Journal of Chromatography A, 1456 (2016) 145–151

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Multimode gradient high performance liquid chromatography mass spectrometry method applicable to metabolomics and environmental monitoring

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ARTICLE INFO

Article history:
Received 28 January 2016
Received in revised form 25 April 2016
Accepted 1 June 2016
Available online 5 June 2016

Keywords:
Gradient multimode separation
HILIC
Anion exchange (AEX)
Cation exchange (CEX)
Reversed-phase
Metabolomics
Complex environmental mixtures

ABSTRACT

Metabolomics or environmental investigations generate samples containing very large numbers of small molecular weight analytes. A single mode chromatographic separation excludes a substantial part of such complex analyte mixtures. For instance, a reversed-phase separation would not retain ionic species, resulting in a correspondingly huge front peak. To address this problem, we used two commercially available mixed-mode ion-exchange reversed-phase columns (WAX-1 and WCX-1) in sequence in a novel multimode separation method.

After trapping hydrophobics on a C_{18}-trap in loop position, hydrophilics passing the trap are separated by a simultaneous gradient for HILIC, anion and cation exchange chromatography. This gradient ends in a washout phase with a high percentage of water, the correct starting conditions for a reversed-phase gradient eluting hydrophobics from the trap in a second step of the run. Amino acids (9), organic acids (2), sugars (8), fatty acid derived compounds (11), antioxidants (4), miscellanea (6) and xenobiotics (4) were analyzed. Compounds were separated after a single sample injection during a 50 min run. Lipids derived small fatty acids up to a chain length of 12 carbons were also accessible within this run time.

1. Introduction

Among today's various analytical fields, metabolomics and environmental monitoring face the most challenging task of having to identify and quantify a multitude of chemicals belonging to a wide variety of chemical classes, covering apolar to polar and ionic species. This requires special care when enriching environmental samples, and metabolites or chemical pollutants from tissues, body fluids or organisms. Solid phase extraction combining materials with different enrichment modes has been used for capturing this multitude of target chemicals [1,2]. Many of the analytes are present at very low concentrations, requiring sensitive detection by MS. Additionally, their excessive diversity in chemical properties cannot be handled in one run by a single-mode chromatography. For this reason, HPLC column fillings that carry different functionalities have been developed, providing LC-columns that can more adequately resolve such diverse multi-component mixtures. Bi-functional and tri-functional solid phases are available, offering additional separation power compared to single group functionalized materials (see Table 1).

LC–MS is increasingly used for the analysis of very complex water samples. It has also become popular for analyzing metabolomes in different research fields, such as drug discovery, medical diagnosis, food and nutrition, forensics and toxicology. Thousands of analytes can be expected to be present in a metabolome or in a complex water sample. A complete separation of all of them is not realistic. However, in order to minimize ion suppression and interferences in the ion source of the MS detector, an even distribution of the compounds over the entire chromatogram is required. An analysis of the *Identification and Evaluation of Metabolomics* (IDEOM) database [3] reveals that, at the physiological pH 7.4, 50.7% of the metabolites are anions, 13.6% cations, 27.4% non-ionic and hydrophilics (see Table 2), including about 20% hydrophobics, thus requiring anion (AEX) and cation exchange (CEX), HILIC and RP chromatography, respectively, to manage the retention of each compound class.

Traditionally, as just recently reviewed [4], the analysis of the metabolome is done using LC–MS based on a single separation mechanisms using either reversed-phase (RP), HILIC or ion

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http://dx.doi.org/10.1016/j.chroma.2016.06.001
0021-9673/

Table 1
Differently functionalized and commercially available solid phases.

Name	Manufact.	Functions	Comment
Acclaim MM WAX-1	Thermo	RP, WAX	weak anion exchanger
Acclaim MM WCX-1	Thermo	RP, WCX	weak cation exchanger
Scherzo SM	Imtakt	(RP, WAX), (RP, WCX)	2 types of particles
Scherzo SS	Imtakt	(RP, SAX), (RP, SCX)	2 types of particles
Obelix N or R	SieLC	RP, IEX imbedded	non-porous particles, separation cell holes
Trinity P1	Thermo	RP, WAX, SCX	in-pore modified and surface agglomerated particles
Trinity P2	Thermo	RP, SAX, WCX	in-pore modified and surface agglomerated particles

MM: mixed-mode.

Table 2
Number of compounds in IDEOM database [15] according to net charge at pH 7.4 (n = 31′940) and logD (n = 34′933).

	Net Charge	# Compounds	(%)	Comment
neutrals	−0.2–0.2	11408	35.7	includes zwitterions
neutrals, charge-free	0	8767	27.4	includes hydrophilics
cations	>0.2	4346	13.6	
anions	<−0.2	16186	50.7	
logD ≤ 1.0		20422	58.5	includes hydrophilics and ionics
logD ≥ 1.0		14511	41.5	

exchange solid phase material. A limited separation is provided by a RP or alternatively by a polar HILIC column. A mono-functionalized solid phase with its specific eluent thus restricts the number of analytes which can be separated by this single chromatographic mode. In case of a RP run, most hydrophilic compounds are not separated, but rather expelled at the front, while in a HILIC-run, hydrophobics are not retained and flushed out at the front. A run using ion exchange (IEX) only, neither retains non-ionic hydrophilics, nor hydrophobics.

The different eluents required for each of these chromatographic modes also influence the ion generation in the electrospray ionization (ESI) source and therefore the sensitivity of the mass spectrometer. It was shown that HILIC, due to the higher organic solvent in the eluent, was more sensitive in ESI MS when analyzing compounds with a pKa ranging from 1.8 to 10.9 and logP values from −1.2 to 5.6 [5]. AEX was evaluated as an additional separation mode and shown to perform better than HILIC for ionic analytes [7]. Despite the fact that more than 50% of the constituents of a metabolome are ionic (see Table 2), AEX has so far not been very popular, except for a recent study of ionic metabolites in cancer cell extracts [8]. Two dimensional orthogonal LC can combine two modes in one run, however at the expense of higher instrumental and handling complexity. The use of a third dimension has been considered impractical [6]. Hence, a single mode procedure cannot provide the separation power needed for complex environmental samples or mixtures of metabolites. On the other hand, to run each chromatographic mode separately would be very laborious and expensive, and therefore mixed-mode chromatography (MMC) is a very interesting alternative. The advantages and applications of MMC have been reviewed by Yang and Geng [9].

Here we present a novel single-run and single-injection mixed-mode chromatography (MMC), making use of several solid phase functionalities of commercially available multimode columns. The objective was to provide RP, HILIC and IEX separation capabilities at physiological pH, all in gradient mode and following one single injection only. All these separation mechanism are needed when different classes of endogenous metabolites (sugars, amino acids, carboxylates, fatty acid metabolites, antioxidants and others) and a few exogenic compounds are to be separated (see Table 3). To our knowledge, the integration of HILIC, IEX and RP in the same chromatographic run has not been exploited to date. The gradient MMC was realized using either one tri-modal column or two dual-mode columns in sequence. The proposed MMC is to be used as a LC–MS platform for complex environmental sample analysis and global and targeted metabolomics. It can retain xenobiotics and metabolites of all classes, spreading them over the entire run, thus reducing the number of co-eluting compounds in the MS ion source and hence the risk of ion suppression and interferences.

2. Material and methods

Commercially available multimode LC columns were coupled to mass spectrometers using electrospray ionization. Multiple reaction monitoring was used for targeted mixture analysis (TSQ Vantage, Thermo Scientific, San Jose, CA, USA) and full scan accurate mass spectra (QExactive, Thermo Scientific) were acquired for non-target approaches, in alternating positive and negative ion mode. A PAL autosampler (CTC, Zwingen, Switzerland) was used to inject 10 μL samples onto a C_{18}-trap column (2 × 10 mm, XTerra, Waters, Baden, Switzerland) connected to the divert valve of the MS, as shown in Fig. 1. Two bi-functional columns (100 × 2 mm), Acclaim MM WAX-1 and WCX-1 (Thermo Scientific), were connected in sequence. The 100 × 2 mm format was obtained by having Thermo's 150 × 3 mm Acclaim columns repacked by Morvay Analytics, Basel. A tri-modal Trinity P1 column (100 × 2.1 mm, courtesy of Thermo Scientific) was also tested as an one-column-one-injection procedure. The isocratic pump (Rheos 2000, Flux Instruments, Basel, Switzerland) delivered eluent C and the sample to the C_{18}-trap column, while the gradient pump (Accela 1250, Thermo Scientific) ran eluents A and B.

Gradient-grade HPLC solvents from Scharlau (Barcelona, E) or Acros Organics (Thermo Scientific) were used. Ammonium hydrogen carbonate was selected as IEX-eluent salt since on the one hand it performs well in IEX chromatography and on the other hand is easily volatilized in the heated ion source of the MS. Eluents were A) ACN with 3% H_2O, 3 mM NH_4HCO_3 (pH 7.3 ± 0.2, adjusted with HNO_3), B) water with 10% ACN, 30 mM NH_4HCO_3 (pH 7.3 ± 0.1, with HNO_3) and C) H_2O with 5% ACN, 10 mM NH_4HCO_3.

A multi-gradient run was applied (see Table 4 and Fig. 2). Stock solutions of single analytes (Sigma Aldrich) were prepared (10–20 mg/mL) in an ACN/H_2O ratio that fully dissolved the analytes. They were stored in amber vials at −20 °C and used for several months. Analyte mixtures (10–20 compounds) were prepared freshly from single stocks and injected in different dilutions (1:20, 1:50, 1:100).