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Journal of Chromatography A, 837 (1999) 281–287

JOURNAL OF CHROMATOGRAPHY A

Short communication

Usefulness of methylated-β-cyclodextrin-based buffers for the separation of highly hydrophobic solutes in non-aqueous capillary electrophoresis
Application to the separation of derivatized phytosterols

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Received 27 August 1998; received in revised form 13 January 1999; accepted 14 January 1999

Abstract

This study describes the capillary electrophoretic separation of highly hydrophobic non-UV absorbing solutes in non-aqueous background electrolyte solution containing neutral cyclodextrins. After a derivatization step, several positively charged derivatives of neutral phytosterols have been partially resolved using a non-aqueous acidic electrophoretic buffer consisting of 20 m*M* ammonium acetate dissolved in methanol–acetonitrile–acetic acid (50:49:1, v/v). The selectivity has been significantly improved by the addition of 10 m*M* heptakis(2,6-di-*O*-methyl)-β-cyclodextrin to the non-aqueous buffer in order to promote hydrophobic interactions to differentiate similar structural molecules. Such non-aqueous buffers will be suitable to capillary electrophoresis–mass spectrometry coupling.

Keywords: Buffer composition; Non-aqueous capillary electrophoresis; Phytosterols; Cyclodextrin

1. Introduction

Phytosterols have been widely studied in phytochemistry and these lipidic constituents of plant cell membranes play an important role to control the membrane fluidity by stabilising the lipid bilayer. Free sterols interact with membrane phospholipids and may affect carrier-mediated ion transport by modifying the permeability of the membrane. Sterols are structurally related cyclic alcohols found in the unsaponifiable fraction of fats and oils. The sterol pattern of an oil is characteristic of the product and, together with the fatty acid methylester profile, constitutes the basis of the official sanctioned method for defining the identity of an edible oil. However, the structural similarity of these highly hydrophobic solutes makes sterol determination in vegetal material a difficult task. A number of gas or liquid chromatography separations have been used for the analysis of sterols [1,2].

In the last five years, capillary electrophoresis (CE) has become an important analytical tool for the separation of natural molecules in plant extracts [3]. The main advantages are high separation efficiencies and short analysis times. Several authors have reported the separation of steroids by capillary zone

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00106-5

electrophoresis (CZE) [4,5] and micellar electrokinetic chromatography (MEKC) [6,7]. The separation of charged conjugated steroids (estriol sulfate, estrone sulfate, estriol glucuronide) has been achieved by CE using borate–sodium hydroxide buffer (pH 10.2) in amniotic fluid [5]. Thus, Görog et al. [4] resolved native steroid hormones (4-ene-3-ketosteroids and 17-ketosteroids) by CE after transformation into the corresponding hydrazones using Girard P and T reagents; then, positively charged derivatives were separated by CZE with an UV detection. In MEKC, Nishi and Matsuo [6] studied the separation of neutral corticosteroids (hydrocortisone, hydrocortisone acetate, cortisone acetate); these lipophilic compounds could not be fully resolved with sodium dodecyl sulfate (SDS) buffers but the addition of γ-cylcodextrin to the SDS buffer remarkably improved the resolution of these UV-absorbing compounds.

The direct coupling of MEKC to mass spectrometry (MS) is hazardous due to the presence of non-volatile surfactants in the electrophoretic buffer. Indeed, the presence of a relatively high concentration of non-volatile surfactant (SDS) results in a significant loss of electrospray efficiency and may cause background ions in the mass spectrometer and then fouling of the ion source, except by using on-line partial filling method [8].

However, the poor solubility of steroids or phytosterols in aqueous buffers prompted us to consider the use of non-aqueous buffer. If CE separations are generally performed in aqueous buffers, the use of non-aqueous media in CE has yet been reported [9–14]. A wide range of solvents including mainly methanol, acetonitrile, formamide, *N*-methylformamide and *N*,*N*-dimethylformamide have been used as non-aqueous media in CE. Non-aqueous media seem to be particularly suitable for the analysis of solutes that are not readily soluble in water or solutes which show very similar electrophoretic mobilities in aqueous media. Thus, it is often possible to obtain different selectivities in non-aqueous media than those found in water. Nevertheless, the separation of highly hydrophobic phytosterols by non-aqueous CE would require an additional separation mechanism based on the differences in hydrophobicity between these solutes. The use of neutral cyclodextrins in solvent mixtures with a relatively low dielectric constant have not previously been described. So, this report focuses on the addition of neutral methylated-β-cyclodextrin to non-aqueous electrophoretic medium in order to increase the selectivity between hydrophobic and closely related derivatized phytosterols.

2. Experimental

2.1. Apparatus

All open-tube electrokinetic capillary chromatographic separations were performed on a Spectraphoresis 1000 instrument (ThermoQuest, San Jose, CA, USA) using a silica capillary tube [70 cm (63 cm to the detector)×75 μm I.D. or 44 cm (37 cm to the detector)×50 μm I.D.×375 μm O.D.]. The capillaries used (Thermoquest) are uncoated fused silica capillaries. Data were processed on an IBM PS/2 Model 70 386 computer. Software operating under IBM OS/2 was supplied by Spectra-Physics. The instrument contains a programmable high-speed scanning, multiple-wavelength UV detector. Using the fast scanning mode from 200 to 360 nm, absorption maxima of studied derivatized phytosterols were determined (284 nm). Electrophoretic separations were performed at positive voltage (+15 kV). Analytes were injected in the hydrodynamic mode using a $5.17 \cdot 10^7$ Pa vacuum (0.75 p.s.i) for 3 s.

The capillary was conditioned daily by washing with 0.1 *M* sodium hydroxide methanolic solution (5 min) and methanol (5 min) and then with buffer (5 min). Between two consecutive analyses, the capillary tubing was flushed with the electrophoretic buffer (5 min).

2.2. Reagents

All chemicals were of analytical-reagent grade. Acetic acid, ammonium acetate, 1-methyl-2-fluoropyridinium *p*-toluenesulphonate, triethylamine, naphthalene were obtained from Fluka (Buchs, Switzerland). Trimethyl-β-cyclodextrin (TM-β-CD) and dimethyl-β-cyclodextrin (DM-β-CD) of derivatization degree (DS) 1.8 were obtained from Wacker (Munich, Germany). Methanol, acetonitrile, di-

chloromethane used for mobile phases were of HPLC-grade (Carlo Erba, Milan, Italy).

For non-aqueous CE under acidic conditions, the buffer composition was 20–100 m*M* ammonium acetate in methanol–acetonitrile–acetic acid (50:49:1, v/v).

2.3. Phytosterol extraction

The phytosterol fraction of rape seed (*Brassica napus*) oil contains three main constituents brassicasterol (1), campesterol (2) and β-sitosterol (3). Their identification has been confirmed by GC–MS at elevated temperature (300°C) on a 95% methyl–5% phenyl bonded fused silica column (DB-5, J&W) whose dimensions are 30 m×0.25 mm I.D, 0.25 μm. Besides, (1) and (2) compounds differ by one double carbon–carbon bond while (2) and (3) differ by one methyl group (Fig. 1).

2.4. Derivatization

A known volume (1 ml) of dichloromethane solution of 1-methyl-2-fluoropyridinium *p*-toluenesulphonate (0.30 mmol) was added to 0.15 mmol sterol extract (dissolved in 0.5 ml of dichloromethane) and 0.30 mmol triethylamine mixture at room temperature [15,16]. Then, sterol consumption was checked by silica gel thin-layer chromatography with $CHCl_3$–MeOH (19:1, v/v) as an eluent. The excess of triethylamine was removed under reduced pressure and the residue washed with HPLC grade acetonitrile to eliminate 1-methyl-2-fluoropyridinium *p*-toluenesulphonate. The stability of 2-alkoxy-1-methylpyridinium π-toluenesulphonate was monitored for one month and no changes in the separations were noticed.

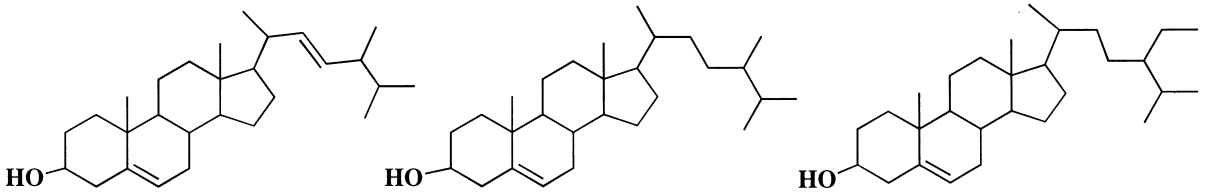
3. Results and discussion

The CE separation of phytosterols, as those described in Fig. 1, has never been previously described in the literature and reveals to be a challenge for the following reasons: (i) studied phytosterols are almost transparent molecules in the UV domain and their direct UV determination would be poorly sensitive and (ii) native phytosterols are hydrophobic non-charged molecules and are not suitable for direct separation by CZE.

To increase the range of compounds which can be analysed by CE with an UV detection, derivatization techniques have been yet used to form solution-ionisable analyte derivatives [15]. Indeed, as in principle only charged solutes can be separated by CZE, neutral solutes can be derivatized by introducing a charge onto the molecule. So, we performed a derivatization reaction before the electrophoretic separation in the off-line mode.

Several authors have mentioned [16,17] that 1-methyl-2-fluoropyridinium *p*-toluenesulphonate reacts quickly and quantitatively and can easily be performed with sterols in the presence of triethylamine to give corresponding 1-methylpyridinium 2-alkoxy *p*-toluenesulphonates (Fig. 2). Derivatized phytosterols bear a positive charge and possess an UV absorbance (at 284 nm) due to the pyridinium group. The detectability improvement is the most pronounced reason for derivatization in CE.

The poor solubility of hydrophobic derivatized



1 2 3

Fig. 1. Structure of studied phytosterols. 1=Brassicasterol (M_r=490); 2=campesterol (M_r=492); 3=β-sitosterol (M_r=506).

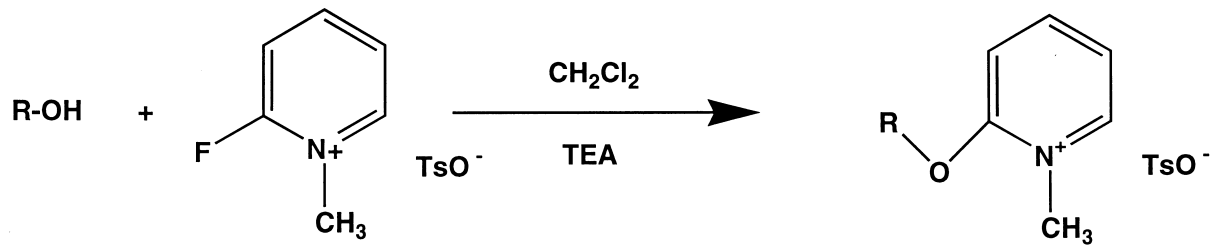


Fig. 2. Derivatization of phytosterol with 1-methyl-2-fluoropyridinium *p*-toluenesulphonate.

phytosterols in aqueous buffers prompted us to consider the use of non-aqueous buffers. So, acetonitrile–methanol mixtures have been used as electrophoretic medium to increase the solubility of the analytes in the separation buffer. Firstly, the CE separation has been investigated in methanol–acetonitrile–acetic acid (50:49:1, v/v) buffer containing 20 m*M* ammonium acetate (Fig. 3); acetic acid was added to adjust the acidity of the medium and avoids the solubility problems. Using this medium, one can solubilize phytosterol derivatives, hence making them amenable to CE analysis. Only two cationic

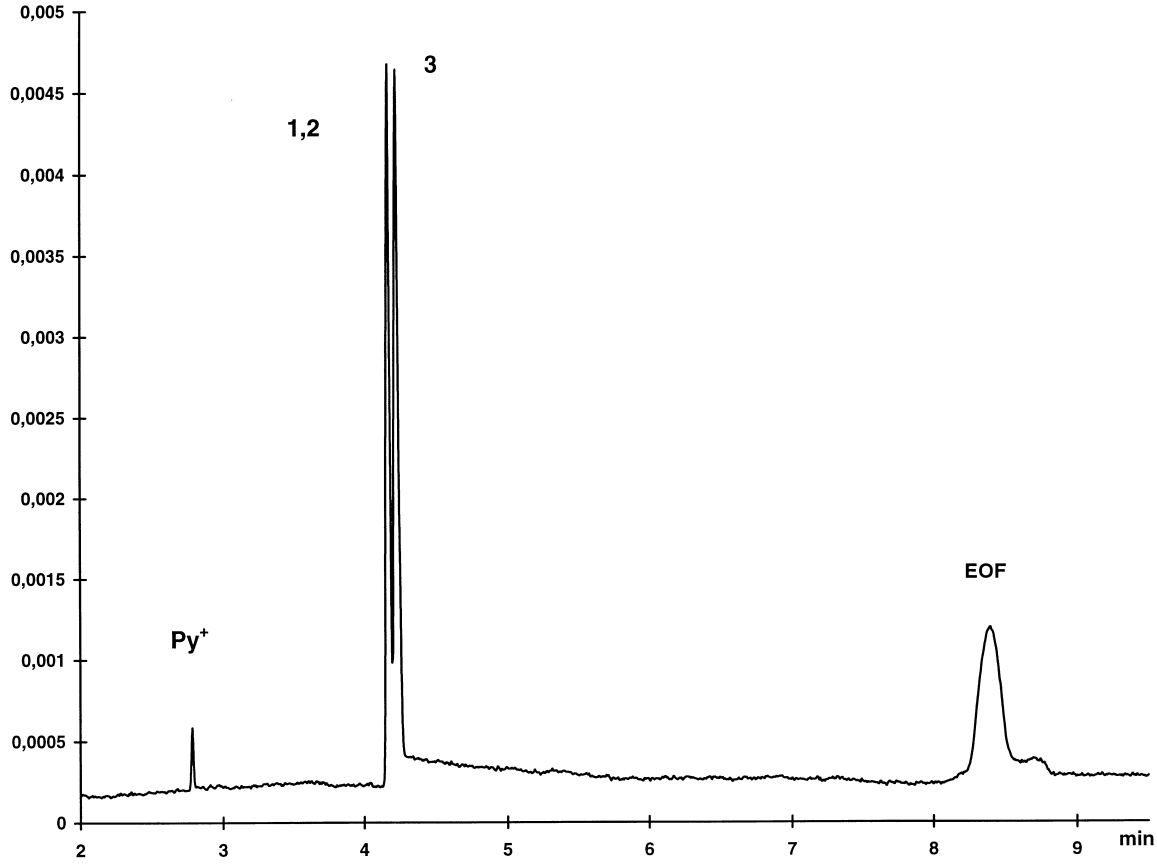


Fig. 3. Separation of phytosterol extract using non-aqueous capillary electrophoresis. Capillary: 44 cm×50 μm; applied voltage: +15 kV; temperature: 20°C; detection at 284 nm; electrolyte: 20 m*M* ammonium acetate dissolved in methanol–acetonitrile–acetic acid (50:49:1, v/v); hydrodynamic injection time: 3 s; solute concentration: 100 μg/ml. Solutes: 1=brassicasterol; 2=campesterol; 3=β-sitosterol.

phytosterol derivatives (at 4.16 and 4.22 min) were observed before the electroosmotic flow (naphtalene peak). The first migrating peak at 2.7 min is relative to the excess of 1-methyl 2-fluoro-pyridinium cationic reagent (noted Py^+) while electroosmotic flow (EOF) appears at 8.4 min. The 1-methyl-pyridinium derivative of campesterol migrates faster than that of β-sitosterol. Indeed, the electrophoretic mobility of charged solute depends on the effective charge–hydrodynamic radius ratio and β-sitosterol molecule contains an additional methyl group. No further improvement of the resolution between brassicasterol and campesterol happens at higher amounts of methanol in the separation buffer.

The effects of varying concentrations of ammonium acetate on the migration time and resolution have been investigated. In aqueous electrolytes, an increase of ionic strength reduces the zeta-potential and causes a reduction of EOF. Such results were also observed in methanol–acetonitrile buffers. Increasing the ionic strength of the electrophoretic buffer induces slower EOF and longer analysis times. A moderate improvement of resolution (+15%) was obtained with an increasing ammonium acetate concentration (from 0.9 at 20 m*M* up to 1.05 for 100 m*M*) at the expense of the analysis time (+30%). Hence, 20 m*M* concentration of ammonium acetate in methanol–acetonitrile–acetic acid (50:49:1, v/v) buffer was selected. Low currents (15 μA) were observed which resulted in reduced Joule heating and high peak efficiencies (315 000 theoretical plates per meter).

Nevertheless, the separation of brassicasterol and campesterol derivatives, which only differ a double carbon–carbon bond, could be improved by another mechanism based on the differences in hydrophobicity between solutes. Thus, neutral β-cyclodextrin was added to the electrophoretic medium in order to promote hydrophobic interactions. The use of cyclodextrins in solvent mixtures with a relatively low dielectric constant have not previously been described.

Cyclodextrins (CDs) are well-known non-reducing oligosaccharides that can form inclusion complexes with hydrophobic molecules. Due to the optical activity of glucopyranose unit, CDs are optically active and are the main discriminating agents employed in the separation of enantiomers in CE [18–21]. Otherwise, CDs have also been proposed as buffer additives in order to improve the selectivity of hydrophobic solutes in MEKC [22,23]. Terabe et al. [22] first highlights the interest of cyclodextrin (CD)–MEKC to resolve highly hydrophobic or closely related solutes such as polycyclic aromatic hydrocarbons or tetrachlorodibenzo-*p*-dioxin isomers. Then, Takeda et al. [23] reported the complete separation of ten aniline derivatives by MEKC after addition of γ-cyclodextrin to the SDS buffer while Valko et al. [10] used cyclodextrin as chiral agent in *N*-methylformamide and reported the potential of some organic solvents for chiral separations by CE.

Two different methylated β-CDs, DM-β-CD and TM-β-CD, were added at two concentrations (5 or 10 m*M*) into the non-aqueous buffer. Either an inclusion of the solute into the cavity of CD or different interaction with the outside of the CD could take place. Experimentally, better resolutions were obtained with DM-β-CD compared to TM-β-CD at different concentrations. We further selected DM-β-CD and investigated the influence of its concentration upon the resolution in the 5–20 m*M* range. Electrophoretic mobilities of cationic derivatized phytosterols decreased since the charge-to-mass ratio lowered due to the complexation with CD. The introduction of 10 m*M* DM-β-CD into the non-aqueous medium improved the resolution between hydrophobic phytosterols (R_S=2). Optimum separation of the mixture of derivatized phytosterols was achieved by adding 10 m*M* DM-β-CD into methanol–acetonitrile–acetic acid (50:49:1, v/v) buffer using a 77 cm×50 μm capillary (Fig. 4). The migration order of derivatized phytosterols inversely depends on sterol moiety hydrophobicity; so, brassicasterol derivative (M_r=490) migrates faster than campesterol one (M_r=492) due to a smaller complexation degree. The migration order both depends on the charge-to-mass ratio and of the sterol hydrophobicity.

4. Conclusion

The hydrophobic nature of phytosterols and their chemical structural similarity preclude the use of usual aqueous electrophoretic buffers which do not provide selectivity enough. At the opposite, the

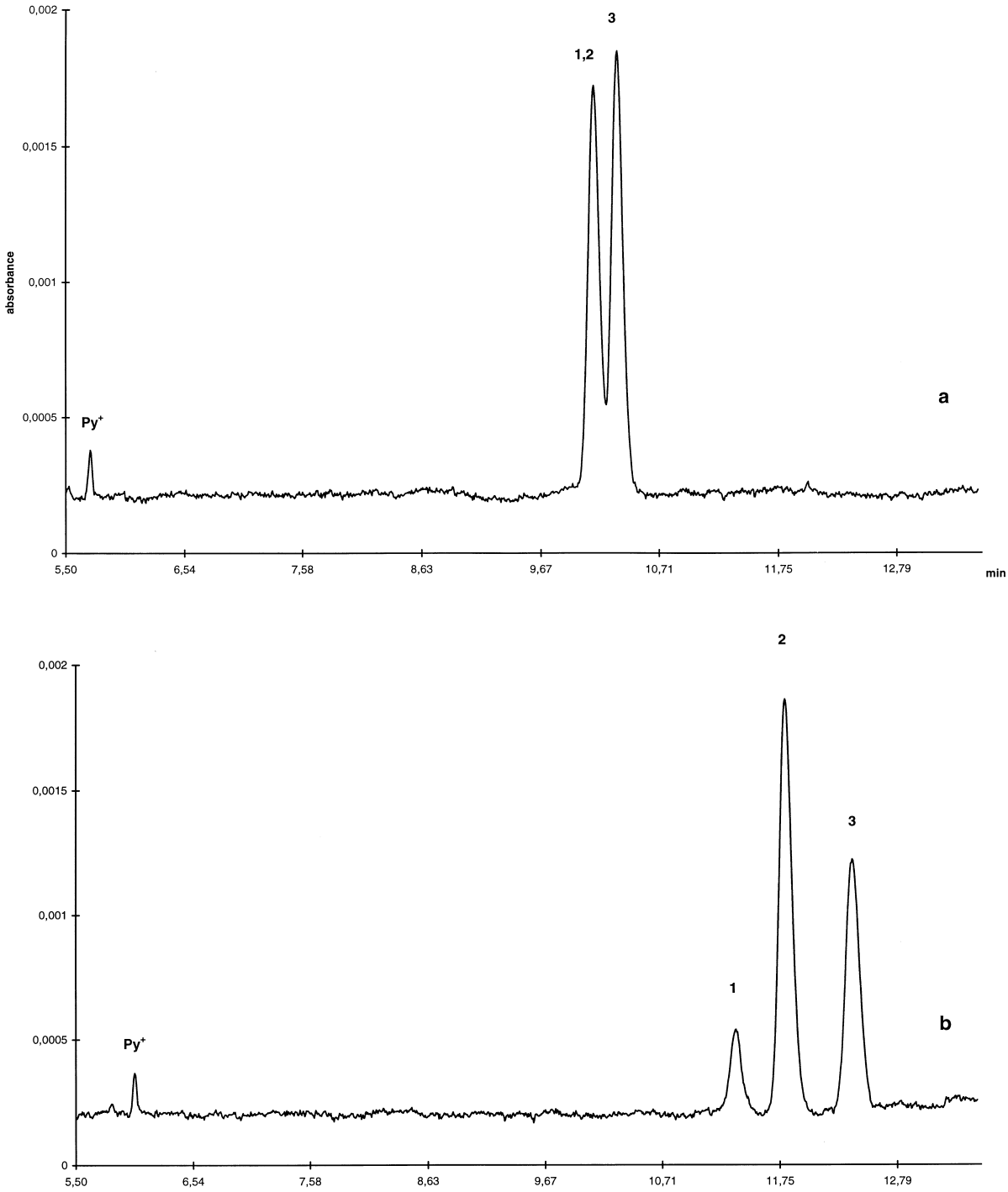


Fig. 4. Influence of the addition of the DM-β cyclodextrin added to the non-aqueous buffer upon the selectivity between derivatized phytosterols. Capillary: 70 cm×75 μm; applied voltage: +15 kV; temperature: 20°C; detection at 284 nm; electrolyte: 20 m*M* ammonium acetate dissolved in methanol–acetonitrile–acetic acid (50:49:1, v/v); hydrodynamic injection time: 3 s; solute concentration: 100 μg/ml. Solutes: 1=brassicasterol; 2=campesterol; 3=β-sitosterol. (a) Without addition of cyclodextrin, (b) with addition of 10 m*M* DM-β-CD.

addition of heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (5–20 m*M*) into non-aqueous acidic electrophoretic buffer (20 m*M* ammonium acetate dissolved in methanol–acetonitrile–acetic acid) allows the separation of closely related hydrophobic molecules.

Acknowledgements

The authors would like to thank ADEME organism (Agence pour le Developpement et la Maitrise de l'Energie, France) for their financial support during this work.

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