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Development and optimization of a system for comprehensive two-dimensional liquid chromatography with UV and mass spectrometric detection for the separation of complex samples by multi-step gradient elution

Mark Eggink *, Wilmar Romero, René J. Vreuls, Henk Lingeman, Wilfried M.A. Niessen, Hubertus Irth

VU University Amsterdam, Faculty of Science, Department of Analytical Chemistry and Applied Spectroscopy, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

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Abstract

Comprehensive two-dimensional liquid chromatography (LC × LC) is a powerful tool for the separation of complex biological samples. This technique offers the advantage of simplified automation and greater reproducibility in a shorter analysis time than off-line two-dimensional separation systems. In the present study, an LC × LC system is developed enabling simultaneous UV and MS detection, and which can be easily converted to a conventional reversed-phase LC-UV/MS system. In LC × LC, a 60-min reversed-phase LC separation with a linear solvent gradient in the first dimension is coupled to a second-dimension separation on a mixed-mode cation-exchange/reversed-phase column with a modulation time of 60 s. The isocratic separation in the second-dimension column is optimized by the use of a multi-step gradient where the organic and the ionic modifier are varied independently. Intraday ($n = 3$) and interday ($n = 4$) variability of the retention times were evaluated with the complete system and found to be 0.5% and 0.7%, respectively. Good linearity was observed in calibration curves for three different compounds varying in polarity.

Keywords: Comprehensive two-dimensional liquid chromatography; LC × LC; Complex mixtures; Multi-step gradient elution

1. Introduction

Comprehensive separation of complex mixtures, *e.g.*, urine samples for metabolomics or biomarker studies, is a difficult challenge due to the presence of thousands of components that vary from polar to non-polar and from picomolar to millimolar concentrations, and that show diverse physico-chemical properties, *e.g.*, acid–base properties, stability, solubility, detectability. The potential of conventional separation techniques such as liquid chromatography (LC) or gas chromatography (GC) and detection approaches like UV–vis or mass spectrometric (MS) detection are limited. In the last decade, comprehensive multidimensional separation techniques such as GC × GC [1,2], LC × GC [3,4], LC × capillary electrophoresis (CE) [5,6], and LC × LC [7–9,31] have been developed and reported. These multidimensional techniques offer an enormous separation power and are therefore ideally suited for the analysis of very complex mixtures. Due to its compatibility with biological matrixes, HPLC is most widely applied in biological applications. Therefore, the use of comprehensive two-dimensional liquid chromatography (LC × LC) would be a logical choice for the separation of complex biological samples where conventional LC does not provide sufficient separation power. In contrast to multidimensional heart-cutting separation techniques (LC–LC), where a particular fraction of the first-dimension separation is transferred and re-separated on the second-dimension column [10], in LC × LC the entire first dimension is analyzed in the second-dimension separation. Comprehensive LC × LC offers various advantages over both multidimensional off-line and conventional separation techniques, especially with respect

* Corresponding author. Tel.: +31 20 598737; fax.: +31 20 59843.
E-mail address: meggink@few.vu.nl (M. Eggink).

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to enhanced peak capacity, automation potential, and reproducibility and shorter analysis time. Given the numerous liquid separation modes, there are numerous possible combinations of first- and second-dimension separation in LC × LC.

In many of the recent papers on LC × LC, compounds are separated by two independent separation techniques featuring the same separation mechanism, especially reversed-phase LC × reversed-phase LC systems (RPLC × RPLC) [11–15,31]. In these cases, the two-dimensional separation is based on the use of different organic modifiers and/or RPLC columns with different properties. Nevertheless, due to the fact that the retention mechanisms in the first and second dimension are largely the same, components with a low retention factor in the first dimension will still have a low capacity factor in the second dimension. As a result, the high theoretical peak capacities can never be attained in such a setup.

Therefore, the use of two different separation mechanisms in the first and second dimension is generally preferred. A variety of LC × LC separation approaches has been described, including RPLC × size-exclusion chromatography (SEC) [16–18], normal-phase (NP) LC × RPLC [7,19], SEC × RPLC [20,21], ion-exchange chromatography (IEC) × SEC [22], IEC × RPLC [23–25]. IEC × RPLC would be a logical column combination for the separation of biological samples because of the presence of many ionic components that can be separated by IEC. Most LC × LC systems based on IEC × RPLC are operated with IEC in the first dimension and RPLC in the second dimension. Perhaps the most widely applied comprehensive LC × LC approach, *i.e.*, the multidimensional protein identification technology (MudPIT) developed by the group of Yates [26] and two-column approaches based on this design [27,28], is based on IEC × RPLC, but in general peptide separation can be considered to be significantly different from the small-molecule separations as under development in the present study. Nevertheless, IEC × RPLC would be attractive because RPLC on C_{18}-materials is the most frequently applied LC mode. RPLC operating conditions are readily compatible with important LC detectors, UV, fluorescence and especially MS. Due to its selectivity and sensitivity, and its ability to confirm compound identity or even elucidate unknown structures, MS detection is nowadays essential in the majority of the LC applications.

From this point of view, the alternative approach of RPLC × IEC, although attractive for its comprehensive two-dimensionality, would be less favorable, because IEC generally requires relatively high salt concentrations and is therefore less compatible with MS detection.

An interesting alternative would be the use of mixed-mode columns featuring both RPLC and IEC separation mechanisms in the second dimension, as these can eliminate some of the shortcomings of either RPLC and IEC.

In the present paper, we proposed a novel approach to overcome these limitations by implementing a mixed-mode RPLC/cation-IEC column in the second dimension in combination with step-gradient elution. Using a test-set of 32 compounds covering a very wide range of polarities, we describe method development and optimization separately for each separation dimension. Optimization of the second-dimension separation indicated the necessity of the use of a step gradient. The fully automated LC × LC system has been coupled to a mass spectrometer.

2. Experimental

2.1. Chemicals and reagents

Alanine, creatinine, dopamine, caffeine, theophylline, paracetamol, desmethyldiazepam, midazolam, aspirin, aniline, 2,6-dimethylaniline, ibuprofen, vanillin, thiobenzamide, nadolol, hydrocortisone, naproxen, diazepam, oxazepam, sulfamethoxypyridazine, sulfaguanidine, 3,4-hydroxybenzoic acid, sulfamic acid, imipramine, tryptophan, tyrosine, nitrazepam, carbamazepine, benzothiophen, metamitron, prometryn, and desethylatrazine were all purchased from Sigma, Zwijndrecht, The Netherlands. Acetonitrile (ACN), formic acid (FA), acetic acid, and trifluoroacetic acid (TFA) were purchased from Biosolve, Valkenswaard, The Netherlands. The test-set was diluted in 1% (v/v) of ACN in water with 0.1% (v/v) FA at concentrations of 125 μM for all compounds. The urine sample was collected from a volunteer; the sample was centrifuged at 13 200 rpm with an eppendorf centrifuge (VWR International, Amsterdam, The Netherlands) directly after collection and stored at −20 °C.

2.2. Instrumentation for LC × LC

A schematic diagram of the setup of the comprehensive LC × LC system is illustrated in Fig. 1. The system consists of two parts which can also be used independently as two separate conventional LC systems.

The first-dimension separation system consisted of a Shimadzu HPLC system (‘s Hertogenbosch, The Netherlands) comprising of two LC-10AD-VP pumps, a SCL-10ADvp

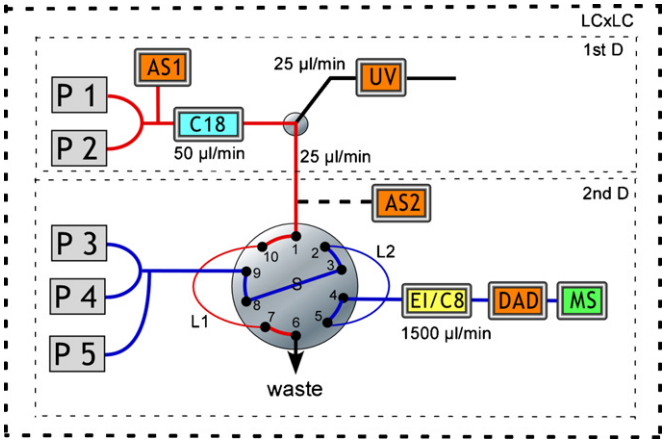

Fig. 1. Schematic diagram of the setup of the LC × LC-UV/MS system. P1, P2: first-dimension gradient pumps. P3, P4, P5: second-dimension gradient pumps. AS1, AS2: autosamplers. S: a ten-port switching valve with two sample loops of 25 μL (L1, L2). The first-dimension column is a Luna C18(2) (50 mm × 1.0 mm), the second-dimension column is a primesep A (30 mm × 4.0 mm). Detectors: UV-detector at 220 nm for the first dimension, DAD for the second dimension, and a Q-TOF2 for APCI-MS.