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The advantage of mixed-mode separation in the first dimension of comprehensive two-dimensional liquid-chromatography☆

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ABSTRACT

Comprehensive two-dimension liquid chromatography (LC × LC) has exhibited its powerful ability to separate complex samples. However, the use of a single chromatographic mode in 1st dimension has been limited to the separation of components by their individual characteristics, such as hydrophobicity, ionic properties etc. The use of mixed-mode stationary phases has revealed opportunities to combine different retention mechanisms. In this respect, stationary phases featuring both RP-like hydrophobic and ion-exchange interactive sites promise great versatility in retaining both polar and more apolar ionic and non-ionic compounds. We have therefore developed an LC × LC system based on mixed-mode (strong anion exchange and reversed phase) in the first dimension and a C18 phase in the second dimension. The system has been evaluated with standard compounds and applied for the separation of white wine and Chinese Herbal Medicine (CHM). The mixed-mode system SAX-PFP × C18 results in a better separation than a single mode system such as SAX × C18 or PFP × C18. Although little improvement in orthogonality (0.91 instead 0.86) is achieved with SAX × C18, the mixed-mode SAX-PFP × C18 gives a much larger effective peak distribution area in the analysis of e. g. white wine. But the analysis of aqueous extracts of CHM (*Hdyotis diffusa* and *Scutellaria barbata*) with SAX-PFP × RP leads to a very long analysis time because of strong hydrophobic interactions with the PFP column. Thus, the system was changed by using a cyano phase instead of a PFP phase. The improved SAX-CN × C18 system shows a better peak distribution and more importantly a reasonable analysis time.

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1. Introduction

The use of comprehensive two-dimensional liquid chromatography (LC × LC) has grown rapidly in the last two decades. Its high peak capacity, selectivity and resolution have attracted much attention from researchers studying proteomics [1,2], natural products [3,4], polymers [5,6] etc. Much effort has been expended in increasing the orthogonality, peak capacity and speed, as has been well described in several reviews [7–9].

The general setup of an LC × LC system consists of two separation columns, which are coupled online via an interface. LC techniques offer a wide variety of separation mechanisms, such as normal-phase (NP), reversed-phase (RP), size-exclusion (SEC), ion-exchange (IEC) or affinity chromatography (AC), characterized by different selectivities. Consequently, two-dimensional liquid chromatography (2D-LC) can theoretically be employed in many multidimensional combinations. However, the combination of certain LC modes can present a series of difficulties, such as mobile-phase immiscibility or incompatibility of the mobile phase of the first dimension with the stationary phase of the second dimension [7].

For practical considerations, reversed-phase (RP) mode has been adopted in the second dimension in most applications. Therefore, the use of a proper separation mode in the first dimension has been a priority in achieving better orthogonality and thus higher effective peak capacity. But each phase combination has its pros and cons. Unfortunately, the use of NP or HILIC is problematic because of the incompatible mobile phase with the second dimension or the reduced selectivity for non-charged compounds, respectively. The use of SEC and IEC have lower resolution thus limited the effective peak capacity. And the use of RP mode in both dimensions leads to a correlated separation mechanism [4].

The use of mixed-mode stationary phases suggested the possibility of combining various selectivity principles [10,11]. After originating in solid-phase extraction materials [12] and capillary

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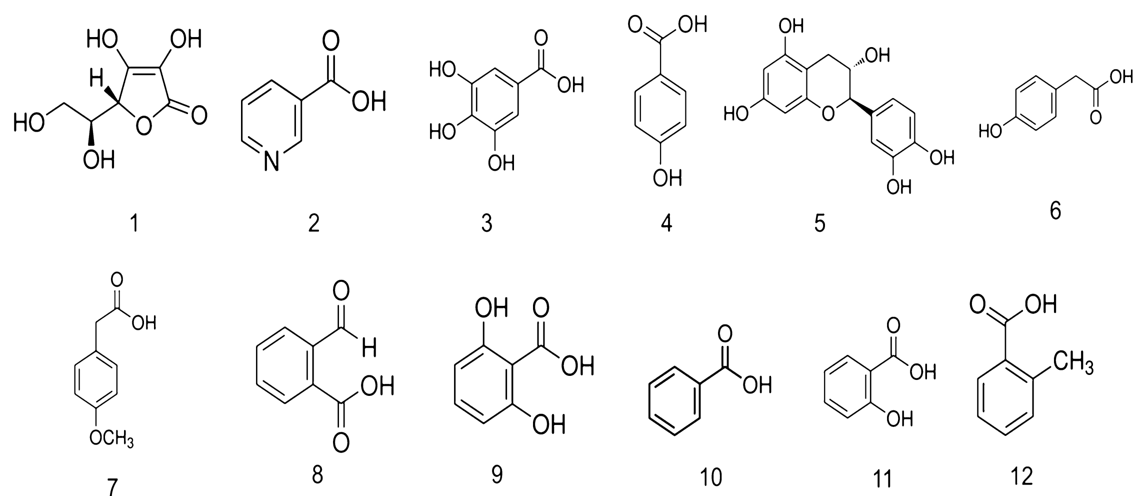

Fig. 1. Reference compounds for LC × LC system evaluation.
L-Ascorbic acid (**1**); nicotinic acid (**2**); gallic acid (**3**); 4-hydroxybenzoic acid (**4**); (+)-catechin (**5**); 4-hydroxyphenylacetic acid (**6**); 4-methoxyphenylacetic acid (**7**); 2-carboxybenzaldehyde (**8**); 2,6-dihydroxybenzoic acid (**9**); benzoic acid (**10**); salicylic acid (**11**); *o*-toluic acid (**12**).

electrochromatography [13,14], the multiple selectivity [15] of mixed-mode stationary phase is attracting considerable attention nowadays [11,16,17]. In general, such mixed-mode chromatographic phases have been achieved with chemically embedded polar functional groups (hydrophilic domains) and/or a terminal quinuclidine ring which accommodates the anion-exchange site (ionic domain) to a single chromatographic ligand, such as an alkyl chain strand (hydrophobic domain) [18]. For lack of commercial chemically bonded mixed-mode stationary phases, physical packing of two or more types of stationary phases into one column was well developed, especially in the method known as multi-dimensional protein-identification technology (MudPIT) in proteomics [19,20]. The packing of more than one stationary phase in a column is, however, often problematic because the optimal slurry conditions for the column packing differ. The simplest way to incorporate different selectivities is to connect two commercial columns in series to form a "tandem column" [17,21–24], such as is used in stationary-phase-optimized-selectivity liquid chromatography (SOSLC) [25,26]. For this, separation materials featuring both RP-like hydrophobic and ion-exchange interactive sites promise great versatility and the capability of retaining polar acidic as well as more apolar ionic and non-ionic compounds [11]. This extends the scope of chromatographic separations to mixtures of compounds with a wide range of retention behavior [21]. In addition, the multi-selectivity obtained by tandem columns facilitates the development of a powerful comprehensive two-dimensional chromatography (LC × LC) application.

Offline two-dimensional liquid chromatography systems using RP column in the second dimension and mixed-mode reversed-phase/weak cation-exchange (RP/WCX) columns [27], reversed-phase/anion-exchange (RP/AX) column [28], and MudPIT technology based mixed-mode reversed-phase/strong anion-exchange (RP-SAX) in the first dimension [29] were reported. MudPIT featured mixed-mode multidimensional separation was not in the discussion scope of this paper. To our best knowledge, there is no work published about online LC × LC using mixed-mode separation in first-dimension.

In the present work, the coupling of IEC and RP in the first dimension was realized with two strong anion-exchange columns (SAX) and one reversed-phase (RP) pentafluorophenyl (PFP) column in series. Both, buffer and organic solvent, were included in the mobile phase in order to elute components from this tandem column in a second-dimension reversed-phase (RP) C18 column and thus achieve an online LC × LC system. A mixture of phenolic and/or polar compounds (Fig. 1) was analyzed to evaluate the performance of the method developed, and the system was then used in the analysis of white wine and modified for the analysis of Chinese Herbal Medicine (CHM). This work is only focused on the development of a powerful LC × LC system with different column combinations and not on the results of the analysis.

2. Experimental part

2.1. Chemicals and reagents

Methanol of LC-MS grade was purchased from VWR (Leuven, Belgium). Ammonium bicarbonate, formic acid, nicotinic acid, 4-hydroxybenzoic acid and 4-methoxyphenylacetic acid were bought from Fluka AG (Buchs, Switzerland); gallic acid, 2,6-dihydroxybenzoic acid, benzoic acid and (+)-catechin were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); salicylic acid and *o*-toluic acid were from Bayer AG (Leverkusen, Germany); L-ascorbic acid was purchased from Acros organics (Geel, Belgium) and other chemicals from Merck-Schuchard OHG (Hohenbrunn, Germany). Fig. 1 shows the structures of the standard compounds.

2.2. Sample preparation

2.2.1. White wine

0.75 mL of 1 M $(NH_4)_2CO_3$ solution was added to 10 mL freshly opened wine (Riesling, feinherb 2011, Mosel, Thörnicher St. Michael). The carbon dioxide was removed by immersion of the sample in an ultrasonic bath. The pH of the sample was approximately 6.5. The sample was filtered through a 0.2 μm PTFE filter before use.

2.2.2. CHM

0.5 g dried *Hdyotis diffusa* and 0.5 g dried *Scutellaria barbata* were soaking in 40 mL water for 30 min. Afterwards, the solution was boiled for 1 h. The extract was collected. And further 30 mL water was added and boiled for another 30 min. The two extracts were combined together and centrifuged at 4000 rpm for 15 min and then filtered through a 0.2 μm PTFE filter before use.

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