



HAL
open science

Separation of Phenolic Acids and Flavone Natural Antioxidants by Two-dimensional Method Combining Liquid Chromatography and Micellar Electrokinetic Capillary Chromatography

Petr Cesla, Jan Fischer, Pavel Jandera

► **To cite this version:**

Petr Cesla, Jan Fischer, Pavel Jandera. Separation of Phenolic Acids and Flavone Natural Antioxidants by Two-dimensional Method Combining Liquid Chromatography and Micellar Electrokinetic Capillary Chromatography. *Electrophoresis*, 2010, 31 (13), pp.2200. 10.1002/elps.200900689 . hal-00599457

HAL Id: hal-00599457

<https://hal.science/hal-00599457>

Submitted on 10 Jun 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Separation of Phenolic Acids and Flavone Natural Antioxidants by Two-dimensional Method Combining Liquid Chromatography and Micellar Electrokinetic Capillary Chromatography

Journal:	<i>Electrophoresis</i>
Manuscript ID:	elps.200900689.R1
Wiley - Manuscript type:	Research Paper
Date Submitted by the Author:	22-Jan-2010
Complete List of Authors:	Cesla, Petr; University of Pardubice, Analytical Chemistry Fischer, Jan; University of Pardubice, Analytical Chemistry Jandera, Pavel; University of Pardubice, Analytical Chemistry
Keywords:	Micellar electrokinetic chromatography, Two-dimensional separation, Flavones, Phenolic compounds



1
2 1 | **Separation of Phenolic Acids and Flavone Natural Antioxidants by Two-Dimensional**
3 2 | **Method Combining Liquid Chromatography and Micellar Electrokinetic Capillary**
4 3 | **Chromatography**
5 4
6 5

7 6
8 7 | Petr Česla, Jan Fischer, Pavel Jandera*

9 8
10 9 | Department of Analytical Chemistry, Faculty of Chemical Technology, University of
11 10 | Pardubice, Studentská 573, CZ-53210 Pardubice, Czech Republic
12 11
13 12

14 13
15 14 | **Correspondence:**

16 15 | Prof. Pavel Jandera, Department of Analytical Chemistry, Faculty of Chemical Technology,
17 16 | University of Pardubice, Studentská 573, CZ-53210 Pardubice, Czech Republic, E-mail:
18 17 | Pavel.Jandera@upce.cz, Tel: +420 466 037 023, Fax: +420 466 037 068
19 18
20 19

21 20
22 21 | **Abbreviations:**

23 22 | MEKC - Micellar Electrokinetic Chromatography, LC - Liquid Chromatography, SDS -
24 23 | Sodium dodecyl sulphate, 2D - two-dimensional, CE - capillary electrophoresis, CD -
25 24 | cyclodextrins, [EOF - electroosmotic flow](#), [C18 - octadecyl](#), [RP - reversed phase](#), [LC-LC two-](#)
26 25 | [dimensional liquid chromatography](#), [LCxLC - comprehensive two-dimensional](#)
27 26 | [chromatography](#), [LC-MEKC - two-dimensional combination of liquid chromatography and](#)
28 27 | [micellar electrokinetic chromatography](#).
29 28
30 29
31 30
32 31

33 32
34 33 | **Keywords:**

35 34 | Flavones, Micellar electrokinetic chromatography, Phenolic compounds, Two-dimensional
36 35 | [separations](#)
37 36
38 37
39 38
40 39
41 40
42 41
43 42
44 43
45 44
46 45
47 46
48 47
49 48
50 49
51 50
52 51
53 52
54 53
55 54
56 55
57 56
58 57
59 58
60 59

25 **Abstract**

26 A two-dimensional method was developed for separation of phenolic acids and
27 flavone compounds combining liquid chromatography with micellar electrokinetic capillary
28 chromatography. The effect of substituted neutral and anionic cyclodextrin additives to the
29 background electrolyte on the quality of MEKC separation was investigated. The best
30 selectivity of the MEKC separation was achieved in 25 mmol/L borate background buffer at
31 pH 9.05 with the addition of 10 g/L SDS and 1.85 g/L heptakis-6-O-sulfo- β -cyclodextrin.
32 These conditions were used in the second dimension of two-dimensional LC-MEKC
33 separation in combination with a (poly)ethylene glycol (PEG) column in the first dimension,
34 providing the best orthogonality (the lowest degree of correlation between the selectivity of
35 separation) in the two dimensions. A CE autosampler was employed as the interface between
36 LC and MEKC steps based on automated fraction collection before the re-analysis of the
37 collected LC fractions in the second, MEKC dimension. The 2-D method under optimized
38 conditions was applied for the separation of natural antioxidants in the samples of green tea.

Deleted: 35

Deleted: mmol

Deleted: mmol

1 Introduction

In recent years, the use of natural occurring compounds, including polyphenolic and flavone compounds, in pharmaceuticals, food supplements and cosmetics was becoming more and more popular. These compounds occur as secondary plant metabolites involved in the production of yellow or red/blue pigmentation or protection of flowers from attack by microbes and insects and show important anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity [1]. The flavones and related natural antioxidants differ by the degree of hydroxylation, methylation, number of double bonds and, in case of glycosides, by the type and position of sugar moieties [2].

Liquid chromatography with different detection modes is frequently used for the analysis and determination of flavones and related compounds [3,4]. Other chromatographic methods - gas chromatography and thin-layer chromatography - are less commonly used for separation of these polar non-volatile compounds [4,5].

Capillary electrophoresis (CE) and micellar electrokinetic chromatography (MEKC) techniques have also been used for separation and determination of flavones and phenolic compounds in tea [6-10], wine [11-13], beer [14], honey [15-17], plants and plant extracts [18-22]. The MEKC separations of flavone compounds are usually carried out using borate background electrolyte at pH > 7, with the addition of sodium dodecyl sulphate micellar reagent [11,12,14,15,18-22]. Capillary zone electrophoresis has been used for separation of quercetin [13,22] and other flavones [16,17]. The applications of various separation techniques for the separation of polyphenolic and flavone natural antioxidants has been covered in several review articles [4,5,23,24].

For the separation of complex sample mixtures, two-dimensional separation methods considerably increase the numbers of resolved components due to increased peak capacity in comparison to one-dimensional separations. Recently, 2-D liquid chromatographic (LC-LC) methods for separation of flavones in food, beverages and plant extracts have been developed [25-27]. In most applications, two reversed-phase systems with different types of stationary phases are combined in the first and in the second dimension. Natural compounds in the extracts of umbelliferae herbs were separated using gradients of acetonitrile in water on a bonded CN column in the first dimension and of methanol in water on a monolithic C18 column in the second dimension [28]. Gradient elution provides significant improvement in peak capacity with respect to isocratic conditions. Natural antioxidants were analyzed using simultaneous gradients of acetonitrile in ammonium acetate buffer on a polyethylene glycol or

1
2 72 [on a phenyl column in the first dimension and on a C18 column in the second dimension \[29\].](#)
3 73 [Acidic compounds were separated using a cation-exchange column in the first dimension and](#)
4 74 [a C18 column in the second dimension with a gradient of acetonitrile in acetate buffer \[30\].](#)
5 75 [Recently, porous-shell fused-core C18 columns were combined in the second dimension with](#)
6 76 [a microbore column packed with phenyl-silica \[31\] or with PEG-silica \[32\] particles in the](#)
7 77 [first dimension for separation of phenolic antioxidants in wine, beverages and other food](#)
8 78 [samples in two-dimensional reversed-phase liquid chromatography.](#)

9 79 [The best improvement in the number of separated compounds, i.e. the highest increase](#)
10 80 [in the peak capacity with respect to single-dimension separations is achieved in highly](#)
11 81 [orthogonal two-dimensional systems with non-correlated selectivities in the first and in the](#)
12 82 [second dimension. Two reversed-phase systems are easy to connect on-line, but show more or](#)
13 83 [less correlated separation selectivities and consequently lower than maximum peak capacity](#)
14 84 [can be achieved in practice. On the other hand, combinations of reversed-phase and ion-](#)
15 85 [exchange systems are not very suitable for two-dimensional separations of non-ionic](#)
16 86 [compounds, for which ion-exchange columns usually show low retention and separation](#)
17 87 [selectivity. Finally, on-line two-dimensional combinations of reversed-phase and normal-](#)
18 88 [phase \(adsorption\) systems show high degree of orthogonality, but poor compatibility of](#)
19 89 [aqueous-organic RP mobile phases with purely organic solvents used as the mobile phase in](#)
20 90 [normal-phase chromatography. Therefore, combinations of HPLC in one dimension with](#)
21 91 [electromigration separation systems in the other one offer attractive possibilities of highly](#)
22 92 [orthogonal separations, complementary to LC-LC two-dimensional systems. Moreover,](#)
23 93 [electromigration methods are generally like to provide narrow peaks and hence high peak](#)
24 94 [capacity.](#)

25 95 [Two-dimensional capillary electrophoresis has been applied to separation of proteins](#)
26 96 [\[33,34\]. 2D combinations of liquid chromatography and capillary electrophoresis offer](#)
27 97 [possibility for increasing the peak capacity for separation of multi-component samples](#)
28 98 [containing ionic compounds. The LC-CE methods have been applied mainly in the analysis of](#)
29 99 [peptides in protein digests and of proteins in various biological samples \[31,35-44\].](#) On-line
30 100 [connection of RPLC to capillary zone electrophoresis \(CZE\) has been introduced by Bushey](#)
31 101 [and Jorgenson \[35\] and further applied by Jorgenson's group \[31,36,37\].](#) Off-line connections
32 102 [of LC and CE have been described by several research groups \[38,39,45\].](#) The off-line
33 103 [approach is slower than the on-line 2D methods, as it involves the collection of fractions from](#)
34 104 [the first dimension and then the analysis of isolated fractions in the second dimension. On the](#)
35 105 [other hand, it is simple to perform, the volume or the number of the collected fractions is not](#)

Deleted: The c

Deleted: in a 2-D setup

Deleted: s another

Deleted: studied

Deleted: et

Deleted: al

1
2 106 [that strictly limited and does not impose restrictions on the dimension of HPLC column in the](#)
3 107 [first dimension. On-line \(comprehensive\) methods analyze the whole applied amount of](#)
4 108 [sample, offer better automation possibilities and higher sample throughput, however they](#)
5 109 [require special instrument modification, micro- or capillary HPLC columns that can](#)
6 110 [efficiently handle only limited sample volumes and put severe time limitations on the second-](#)
7 111 [dimension separation, which must be accomplished in collection time of the next HPLC](#)
8 112 [fraction from the first dimension.](#)

9 113 Applications of 2D LC-CE for the analysis of naturally occurring compounds are less
10 114 common. Garcia-Villalba et al. used HPLC-CE for the analysis of extra-virgin olive oil
11 115 samples [45]. Selected fractions containing phenolic compounds were isolated for
12 116 quantification and *in vitro* studies of anti-carcinogenic capacity. [Like ion-exchange liquid](#)
13 117 [chromatography, capillary electrophoresis is not suitable for separations of non-ionic](#)
14 118 [compounds, which can be better separated by micellar electrokinetic chromatography, usually](#)
15 119 [in buffers containing sodium dodecylsulphate \(SDS\).](#) Zhang et al. [46] employed
16 120 comprehensive 2-D HPLC-MEKC for the separation of natural compounds in traditional
17 121 Chinese medicines. The HPLC instrument was connected on-line to the CE system through
18 122 interface based on effluent stacking-injection [of fractions collected from a micro-LC column](#)
19 123 [in a 0.25 \$\mu\$ l chamber during 50-60 s, which was the time for the second-dimension MEKC](#)
20 124 [analysis.](#)

21 125 In the present work, the effects of the addition of substituted cyclodextrins to the
22 126 background electrolyte on the separation selectivity of phenolic acids and flavone related
23 127 compounds in MEKC were investigated. A 2-D method combining liquid chromatography
24 128 with MEKC using automated off-line coupling with CE autosampler as the fraction collector
25 129 interface was developed, [to avoid time limitations in the second, MEKC dimension and to](#)
26 130 [allow using a previously optimized HPLC separation on a polyethylene glycol \(PEG\) column,](#)
27 131 [whose dimensions are not on-line compatible with the MEKC separation in the second](#)
28 132 [dimension \[29,47,48\]. The main objective was to investigate possible improvement of the](#)
29 133 [overall performance of the LC-MEKC method with earlier reported LCxLC 2D separations of](#)
30 134 [phenolic and flavone natural antioxidants \[32,49\].](#) The HPLC-MEKC 2-D method was
31 135 applied for the [analysis](#) of green tea samples.

Deleted: separations

136

2 Materials and methods

2.1 Materials and reagents

A fused silica capillary with extended 125 μm light path cell (25 μm I.D., 48 cm total length, 40 cm to the detector) was obtained from Agilent (Palo Alto, CA, USA). The Discovery HS PEG, 5 μm , 50 mm x 2.1 mm i.d. column was obtained from Supelco (Bellafonte, PA, USA).

The standards of flavone related compounds and phenolic acids (Figure 1) were obtained from Fluka (Buchs, Switzerland) and Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate, boric acid, sodium tetraborate, sodium dodecyl sulphate and substituted cyclodextrins, methyl- β -cyclodextrin, heptakis(2,6-di-O-methyl)- β -cyclodextrin, heptakis(2,3,6-tri-O-methyl)- β -cyclodextrin, and heptakis(6-O-sulfo)- β -cyclodextrin, were obtained from Fluka (Buchs, Switzerland), acetonitrile was from Sigma-Aldrich (St. Louis, MO, USA), methanol from Merck (Darmstadt, Germany) and formic acid from Lachema (Brno, Czech Republic).

The extract of Jasmine Green Tea, (Twining & Company Ltd., London, England) was prepared by keeping the tea bag in 100 mL of 95°C hot water for 3 minutes and cooling to the laboratory temperature. Before injection, the extract was filtrated using Millipore 0.45 μm syringe filter (Bedford, MA, USA).

2.2 Equipment

All MEKC experiments were carried out using Agilent ^{3D}CE (Agilent, Palo Alto, CA, USA) instrument at 20 kV. pH of the background electrolyte solutions was measured using an Orion 3 Start pH meter (Thermo Scientific, Waltham, MA, USA). Water was purified on an Ultra Clear UV apparatus (SG, Hamburg, Germany). The LC step of the 2-D method was carried out using an Agilent 1200 series Rapid Resolution liquid chromatograph in the first dimension. The outlet of the LC column was connected through a switching valve with the CE autosampler for automated collection of the fractions from first dimension. Figure 2 shows the experimental setup used for the 2D LC-MEKC setup.

Deleted: HPLC separation

2.3 Methods

The LC separations were carried out on a Discovery HS PEG column at 40°C using gradient elution with 10 mmol/L ammonium acetate in water (pH = 3.0 adjusted by the addition of formic acid) as the solvent A and acetonitrile as the solvent B. The mobile phase gradient program was from 1% to 30%B in 15 min at 0.4 mL/min. After the end of each 2-D run, the column was equilibrated with the initial gradient mobile phase for 10 min. The injection sample volume was 5 µL.

The detection wavelengths of the CE instruments were set to 220, 254, 280 and 350 nm. All electropherograms were extracted at wavelength 280 nm being the most general for the different classes of studied compounds. The capillary temperature was kept at 25 °C.

Samples were injected by applying overpressure of 50 mBar on the inlet sample vial for 10 s in single CE analyses. New capillaries were preconditioned by rinsing subsequently with 0.5 mol/L sodium hydroxide for 10 min, with water for the next 10 min and finally with the background electrolyte for 10 min. Further, the capillaries were rinsed prior to every injection for 1 min with the background electrolyte. In the LC-MEKC 2D experiments, 25 mmol/L borate buffer pH 9.05 with addition of 10 g/L SDS and 1.85 g/L heptakis(6-O-sulfo)-β-cyclodextrin was used as the background electrolyte in the second, MEKC dimension. The background electrolytes and mobile phases were filtered using a Millipore (Bedford, MA, USA) 0.45 µm filter and degassed by ultrasonication before use.

In the two-dimensional setup, an Agilent^{3D} CE autosampler was used as the interface between the LC and the MEKC separation systems (Figure 2). The outlet of the LC column was connected to the autosampler via a six-port switching valve, which was programmed to collect the fractions of the effluent from the LC column during the analysis in the first dimension (Figure 2A) into the vials equipped with 250 µL glass inserts. The CE autosampler was rotated each 15 s, thus the volume of the fractions was 100 µL. In total, 42 1st dimension fractions containing analytes were collected during the gradient elution, from 0.5 min to 11 min, for the analysis in the MEKC 2nd D system.

The fractions collected from the LC dimension were stored in the autosampler vials until the LC analysis run has been finished. After the end of the LC step, the valve was switched into the second position and the effluent from the first dimension was redirected to the waste while the LC column was re-equilibrated with the mobile phase used at the start of the gradient. Meanwhile, the collected fractions were subsequently analyzed in the MEKC second dimension (Figure 2B).

Deleted: and

Deleted: ; t

Deleted: 35 mmol

Deleted: mmol

Deleted: m

The UV detector signal of the CE instrument was processed using the export function of the CE Chemstation software. The exported ASCII data were converted into a matrix with the rows corresponding to the fraction cycle periods. The 2D chromatograms were plotted as the contour plots with the first dimension times on the x-axis and the second dimension times on the y-axis coordinates, respectively, using proprietary software developed for the evaluation of 2D LCxLC chromatograms [32]. The identification of the sample compounds was based on the comparison of the LC elution times and the CE migration times in the individual dimensions and the signal ratio at the four pre-set wavelengths (220, 254, 280 and 350 nm).

Deleted: three

Deleted: and

3 Results and discussion

3.1 Optimization of the MEKC separation of phenolic and flavone compounds.

In continuation of our earlier studies of the applications of two-dimensional LCxLC methods for separation of flavones and phenolic acids [26,29,32,47], we investigated possibilities of using electromigration separation instead of LC separation in the second dimension of a two-dimensional system. Most flavone compounds are not-ionized in background electrolytes commonly used in CZE separations. To obtain successful separation of these compounds, we used MEKC with sodium dodecyl sulphate additive (SDS). For this purpose, we first optimized the MEKC step for best separation of 25 selected representative standard compounds (Fig. 1). Based on the previously published results [14], we used 25 mmol/L borate background electrolyte with pH adjusted to 9.05 to obtain appropriate electroosmotic flow velocity. On the basis of the experimental migration times, t_{mi} , we calculated the MEKC retention factors, k_i , of sample compounds using the well-known Eq. (1):

$$k = \frac{t_{m,i} - t_{EOF}}{t_{EOF} \left(1 - \frac{t_{m,i}}{t_{MIC}} \right)} \quad (1)$$

Here, t_{EOF} is the migration time of the electroosmotic flow, measured as the migration time of thiourea and t_{MIC} is the migration time of the SDS micelles, measured using the Sudan III dye as the marker compound, which is completely retained in the SDS micellar pseudo-stationary phase. All sample compounds appear between t_{EOF} and t_{mic} . Table 1 shows the experimental t_{mi} of the standard compounds in various working electrolytes tested.

The mobility and hence the migration times and the retention factors of flavones and phenolic compounds in MEKC depend on their structures. Generally, the mobility increases

Deleted: s

1
2 231 with increasing number of –OH groups (myricetin, 6 < quercetin, morin, 5 < apigenin, 3 < 7-
3 232 hydroxyflavone, 1). Further, catechins with five hydroxy groups ((+)-catechin and (-)-
4 233 epicatechin) migrate faster than quercetin with the same number and position of hydroxy-
5 234 groups, but flavone structure (Fig. 3A). Retention of flavonoid glycosides is affected by the
6 235 position of glycosylation: hesperidin and naringin (glycosylated at the position 7) elute later
7 236 than corresponding aglycones hesperetin and naringenin, but rutin (glycosylated at the
8 237 position 3) migrates faster than quercetin. First, we investigated the effect of the SDS on the
9 238 separation in the concentration range 4.3 – 20.2 g/L. Generally, the migration times and the
10 239 resolution increase at higher concentrations of the SDS micelles in the background
11 240 electrolyte. This effect is stronger for the compounds with lower migration velocities. The
12 241 electrolytes with 10 g/L SDS provided the best compromise between the resolution of the
13 242 standards and the time of analysis, hence we used this concentration throughout this work
14 243 with various electrolytes (Fig. 3A).
15 244 Many flavone and phenolic acid standards could be separated by MEKC with 10 g/L SDS in
16 245 the background electrolyte, however some critical pairs could not be resolved in this system
17 246 and some standards were only partially separated (e.g., hesperetin/4-hydroxyphenylacetic
18 247 acid, syringic acid/myricetin, chlorogenic acid/vanilic acid/salicylic acid,
19 248 quercetin/morin/hesperidin, p-hydroxybenzoic acid/apigenin, 7-hydroxyflavone/biochanin A)
20 249 . To improve the resolution, we tested the effects of the organic solvents and various
21 250 substituted cyclodextrin additives (CD) to the background electrolyte. The addition of
22 251 methanol or acetonitrile up to 20% (v/v) did not improve the separation, but only increased
23 252 the time of analysis. The increase in the separation time was due to the effect of the organic
24 253 solvent on decreased experimental EOF mobility, which was $36.0 \times 10^{-8} \text{ m}^2 \text{V}^{-1} \text{ s}^{-1}$, in purely
25 254 aqueous electrolyte without an organic solvent additive; $23.8 \times 10^{-8} \text{ m}^2 \text{V}^{-1} \text{ s}^{-1}$ in the electrolyte
26 255 with 15% methanol and $27.9 \times 10^{-8} \text{ m}^2 \text{V}^{-1} \text{ s}^{-1}$ in the electrolyte with 15% acetonitrile.
27 256 In the background electrolytes with cyclodextrins, the partitioning equilibrium in
28 257 MEKC is affected by the formation of complexes of samples with cyclodextrins in the
29 258 aqueous phase, which compete for the analytes with the micellar pseudostationary phase. We
30 259 compared three non-charged cyclodextrins (methyl- β -cyclodextrin, heptakis(2,6-di-O-
31 260 methyl)- β -cyclodextrin, heptakis(2,3,6-tri-O-methyl)- β -cyclodextrin) and negatively charged
32 261 heptakis(6-O-sulfo)- β -cyclodextrin) additives to the background electrolyte. The neutral CDs
33 262 migrate along the capillary towards the detector (cathode) at the electroosmotic flow (EOF)
34 263 migration velocity, whereas the negatively charged CD moves in the same direction, but at

Deleted: in

Deleted: in

Deleted: 15

Deleted: 70

Deleted: mmol

Deleted: 35

Deleted: mmol

Deleted: 35

Deleted: mmol

Deleted: (e.g., hesperetin/4-hydroxyphenylacetic acid, myricetin/p-coumaric acid, hesperidin/p-hydroxybenzoic acid)

different migration velocity as the SDS micelles, i.e., against the direction of EOF. The resulting migration velocities are further contributed to by the own electrophoretic mobilities of phenolic compounds, which are more or less ionized in the running buffers at pH = 9. This applies mainly to phenolic acids, but even other compounds may be present in the form of anions. Taking into account all these complex equilibria, it is not possible to predict a-priori general effects of the CD additives on the migration times, separation selectivity and resolution, which strongly depend on the sample structures.

Table 1 shows the retention factors of the test standards in running buffers with 1.3 – 7.2 g/L cyclodextrins and 10 g/L SDS. The values and even the order of migration times of flavones and phenolic acids depend significantly on the type and concentration of cyclodextrin in the running buffer. Cyclodextrin additives affect also the bandwidths and the separation efficiency characterized by the number of theoretical plates per meter, N (Table 1). The methyl- β -cyclodextrin additive decreases the plate number by 20-25% with respect to the buffer without the CD additive. The other CD additives moderately decrease the separation efficiency only at the concentrations of 4 g/L or more.

To compare the performance of various running buffers, we selected the "critical pairs" of standard phenolic and flavone compounds with adjacent peaks, i and j , which are most difficult to resolve in various MEKC systems. For these "critical pairs" we determined the separation selectivity, $\alpha_{i,j}$, defined as the ratio of the corresponding retention factors, k_i (of earlier migrating compound i) and k_j (of later migrating compound j), respectively:

$$\alpha_{i,j} = \frac{k_j}{k_i} = \frac{t_{m,j} - t_{EOF}}{t_{m,i} - t_{EOF}} \cdot \frac{1 - \frac{t_{m,i}}{t_{MIC}}}{1 - \frac{t_{m,j}}{t_{MIC}}} \quad (2)$$

The separation selectivity $\alpha_{i,j} = 1$ means that the analytes i and j co-elute in a single peak; the higher is $\alpha_{i,j}$, the better is the separation selectivity and the resolution of analytes.

The separation selectivity for "critical pairs" of flavones and phenolic acids significantly depends on the type of cyclodextrin (Table 2). The number of "critical pairs" with resolution $R_{S,i,j} < 1$ significantly decreases in working buffers containing CD additives (10 in the buffer without CD, 9 in the buffer with methyl- β -cyclodextrin, 7 in the buffer with heptakis(2,6-di-O-methyl)- β -cyclodextrin and 4 in the buffer with heptakis(2,3,6-tri-O-methyl)- β -cyclodextrin). The improvement of separation selectivity and resolution is even

Deleted: 5

Deleted: mmol

Deleted: 35 mmol

Deleted: 3

Deleted: mmol

Deleted: standards of

1
2 294 better in the buffers containing negatively charged heptakis(6-O-sulfo)- β -cyclodextrin), where
3
4 295 there are only three "critical pairs" with $R_{S,i,j} < 1$. The concentration of the CDs also strongly
5 296 affects the selectivity of separation and the resolution. It should be noted that the change in
6
7 297 the type and concentration of the CD additive to the running buffer may cause change in the
8 298 order of migration times of "critical pairs". The reversed migration order is marked by asterisk
9
10 299 in Table 2.

11 300 Fig. 4 shows the plots of resolution of some critical pairs of flavones and phenolic
12 301 acids *versus* the concentration of heptakis(6-O-sulfo)- β -cyclodextrin in background
13 302 electrolyte. Most significant improvement in resolution was achieved in the background
14 303 electrolyte with 1.85 g/L anionic heptakis(6-O-sulfo)- β -cyclodextrin.

Deleted: mmol

17 304 The separation of 25 test compounds in this background electrolyte shown in Fig. 3B
18 305 is only slightly longer than in the buffer without CD (Fig. 3A), but the resolution of the pairs
19 306 of compounds 1/2, 11/21, 6/13 improves considerably and there are 20 resolved peaks in the
20 307 sample in comparison to 18 in the buffer without CD. The addition of 1.85 g/L heptakis(6-O-
21 308 sulfo)- β -cyclodextrin does not affect the efficiency (the number of theoretical plates, Table 1).
22 309 Hence, this optimized running buffer composition was applied for the separation of flavones
23 310 and phenolic acids in the two-dimensional LC-MEKC system.
24
25
26
27
28

Deleted: mmol

29 312 3.2 Correlations between the LC and MEKC methods

30 313 The best improvement of resolution and peak capacity in 2D systems is achieved in
31 314 so-called "orthogonal" systems, where the separation principles in both dimensions are
32 315 completely different. The degree of orthogonality of a two-dimensional separation system can
33 316 be described by the correlation coefficient of the retention (migration) times, or of the
34 317 retention factors in the two dimensions [49]. We compared the retention times of flavones and
35 318 phenolic acids on four HPLC columns with different stationary phases and dimensions with
36 319 the MEKC migration times in the running buffer with 1.85 g/L heptakis(6-O-sulfo)- β -
37 320 cyclodextrin (Table 3). Other MEKC separation systems showed similar correlations with the
38 321 HPLC separations and are not shown here. There are generally low correlations between the
39 322 MEKC and HPLC characteristics. The lowest correlations of the migration times for both
40 323 flavone compounds and phenolic acids was observed for the column 2 (Discovery HS PEG),
41 324 with a polyethyleneglycol bonded stationary phase, higher for the column 3 (Novapak
42 325 Phenyl) with a phenyl bonded stationary phase and for the columns 1 and 4 with C18 bonded
43 326 stationary phases, which can be possibly attributed to some similarities in the separation

Deleted: 43

Deleted: mmol

Deleted: slightly

Deleted: than

Deleted: Slightly higher correlation coefficients observed with the C18 columns

1
2 327 selectivity of non-charged compounds in the MEKC systems with SDS micelles in the
3 328 background electrolyte and the non-polar stationary phases in reversed-phase liquid
4 329 chromatography.

5 330 This trend in system correlations agrees with our previously published work[48] -
6 331 [50], where the combinations of a PEG column with a C18 column provide highly different
7 332 chromatographic selectivity and high degree of orthogonality in comprehensive two-
8 333 dimensional LCxLC. [The correlation coefficients between the retention data of flavone and](#)
9 334 [phenolic acid standards on a PEG column and on various C18 columns, including monolithic,](#)
10 335 [fully porous particle and superficially porous solid core particles, \$R^2 = 0.07 - 0.43\$, higher](#)
11 336 [than in the LC-MEKC two-dimensional setup described in the present work \(0.049, Table 3\),](#)
12 337 [which therefore shows higher orthogonality.](#) Moreover, a lower concentration of acetonitrile
13 338 in the mobile phase used for the separation of flavones and phenolic acids on the PEG column
14 339 in comparison to the C18 column is beneficial for the MEKC separation in the second
15 340 dimension, where the injection of sample dissolved in mobile phase with high concentration
16 341 of organic solvent can decrease the concentration of micelles and impair the resolution of
17 342 sample compounds.

27 344 3.3 Two-dimensional LC-MEKC separations

28 345 To match the column dimensions with the separation capillary in the MEKC system
29 346 and to minimize the dilution of the transferred fractions, a narrow-bore polyethyleneglycol
30 347 column was selected for the first dimension in the LC-MEKC separations (Discovery HS
31 348 PEG, 50x2.1 mm i.d., 5 μ m particles), with a gradient of acetonitrile in aqueous ammonium
32 349 acetate buffer. Fig. 5 shows the two-dimensional separation of the standard compounds (as a
33 350 contour plot) with the HPLC elution times on the X-axis and the MEKC migration times on
34 351 the Y-axis in the optimized 2D off-line separation system. All 25 standards are resolved and
35 352 there are some spots of minor impurities in the two-dimensional retention plane. The
36 353 estimated maximum theoretical peak capacity, based on the average experimental bandwidths
37 354 is approximately 450 in the present automated off-line 2D LC-MEKC system, in comparison
38 355 to 30 in the MEKC and 20 in the HPLC one-dimensional systems. The overall time necessary
39 356 for the subsequent MEKC analysis of all HPLC fractions is long, 10.5 hours, but the analysis
40 357 can run unattended overnight. [In the recently reported comprehensive LCxLC 2D method](#)
41 358 [with a PEG column in the first dimension and a porous-shell fused core Ascentis C18 column](#)
42 359 [in the second dimension \[32\], we could completely resolve 24 compounds of 27 phenolic](#)
43 360 [acids and flavone standards in 30 min total separation time. However, the second dimension](#)

Deleted: in the form of the

1
2 361 | peak capacity was only 12 and the total two-dimension peak capacity was cca 280, slightly
3 362 | more than half of the peak capacity in the present work. We are presently working on the
4 363 | reduction of the second dimension MEKC separation time. Fig. 6 shows an example of
5 364 | practical applications of the present two-dimensional LC-MEKC system for the separation of
6 365 | a sample of green tea extract.

Deleted: optimized

11 367 **4 Concluding remarks**

12 368 | MEKC separation of 25 flavone related compounds and phenolic acid standards was
13 369 | achieved in 15 min using an optimized MEKC system employing the background electrolyte
14 370 | containing 25 mmol/L borate buffer (pH 9.05) with the addition of 10 g/L SDS and 1.85 g/L
15 371 | heptakis(6-O-sulfo)- β -cyclodextrin. The presence of anionic cyclodextrin additive allows
16 372 | improving the resolution of the MEKC method. An automated off-line two-dimensional
17 373 | separation system was developed coupling a HPLC gradient separation on a bonded
18 374 | polyethylene glycol column in the first dimension and a MEKC separation in a borate buffer
19 375 | with SDS and cyclodextrin additives.

Deleted: 35

Deleted: mmol

Deleted: mmol

Deleted:

20 376 | The experimental setup developed in this work has several advantages over the
21 377 | comprehensive on-line real-time LC-MEKC 2D system. The off-line system does not require
22 378 | that the analysis of the collected fraction in the second dimension should be accomplished in
23 379 | the time of collection of the next fraction. Less stringent limitations of the second-dimension
24 380 | separation time considerably increase the available second-dimension peak capacity and the
25 381 | number of peaks that can be resolved during a single analysis run, of course at the cost of a
26 382 | longer total analysis time. Further, using an external fraction collector to store the fractions of
27 383 | the HPLC column effluent in vials, matching the lengths and inner diameters of the HPLC
28 384 | column and the MEKC capillary and the flow rates in both separation systems is not that
29 385 | critical as in the comprehensive setup. The whole 2D system is closed and fully automated, so
30 386 | that there is less risk of losing some sample during the manipulation with the collected
31 387 | fractions, which improves the reliability of the system. Last but not least, the analysis of the
32 388 | individual fractions in the second dimension can be repeated and (or) the on-line pre-
33 389 | concentration step of the sample in the capillary before the MEKC analysis can be employed
34 390 | to improve the sensitivity and the detection limits.

35 391 | Further research is in progress to investigate the possibilities of reducing the overall
36 392 | analysis time in a high peak capacity two-dimensional LC/MEKC system.

394 **Acknowledgements**

395 This work was supported by the Ministry of Education, Youth and Sports of the Czech
396 Republic, project No. MSM0021627502 and by the Czech Science Foundation, projects Nos.
397 203/09/P199 and 203/07/0641.

399 **5 References**

- 400 1. Di Carlo, G., Mascolo, N., Izzo, A. A., Capasso, F., *Life Sciences* 1999, 65, 337-353.
- 401 2. [Heim, K. E., Tagliaferro, A. R., Bobilya, D. J., *J. Nutr. Biochem.* 2002, 13, 572-584.](#)
- 402 3. Molnar-Perl, I., Fuzfai, Z., *J. Chromatogr. A* 2005, 1073, 201-227.
- 403 4. de Rijke, E., Out, P., Niessen, W. M. A., Ariese, F., Gooijer, C., Brinkman, U. A. Th.,
404 *J. Chromatogr. A* 2006, 1112, 31-63.
- 405 5. Stalikas, C. D., *J. Sep. Sci.* 2007, 30, 3268-3295.
- 406 6. Nelson, B. C., Thomas, J. B., Wise, S. A., Dalluge, J. J., *J. Microcol. Sep.* 1998, 10,
407 671-679.
- 408 7. Barroso, M. B., Van de Werken, G., *HRC J. High Resolut. Chromatogr.* 1999, 22,
409 225-230.
- 410 8. Bonoli, M., Pelillo, M., Toschi, T. G., Lercker, G., *Food Chemistry* 2003, 81, 631-
411 638.
- 412 9. Aucamp, J. P., Hara, Y., Apostolides, Z., *J. Chromatogr. A* 2000, 876, 235-242.
- 413 10. Horie, H., Kohata, K., *J. Chromatogr. A* 1998, 802, 219-223.
- 414 11. Sun, Y., Fang, N., Chen, D. D. Y., Donkor, K. K., *Food Chemistry* 2008, 106, 415-
415 420.
- 416 12. Rodriguez-Delgado, M. A., Perez, M. L., Corbella, R., Gonzales, G., Montelongo, F. J.
417 G., *J. Chromatogr. A* 2000, 871, 427-438.
- 418 13. Wang, S. P., Huang, K. J., *J. Chromatogr. A* 2004, 1032, 273-279.
- 419 14. Cortacero-Ramirez, S., Segura-Carretero, A., Cruces-Blanco, C., de Castro, M. H. B.,
420 Fernandez-Gutierrez, A., *Electrophoresis* 2004, 25, 1867-1871.
- 421 15. Ferreres, F., Blazquez, M. A., Gil, M. L., Tomas-Barberan, F. A., *J. Chromatogr. A*
422 1994, 669, 268-274.
- 423 16. Andrade, P., Ferreres, F., Gil, M. L., Tomas-Barberan, F. A., *Food Chem.* 1997, 60,
424 79-84.
- 425 17. Fu, S. H., Yang, M. H., Wen, H. M., Chern, J. C., *J. Food Drug Anal.* 2005, 13, 43-50.

Formatted: Italian (Italy)

- 1
2 426 18. Dadáková, E., Procházková, E., Křížek, M., *Electrophoresis* 2001, 22, 1573-1578.
3
4 427 19. Pietta, P. G., Mauri, P. L., *J. Chromatogr.* 1991, 549, 367-373.
5 428 20. Cheung, H. Y., Lai, W. P., Cheung, M. S., Leung, F. M., Hood, D. J., Fong, W. F., *J.*
6 429 *Chromatogr. A* 2003, 989, 303-310.
7
8 430 21. Zhu, J. H., Yu, K., Chen, X. G., Hu, Z. D., *J. Chromatogr. A* 2007, 1166, 191-200.
9
10 431 22. Cao, Y. H., Lou, C. G., Fang, Y. Z., Ye, J. N., *J. Chromatogr. A* 2002, 943, 153-157.
11 432 23. Liu, E. H., Qi, L. W., Cao, J., Li, P., Li, C. Y., Peng, Y. B., *Molecules* 2008, 13, 2521-
12 433 2544.
13
14 434 24. Watanabe, T., Terabe, S., *J. Chromatogr. A* 2000, 880, 311-322.
15 435 25. Dugo, P., Kumm, T., Cacciola, F., Dugo, G., Mondello, L., *J. Liq. Chromatogr.*
16 436 *Related Technol.* 2008, 31, 1758-1807.
17 437 26. Hájek, T., Škeřfíková, V., Česla, P., Vyňuchalová, K., Jandera, P., *J. Sep. Sci.* 2008,
18 438 31, 3309-3328.
19
20 439 27. Dugo, P., Cacciola, F., Herrero, M., Donato, P., Mondello, L., *J. Sep. Sci.* 2008, 31,
21 440 3297-3308.
22
23 441 28. [Hu, L., Chen, X., Kong, L., Su, X., Ye, M., Zou, H., *J. Chromatogr. A* 2005, 1092,](#)
24 442 [191-198.](#)
25
26 443 29. [Cacciola, F., Jandera, P., Hajdú, Z., Česla, P., Mondello, L., *J. Chromatogr. A* 2007,](#)
27 444 [1149, 73-87.](#)
28
29 445 30. [Pól, J., Hohnová, B., Jussila, M., Hyötyläinen, T., *J. Chromatogr. A* 2006, 1130, 64-](#)
30 446 [71.](#)
31
32 447 31. [Larmann, J. P., Lemmo, A. V., Moore, A. W., Jorgenson, J. W., *Electrophoresis* 1993,](#)
33 448 [14, 439-447.](#)
34
35 449 32. [Česla, P., Hájek, T., Jandera, P., *J. Chromatogr. A* 2009, 1216, 3443-3457.](#)
36 450 33. [Michels, D. A., Hu, S., Schoenherr, R. M., Eggertson, M. J., Dovichi, N. J., *Moll. Cell.*](#)
37 451 [Proteomics 2002, 1, 69-74.
38
39 452 34. \[Kraly, J. R., Jones, M. R., Gomez, D. G., Dickerson, J. A., Harwood, M. M.,\]\(#\)
40 453 \[Eggertson, M., Paulson, T. G., Sanchez, C. A., Odze, R., Feng, Z., Reid, B. J.,\]\(#\)
41 454 \[Dovichi, N. J., *Anal. Chem.* 2006, 78, 5977-5986.\]\(#\)
42
43 455 35. Bushy, M. M., Jorgenson, J. W., *Anal. Chem.* 1990, 62, 978-984.
44
45 456 36. Moore, A. W., Jorgenson, J. W., *Anal. Chem.* 1995, 67, 3456-3463.
46
47 457 37. Lewis, K. C., Opiteck, G. J., Jorgenson, J. W., Sheeley, D. M., *J. Am. Soc. Mass*
48 458 *Spectrom.* 1997, 8, 495-500.
49
50
51
52
53
54
55
56
57
58
59
60](#)

- 1
2 459 38. Issaq, H. J., Chan, K. C., Janini, G. M., Muschik, G. M., *Electrophoresis* 1999, 20,
3 460 1533-1537.
4
5 461 39. Mikulikova, K., Eckhardt, A., Miksik, I., *J. Sep. Sci.* 2006, 29, 1126-1131.
6
7 462 40. [Yang, X., Zhang, X., Li, A., Zhu, S., Huang, Y., *Electrophoresis* 2003, 24, 1451-1457.](#)
8 463 41. [Michels, D. A., Hu, S., Dambrowitz, K. A., Eggertson, M. J., Lauterbach, K., Dovichi,](#)
9 464 [N. J., *Electrophoresis* 2004, 25, 3098-3105.](#)
10 465 42. [Janini, G. M., Chan, K. C., Conrads, T. P., Issaq, H. J., Veenstra, T. D.,](#)
11 466 [Electrophoresis 2004, 25, 1973-1980.](#)
12
13 467 43. [Evans, Ch. R., Jorgenson, J. W., *Anal. Bioanal. Chem.* 2004, 378, 1952-1961.](#)
14 468 44. [Tempels, F. W. A., Wiese, G., Underberg, W. J. M., Somsen, G. W., de Jong, G. J., *J.*](#)
15 469 [Chromatogr. B 2006, 839, 30-35.](#)
16
17 470 45. Garcia-Villalba, R., Carrasco-Pancorbo, A., Vazquez-Martin, A., Oliveras-Ferraro,
18 471 C., Menendez, J. A., Segura-Carretero, A., Fernandez-Gutierrez, A., *Electrophoresis*
19 472 2009, 30, 2688-2701.
20
21 473 46. Zhang, X., Hu, H., Xu, S., Yang, X., Zhang, J., *J. Sep. Sci.* 2001, 24, 385-391.
22
23 474 47. Blahová, E., Jandera, P., Cacciola, F., Mondello, L., *J. Sep. Sci.* 2006, 29, 555-566.
24
25 475 48. [Cacciola, F., Jandera, P., Blahová, E., Mondello, L., *J. Sep. Sci.* 2006, 29, 2500-2513.](#)
26
27 476 49. Jandera, P., Česla, P., Hájek, T., Vohralík, G., Vyňuchalová, K., *J. Chromatogr. A*
28 477 2008, 1189, 207-220.
29
30 478 50. [Jandera, P., Vyňuchalová, K., Hájek, T., Česla, P., Vohralík, G., *J. Chemometrics*](#)
31 479 [2008, 22, 203-217.](#)

1
2 480 **Figure captions**

3 481

4
5 482 **Figure 1**

6 483 Structures of flavone compound and phenolic acid standards.

7 484

8
9 485 **Figure 2**

10 486 Experimental setup used in the automated off-line LC-MEKC two-dimensional method.

11 487 LC separation and fraction collecting step (A), MEKC analysis of collected fractions (B). 1 –

12 488 HPLC separation column, 2 – switching valve, 3 – fused silica capillary, 50 μm i.d., 200 μm

13 489 o.d., distance approx. 1 mm between the end of the capillary and the cap of the vial, 4 – CE

14 490 autosampler, vials with 100 μL glass insert and with adapted caps with 4 mm hole, 5 – CE

15 491 separation capillary, 6 – background electrolyte vials, 7 – sample inject vial.

16 492

17 493 **Figure 3**

18 494 MEKC separations of 25 flavones and phenolic acids in background electrolyte containing 10
19 495 g/L SDS (A) and with addition of 1.85 g/L heptakis(6-O-sulfo)- β -cyclodextrin (B).

20 496 Capillary 48 cm (40 cm to the detector) x 25 μm I.D. with bubble cell extended to 125 μm

21 497 optical path length. Background electrolyte: 25 mmol/L borate pH 9.05 containing 10 g/L

22 498 SDS; applied potential 20 kV, overpressure injection at 30 mBar/10 s, detection: UV, 280 nm,

23 499 25°C. The numbers of compounds are as in Figure 1.

24 500

25 501 **Figure 4**

26 502 The effect of heptakis(6-O-sulfo)- β -cyclodextrin concentration, c_{CD} , in background
27 503 electrolyte on resolution, R_s , of some critical pairs of flavones.

28 504 Capillary, background electrolyte and other separation conditions as in Figure 3, except for 0

29 505 – 5.6 g/L of heptakis(6-O-sulfo)- β -cyclodextrin, 20 kV. The numbers of compounds are as in

30 506 Figure 1.

31 507

32 508 **Figure 5**

33 509 2-D separation of 25 flavone and phenolic acid standards. The numbers of compounds are as

34 510 in Figure 1. See text for experimental conditions.

35 511

36 512 **Figure 6**

37 513

38 514

39 515

40 516

41 517

42 518

43 519

44 520

45 521

46 522

47 523

48 524

49 525

Deleted: 35

Deleted: mmol

Deleted: mmol

Deleted: 35

Deleted: mmol

Deleted: 2

Deleted: 3

Deleted: mmol

1
2 513 | 2-D separation of a green tea sample. [The numbers of compounds are as in Figure 1.](#)
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

ELECTROPHORESIS

Retention factors, k , calculated using Eq. (1), electroosmotic flow time, t_{EOF} , migration time of micelles, t_{MIC} , and efficiencies of the separation of flavones and phenolic acids in the 25 mmol/L borate background electrolyte pH 9.05 with 10 g/L SDS and addition of various substituted cyclodextrins.

Compound		Without CD	methyl- β -CD			heptakis(6-di-O-methyl)- β -CD			heptakis(6-tri-O-methyl)- β -CD			heptakis(6-O-sulfo)- β -CD	
Cyclodextrin concentration [g/L]		0	1.31	3.93	6.6	1.33	4.00	6.66	1.43	4.29	7.15	1.85	5.55
t_{EOF} [min]		4.96	5.10	5.19	5.30	5.22	5.29	5.37	5.20	5.26	5.35	5.12	5.54
t_{MIC} [min]		15.29	15.25	15.21	15.26	16.09	16.16	16.15	15.69	15.71	15.55	18.08	20.19
1	(-)-Epicatechin	3.99	4.23	4.41	4.64	4.53	4.69	4.90	4.60	4.80	5.13	4.15	5.57
2	(+)-Catechin	4.29	4.54	4.73	4.93	4.86	5.05	5.27	4.95	5.19	5.57	5.15	6.34
7	Rutin	5.30	5.80	6.10	6.44	6.22	6.51	6.76	6.39	6.70	7.14	5.76	9.00
10	Naringenin	6.22	6.70	6.88	6.98	7.29	7.57	7.47	7.32	7.16	7.68	6.35	8.77
11	Hesperetin	6.85	7.22	6.88	6.72	7.83	7.57	7.73	7.77	7.34	7.14	7.11	11.06
21	4-Hydroxyphenylacetic acid	6.85	7.59	8.12	8.72	8.18	8.67	9.18	8.43	8.98	9.85	6.68	8.58
24	Ferulic acid	8.02	8.64	9.22	9.94	9.33	9.86	10.46	9.58	10.22	11.22	7.76	9.60
20	Syringic acid	8.46	8.64	9.22	9.94	9.33	9.86	10.46	9.58	10.22	11.22	7.76	9.60
14	Myricetin	8.46	9.48	10.22	10.81	10.23	11.20	11.39	10.72	11.42	12.36	12.42	16.21
23	p-Coumaric acid	9.58	10.41	12.28	12.21	11.27	13.16	12.83	11.60	12.48	14.27	9.26	12.57
25	Chlorogenic acid	10.43	8.64	10.22	10.81	10.23	11.20	11.39	10.58	11.26	12.36	8.06	10.39
19	Vanilic acid	10.43	10.41	12.28	13.95	12.33	13.16	14.09	12.70	13.71	15.45	9.95	12.57
18	Salicylic acid	10.43	11.82	12.87	13.95	12.78	13.81	15.14	13.41	14.44	16.31	9.95	13.46
6	Quercetin	13.12	14.50	16.76	16.99	14.05	13.81	11.39	13.41	16.51	13.81	14.89	11.06
13	Morin	13.12	17.94	17.48	16.99	22.74	23.62	25.39	22.72	24.95	29.02	15.54	16.21
12	Hesperidin	13.12	14.50	16.42	14.59	16.72	17.16	12.83	17.47	14.44	16.31	17.79	21.50
17	p-Hydroxybenzoic acid	15.95	14.50	17.48	17.73	15.81	19.11	18.84	16.39	17.85	21.48	12.06	17.07
5	Apigenin	15.95	20.33	22.07	23.22	20.66	19.11	19.48	20.72	17.85	20.42	16.15	16.21
4	7-Hydroxyflavone	17.26	12.69	10.22	8.72	14.05	12.00	9.18	13.41	9.45	9.85	12.42	17.07
9	Biochanin A	17.26	17.94	16.42	12.21	20.05	20.03	19.48	20.72	24.95	29.02	16.15	27.71
8	Naringin	18.99	17.94	16.42	14.59	21.50	17.90	15.51	22.72	16.51	13.81	15.54	27.71
22	Caffeic acid	25.57	34.18	36.19	47.57	36.82	46.92	49.55	40.02	45.81	62.57	22.03	33.08
15	Gallic acid	32.10	45.00	58.30	76.40	59.44	57.26	78.46	63.82	76.47	107.92	29.69	49.06
16	Protocatechuic acid	52.68	77.95	106.28	179.83	96.12	113.83	180.66	112.56	160.52	499.58	39.77	78.79
3	Flavone	84.97	121.71	106.28	97.90	180.65	152.06	118.93	220.57	160.52	123.38	64.50	1408.6
Average number of theoretical plates per meter of capillary		196 000	153 000	147 000	160 000	190 000	170 000	136 000	202 000	186 000	172 000	191 000	151 000

Table 2

Separation selectivities, $\alpha_{i,j}$, calculated according to the Eq. (2) and resolution, $Rs_{i,j}$, of the critical pairs of flavones and phenolic acids in 25 mmol/L borate background electrolyte pH 9.05 with 10 g/L SDS and addition of various cyclodextrins.

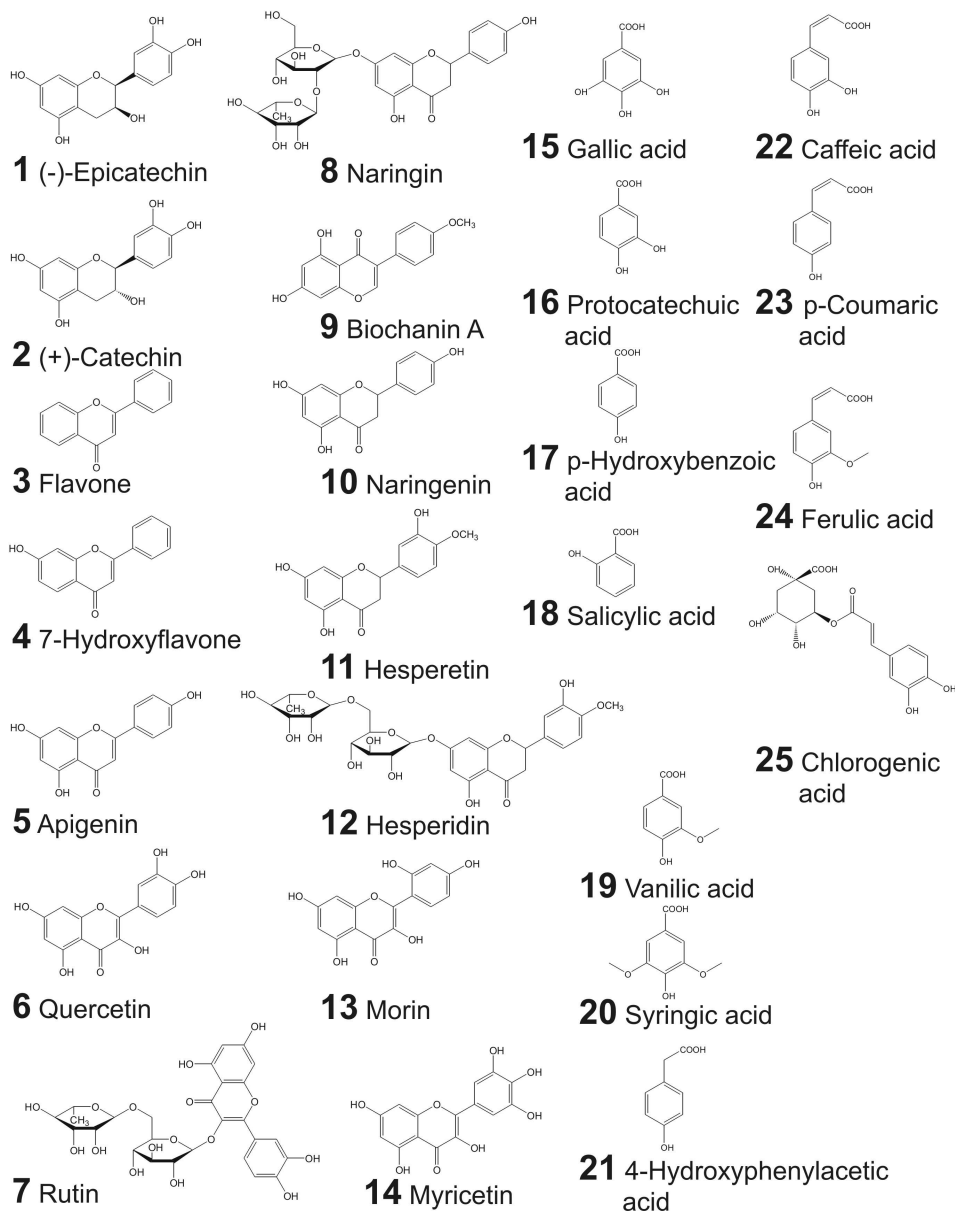
Critical pairs i,j	Without CD		3.93 g/L methyl- β -CD		4.0 g/L heptakis(6-di-O-methyl)- β -CD		4.29 g/L heptakis(6-tri-O-methyl)- β -CD		1.85 g/L heptakis(6-O-sulfo)- β -CD	
	$\alpha_{i,j}$	$Rs_{i,j}$	$\alpha_{i,j}$	$Rs_{i,j}$	$\alpha_{i,j}$	$Rs_{i,j}$	$\alpha_{i,j}$	$Rs_{i,j}$	$\alpha_{i,j}$	$Rs_{i,j}$
4/9	1	0	1.61	5.19	1.67	10.63	2.64	21.38	1.30	5.22
5/9	1.08	1.98	*1.34	5.90	1.05	0.92	1.40	4.72	1	0
6/12	1	0	*1.02	0.41	1.24	3.16	*1.14	2.36	1.20	4.63
6/13	1	0	1.04	0.83	1.71	8.48	1.51	8.84	1.04	1.08
8/9	*1.10	2.41	1	0	1.12	1.43	1.51	8.84	1.04	1.13
10/11	1.10	2.69	1	0	1	0	1.03	0.71	1.12	3.65
11/21	1	0	1.18	4.14	1.15	3.81	1.22	3.68	*1.06	1.93
12/13	1	0	1.07	1.24	1.38	5.42	1.73	7.98	*1.15	3.58
13/17	1.22	2.73	1	0	*1.24	3.19	*1.40	4.72	1.29	6.57
14/25	1.23	4.45	1	0	1	0	* 1.01	0.49	*1.54	7.25
17/5	1	0	1.26	4.34	1	0	1	0	1.34	8.67
18/25	1	0	*1.26	2.20	*1.23	1.92	*1.28	3.61	1.23	5.00
19/18	1	0	1.05	0.83	1.05	0.97	1.05	1.05	1	0
19/25	1	0	*1.20	1.91	*1.17	1.61	*1.22	3.34	*1.23	5.00
20/14	1	0	1.11	1.12	1.14	1.34	1.12	2.86	1.60	7.55
20/24	1.06*	1.04	1	0	1	0	1	0	1	0
23/19	1.09	2.19	1	0	1	0	1.10	1.96	1.08	2.03

* - reversed migration order of the i,j compounds in critical pair

1 **Table 3.**2 Correlation coefficients, R^2 , between the LC retention times and the MEKC migration times of flavones, related compounds and phenolic acids.

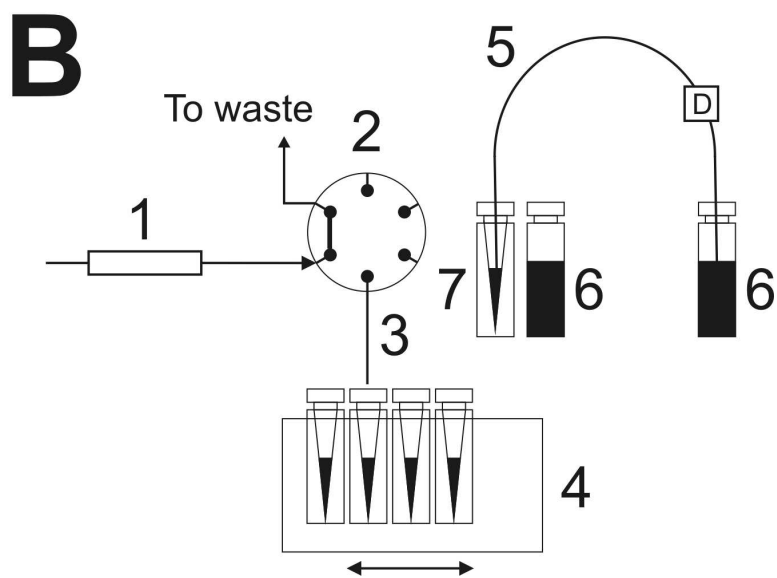
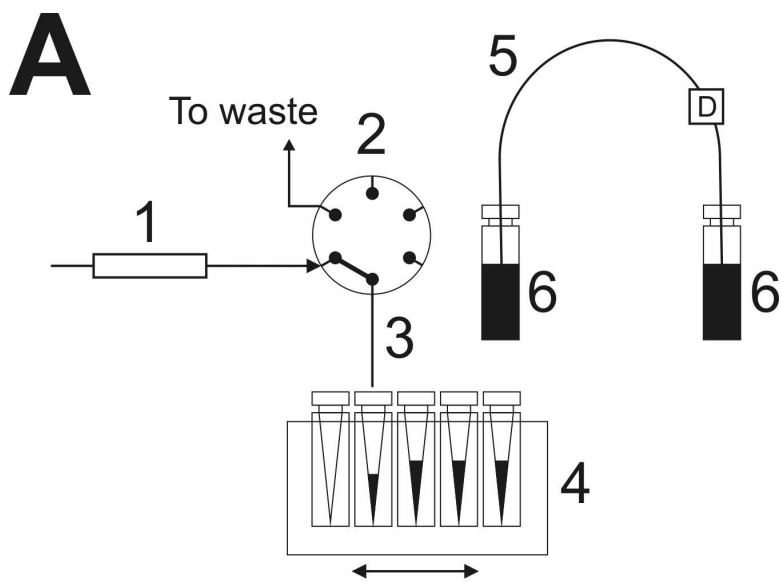
3 For LC separation conditions see the references [26,29,32,49]; MEKC conditions as in Figure 3B.

LC column	LC mobile phase	Without CD			1.85 g/L heptakis-(6-O-sulfo)- β -CD			LC conditions
		Flavones	Phenolic acids	All compounds	Flavones	Phenolic acids	All compounds	
1 Ascentis Express C18, (Supelco, Bellefonte, PA, USA), 30 x 3 mm, 3 μ m	15% (v/v) ACN / water, 0.5 ml/min, 40°C	0.564	0.232	0.196	0.425	0.188	0.189	[26,32]
2 Discovery HS PEG, (Merck, Darmstadt, Germany), 50 x 2.1 mm, 5 μ m	10% (v/v) ACN / water, 0.4 ml/min, 40°C	0.162	0.049	0.049	0.153	0.037	0.047	[29]
3 Novapak Phenyl, (Waters, Milford, MA, USA), 50 x 3.9 mm, 5 μ m	15% (v/v) ACN / water, 1 ml/min, 40°C	0.417	0.002	0.155	0.332	0.001	0.140	[29,49]
4 Purospher STAR, (Merck, Darmstadt, Germany), 125 x 4 mm, 5 μ m	30% (v/v) ACN / water, 1 ml/min, 40°C	0.506	0.045	0.200	0.480	0.034	0.178	[49]

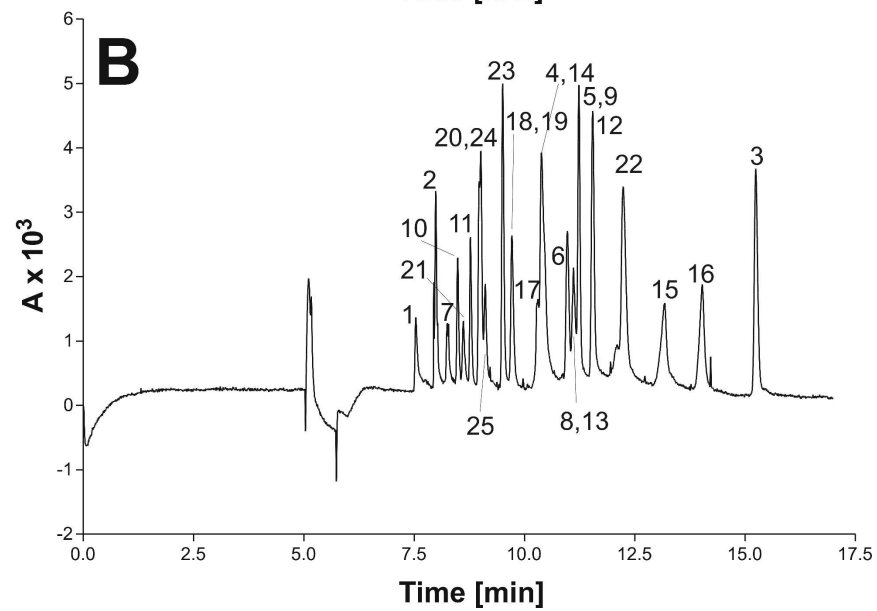
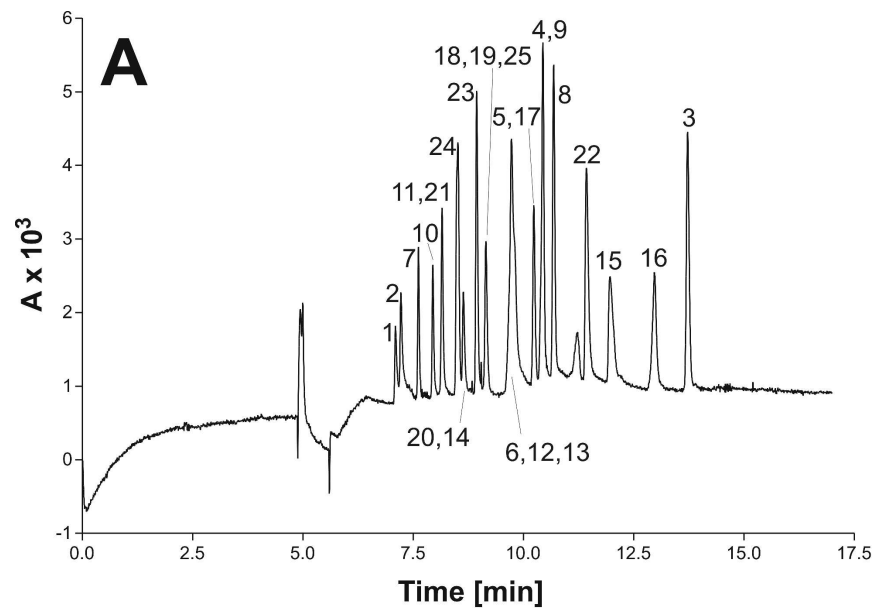


231x291mm (300 x 300 DPI)

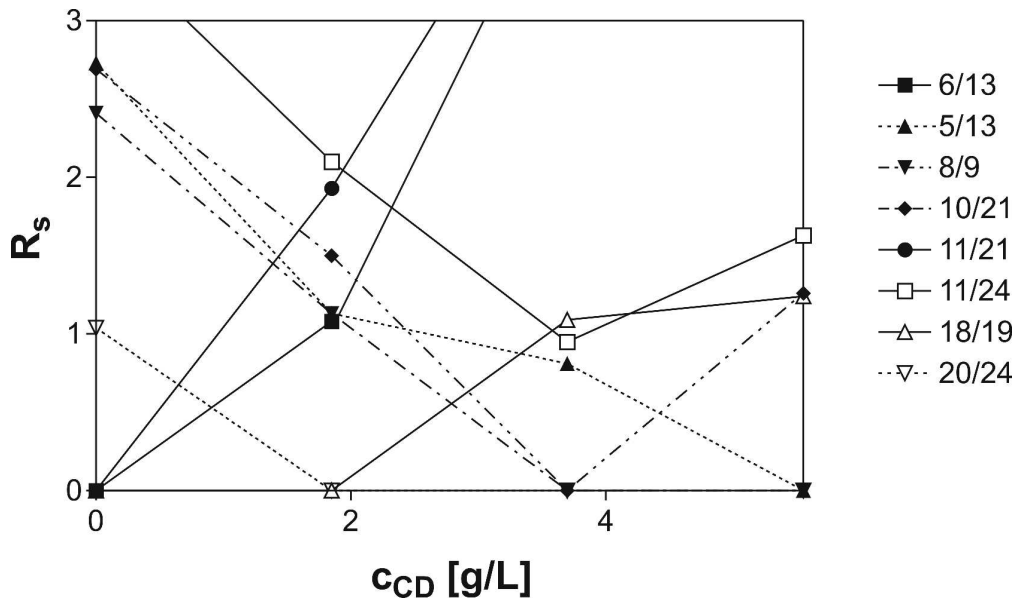
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



114x177mm (300 x 300 DPI)



166x232mm (600 x 600 DPI)



141x83mm (600 x 600 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



1165x1138mm (72 x 72 DPI)



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



1161x1144mm (72 x 72 DPI)

