}B + H_2O$

The measured association constant K for the binding of the sugar L with borate B is then defined as:

$$K = \frac{[L-B]}{[L][B]} \tag{a}$$

(where [L] is the concentration ($g\,l^{-1}$) of free L ligand fraction, [B] is the concentration ($mol\,l^{-1}$) of borate, [L–B] the concentration of ligand–borate complex, with the total ligand concentration $[L]_{tot} = [L]+[L-B]$; the dissociation constant K_d can be defined as $K_d = 1/K$).

The effective electrophoretic mobility μ_{eff} (calculated from the migration times and deducing of the endoosmotic flow) is directly proportional to the velocity of the complex and is a weighted average of the effective electrophoretic mobilities of all the free and bound forms of the analyte in the studied system.

$$\mu_{eff} = \frac{[L]}{[L]_{tot}}\mu_o + \frac{[L-B]}{[L]_{tot}}\mu_{cn} \tag{b}$$

(μ_o = electrophoretic mobility of the free analyte for [B] = 0, μ_c is the electrophoretic mobility of the formed complex with borate)

Combining Eq. (a) with Eq. (b) gives:

$$\mu_{eff} = \frac{\mu_o + K[B]\mu_c}{1 + K[B]} \tag{c}$$

The experimental data were fitted with the above 1:1 binding model to Eq. (c) for the determination of the measured binding constants K (Fig. 3). One can see from the structures in Fig. 1(a) that both D-erythrono-1,4-lactone and L-mannono-1,4-lactone allow the complexation with borate ions of the two 1,2-diol groups in the *cis*-position on carbon C_2 and C_3, leading to the formation of the negatively charged BL^- complex. *cis*-1,2-Diols are generally preferred in complexation to *trans*-1,2-diols [20]. 1,2-Diols were also found to have generally higher association constants than 1,3-diols [21]; this implies

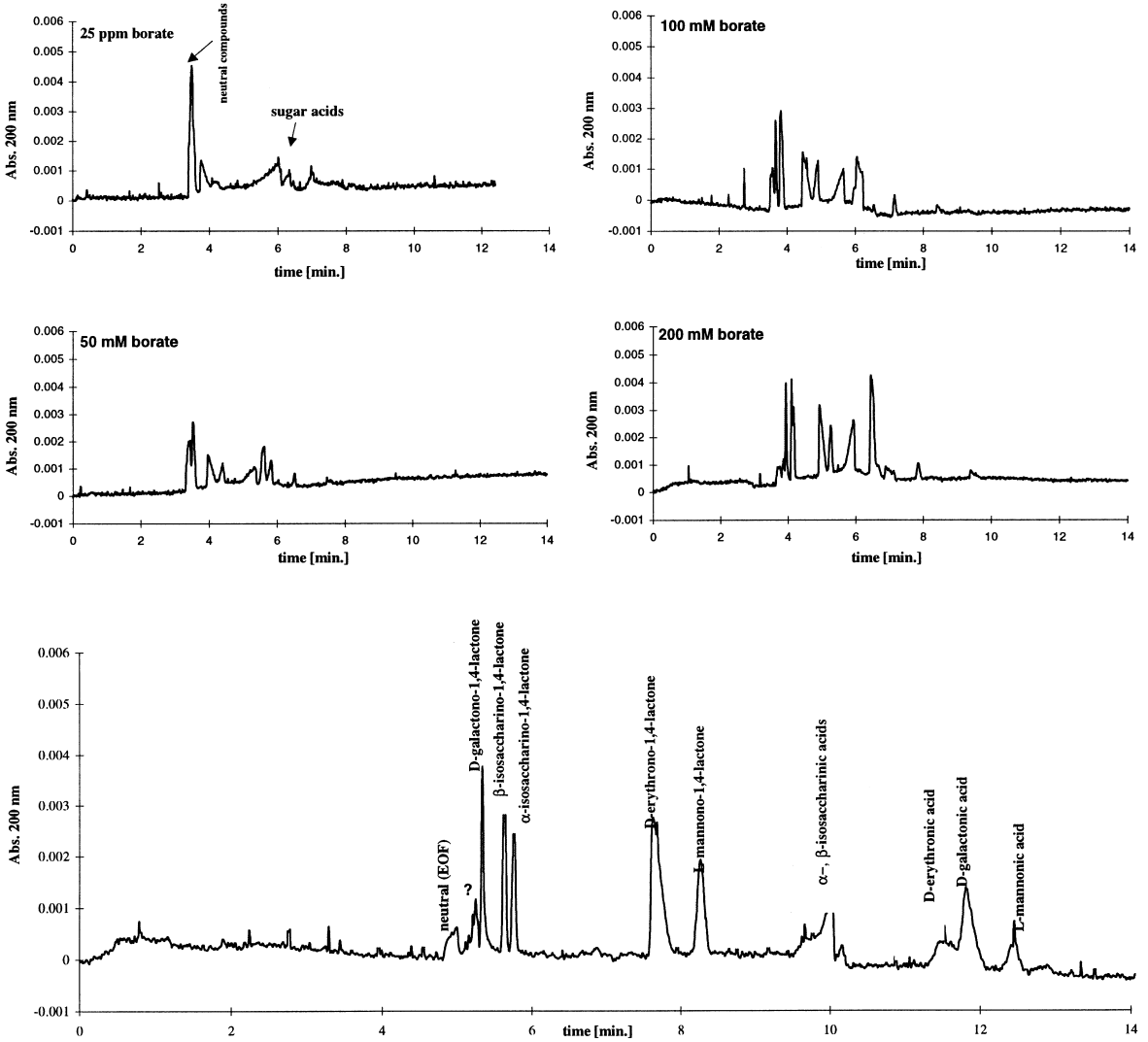


Fig. 2. Influence of the borate concentration in the separation buffer on the electrophoretic separation of selected 1,4-lactones and their saponification products (pH 7.1, 30°C, 20 kV, 200 nm).

from Eq. (c) that within the same variation in borate concentration (Δ[B]) the corresponding variation in mobility ($\Delta\mu_{eff}$) is lower for 1,3-diols than for 1,2-diols and the calculated *K* of the 1,3-diols respectively smaller. D-Galactono-1,4-lactone possesses the 1,2-diols (C_2 and C_3) and 1,3-diols (C_3 and C_5) only in *trans*-position and this explains the smallest *K* and low mobility. D-Erythrono-1,4-lactone possess one 1,2-*cis*-diol complexing site at carbon C_2 and C_3; L-mannono-1,4-lactone possesses two potential binding sites in *cis*-position (see Fig. 1a) and this explains the highest *K* value of this lactone. The low mobility of D-galactono-1,4-lactone implies a low complexation of this analyte; it possesses two hydroxyl groups in the side chain on carbon C_5 like L-mannono-1,4-lactone (hydroxyl group on terminal carbon shown by arrows (1) in Fig. 1a); the hydroxyl groups in terminal carbon position of the lactones seem not to be as reactive under these borate concentration and pH conditions. The α-isosac-

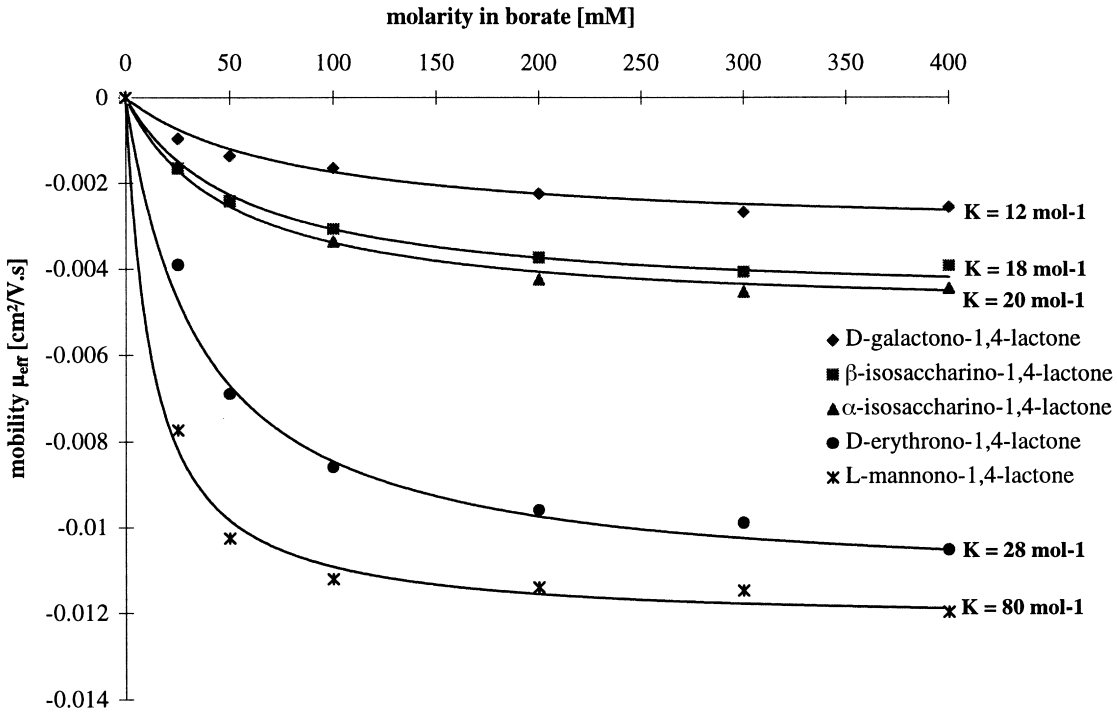


Fig. 3. Experimental data points and fitted curves (according to Eq. (c)) of the changes in effective mobility as a function of the addition of borate to the buffer for selected 1,4-lactones.

charino-1,4-lactone isomer is described to be the (*S*,*S*) (the four possible isomers are shown in Fig. 1b). It has a higher mobility than the other measured unknown isomer, supposed to be β-isosaccharino-1,4-lactone. Because the α-isosaccharino-1,4-lactone does not possess hydroxyl groups in *cis*- or *trans*-position, the separation in the presence of borate ions may be due to the possible complexation of the hydroxyls from the two ethanoyl in *cis*-position [see arrows (a) in Fig. 1b]. The isomers (*R*,*R*) would have a similar borate complexation possibility as α-isosaccharino-1,4-lactone and would migrate with a close velocity. Because the mobility of α-isosaccharino-1,4-lactone compared to its isomer is higher, the binding constant *K* of borate with the other isomer should be smaller. The structure of the unknown isomer may be (*R*,*S*) or (*S*,*R*). The two compounds are obtained from the hydrolysis of xylan (identical source), the most probable structure proposed for this isomer is the (*S*,*R*) configuration (Fig. 1b); this is in accordance with the supposed structure of β-isosaccharino-1,4-lactone.

Changing the pH of the separation buffer from pH 5.5 to pH 8.5 (keeping the borate molarity constant at 400 m*M*) is illustrated in Fig. 4. At pH 5.5 the 1,4-lactones coelute in the neutral peak [electroosmotic flow (EOF)]. By increasing the pH and thus the fraction of tetraborate species the selectivity of the separation is increased; choosing a pH over 7.0 increases the amount of charged complexed fractions and the mobility is thus increased, leading again to coelutions as anions. In our neutral pH conditions the boric acid species is predominant; but even with this low fraction in borate ions ($<1\%$), with 400 m*M* borate concentration, the selectivity of the separation of the chosen 1,4-lactones is sufficient for their analysis.

According to the literature [21], the following rules govern the stability of borate esters with sugar acids: (1) the increase of the number of OH in the molecule increases the stability of the complex (high *K* values); (2) diols in threo-position are more stable than diols in terminal position; (3) diols in terminal position are more stable than diols in erythro-posi-

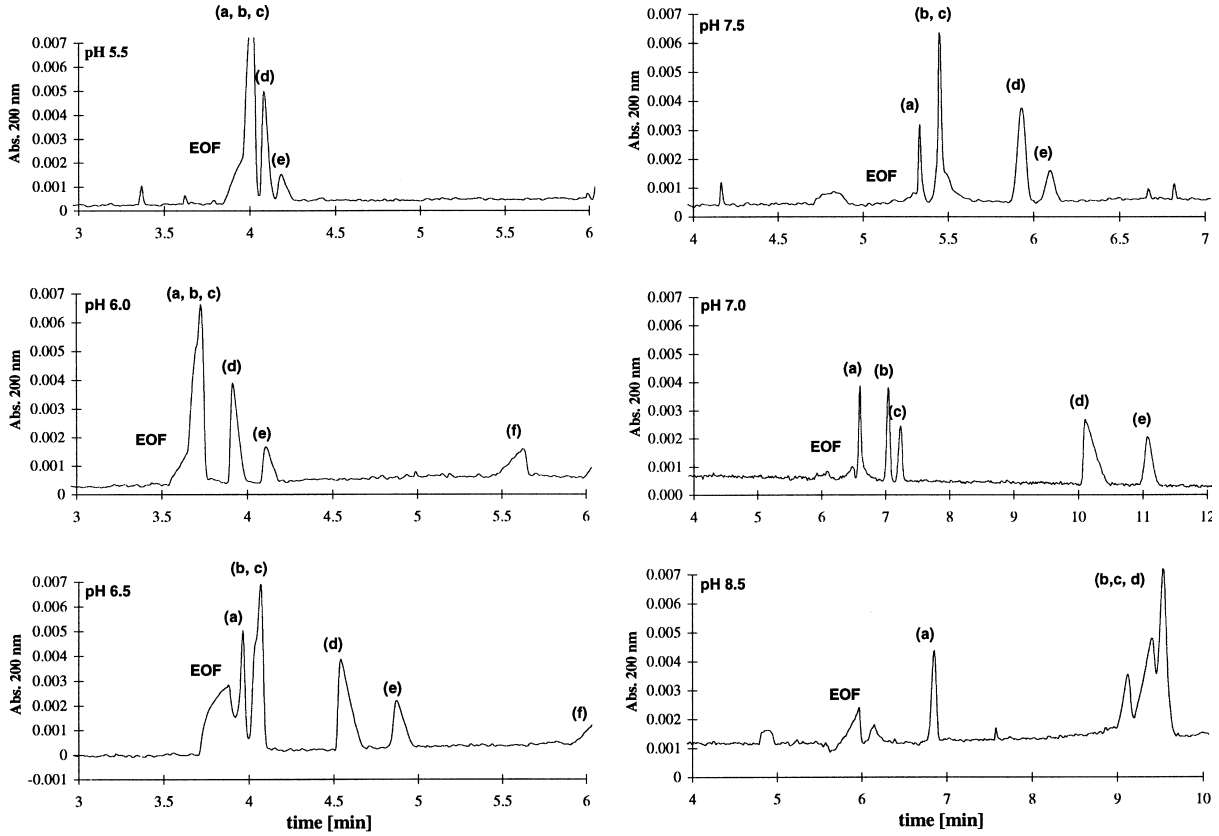


Fig. 4. Influence of the buffer pH on the separation of (a) D-galactono-1,4-lactone, (b) α-isosaccharino-1,4-lactone, (c) β-isosaccharino-1,4-lactone, (d) D-erythrono-1,4-lactone, (e) L-mannono-1,4-lactone and (f) isosaccharinic acid (400 m*M* borate buffer, 30°C, 20 kV, 200 nm).

tion. Furthermore, the presence of Coulomb repulsion (carboxyl group on adjacent carbon) decreases drastically the stability of the borate esters. The dissociation constant of the carboxylic groups of the sugar acids are around pK_a 3.2±0,1, and the hydroxyl groups have all pK_a higher than 12. These compounds have all the same charge at the separation buffer pH; their mobility is thus only governed by their differences in size (the higher the amount of carbons, the lower the mobility) and the stability of the formed borate complexes in the separation buffer.

Following these simple rules one can prognose the stability of the formed complexes assuming that the hydroxyl groups involved in the borate esters are only positioned on carbon C_3 to C_6 (electronic repulsions between the negatively charged COO and the borate moieties). The binding sites with the highest stability (threo>terminal>erythro) are illustrated with arrows in Fig. 1(c). The migration order of the sugar acids (migration times) according to the rules should then follow the order:

C4 sugar acids:

D-erythronic acid < L-threonic acid < tartaric acid

C5 sugar acids:

D-ribonic acid < D-xylonic acid

C6 sugar acids:

isosaccharinic acid < glucuronic acid
< D-galactonic acid < L-mannonic acid
= D-gluconic acid < glucaric acid

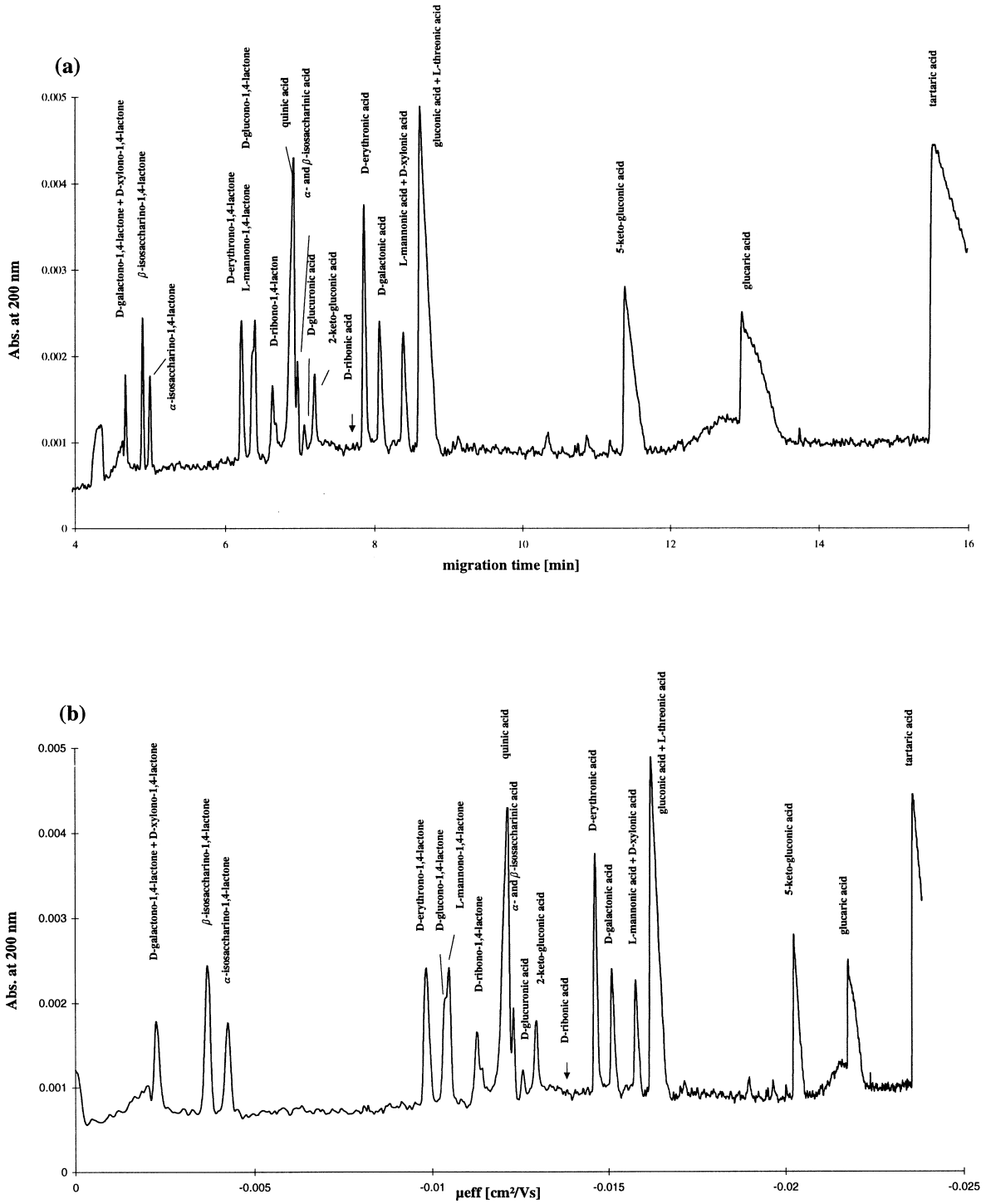


Fig. 5. (a) Electropherogram of the separation of selected 1,4-lactones and sugar acids. (b) The lower electropherogram is a representation of the same electrophoretic data as in (a) the mobility domain (pH 7.1, 20°C, 20 kV, 200 nm).

The separation of the compounds (electropherogram in Fig. 5) verify these rules except for D-gluconic acid and L-mannonic acid, where the migration order corresponds to a higher stability of the borate ester with terminal hydroxyls in threo-position compared to the borate ester with terminal hydroxyls in erythro-position. It seems that the presence of the keto group on C_2 (near the carboxylic group) strongly inhibits the borate ester formation in threo-position compared to 5-keto-gluconic acid.

Best resolution was obtained by increasing the borate ion molarity to 600 m*M*. The addition of organic modifiers (methanol and acetonitrile) only affected the migration times (because of changes in the EOF) but not the resolution. Lowering the temperature from 30°C to 20°C gave better separation of all compounds (Fig. 5a).

Fig. 5b visualizes the same electropherogram as Fig. 5a in the mobility domain (μ_{eff} as *x*-axis). This plotting of the data is very useful when comparing electropherograms (in our case after spiking the sample with standards) or when analysing real probes where the EOF can be affected. If there are some changes in the EOF (when analysing real samples, changing the applied voltage or changing to a new capillary), with great effects on the migration times of the analytes (especially with high migration times), the effective mobilities stay the same and these changes are not visible with this representation. The effective mobility is an absolute value which is a direct function of the physicochemical properties of the analyte; a given analyte has always the same μ_{eff} when the experimental separation conditions stay constant, but its migration time is always dependant on the stability of the EOF (little EOF variations can have drastic time variations, especially at higher retention times). The usefulness of a similar data representation in the 1/time domain has already been presented by other authors [22] and modifications of the CE software to accommodate this absolute way of representation should be proposed.

4. Conclusion

A method for the simultaneous analysis of 1,4-lactones and their related sugar acids has been proposed. It allowed the separation of α-isosaccharino-1,4-lactone and one of its isomers; furthermore the structure of this isomer could be verified being asthe (*S,R*) β-isosaccharino-1,4-lactone isomer from the stability measurement of its borate ester. Simple rules relating the stability of borate esters to the structure of the analytes can be used to predict the migration time of analogue sugar acids in the electrophoretic run. This tool can be useful when developing analytical strategies for the identification or confirmation of structures of unknown compounds from real matrices.

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