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Comprehensive two-dimensional liquid chromatography: Ion chromatography × reversed-phase liquid chromatography for separation of low-molar-mass organic acids

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ABSTRACT

In the work presented here a novel approach to comprehensive two-dimensional liquid chromatography is evaluated. Ion chromatography is chosen for the first-dimension separation and reversed-phase liquid chromatography is chosen for the second-dimension separation mode. The coupling of these modes is made possible by neutralising the first-dimension effluent, containing KOH, prior to transfer to the second-dimension reversed-phase column. A test mixture of 24 low-molar-mass organic acids is used for optimisation of the system. Three food and beverage samples were analysed in order to evaluate the developed methodology, the resulting two-dimensional separation is near-orthogonal, the set-up is simple and all instrumental components are available commercially. The method proved to be robust and suitable for the analysis of wine, orange juice and yogurt.

1. Introduction

Comprehensive two-dimensional liquid chromatography (LC × LC) has found its way into many application areas where very complex mixtures need to be separated. Examples of such areas include food analysis [1–5], environmental analysis [1], separation of biological samples, such as peptides [6,7] and proteins [8], pharmaceutical analysis [9], traditional Chinese medicines [10,11], synthetic polymers [12–14], and many more [1].

In LC × LC every part of the sample is subjected to two different separations. Ideally the separation mechanisms in each of these separation dimensions should be completely independent ('orthogonal'). Maximising differences in the separation mechanisms generally leads to more of the available two-dimensional separation space being occupied by peaks. Practical considerations can stand in the way of coupling completely independent separation mechanisms and quite often a truly orthogonal system is not employed [15]. Several approaches to LC × LC have been described in the literature, including reversed-phase liquid chromatography (RPLC) coupled to size-exclusion chromatography (SEC) [12,16], RPLC to RPLC [2,4,7,17,18], ion chromatography (IC) to SEC [14], ion-exchange chromatography with a strong cation-exchange column (SCX) to RPLC [8], normal-phase liquid chromatography (NPLC) to RPLC [19,20], or IC to IC [21].

None of the combinations are likely to offer true orthogonality in the mathematical sense, but in some cases orthogonality can be closely approached. For instance, orthogonality is closely approximated for peptide analysis using SCX × RPLC or with $RPLC_{pH=2.6} \times RPLC_{pH=10}$. In the latter case different pH values (*e.g.* 2.6 and 10) are chosen for the different dimensions and the differences in separation are strongly analyte dependent [15]. The combination of hydrophilic interaction chromatography (HILIC) and RPLC may provide a high degree of orthogonality. However, compatibility of the effluent from the first-dimension separation column with the second-dimension separation system may be problematic [15]. A number of papers describing theoretical and practical considerations for comprehensive two-dimensional liquid chromatography have been published [1,22–25].

Low-molar-mass organic acids (LMMOAs) are found in food and beverages where they contribute to the flavour, colour and aroma [26,27]. Thus LMMOAs can be used as indicators of product quality. Methods such as RPLC [26,28–30], IC [26], capillary electrophoresis [26,27] and gas chromatography [26] have been utilised for the analysis of LMMOAs. However, for complex matrices one-dimensional separations are not always able to fully resolve all the compounds. In the present study ion chromatography was coupled to reversed-phase liquid chromatography to exploit the differences in separation mechanism for the two-dimensional analysis of LMMOAs in wine, orange juice and yogurt.

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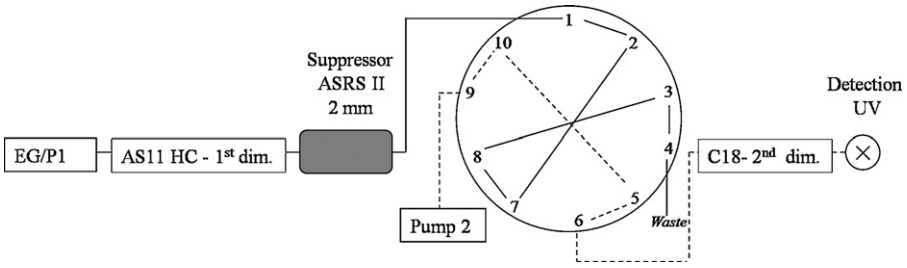

Fig. 1. Schematic of the instrument set-up used for IC × RPLC.

IC and RPLC are governed by very different separation mechanisms. IC is chosen as the first-dimension separation mode. The separation is achieved by employing a gradient of increasing hydroxide concentration. Upon completion of the first-dimension separation, the pH of the first-dimension eluent reaches 12.7. This causes a compatibility issue, since the RPLC second-dimension column is not suited to operation beyond pH 8. To overcome this problem and to allow transfer of fractions of the first-dimension effluent to the second-dimension RPLC column without destroying the C18 stationary phase, the effluent is neutralised using a membrane suppressor. This device provides electrolytically regenerated suppression of the hydroxide eluent by converting the eluent to essentially pure water. This has an important secondary advantage under the low-pH conditions chosen for the second dimension – namely the analytes concentrate in a narrow band at the head of the RPLC column. In this way, any band broadening that is incurred by the suppressor – or by the relatively large injection volume (200 μL) in the second dimension – can be effectively negated before the second-dimension separation commences. In comparison with LC × LC systems, in which this focussing effect on the top of the second-dimension column does not exist [22], we have the significant added advantage of a relatively large internal diameter (and flow rate) for the first-dimension column. The second-dimension column does not need to be much wider than the first-dimension column, which allows the sensitivity of the LC × LC system to be greatly increased.

2. Experimental

2.1. Instrumentation

A schematic of the instrument setup is provided in Fig. 1. A Dionex ICS-3000 ion chromatograph (Sunnyvale, CA, USA) was used throughout this work. Instrument control and data acquisition were performed using Chromeleon® software (Dionex). The first-dimension separation was performed on a Dionex IonPac AS11-HC column (250 mm × 2 mm I.D.) with an IonPac AG11-HC (50 mm × 2 mm I.D.) guard column. The hydroxide eluent was generated online utilising a Dionex Eluent Generator (EG) module fitted with a EluGen II KOH cartridge. A continuously regenerated anion-trap column (Dionex CR-ATC) was inserted after the KOH cartridge. The second-dimension column was a Dionex Acclaim C18 column (150 mm × 4.6 mm I.D., 5 μm packing material). Prior to transfer of the first-dimension effluent to the second dimension, the effluent was neutralised using a Dionex ASRS Ultra II 2-mm membrane suppressor. The suppressor was set at 8 mA throughout and the pH was measured off-line to confirm complete suppression of the hydroxide from the first-dimension separation, before it was connected to the second dimension. The two dimensions were interfaced by a high-pressure 10-port valve (Dionex HP valve) the 10-port valve was equipped with two 400-μL sample loops for storage and transfer of the first-dimension effluent to the second-dimension separation column. An external pump (Dionex I.C 25) was used to provide high-purity water (external mode) for the membrane suppressor. A Dionex AS autosampler equipped with a 25-μL sample loop was used for sample injection. A UV detector (AD Absorbance Detector, Dionex) was used throughout.

2.2. Reagents

The following 24 standard solutions were prepared (analytical reagent grade wherever possible): Quinate (D(-)quinic acid), lactate (DL-lactic acid sodium salt), butyrate (isobutytic acid), pyruvate (sodium pyruvate), galacturonate (D-galacturonic acid sodium salt), gluconate (sodium D-gluconate), maleate (maleic acid), α-ketoglutarate (α-ketoglutaric acid disodium salt), all from Fluka, St. Louis, MO, USA. Glucolate (glucolic acid), cis-aconitate (cis-aconitic acid), malate (DL-malic acid), and fumarate (fumaric acid) from Sigma, St. Louis, MO, USA. Glutarate (glutaric acid), tartarate (L-tartaric acid), iso-citrate (DL-isocitric acid tri-sodium salt, and trans-aconitate (trans-aconitic acid) from Aldrich, St. Louis, MO, USA. Formate (sodium formate) form Sigma–Aldrich, AUSTRALIA; Acetate (Sodium acetate) and adipate (adipic acid), Ajax Chemicals, Unilab, Auckland, New Zealand. Propionate (propoionic acid) fom Chem Supply, Adelaide, Australia. Succinate (sodium succinate), malonate (sodium malonate), oxalate (potasium oxalate), citrate (tri-sodium salt citrate) all from BDH, West Chester, PA, USA. All analytes were dissolved in deionized water and prepared as stock solutions at concentrations of 0.9 to 730 mg/L. The food and beverage samples, orange juice (freshly squeezed), yoghurt (Tamar Valley, Classic Natural, 98% fat free) and white wine (Sauvignon Blanc, Rosemount 2008) were all purchased from a local store.

2.3. Methods

All separations were performed at 30 °C. The detector was set at 210 nm throughout. The first-dimension gradient was generated on-line by programming the current applied to the eluent generator. The optimised first-dimension hydroxide gradient was found to be as follows: 0–22.5 min, gradient from 1 to 15 mM KOH; 22.5–25 min, isocratic at 15 mM; 25–50 min, gradient from 15 to 48 mM; 50–55 min, 48–50 mM; 56–95 min, isocratic re-equilibration at 1 mM. The first-dimension flow rate was 0.1 mL/min throughout. The second-dimension mobile phase (20 mM phosphate buffer at pH 2.25), was prepared by dissolving 20 mmol sodium dihydrogen phosphate monohydrate (Fluka, Puriss p.a, Steinheim, Germany) and about 22 mmol phosphoric acid (BDH, 88%, AnalaR) to reach a measured pH of 2.25 ± 0.02 in 1 L deionized water. Prior to use the mobile phase was filtered (Nylon membrane filters, 0.2 μm; Grace, Rowville, Australia). The phosphate buffer was mixed with methanol (LiChrosolv for Chromatography, Merck, Darmstadt, Germany) at a ratio of 90:10 phosphate buffer: methanol and was applied isocratically at 1.5 mL/min throughout. The modulation time (between two fractions injected in the second dimension) was 2 min. All water used