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## Microemulsion electrokinetic chromatography of diphenylhydrazones of dicarbonyl sugars

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### Abstract

A mixture of diphenylhydrazones of dicarbonyl sugars was separated by microemulsion electrokinetic chromatography. Separations were carried out with a laboratory-made capillary electrophoresis apparatus using an untreated fused-silica capillary [70 cm (effective length 65 cm) × 50 μm I.D.] at 20 kV; detection was at 220 nm. The microemulsion system was composed of 5 mM borate buffer, pH 8.0 (89.27%, w/w), sodium dodecyl sulphate (SDS; 3.31%, w/w), *n*-butanol (6.61%, w/w) and *n*-octanol (0.81%, w/w). Separation by microemulsion electrokinetic chromatography was significantly better than separation obtained by routine micellar electrokinetic chromatography with SDS and was characterized by a much higher efficiency (theoretical plates ranged from 100 · 10<sup>3</sup> to 254 · 10<sup>3</sup>). SDS–micellar electrokinetic chromatography resolved only one compound from a joint peak of two sugars and from a cluster peak consisting of seven compounds. Applicability to the analysis of biological samples was also demonstrated.

**Keywords:** Microemulsion electrokinetic chromatography; Derivatization, electrophoresis; Sugars; Carbonyl compounds

### 1. Introduction

Dicarbonyl monosaccharides are known as intermediates of many metabolic pathways. They have been detected in bacteria, yeasts, fungi, plants and animals [1]. D-*arabino*-2-Hexosulose (D-glucosone) plays an important role, mainly in the sugar metabolism of numerous wood-decaying Basidiomycetes. Pyranose oxidase, the enzyme responsible for D-glucosone formation, has been found in e.g. *Corticium caeruleum* [2], *Oudemansiella mucida* [3], *Phanerochaete chrysosporium* [4] and in *Trametes versicolor* [5]. There is also increasing evidence about the role of tricarbonyl sugar derivatives in monosaccharide metabolism in fungi [5,6]. Sugar oxidation by pyranose oxidase represents an im-

portant source of hydrogen peroxide, the compound necessary for lignin degradation in wood-decaying fungi. Spontaneous formation of 3-deoxy-2-hexosuloses was reported from various biological materials in connection with sugar degradation in the presence of amino acid residues — Maillard reaction (or non-enzymatic glycation) [7,8].

Separation of colourless dicarbonyl sugars in an underivatized form, devoid of distinct UV absorbance in the near UV region brings about many difficulties. Thin-layer chromatography (TLC) with limited possibilities of quantification is acceptable only for screening purposes. In high-performance liquid chromatographic (HPLC) separations, problems with irreversible binding to the stationary (amino-bonded) phase occurs frequently. Moreover, spectrophotometric detection of dicarbonyl sugars is possible only in the short-wavelength UV region

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(190–210 nm), where many of the accompanying compounds from biological matrices interfere. Infra-red (IR) detection did not lead to satisfactory detection limits for biomedical purposes. Formation of tautomeric forms under neutral conditions, and easy decomposition under acidic or alkaline conditions, generally limit the use of liquid–solid chromatography (LSC), both in planar and column arrangement. Finally, instability of the trimethylsilyl derivatives under the conditions of analysis precludes the use of gas chromatography (GC) [1].

The above hindrances can be circumvented by pre-column derivatization. Dicarbonyl compounds, when treated with *o*-phenylenediamine, yield stable quinoxaline derivatives, the separation of which was investigated earlier [9,10]. Reaction of carbonyl compounds with substituted phenylhydrazine resulted in the formation of stable crystalline derivatives. HPLC determination of D-glucosone after reaction with 2,4-dinitrophenylhydrazine on a silica gel column [11] and separation of derivatized products of the Maillard reaction on a reversed-phase column [12] were also described.

Capillary electrophoretic separation of sugar derivatives is an alternative to the existing HPLC technology; it is a rapidly developing area at the moment (for reviews see Refs. [14–16]). Separation of 2,4-dinitrophenylhydrazones of oxo-compounds arising during the Maillard reaction [13], as well as the separation of quinoxalines (*o*-phenylenediamine derivatives) of dicarbonyl sugars [9], represent typical examples of the application of capillary electrophoresis in this context.

Microemulsion electrokinetic chromatography (MEEKC) has emerged only recently. It was first used by Watarai [17] in 1991 for the separation of fluorescent aromatic compounds; Terabe et al. [18] used it for the separation of test mixtures of some aromatics and drugs and compared this separation mode with micellar electrokinetic chromatography (MEKC) [with sodium dodecyl sulphate (SDS)]. Recently, Boso et al. [19] used this system for the separation of fat- and water-soluble vitamins. Vomastová et al. [20] separated steroids and Fu et al. [21] separated antipyretic analgesic ingredients. In general, the microemulsions [oil-in-water, (o/w)] are formed from oil (named the core phase, usually a hydrocarbon or other hydrophobic substance), water

(buffer), surfactant and cosurfactant (such as a medium alkyl-chain alcohol). It appears that the structure of the microemulsion is similar to the structure of the micelle — the oil droplet is stabilised by the surfactant and cosurfactant located on the surface of the droplet [18].

In this work, we attempted to develop a method for the separation of diphenylhydrazine derivatives of dicarbonyl (and one tricarbonyl) carbohydrates. The hydrazones studied exhibited antibacterial effects, as do other hydrazones [22]. 2-Furylglyoxal, as a product of the spontaneous degradation of sugars, has been included in the set of compounds studied.

## 2. Experimental

### 2.1. Capillary electrophoresis

Fused-silica capillaries, untreated, UV transparent (CElect-UVT; Supelco, Bellefonte, PA, USA), 70 cm long (65 cm to the detector) × 50 μm I.D. were mounted into a laboratory-made capillary electrophoresis apparatus [23]. Stock solutions of standards were prepared as 1 mg ml<sup>-1</sup> solutions in methanol. The test mixture was prepared by mixing 30 μl of most of the stock solutions (60 μl of 5-keto-D-fructose bisdiphenylhydrazone; D-glucosone, monodiphenylhydrazone and 2-furylglyoxal, bisdiphenylhydrazone were also added along with 15 μl of the 2-furylglyoxal, monodiphenylhydrazone solution). Injections were made electrokinetically (10 kV, 5 s). Separations were routinely run at 20 kV and monitored at 220 nm at ambient temperature. In between runs, the capillary was washed by 0.5 M NaOH for 4 min.

#### 2.1.1. Microemulsion electrokinetic chromatography

Microemulsions were prepared according Boso et al. [19] and Vomastová et al. [20] by mixing the microemulsion-forming organic solutes *n*-octanol (0.81%, w/w), SDS (3.31%, w/w) and *n*-butanol (6.61%, w/w) with 5 mM borate buffer (89.28%, w/w) and adjusting the pH to 7–11 with 0.1 M NaOH, where necessary. The optimized pH value was 8.0 (apparent pH of 8.2 of the microemulsion mixture).

### 2.1.2. SDS–micellar electrokinetic chromatography

Separations were carried out in the same manner with 5 mM borate buffer, pH 8.0 (96.42%, w/w), and SDS (3.58%, w/w).

### 2.2. Chemicals

SDS was purchased from Merck (Darmstadt, Germany) and N,N-diphenylhydrazine was from Koch-Light Labs. (Colnbrook, UK). All other chemicals were obtained from Lachema (Brno, Czech Republic) and were of the highest available purity. All buffers were prepared in Milli-Q water. D-arabino-2-Hexosulose (D-glucosone), D-lyxo-2-hexosulose (D-galactosone), D-threo-2-pentosulose (D-xylosone), 6-deoxy-D-arabino-2-hexosulose (6-deoxy-D-glucosone) and D-threo-2,5-hexodiulose (5-keto-D-fructose) were prepared enzymatically from the corresponding aldoses [3]. The tricarbonyl compound, 5-hydroxy-2,3-dioxopentanal, was prepared enzymatically from 6-deoxy-D-xylose [6]. 2-Furylglyoxal was prepared by spontaneous cyclization of the unsaturated  $\beta$ -pyrone, cortalcerone [24].

### 2.3. Preparation of derivatives

N,N-Diphenylhydrazones were prepared by condensing the individual di- and tricarbonyl sugars and 2-furylglyoxal with freshly distilled N,N-diphenylhydrazine in the presence of acetic acid. The respective mono- and bisdiphenylhydrazones were separated and purified by preparative TLC on silica gel sheets (Silufol 20×20 cm; Kavalier, Votice, Czech Republic) using chloroform–methanol (20:1, v/v) as the eluent, followed by recrystallization from ethanol. Glycosulose hydrazones were acetylated using a mixture of acetic anhydride–pyridine (1:1, v/v) for 24 h at room temperature and then were crystallized from 50% aqueous ethanol [3].

### 2.4. Application

The basidiomycete *Daedalea quercina* was cultivated submerged in liquid, as previously described [25]. Cultures from an eight day cultivation were harvested, culture liquid was centrifuged (500 g) and 50 ml were condensed with 0.5 g of freshly distilled N,N-diphenylhydrazine in 2 ml of 40% acetic acid.

Unreacted derivatization reagent was removed by TLC (see Section 2.3). No detectable amounts of glucose were detected using the glucose oxidase–peroxidase method and TLC [3] in underivatized culture liquid.

### 2.5. Calculations

The number of theoretical plates,  $N$ , was calculated according to the equation  $N=5.545 (t_m/W_h)^2$ , where  $t_m$  is the migration time and  $W_h$  is the peak width at half height of the peak (in min); resolution,  $R$ , was calculated from the equation  $R=2 \Delta_t (W_1 + W_2)^{-1}$ , where  $\Delta_t$  is the difference between the migration times of the two solutes involved and  $W_1$  and  $W_2$  are their peak widths (in time units) at the baseline.

## 3. Results and discussion

The separation conditions for mono- and bisdiphenylhydrazones of dicarbonyl (and also tricarbonyl) sugars were optimized with respect to buffer pH and its concentration. Unsatisfactory separations are not shown; the optimum results were obtained with 5 mM borate buffer, pH 8.0 (89.27%, w/w), SDS (3.31%, w/w), *n*-butanol (6.61%, w/w) and *n*-octanol (0.81%, w/w) (see Fig. 1). Separation was unsuccessful if a buffer concentration exceeding 25 mM was used. In this case, most of the compounds (particularly the last seven, see Table 1) were not eluted, even after a run time of 120 min; this means that the electroosmotic flow was too slow to bring the compounds to the window of the detector. *n*-Octanol was used as the organic core phase on the basis of our previous work on MEEKC [19,20], where we compared various core phases, namely *n*-hexane, cyclohexane, diethyl ether, *n*-amylalcohol, *n*-hexanol and *n*-octanol.

The microemulsion system used was able not only to separate the hydrazones of the compounds investigated (Fig. 1) (both mono- and bis derivatives), but also the respective derivatives of 2-furylglyoxal; this is quite important because 2-furylglyoxal represents a product of the spontaneous degradation of carbohydrates. Under the optimized conditions, quite high numbers of theoretical plates were achieved, ranging

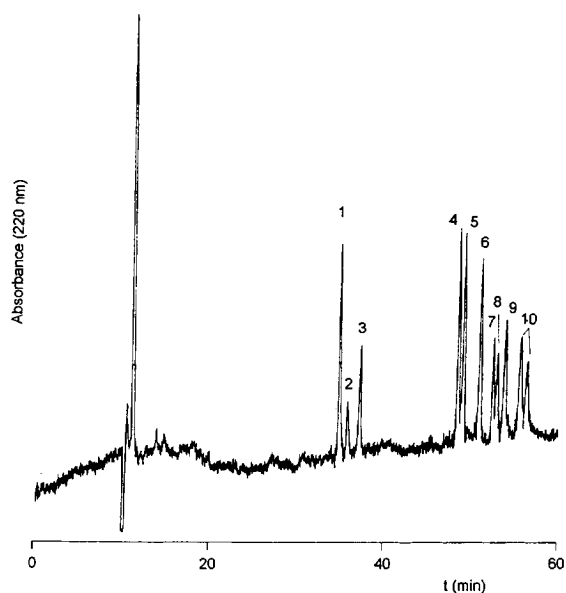


Fig. 1. Separation of mono- and bisdiphenylhydrazones of sugars by MEEKC. Conditions: untreated fused-silica capillary [70 cm (effective length 65 cm)  $\times$  50  $\mu$ m I.D.] at 20 kV; detection was at 220 nm; the microemulsion system was composed of 5 mM borate buffer, pH 8.0 (89.27%, w/w), SDS (3.31%, w/w), *n*-butanol (6.61%, w/w) and *n*-octanol (0.81%, w/w). Peak identification: 1 = D-galactosone, monodiphenylhydrazone; 2 = 5-keto-D-fructose, bisdiphenylhydrazone; 3 = D-glucosone, monodiphenylhydrazone; 4 = D-galactosone, monodiphenylhydrazone, acetyl derivative; 5 = D-xylosone, monodiphenylhydrazone, acetyl derivative; 6 = 6-deoxy-D-glucosone, monodiphenylhydrazone, acetyl derivative; 7 = 2-furylglyoxal, monodiphenylhydrazone; 8 = D-glucosone, bisdiphenylhydrazone; 9 = 5-hydroxy-2,3-dioxopentanal, trisdiphenylhydrazone and 10 = 2-furylglyoxal, bisdiphenylhydrazone. The full scale on the absorbance axis was 0.05 mV.

from  $100 \cdot 10^3$  to  $254 \cdot 10^3$  (see Table 1). Resolution of all examined peaks (relative to the preceding peak) was also satisfactory — the critical pairs were 2-furylglyoxal (monodiphenylhydrazone)–D-glucosone (bisdiphenylhydrazone) ( $R=0.88$ ), 2-furylglyoxal (bisdiphenylhydrazone) double peak ( $R=1.03$ ) and D-galactosone (monodiphenylhydrazone, acetyl derivative)–D-xylosone, (monodiphenylhydrazone, acetyl derivative) ( $R=1.25$ ). With all other components of the mixture,  $R$  was  $>1$ . Derivatization with *N,N*-diphenylhydrazine generally yields mono- and bis derivatives. The latter are generally less polar and, consequently, their affinity to the micellar phase should be higher. Indeed, in the electrophoretic patterns obtained, monodiphenylhydrazones were eluted first, followed by the bis derivatives. Limits of detection were 10  $\mu$ g for D-galactosone, monodiphenylhydrazone; D-galactosone, monodiphenylhydrazone, acetyl derivative; D-xylosone, monodiphenylhydrazone, acetyl derivative; 6-deoxy-D-glucosone monodiphenylhydrazone, acetyl derivative and 2-furylglyoxal, monodiphenylhydrazone, were 20  $\mu$ g for D-glucosone, bisdiphenylhydrazone and 5-hydroxy-2,3-dioxopentanal, trisdiphenylhydrazone and were 50  $\mu$ g for 5-keto-D-fructose, bisdiphenylhydrazone; D-glucosone, monodiphenylhydrazone and 2-furylglyoxal, bisdiphenylhydrazone.

In sharp contrast to this complete separation obtained by MEEKC was the poor separation obtained by MEKC (Fig. 2). Conditions for separation by MEKC were, in principle, the same as with

Table 1  
Electrophoretic characteristics of separated compounds

Compound	<i>t</i> (min)	<i>N</i> ( $\times 10^3$ )	<i>R</i>
D-Galactosone, monodiphenylhydrazone	28.8	162	51.33 <sup>a</sup>
5-Keto-D-fructose, bisdiphenylhydrazone	29.7	100	1.80
D-Glucosone, monodiphenylhydrazone	30.7	127	2.60
D-Galactosone, monodiphenylhydrazone, acetyl derivative	40.0	254	25.95
D-Xylosone, monodiphenylhydrazone, acetyl derivative	40.5	187	1.25
6-Deoxy-D-glucosone, monodiphenylhydrazone, acetyl derivative	42.1	201	3.65
2-Furylglyoxal, monodiphenylhydrazone	43.5	252	3.00
D-Glucosone, bisdiphenylhydrazone	43.8	188	0.88
5-Hydroxy-2,3-dioxopentanal, trisdiphenylhydrazone	44.5	170	1.48
2-Furylglyoxal, bisdiphenylhydrazone	46.0, 46.6	113, 105	2.50, 1.03

migration time *t* (min), number of theoretical plates *N* ( $\times 10^3$ ) and resolution *R* (to the preceding peak).

<sup>a</sup>Refers to the peak of endosmotic flow.

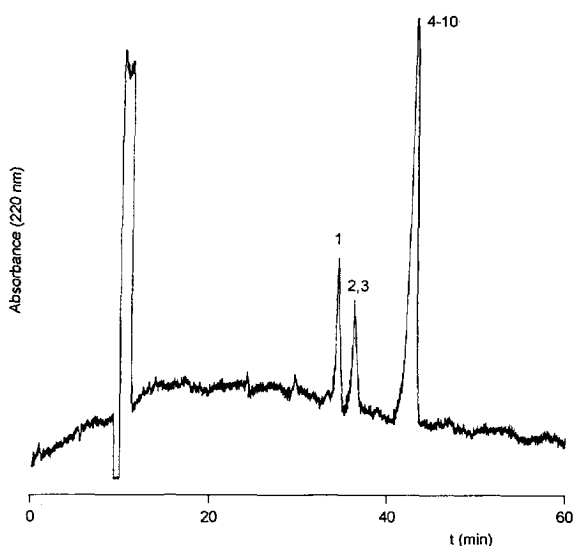


Fig. 2. Separation of mono- and bisdiphenylhydrazones of sugars by MEKC. Conditions were the same as in Fig. 1, except that the buffer consisted of 5 mM borate buffer, pH 8.0 (96.42%, w/w) and SDS (3.58%, w/w) only, with no alcohol added. Peak identification and other conditions are the same as in Fig. 1.

MEEKC, i.e., the pH and concentration of the buffer as well as the amount of SDS were identical in both cases. The difference between MEEKC and MEKC is quite obvious: MEKC was able to separate D-galactosone monodiphenylhydrazone from a fused peak of 5-keto-D-fructose, bisdiphenylhydrazone and D-glucosone, monodiphenylhydrazone and no separation occurred with the next seven compounds (D-galactosone, monodiphenylhydrazone, acetyl derivative; D-xylosone, monodiphenylhydrazone, acetyl derivative; 6-deoxy-D-glucosone, monodiphenylhydrazone, acetyl derivative; 2-furylglyoxal, monodiphenylhydrazone; D-glucosone, bisdiphenylhydrazone; 5-hydroxy-2,3-dioxopentanal, trisdiphenylhydrazone and 2-furylglyoxal, bisdiphenylhydrazone). This poor separation of diphenylhydrazones of dicarbonyl sugars by MEKC is in sharp contrast to the good resolution of 2,4-dinitrophenylhydrazones of oxo-compounds arising during the Maillard reaction [13] or of lower aliphatic aldehydes found in water [26]; 2,4-dinitrophenyl derivatives are apparently more readily separated by MEKC with SDS in spite of the fact that there are considerable differences in the chemi-

cal structures of the aldehydes involved (formaldehyde, acetaldehyde and propionaldehyde). The enhanced selectivity of the microemulsion system for carbohydrate carbonyls is apparently due to the different derivatization reagent (N,N-diphenylhydrazine vs. 2,4-dinitrophenylhydrazine) [13,26]. It is feasible to assume that, in our case, derivatization with a more hydrophobic reagent favourably influenced partitioning between the microemulsion pseudophase and the surrounding buffer. Extremely increased migration times, as found with a higher background electrolyte concentration (25 mM), are indicative of a high affinity of this kind of derivative to the microemulsion pseudophase.

Applicability of the examined MEEKC method for biologically relevant samples is shown in Fig. 3. Production of the extracellular enzyme pyranosa 2-oxidase by the wood-decaying basidiomycete *Daedalea quercina* was examined. Derivatization of the culture liquid N,N-diphenylhydrazine gave the electrophoretic profile seen in Fig. 3A. The position of the D-glucosone derivative on the electropherogram was revealed by spiking with appropriate standards (Fig. 3B).

#### 4. Conclusions

The advantage of using a microemulsion system for the separation of sugars in the form of diphenylhydrazones was clearly demonstrated: The separation obtained was considerably better than results obtained with MEKC. Using the microemulsion pseudophase together with a more hydrophobic derivatization reagent resulted in better selectivity and rather high plate numbers. While SDS-micellar separations were capable of resolving only D-galactosone, monodiphenylhydrazone from a joint peak of 5-keto-D-fructose, bisdiphenylhydrazone and D-glucosone, monodiphenylhydrazone, and a cluster peak consisting of seven compounds (D-galactosone, monodiphenylhydrazone, acetyl derivative; D-xylosone, monodiphenylhydrazone, acetyl derivative; 6-deoxy-D-glucosone, monodiphenylhydrazone, acetyl derivative; 2-furylglyoxal, monodiphenylhydrazone; D-glucosone, bisdiphenylhydrazone; 5-hydroxy-2,3-dioxopentanal, trisdiphenylhydrazone and 2-furylglyoxal, bisdiphenylhydrazone), use of

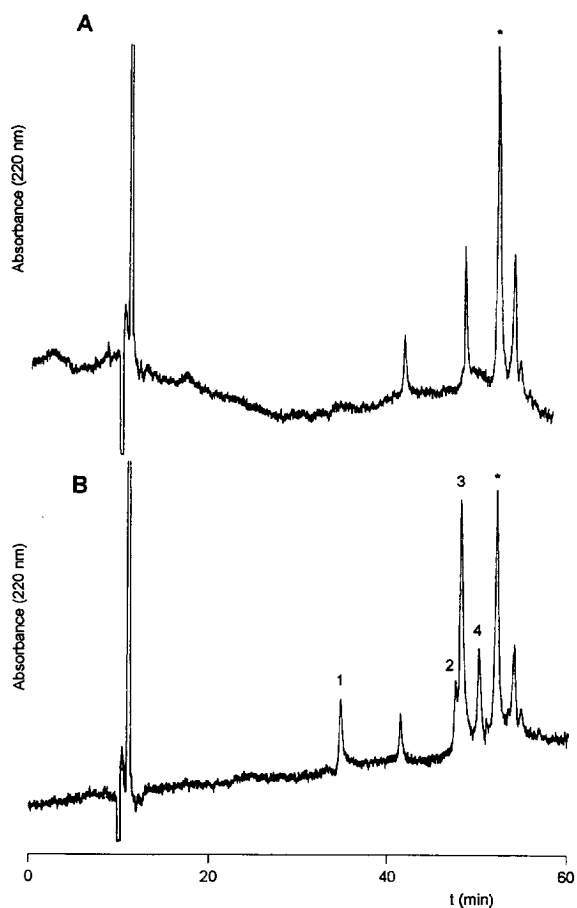


Fig. 3. An example of the application of the MEEKC separation of diphenylhydrazones of sugars. (A) Profile of the derivatized extract from the *Daedalea* sp.; (B) the same as (A) but spiked with (1) D-galactosone, monohydrazone; (2) D-galactosone, monohydrazone, acetyl derivative; (3) D-xylosone, monohydrazone, acetyl derivative and (4) 6-deoxy-D-glucosone, monohydrazone, acetyl derivative. Asterisk indicates the retention of D-glucosone, bishydrazone; The full scale on the absorbance axis was 0.05 mV.

the microemulsion pseudophase resulted in a practically complete separation of all members of the test mixture. It is feasible to assume that the better results obtained are due to the more hydrophobic nature of the derivatizing reagent, along with the more hydrophobic nature of the pseudophase used. It appears that the use of more hydrophobic derivatives with microemulsion pseudophases can generally result in good separations of mixtures that are difficult to separate by MEKC.

Applicability of the method extrapolated to real samples was demonstrated.

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