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Multimode Gradient High Performance Liquid Chromatography Mass Spectrometry Method for Metabolomics and Environmental Monitoring

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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 a complex analyte mixture. For instance, a reversed-phase separation would not retain ionic species, resulting in one 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 multimode separation LC-MS 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 RP-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), miscellaneous (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.

Keywords

Gradient multimode separation, HILIC, anion exchange (AEX), cation exchange (CEX), reversed phase, metabolomics, complex environmental mixtures

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 the metabolome 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, an optimal distribution of the compounds over the entire chromatogram is all the more urgently needed in order to minimize ion suppression and interferences in the ion source of the MS detector. 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 exchange solid phase material. A limited separation is provided by a reversed phase (RP) or alternatively by a polar HILIC column. A mono functionalized solid phase with its specific eluent restricts the number of analytes which can be separated by athis single chromatographic mode. In case of a RP-run, most hydrophilic compounds are not separated and 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 cannot retain non-ionic hydrophilics nor hydrophobics. The different eluents required for each of these chromatographic modes also influences the ion generation in the 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 when analyzing compounds with pKa ranging from 1.8 to 10.9 and logP values from -1.2 to 5.6 by ESI MS[5]. Two dimensional orthogonal LC can combine two modes in one run at the expense of higher instrumental and handling complexity, but the use of a third

dimension is considered impractical [6]. Furthermore, IEX was evaluated as an additional separation mode and shown to perform better than HILIC for ionic analytes (Burgess et al. [7]). However, IEX was not further investigated for the separation of ionic metabolites, despite the fact that they account for more than 50% of a metabolome (see Table 2).

Hence, a single mode procedure cannot fulfil the required chromatographic task of preventing a large part of a metabolome eluting in one huge peak. To run each chromatographic mode separately would be too laborious and expensive, and therefore, a single-run and single-injection mixed-mode chromatography (MMC) was developed. The advantages and applications of MMC have been reviewed by Yang and Geng [8].

Here we present a new type of MMC, making use of several solid phase functionalities of commercially available multimode columns. The objective was to provide RP, HILIC and IEX separation capabilities, all in gradient mode and in the same one injection run. All these separation mechanism are active when different classes of endogenous metabolites (sugars, amino acids, carboxylates, fatty acids metabolites, antioxidants and others) and a few exogenic compounds are injected (see Table 3). To our knowledge, the integration of HILIC, IEX and RP in the same run has not ben expoited so far. The gradient MMC was realized using either one trimodal column or two dual-mode columns in sequence. The MMC is proposed to be used as a HPLC MS platform for complex environmental sample analysis and untargeted and targeted metabolomics approaches. It can retain xenobiotics and metabolites of all classes and thereby spreading them over the entire run reduces 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. In a targeted approach multiple reaction monitoring (Vantage, Thermo Scientific, San Jose, CA, USA) and in an untargeted approach full scan accurate mass spectra (QExactive, Thermo Scientific) were acquired in alternating positive and negative ion mode. The divert valve of the instruments fitted with a C_{18}-trap column (2x10 mm, XTerra) were used to switch the flow in connection with a PAL auto sampler (CTC, Zwingen, Switzerland) injection valve, as shown in Figure 1. Ten µL were injected through the PAL injection port.

Two bi-functional columns (100x2 mm), Acclaim MM WAX-1 and WCX-1 (Thermo), were connected in sequence. , The 100x2 mm format was obtained by repacking Thermo's 150x3 mm Acclaim columns by Morvay Analytics, Basel. A trimodal Trinity P1 column (100x2.1 mm, courtesy of Thermo) was also tested as a one column one injection procedure. The isocratic pump (Rheos 2000) delivered eluent C and the sample segment to the C_{18}-trap column, while the gradient pump (Accela 1250) ran eluent 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

one hand it performs well as eluent ions in IEX chromatography and on the and on the other hand are volatilized in the heated ion source of the MS. Eluents were A) ACN with 3% H_2O, 3 mM H_4NHCO_3 (pH 7.3±0.2, adjusted with HNO_3), B) water with 10% ACN, 30 mM H_4NHCO_3 (pH 7.3±0.1, with HNO_3) and C) H_2O with 5% ACN, 10 mM H_4NHCO_3.
A multi-gradient run (details see Table 4) was applied which is also graphically depicted in Figure 2.
Stock solutions of single analytes (Sigma Aldrich) were prepared (10-20 mg/mL) in ACN / H2O in a ratio that dissolved the analyte. 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).

3. Results and discussion

3.1. Combining HILIC, IEX and RP

Charged solid phases have frequently been used in HILIC separations as reviewed in [9], providing additional ionic interaction for ionic analytes. Ion exchange columns provide HILIC separation modes [10-12] due to their ionic groups which maintain a water layer, enabling hydrophilic interaction under a high organic solvent regime. Lämmerhofer et al. [10] compared the RP and HILIC performance of a large number of mixed-mode solid phase materials, demonstrating the HILIC behavior of RP/weak anion-exchangers at 90 % ACN and the RP separation at 40% ACN. Lämmerhofer et al. and others [11, 13] showed that the Aclaim MM® WAX-1 ion exchanger column provides good RP and HILIC separation capabilities. Thus, with respect to a gradient multimode LC, the separation modes HILIC, AEX and CEX can be executed simultaneously on one and the same trimodal column or on two bimodal columns connected in sequence.
A single column procedure using the Trinity P1 column was tested first. Its trimodal functionalized solid phase (WAX, SCX and RP) showed that the multimode chromatography worked well except that the apparent retention times (t_R) for cationic compounds were too long in order to appear as an integrable peak. To reduce these t_R, the strong cation exchange sites (SCX) responsible for this shortcoming require much higher eluent salt concentrations which are not compatible with the salt-sensitive ESI without an ion suppressor device. Such a device, however, would be a sink for a fraction of possible analytes. Therefore, strong ion exchangers are inappropriate and, unfortunately, a trimodal phase without strong ion exchanger was not available, so two dual mode weak ion exchanger columns, WAX-RP and WCX-RP, operated in sequence had to be selected. A short piece of narrow tubing connected the two columns without a significant dead volume increase.

3.2. Single run gradient multimode chromatography

The multimode gradient starts with HILIC and IEX initial conditions (see Figure 2). For the HILIC gradient the starting high ACN content was gradually linearly decreases down to 10% (100% B). The lower decrease between 3 and 12 min was chosen due to the fact that HILIC only works with ACN contents higher than 70% on these columns. By increasing B however, the initial low ion concentrations (3 mM NH_4HCO_3) for IEX is increased simultaneously to 30 mM. Together, simultaneously a HILIC-gradient, an anion (HCO_3^-) and a cation (NH_4^+) gradient for IEX are produced. The NH_4HCO_3 concentrations in eluent A and B were optimal for IEX on the weak ion exchange functionalities on these columns. A somewhat higher NH_4HCO_3 concentration was chosen for C (10 mM) for a better hydrophobic interaction in support of the trapping function and the fact that C is strongly diluted on the separator columns.

After a washout phase (18-21 min), the valve was switched (see Figure 2) and the gradient was reversed. The increasing ACN amount starts the RP-separation in the second part of the run. At the end of the run starting conditions for another sample injection are reached, starting again with HILIC and IEX gradients.

By executing HILIC together with IEX before RP in a consecutive manner, the two counter acting gradients are complementing each other in a single-injection run, provided the hydrophobics are retained in the beginning, prior to being eluted onto the separator columns. Therefore, upon injection (see Figure 1A), hydrophobics are trapped on a C_{18}-column (0-3 min, 100% C), while hydrophilics are flushed to the separators where they are chromatographically separated. After hydrophilics have reached the separator column, the flow through the trap column is stopped (3 min), preventing weakly adsorbed compounds from leaking off the trap during HILIC / IEX separation.

The incompatibility of eluent C (H_2O, 5% ACN flowing over the C_{18}-trap onto the separator) with HILIC start conditions was overcome by the ratio of the two flows. The low flow (25 μL/min) through the C_{18} introduces only minor changes in the composition of the much higher flow through the separators (260 μL/min), so that optimal HILIC start conditions are maintained on the separators. The combined eluent composition hitting the top of the separator was calculated to be 90% ACN and 3.5 mM NH_4HCO_3 after a presumed mixing by the T-piece, which still are acceptable HILIC starting conditions without compromising too much the ionic strength increase for the IEX-gradient separation.

Before the RP washout (33-46 min) ends, the MS divert valve is turned back (at 36 min) to keep the C_{18}-trap out of the gradient pump flow (see Fig. 1A) which, at this time, delivers a high percentage of ACN, incompatible for the C_{18}-trap re-equilibration. At the same time the isocratic pump flow is resumed to equilibrate the C_{18}-trap before the next injection onto the trap.

The flow of the gradient pump is at no time diverted to waste, thus all compounds retained either by the C_{18}-trap or the ion exchange columns reach the detector according to their chromatographic behavior. The overall run time corresponds to two single runs each in single

mode chromatography but makes the exchange of columns and flushing the whole system obsolete. The data collected in one file also reduces significantly the data evaluation effort.

3.3. Chromatographic behavior of metabolites and xenobiotics

Model compounds were selected to represent (polarity, hydrophobicity etc.) several class of endogenous metabolites and xenobiotics as shown in Table 3 to test the proposed multi-mode gradient separation: amino acids (9), organic acids (2), sugars (8), fatty acids derived compounds (11), antioxidants (4), miscellaneous (6) and xenobiotics (4). Mixtures of 10-20 compounds were injected after having optimized source conditions for maximal molecular ion and / or fragment ion intensity. Figure 3 shows the separation of an 18 component mixture.

The two columns provided good retention properties, judged by the t_R = 5.5 min and an apparent retention factor (k*) of 3.2 of the neutral but highly hydrophilic uracil, a compound usually difficult to retain [11]. Because of the total C_{18}-length (200 mm) of the two columns in sequence, metabolites of very high hydrophobicity will not elute within a reasonable run time. However, unbranched fatty acids bearing an apolar carbon chain length up to C_{12} were tested and eluted in less than 36 min, including tocopherol another lipophilic metabolite. Limitations of the method are indicated by the high retention time of dodecyl sulfate (t_R = 41.2 min, k*= 33.8), caused by the higher anion charge density of the sulfate group compared to carboxylate, which contributes significantly to retention by anion exchange in addition to RP separation mechanism.

Compounds only partially retained on the trap will split into two peaks, since the flow through the trap was stopped after 3 minutes. The three minutes are the time it takes to transport non-retained compounds to the separator columns. Compounds retained or only partially retained on the trap will appear after 22 min, when the RP-gradient has started and flows through the trap and separators. The ratio between the two peaks depends on the amount of organic solvent in the injection solution. At 23 % ACN in the injection solution, amino acids were eluted within the first, the HILIC /IEX part, of the run, except cysteine which was eluted exclusively in the second, the RP part. The larger amount (82%) of the less polar tryptophan also eluted in the second peak whereas only a small amount (12%) of phenylalanine and the tripeptide glutathione appeared in the second peak. Glutamate was distributed over two peaks to about the same amount. All other tested compounds eluted as a single peak. This demonstrates that there is a sharp splitting by the trap, which allows to elute most compounds in one single peak. Only a minority of the analytes are split into two peaks. A second retention time for these compounds can be considered beneficial for their identification.

Apparent retention factors (k*) of all metabolites were calculated for a comparison with metabolites in the IDEOM-data base. Apparent retention times given in the data base were used to calculate k^*_{IDEOM} for measured and predicted t_R. Retention factors from measured t_R of

both MMC and IDEOM-standards correlated well (see Figure 4, filled diamonds ♦). The slope of this correlation was almost identical to the one obtained from predicted versus measured t_R for the IDEOM-metabolites (open squares □), except for a systematic and constant shift towards larger k* (longer t_R) for the MMC compared to IDEOM. IDEOM-metabolites were separated by HILIC only. So it can be concluded that the additional separation forces active during the MMC separation are causing larger k^*_{MMC}.

The xenobiotic compounds clotrimazole (ESI^+), fluconazole (ESI^+) and sucralose ($ESI^\pm$) were used as internal standards, retention time markers and sample preservatives. Sucralose was particularly useful since it produced a detector response in ESI^+ ($M+NH_4^+$) and in ESI^- ($M+HCO_3^-$) mode and it was also sensitive to the ACN content of the injected sample. At 80% ACN in the injection solution, 80 - 90 % of sucralose was not trapped and eluted in the HILIC-part (t_R=9.2), while at 23% ACN it was completely trapped. The entire behavior as a hydrophobic compound is noteworthy since in the di-saccharide saccharose only three hydroxyl groups have been replaced by chlorine atoms leaving still five OH-groups in the molecule. Such an ambivalent behavior makes sucralose an important indicator for the real conditions on the trap column which also depends on the sample composition.

4. Conclusions

Using only one single injection, the suggested MMC procedure allows to perform four different chromatographic modes: HILIC, AEX and CEX in the first part and RP in the second part, all in gradient mode and in one run. Compounds can only escape detection by very strongly binding to the column (highly hydrophobic compounds), but not through loss by a flow diverted to waste. Eluents and the columns used are ideally suited for this multimode separation and the ESI MS detection since the salt used for IC does not need a special device to remove or neutralize the eluent ions before ESI.

The selected metabolites were all retained by MMC and reached systematically longer t_R than through a separation by HILIC only. Although a limited number of compounds have been analyzed, but as they are typical for different class of metabolites, it can be concluded that a metabolome can be much more evenly spread over the whole chromatogram than a single mode chromatographic run can achieve. Only a few metabolites may appear at two t_R, which keeps the database search simple. However, a second t_R increases the identification correctness remarkably.

Limitations on detectable metabolites arising from the column used were evaluated and the results show that lipid derived fatty acids up to C_{12} chain length are well accessible. Compounds bearing a hydrophobic carbon chain longer than C_{12} and one or more charged groups will appear at t_R longer than 50 min. For such compounds a dedicated lipidomics platform would be the better choice.

Nevertheless, the simple procedure can provide an efficient and effective separation platform for metabolomics and environmental monitoring.

Acknowledgement

We thank Thermo for lending the P1 multimode column.

Table captions

Table 1 Differently functionalized and commercially available solid phases.

Table 2 Number of compounds in IDEOM database [3] according to net charge at pH 7.4 (n = 31’940) and logD (n = 34’933).

Table 3 Compounds investigated (biogenic and xenobiotic), their t_R and ions detected.

Table 4 Gradients and eluent flow program of the isocratic and the gradient pump.

Tables

Table 1.

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 particle
Trinity P2	Thermo	RP, SAX, WCX	in-pore modified and surface agglomerated particle

MM: Mixed-Mode

Table 2

	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	

Table 3

Metabolite / Compound	Formula	Mass [Da] monoisotope	Retention times and detected ions				
			$M+H]^+$ t_{R1}	$M+H]^+$ t_{R2} [a]	$M+NH_4]^+$	$M-H]^-$	$M+HCO_3]^-$
Amino acids							
Arginine	$C_6H_{14}N_4O_2$	174.11	27.7		-	-	
Aspartate	$C_4H_7NO_4$	133.04	18.1 (85%)	-	-	18.1 (15%)	-
Cysteine	$C_3H_7NO_2S$	121.02	30.2		-	-	
Cystine	$C_6H_{12}N_2O_4S_2$	240.02	18.3		-	-	-
Glutamate	$C_5H_9NO_4$	147.05	18.1 (42%)	25.0 (58%)	-	-	
Lysine	$C_6H_{14}N_2O_2$	146.11	25.0		-	-	
Phenylalanine	$C_9H_{11}NO_2$	165.08	15.0 (88%)	25.0 (12%)	-	-	
Tryptophan	$C_{11}H_{12}N_2O_2$	204.09	15.6 (18%)	26.6 (82%)	-	-	
Tyrosine	$C_9H_{11}NO_3$	181.07	15.5 (96%)	24.4 (4%)	-	-	
Acids							
Citrate	$C_6H_8O_7$	192.03	-	-	-	24.3	
Glucose 1-phosphate	$C_6H_{13}O_9P$	260.03	-	-	-	21.4	
Sugars							
Galacturonic acid	$C_6H_{10}O_7$	194.04			18.4		
Glucose / Fructose / Galactose [b]	$C_6H_{12}O_6$	180.06	-	-	13.8		
Ribose / Xylose [b]	$C_5H_{10}O_5$	150.05	-	-	12.9		
Sorbitol / Mannitol	$C_6H_{14}O_6$	182.08	-	-	15.5	-	
Sucrose	$C_{12}H_{22}O_{11}$	342.12					
Fatty acids derived compounds							
Butanoic acid	$C_4H_8O_2$	88.05	16.8		-		
Butyrate, 3-HO-	$C_4H_8O_3$	104.05	-		30.4		
Decalanoic acid-4-ctone	$C_{10}H_{18}O_2$	170.25	30.5 (83%)		30.5 (17%)		
Decanoic acid	$C_{10}H_{20}O_2$	172.15	26.1		-		
Dodecanoic (lauric) acid	$C_{12}H_{24}O_2$	200.18				26.1	
Dodecylsulfate	$C_{12}H_{26}SO_4$	266.16				41.4	
Hexane-1-sulfonate	$C_6H_{13}SO_3$	166.07				30.2	
Hexanoic acid	$C_6H_{12}O_2$	116.08	-		18.5		
Hexanoic acid 4-lactone	$C_6H_{10}O_2$	114.07	26.1		-	-	
Octanoic (caprylic) acid	$C_8H_{16}O_2$	144.12			29.3	-	
Octanoic acid-4-lactone	$C_8H_{14}O_2$	142.10			23.4		
Antioxidants							
Ascorbate	$C_6H_8O_6$	176.03	-	-	-	17.3	
Glutathione	$C_{10}H_{17}N_3O_6S$	307.08	19.0 (88%)	27.7 (12%)			
Glutathionedisulfide (GSSG)	$C_{20}H_{32}N_6O_{12}S_2$	612.15	-	-		22.1	
Tocopherol, α- (Vit. E)	$C_{29}H_{50}O_2$	430.38	35.9				
Miscellous							
Corticosterone	$C_{21}H_{30}O_4$	346.21	29.10				
Methylpyruvate	$C_4H_6O_3$	102.03	-	-	22.3	-	
Progesterone	$C_{21}H_{30}O_2$	314.22	31.20				
Putrescine	$C_4H_{12}N_2$	88.10	22.2				
Spermidine	$C_7H_{19}N_3$	145.16	28.8				
Uracil	$C_4H_4N_2O_2$	112.03	5.6				
Xenobiotics							
Clotrimazole [c]	$C_{22}H_{17}ClN_2$	344.11	32.1				
Fluconazole	$C_{13}H_{12}F_2N_6O$	306.10	27.7				
Sucralose	$C_{12}H_{19}O_8Cl_3$	396.01	-		26.9 (13%)		26.9 (87%)
EDTA	$C_{10}H_{16}N_2O_8$	292.09	-		-	24.2	

a) partially trapped compounds produce a second peak (see text)

b) stereoisomeres not separated

c) only ion $M-C_3H_3N_2]^+$ was observed

Table 4.

Time min	**Pump1** (isocratic) C flow µL/min	**Pump 2** (gradient) A %	B %	flow µL/min	Comment
0	25	100	0	260	Sample injection by auto sampler
3	25	100	0	260	Hydrophobics trapped on C_{18}, delivery pump stop
3	0	100	0	260	HILIC and IC gradients start
12	0	70	30	260	End of moderate (2%/min) gradient
18	0	0	100	260	End of steep HILIC and IC gradients (10%/min), start washout
21	0	0	100	260	End of wash out, MS divert valve switch and start RP-gradient
33	0	100	0	260	End of RP gradient, start of washout.
36	25	100	0	260	Divert valve switch and delivery pump 1 start, reconditioning C_{18} –trap by eluent C
50	25	100	0	260	End of wash out and columns reconditioned, ready for injection

Figures

Figure 1 Valves, connectivities, and flow paths applied in MMC. Valve positions and flows during HILIC and IEX run (1A, 3-21 min) and during RP run (1B, 21-36 min). The isocratic delivery pump flow was reduced to 0 during 3 – 36 min (see Table 4).

Figure 2 Nominal ACN-percentages on separators during gradients is shown. The programming starts with HILIC and IEX initial conditions (100% A) and decreases the ACN content down to 10% (100% B). After the washout period (18-21 min) the MS divert valve is switched and the RP-gradient starts.

Figure 3 Typical multimode separation chromatogram obtained from 18 standard compounds (1 phenylalanine, 2 ascorbate, 3 galacturonic acid, 4 glutamate, 5 cystine, 6 hexanoic acid, 7 glutathione, 8 glucose-1-posphate, 9 glutathionedisulfide, 10 lysine, 11 tryptophan, 12 sucralose, 13 fluconazole, 14 arginine, 15 cysteine, 16 clotrimazole, 17 tocopherol, 18 dodecylsulfate).

Figure 4 Apparent retention factors (k*) correlation between MMC and HILIC separation used for IDEOM data base. k* obtained from MMC are plotted (dashed line) against k* from IDEOM – HILIC measurements (♦) and compared to the correlation (solid line) between the IDEOM measured and predicted data (□).

IDEOM-HILIC measured and predicted t_R-entries were transformed to pH 7.4 and k* were calculated assuming t_0=1.85 min.

Figure 1 A

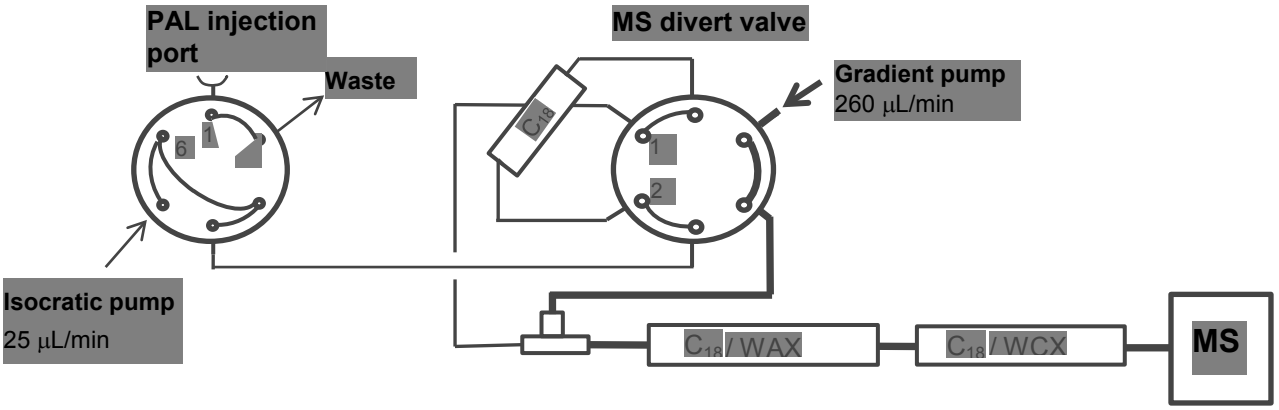


Figure 1 B

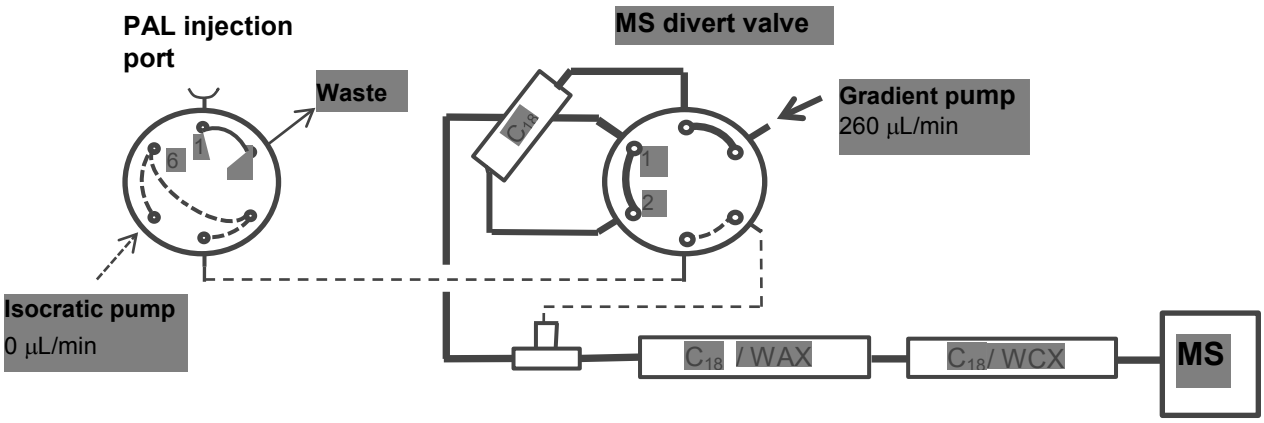


Figure 2

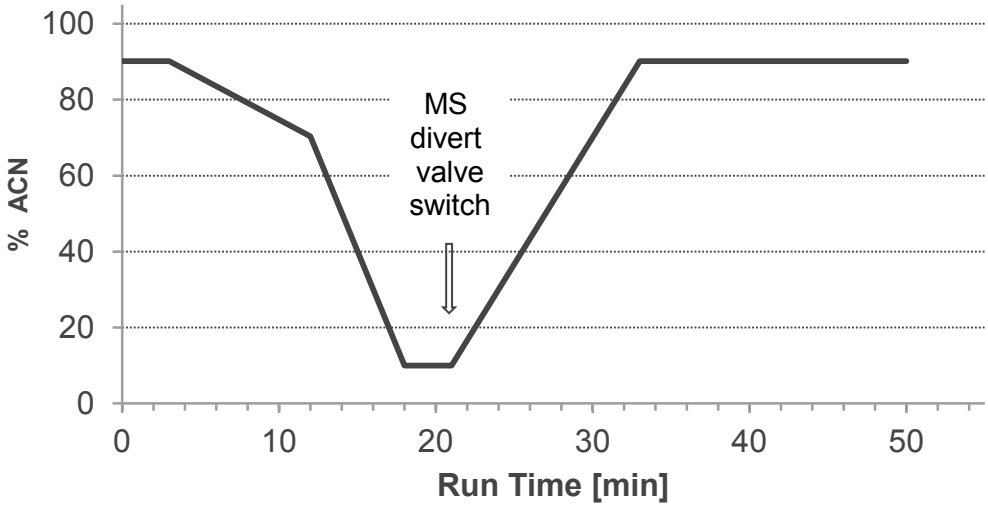


Figure 3

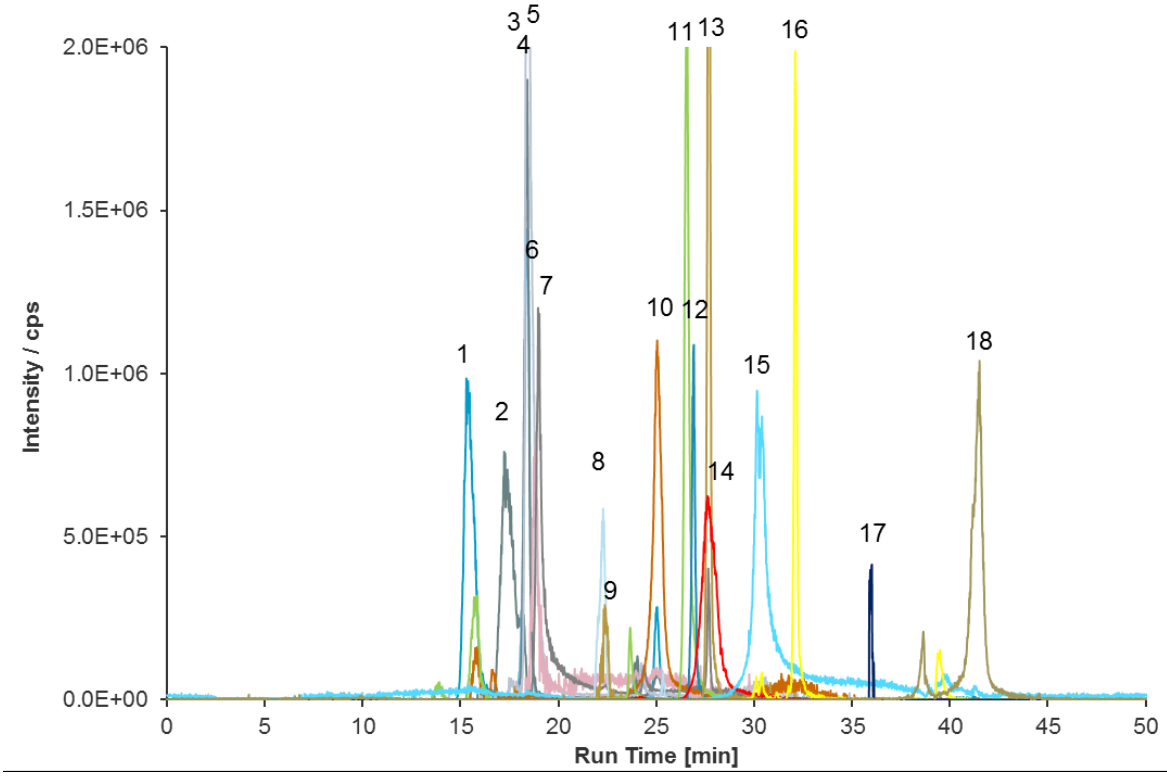
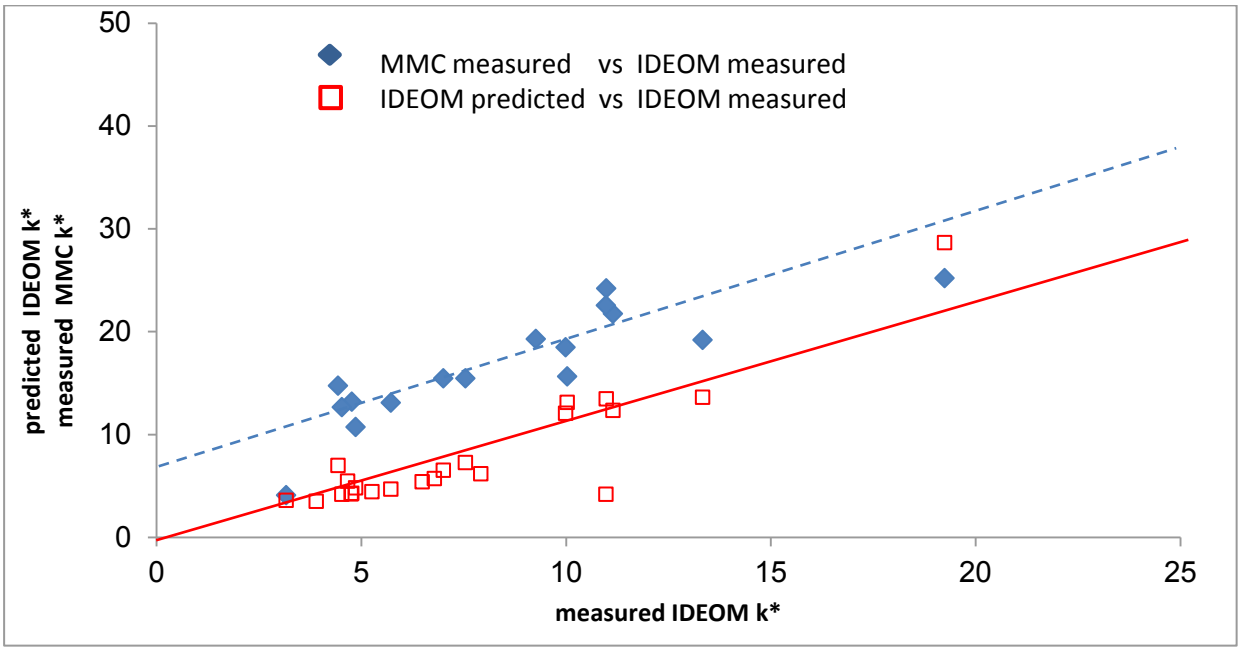


Figure 4



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