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Automated dual two-dimensional liquid chromatography approach for fast acquisition of three-dimensional data using combinations of zwitterionic polymethacrylate and silica-based monolithic columns

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ABSTRACT

A monolithic sulfobetaine polymethacrylate micro-column BIGDMA-MEDSA designed in our laboratory, shows dual retention mechanism: In acetonitrile-rich mobile phase, hydrophilic interactions control the retention (HILIC system), whereas in more aqueous mobile phases the column shows essentially reversed-phase behavior with major role of hydrophobic interactions. The zwitterionic polymethacrylate micro-column can be used in the first dimension of two-dimensional LC in alternating reversed-phase (RP) and HILIC modes, coupled with an alkyl-bonded core-shell or silica-based monolithic column in the second dimension, for HILIC × RP and RP × RP comprehensive two-dimensional separations. During the HILIC × RP period, a gradient of decreasing acetonitrile gradient is used for separation in the first dimension, so that at the end of the gradient the polymeric monolithic micro-column is equilibrated with a highly aqueous mobile phase and is ready for repeated sample injection, this time for separation under reversed-phase gradient conditions with increasing concentration of acetonitrile in the first dimension. The fast repeating reversed-phase gradients on a short silica-monolithic or core-shell column in the second dimension can be optimized independently of the actual running first-dimension gradient program. As the alternating HILIC and RP separations on the first-dimension zwitterionic methacrylate column are based on complementary retention mechanisms, the instrumental setup essentially represents two coupled two-dimensional systems. It is first time that such an automated dual LCxLC approach is reported. The novel system allows obtaining three-dimensional data in a relatively short time and can be applied not only to multidimensional gradient separations of flavones and related polyphenolic compounds.

1. Introduction

Usually, samples containing up to a few tens of compounds are separated on a single HPLC column in one-dimensional liquid chromatography [1]. A system consisting of several columns can be regarded as a multi-dimensional, when the mechanism of the separation in each dimension is different [2,3]. A two-dimensional LC system consisting of two columns connected on-line via a regularly operated multiport switching valve enables collecting small-volume fractions from the first-dimension column for immediate subsequent analysis on a short column in the second dimension, where the separation should be accomplished while the next fraction from the first dimension is collected, often in less than 1 min. In this way, the whole sample is analyzed in the two dimensions [4]. The comprehensive approach has become popular for separations of medium-complexity samples, providing theoretical 2D peak capacities up to 1500 peaks in about 30–120 min, but still this is not enough for many complex samples. The straightforward solution seems to be adding another dimension to the separation system [5], resorting to spatial multidimensional techniques [2,6].

The crucial point affecting the separation time in comprehensive two-dimensional liquid chromatography highly efficient fast chromatographic separation in the second dimension, which can be accomplished on short columns packed with sub-2 μm particles, at the cost of very high operation pressure and special UHPLC equipment [7], or with conventional liquid chromatographic instrumentation and core-shell or silica monolithic columns, showing better permeability [8–15].

In reversed-phase LC, polar, weakly acidic or basic samples are often not sufficiently retained for successful separation. This problem can often be alleviated using aqueous normal-phase chromatography, which employs a polar stationary phase in com-

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bination with a mobile phase, containing more than 50% organic solvent in water—hydrophilic interaction liquid chromatography (HILIC) [16]. Various columns can be used in the HILIC mode for separations of peptides, proteins, oligosaccharides, drugs, metabolites and various natural compounds [17].

Because of the complementary effects of sample polarity on the retention, combinations of aqueous normal-phase (HILIC) and reversed-phase (RP) systems in 2D systems principally provide highly orthogonal 2D systems with largely different selectivities in the two dimensions, but RP systems provide largely different elution strengths, which may cause compatibility problems when coupling on-line the RP and HILIC separation modes [18].

On some polar chemically bonded phases, dual retention mechanism was observed for polar compounds, with a retention minimum at the "U turn" composition of the aqueous-organic mobile phase, corresponding to the transition from the RP (at high water concentrations) to the HILIC mechanism (at high organic solvent concentrations) [19,20].

Recently stable and reproducible zwitterionic polymethacrylate monolithic columns were prepared in capillary format with efficiencies up to 70 000 theoretical plates/m, using (*N*,*N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl) ammonium betaine – (MEDSA) monomer cross-linked with bisphenol A glycerolate dimethacrylate – (BIGDMA) [21]. The BIGDMA-MEDSA columns contain well solvated pores, well accessible in aqueous-organic mobile phases, which provide dual retention mechanism, HILIC at high concentrations of acetonitrile and reversed-phase (RP) in water rich mobile phases [21]. The BIGDMA-MEDSA monolithic columns were used in the first dimension of 2D HILIC × RP separations of polyphenolic compounds [22], combined with short core-shell or monolithic silica columns in the second dimension, in independent off-line two-dimensional RP × RP and HILIC × RP runs [8].

In the present work, we investigated possibilities of combining two-dimensional systems including a single zwitterionic polymethacrylate BIGDMA-MEDSA monolithic column operated both in the HILIC and in the RP modes, in combination with silica monolithic or core-shell alkyl bonded short columns.

2. Experimental

2.1. Materials

Acetonitrile, LiChrosolv grade, was purchased from Merck (Darmstadt, Germany). Water was purified using a SG Ultra Clear UV water purification system (SG, Hamburg, Germany). Ammonium acetate and formic acid (≥98%) were purchased from Sigma-Aldrich (St. Louis, MI, USA). Buffered mobile phases containing 10 mM CH_3COONH_4 were prepared by dissolving the appropriate weighed mass of ammonium acetate in water (A) and in acetonitrile (B), before adjusting the pH to 3.1 by adding a few drops of formic acid. The mobile phases were filtered using a Millipore (Bedford, MA, USA) 0.45 μm filter and degassed by ultrasonication.

Phenolic acids and flavonoid compounds (Table 1) were obtained from Sigma-Aldrich (St. Louis, MI, USA). The stock solutions were prepared by dissolving phenolic acids and flavonoid standards (100 mg/L) in 1:1 aqueous methanol. The working standard solutions were obtained by dilution with the mobile phase.

The monolithic zwitterionic polymethacrylate 0.53 mm, i.d. BIGDMA–MEDSA micro-column used in the first dimension either in HILIC or in RP mode was fabricated in laboratory as described previously [22] and combined with any of five short commercial core-shell or silica-based monolithic columns in multidimensional LC setups (Table 2): three commercial silica-based monolithic columns with octadecyl stationary phases: Chromolith Flash RP-18e 25 × 4.6 mm i.d. column (V_0 = 0.383 mL), Chromolith Fast-Gradient RP-18e 50 × 3.0 mm i.d (V_0 = 0.321 mL) and Chromolith HighResolution RP-18e 50 × 4.6 mm i.d. column (V_0 = 0.760 mL), all from Merck, Darmstadt, Germany; and two core-shell columns with octadecyl stationary phases: Kinetex 2.6 μm XB-C18 100 Å 30 × 3.0 mm i.d (V_0 = 0.148 mL) and 50 × 3.0 mm i.d. (V_0 = 0.218 mL) from Phenomenex, Torrance, CA, USA. (V_0 are the column hold-up volumes).

2.2. Equipment

An Agilent 1290 Infinity 2D-LC Solution liquid chromatograph (Agilent, Palo Alto, CA, USA) equipped with a micro-flow binary pump, a binary pump, a degasser, an auto-sampler, a diode-array UV detector and a thermostatted column compartment was used in the comprehensive 2D LC × LC setup. The first- and second-dimension columns were connected by an electronically controlled high-pressure 2-position/4-port-duo valve (Agilent, Palo Alto, CA, USA) equipped with two identical 10 μL sampling loops, as the fraction transfer interface.

2.3. Methods

Table 2 presents the overview of the two-dimensional setups of core-shell and monolithic C_{18} columns used in the second dimension, in combination with the BIGDMA-MEDSA micro-column used in the first dimension. In the comprehensive LC × LC experiments, the whole effluent from the first-dimension micro-column was transferred in-line to the second-dimension column in subsequent fractions collected alternately in two loops (10 μL) using a 2-position/4-port-duo valve interface between the first- and the second-dimension columns. The collecting loops were only partially filled and the exact volume of the collected fractions was set by the switching valve cycle time, to match the desired fraction volume at the pre-set second-dimension gradient time (0.7 – 1.0 min + 0.5 min column re-equilibration time). The flow rate in the first dimension was set to 3–5 μL/min and the flow rate in the second dimension was set at 3–4.5 mL/min, close to the column backpressure limit recommended by the manufacturer (60 MPa for the core-shell columns, or 20 MPa for the Chromolith colums).

The main novelty of this technique was that after the end of the HILIC × RP 2D run with decreasing (RP) gradient of acetonitrile, the experiment was not immediately stopped and the starting conditions reset, but the mobile phase flow through the BIGDMA-MEDSA column continued for a short period (e.g., 5 min), until sufficiently low concentration of acetonitrile (e.g., 15%) is accomplished, suitable for the next repeated sample injection, without interrupting the experiment, but this time starting elution with a gradient of increasing acetonitrile concentration, until an original acetonitrile concentration in the mobile phase is achieved (e.g., 98%) and the MD system is again ready to repeat the whole serial experiment (Fig. 1).

The column temperatures were set at 50 °C. The signal of the diode-array UV detector was monitored in the effluent from the second column at 254, 280, 300, 320, and 340 nm; full UV spectra were recorded for peak identification.

The detector signal was exported in the ASCII format using the LC ChemStation software. The ASCII data were converted into a matrix with rows corresponding to the fraction cycle periods, using a proprietary synchronization program. The 2-D chromatograms were plotted as the contour plots with the coordinates calibrated in the first-dimension elution times on the x-axis and the second-dimension elution times on the y-axis (with the time scale range