ONLINE BIOANALYTICAL MASS SPECTROMETRY METHODS FOR ANALYSIS OF SMALL AND LARGE BIOMOLECULES USING ONE- AND TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY

by

Yehia Zakaria Baghdady

DISSERTATION

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Supervising Committee:

Professor Kevin Schug, Chair

Professor Daniel Armstrong

Professor Krishnan Rajeshwar

Associate Professor Kayunta Johnson-Winters

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Abstract

Online Bioanalytical Mass Spectrometry Methods for Analysis of Small and Large Biomolecules Using One- and Two-Dimensional Liquid Chromatography

Yehia Baghdady, Ph.D.

The University of Texas at Arlington, 2018

Supervising Professor: Kevin A. Schug

In recent years, the innovation in biomarkers and biotherapeutics has increased the demand for state-of-the-art analytical platforms to achieve reliable and accurate discoveries and quantitative analysis methods. The challenges in their determinations are not limited to the large number of small and large biomolecules but also involve the great heterogeneity in chemical properties and abundance of those analytes. Liquid chromatography and mass spectrometry have advanced significantly to be the most common platform for analyzing biological samples. However, there are still many unmet challenges that that require application of novel bioanalytical methods to fill many gaps in online sample preparation, chromatographic separation, and mass spectrometric detection of different analyte sets. Frontal chromatography was used to investigate the trapping efficiency and capacity of online restricted access media trap columns to streamline sample preparation for online enrichment of small molecule analytes in complex biological samples. To enhance the sensitivity and selectivity for determination of 14 biomarkers of exposure to BTEX in urine, we developed a novel one pot charge reversal derivatization kit, optimized its performance through multivariate analysis using central composite design, and then validated its performance by using isotope dilution mass spectrometry. We further developed a novel high pH

reversed phase chromatographic method for orthogonal separation of intact proteins using mass spectrometry compatible buffers. Finally, we adopted this method as the first dimension in a comprehensive two-dimensional reversed phase x reversed phase-mass spectrometry platform for intact protein separations.

Contents

CHAPTER ONE: INTRODUCTION TO DISSERTATION

1.1 Introduction

Liquid chromatography mass spectrometry (LC–MS) has been the workhorse platform for bioanalysis due to its inherent sensitivity and selectivity, and its compatibility with analytes in biological matrices [1, 2]. Chemical derivatization can be online or offline integrated with LC–MS to reach the desired high detection sensitivity via changing the physicochemical properties of analytes and improving their ionization efficiencies [3, 4]. Through derivatization, the chromatographic behavior of the target analytes can also be intentionally altered to improve the separation selectivity prior to MS detection, and thus minimize ion suppression caused by coeluting matrix interferences [5-7]. *In situ* derivatization (ISD), where analytes are derivatized in their native biological matrices without any sample pretreatment, is an efficient and attractive way to achieve an ultrahigh sensitivity in a high throughput fashion. Chapter 2 reviews the literature on the ISD reagents that have been integrated with LC–MS. Analytes were divided into different chemical classes according to their target functional groups. In each class, we discussed some characteristic features and the useful outcomes achieved by adopting these ISD reagents.

Biological fluids are highly complex matrices with different classes of biomolecules (e.g. metabolites, lipids and proteins) [8]. Hence, sample preparation is usually the bottle neck of fast bio-analysis and the most error-prone part of the developed methods. To streamline sample preparation, incorporation of a restricted access media (RAM) trap column, which can be considered as an enhanced online solid phase extraction column, will allow segregation of sample classes to achieve the required cleanup and preconcentration of the analytes of interest [9]. The important chromatographic parameters to better understand the fundamentals and the optimization of online enrichment using RAM are covered in Chapter 3.

As previously mentioned, even with the current state-of the-art instruments, biological fluids are not suitable for direct analysis and ISD reagents alone or together with RAM columns can be integrated online with LC-MS to achieve the desired goals of a high throughput bioanalytical method. This motivated us to develop in our lab a novel charge reversal ISD kit for the bulk derivatization of 14 biomarkers of occupational exposure to benzene, toluene, ethylbenzene, and xylenes (BTEX) in human urine. To optimize the performance of this kit, we used a response surface method based on central composite design. BTEX metabolites, which have less responsive phenolic and carboxylic functional groups, were derivatized to highly responsive analytes with permanently positively-charged functional groups. Isotope dilution mass spectrometry, using deuterated internal standards, was then applied for their quantitative determination. Experimental details and results for this study are included in Chapter 4.

Intact proteins are large biomolecules with three dimensional structures that impose new challenges for their qualitative and quantitative determination using LC-MS. Increased recognition of their primary importance as potential candidates for biomarker discovery and new therapeutics has increased the demand for reliable LC-MS methods capable of their accurate characterization and/or quantitation [10]. Chromatographic challenges inherent to proteins include their low diffusivities and sorption kinetics, low recovery and carry over (memory effect), restricted diffusion, and mixed mode interactions [11]. Low pH reversed phase (RP) is among the most common LC modes used for intact protein separations because of its high resolving power and direct compatibility with MS detection. However, low pH and one-dimensional separation is usually insufficient for complex proteomic samples. Therefore, we investigated the use of MS-compatible high pH reversed phase for altered selectivity and efficient separation of intact proteins. The chromatographic details and the results of this work are provided in Chapter 5.

Comprehensive two-dimensional liquid chromatography (LC x LC) is currently the most powerful analytical platform capable of achieving very high peak capacities in the shortest possible time [12]. We successfully developed and optimized LC x LC-MS using the optimized high pH RP (Chapter 5) as the first dimension and low pH RP as the second dimension for the separation of a mixture of seven standard proteins and a proteome sample of *Escherichia coli*. Chapter 6 discuss the details and the results of this method.

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CHAPTER TWO: *IN SITU* DERIVATIZATION TECHNIQUES FOR ENHANCED BIOANALYSIS USING LIQUID CHROMATOGRAPHY WITH MASS SPECTROMETRY

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In Situ Derivatization Techniques for Enhanced Bioanalysis Using Liquid Chromatography with Mass Spectrometry

Yehia Z. Baghdady, Kevin A. Schug*

Department of Chemistry & Biochemistry, The University of Texas at Arlington, Arlington TX USA

*Correspondence to: 700 Planetarium Pl.; Box 19065; Arlington TX 76019-0065; (ph) 817-272-3541; (fax) 817-272-3808; (email) kschug@uta.edu

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2.1 Abstract

Accurate and specific analysis of target molecules in complex biological matrices remains a significant challenge, especially when ultra-trace detection limits are required. Liquid chromatography–mass spectrometry is often the method of choice for bioanalysis. Conventional sample preparation and clean-up methods prior to analysis of biological fluids such as liquid-liquid extraction, solid phase extraction, or protein precipitation are time-consuming, tedious, and can negatively affect target recovery and detection sensitivity. An alternative or complementary strategy is the use of an off-line or on-line *in situ* derivatization technique. *In situ* derivatization can be incorporated to directly derivatize target analytes in their native biological matrices, without any prior sample clean-up methods, to substitute or even enhance the extraction and pre-concentration efficiency of these traditional sample preparation methods. Designed appropriately, it can reduce the number of sample preparation steps necessary prior to analysis. Moreover, *in situ* derivatization can be used to enhance the performance of the developed liquid chromatography-mass spectrometry-based bioanalysis methods regarding stability, chromatographic separation, selectivity, and ionization efficiency. This review presents an overview of the commonly used *in situ* derivatization techniques coupled to liquid chromatography-mass spectrometry-based bioanalysis to guide and to stimulate future research.

2.2 Introduction

Liquid chromatography-mass spectrometry (LC-MS) is one of the most widely used analytical methods for bioanalysis due to its inherent selectivity and sensitivity, and its compatibility with biological matrices and analytes [1]. Atmospheric pressure ionization (API) sources have contributed to the successful application of LC-MS for the analysis of complex biological samples. Atmospheric pressure chemical ionization (APCI) is applicable for low to medium polarity compounds having high proton-affinity atoms, such as oxygen, while electrospray ionization (ESI) can be applied for more polar compounds and those amenable to form ionic species in solution or during the electrospray process. However, some major limitations still exist, often due to very low concentrations in biological matrices, poor retention, and/or weak ionization efficiency of some analytes, which affect sensitivity and applicability of the developed bioanalytical method [2, 3].

To achieve ultra-high sensitivity in various ionization modes, chemical derivatization can be integrated with LC-MS either online or offline and pre- or post-column to change the structure of the analyte and to affect its chemical and physical properties to reach the desired sensitivity [4, 5]. Chemical derivatization can also be integrated to affect chromatographic behavior to improve selectivity and efficiency of separated analytes before reaching the MS detector, and thus minimize ion suppression caused by co-eluting matrix interferences [6-8]. Extraction efficiency in the case of LLE and SPE can also be increased by using derivatization prior to analyte extraction and concentration [9-11]. The stability of some analytes during collection, storage, and handling has been shown to be preserved by derivatization [12].

Despite all the advantages mentioned above, the integration of derivatization with LC-MS for bioanalysis is still limited by some shortcomings, such as formation of byproducts after

derivatization, stability of formed derivatives, loss of analytes due to multistep sample preparation, and time consumption. Most of these disadvantages can be overcome by the proper selection and optimization of the derivatization parameters and testing the derivatization yield and products before integration with LC-MS. The increase in number of steps of sample preparation caused by adopting derivatization in the sample preparation procedures, will still be a problem. Thus, a major advantage of performing *in situ* derivatization (ISD), where the analytes are derivatized in their native biological matrices, is that it can obviate the need for clean-up procedures prior to derivatization and allow for sensitive and selective measurements in a reasonable time frame.

Chemical derivatization reagents should have two unique characteristics to be used for ISD. They need the ability to be added in high concentrations without causing significant ion suppression, and they need to have high selectivity for target analytes to allow for complete derivatization in the presence of other matrix components. The reaction profile for the derivatization reagents should also be compatible with the generally aqueous biological matrix.

ISD is an efficient way to improve the sensitivity of detection, and at the same time, decrease the required number of sample preparation steps as compared with ex situ derivatization (i.e., derivatization of previously purified or extracted analytes). However, adding large amounts of derivatization reagent or prolonging the time of derivatization reaction to bulk derivatize the biosamples can cause a dilution effect and sometimes formation of byproducts. This is exemplified in work by Yang and Attygale, as shown in Fig. 2-1 [13]. An additional LLE or SPE step, or use of online restricted access media, may be required prior to LC separation to remove this excess reagent, to overcome dilution effect by pre-concentration, and to reduce any associated ion suppression. However, the ultra-high sensitivity that can be reached can balance these disadvantages.

So far, a number of derivatization reagents have been reported for LC-MS and they are summarized in several review papers [14-21]. In this review we focused only on the derivatization reagents which have been utilized for ISD and have been integrated with LC-MS. Analytes targeted for ISD in complex biological fluids were divided into different classes according to their target functional groups. In each chemical class, we discussed some of the features that characterize ISD reagents and the useful outcomes achieved by integrating these reagents with the analysis. We did not discuss the specific conditions associated with each adopted ISD reaction. These details were excluded for the sake of brevity. Detailed information can be found in individual references. We hope that this review article will guide and stimulate further research concerns in this particular area. While we tried to be comprehensive in our review, we apologize if we inadvertently overlooked a published work that should have been incorporated.

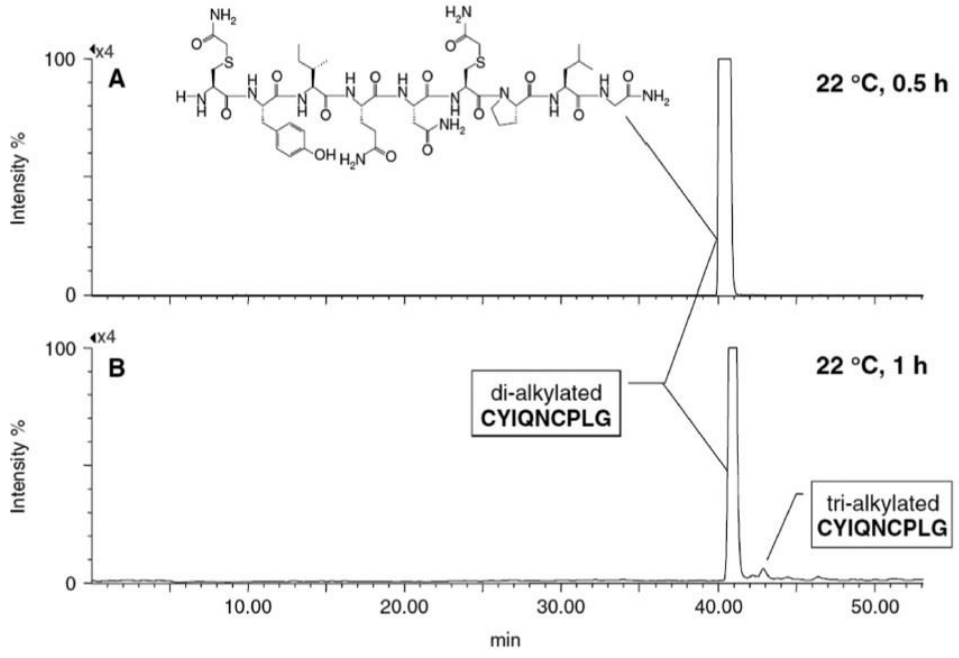

Figure 2-1. LC/MS profiles showing the formation of tri-alkylated by-product obtained from aliquots withdrawn from the reaction mixture of reduced oxytocin and iodoacetamide after 0.5 (A) and 1.0 h (B). Reproduced with permission from John Wiley & Sons, Ltd (copyright 2007) [13].

2.3 Aldehydes and ketones

Aldehydes and ketones have neutral carbonyl groups which decrease their ionization efficiencies when using API. To enhance ionization efficiency, a chargeable moiety can be introduced via ISD. Such derivatization strategies can be used to eliminate the volatile and reactive nature of aliphatic-chain-based aldehydes and ketones to make them more amenable for LC-MS analysis. A schematic diagram for the reaction of some common ISD reagents with aldehydes and ketones is shown in Fig. 2-2. ISD reagents and their applications for aldehydes and ketones are summarized in Table 2-1.

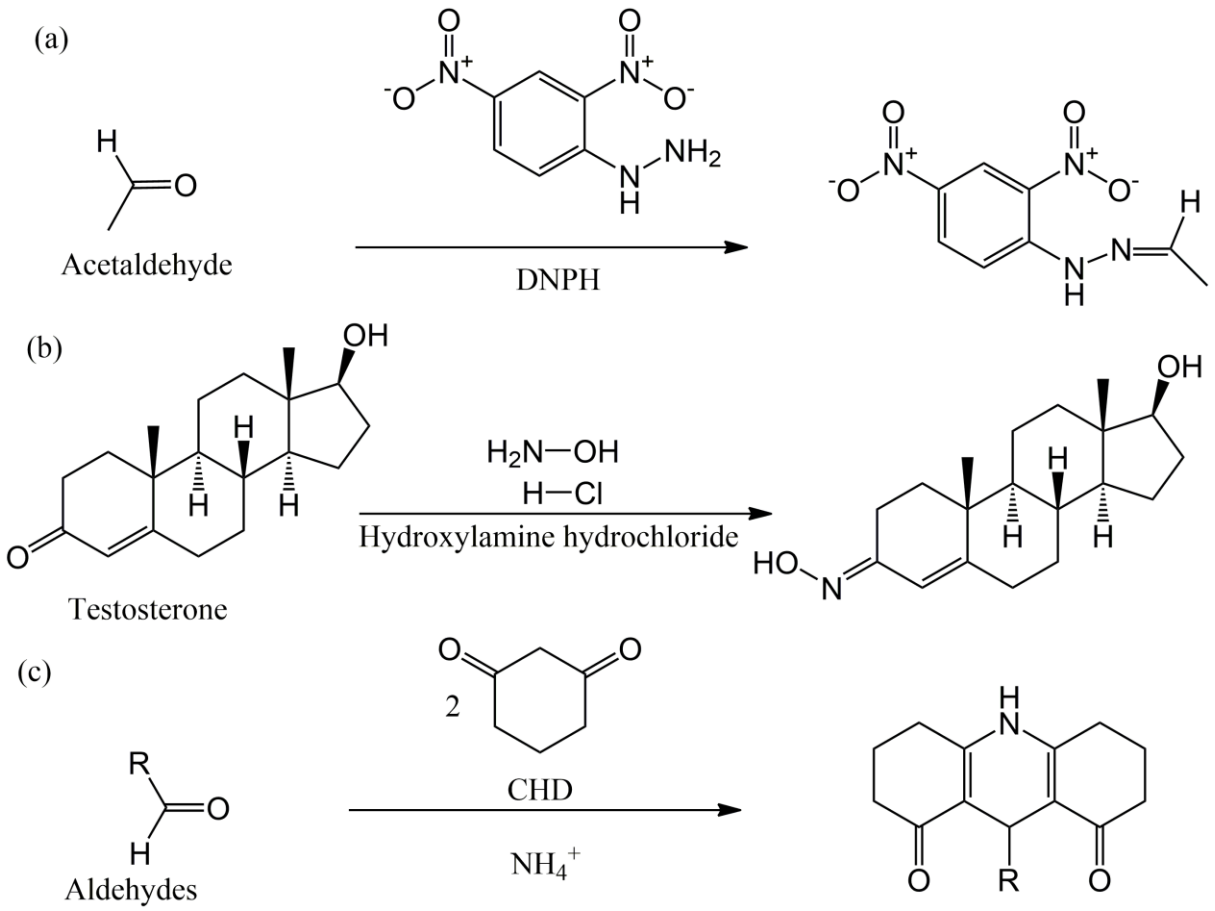

Figure 2-2. Example derivatization reactions for aldehydes and ketones: (a) DNPH; (b) Hydroxylamine hydrochloride; and (c) CHD.

2,4-Dinitrophenylhydrazine (DNPH, Brady's reagent) can react with aldehydes and ketones to form dinitrophenylhydrazone derivatives. The formed derivatives showed high sensitivity due to introduction of readily ionizable groups. DNPH can be added directly to biosamples [22-25] or can be impregnated in SPE column materials to facilitate cleanup, *in situ* derivatization, and preconcentration of aldehydes from matrices, such as urine [26].

Table 2-1. ISD reagents for aldehydes and ketones

Analyte (s)	Bio-matrix	Derivatization reagent	Ionization source	Reference
3-oxo sterols	Human and mouse plasma	Girard's reagent P	+ ESI	[27]
Succinic semialdehyde	Human urine and cerebrospinal fluid	2,4-dinitrophenyl hydrazine (DNPH)	- ESI	[22]
Malondialdehyde	Human urine	2,4-dinitrophenylhydrazine (DNPH)	+ ESI	[25]
Malondialdehyde, pentanal, hexanal, heptanal, octanal, and decanal	Human urine	4-(2-(trimethylammonio)ethoxy)benzenaminium halide (4-APC)	+ ESI	[31]
Aldehydes (pentanal, hexanal, heptanal, octanal, nonanal, and decanal)	Human urine and plasma	4-(2-((4-bromophenethyl) dimethylammonio)ethoxy) benzenaminium dibromide (4-APEBA)	+ ESI	[32]
Alkanals, alkenals and hydroxyalkenals	Human plasma	Cyclohexanedione (CHD)	+ ESI	[30]
Malondialdehyde (MDA) and 4-hydroxynonenal (HNE)	Human exhaled breath condensate samples	2,4-dinitro-3,5,6-trideuterophenylhydrazine (d_3-DNPH) as "heavy" reagent in combination with commercial "light" DNPH	+ ESI for MDA - ESI for HNE	[24]
Houttuynin (decanoyl acetaldehyde)	Human plasma	2,4-dinitrophenylhydrazine (DNPH)	+ ESI	[23]
Formaldehyde, acetaldehyde, hexaldehyde, acrolein, propionaldehyde, crotonaldehyde, butyraldehyde and valeraldehyde	Human urine	2,4-dinitrophenylhydrazine (DNPH)	- ESI	[26]
Testosterone	Human serum	Hydroxylamine hydrochloride	+ ESI	[28]
Succinylacetone	Human urine	Hydroxylamine hydrochloride	+ ESI	[29]

DeBarber *et al.* used Girard's reagent P (1-(carboxymethyl) pyridinium chloride to derivatize 3-oxo sterols in human and mouse plasma by forming charged hydrazine derivatives. This enabled the ESI-MS analysis of these sterols in the presence of a large excess (3 orders of magnitude) of cholesterol without prior SPE removal of cholesterol. The signal enhancement factor for Girard derivatization relative to analysis of underivatized samples was more than 30-fold [27].

Hydroxylamine hydrochloride can be used to derivatize ketones to form the corresponding oximes. This was successfully applied to the analysis of testosterone [28] in human serum where a limit of quantitation (LOQ) of 0.035 nM was achieved. In another study, for the determination of succinyl acetone for the diagnosis of hepatorenal tyrosinemia, urine samples were oximated with hydroxylamine hydrochloride, extracted by LLE, and followed by derivatization with butanolic HCl. The butylated isoxazole derivatives of succinyl acetone and its internal standard were detected and quantified; a LOQ of 0.063 µM was achieved [29].

Cyclohexanedione (CHD) has been shown to provide high selectivity for lipid aldehydes in human plasma [30], where other carbonyl compounds did not react. CHD derivatives showed excellent ESI response which decreased with increasing chain length. The highest response of 10 pg on column was for derivatized hexanal and the lowest of 100 pg was for derivatized decanal. CHD derivatization caused the formation of common product ions at m/z of 216 under MS/MS conditions for all classes of aldehydes which was useful as a detection strategy when precursor ion scans were performed. However, such a common product ion transition for different derivatized analytes can also be a drawback, since the product ion does not contain any information diagnostic to the initial precursor ion. Thus, while precursor ions selected can still be unique, the lack of

unique product ions can hamper specificity for extremely complex samples. Such situations should be treated with caution and carefully controlled.

4-(2-(trimethylammonio)ethoxy)benzenaminium halide (4-APC) has a quaternary ammonium group, besides an aniline moiety, which can selectively react with aliphatic aldehydes. This permanent positive charge will create enhanced ESI sensitivity and can provide for the use of unique extraction and separation strategies, perhaps through the use of ionic exchange-based media. In one demonstration of the use of 4-APC, limits of detection of 3-33 nM for a series of aldehyde derivatives were achieved. 4-APC derivatives could also be used to monitor the typical losses of m/z 59 and m/z 87 in tandem MS screening for aldehydes [31].

To enhance the ability for detection and screening of unknown aldehydes, as well as carboxylic acids, Eggink *et al.* used 4-(2-((4-bromophenethyl)dimethylammonio)ethoxy) benzenaminium dibromide (4-APEBA) as a derivatizating reagent [32]. It has a bromophenethyl group which exhibits selective fragmentation identifiers and helps to provide an isotopic signature (based on this incorporation of bromine) to the derivatives. 4-APEBA derivatives contain a permanent positive charge enabling them to be readily analyzed in positive ionization mode ESI. This could also be a useful strategy to allow exploration of novel preparation and separation means after derivatization, since cation exchange concepts could be used to selectively isolate the permanently-positively-charged analytes. Furthermore, the total molecular mass of 4-APEBA derivatives was found to be higher than that of 4-APC derivatives resulting in a higher signal-to-noise ratio of analytes in analyses performed from biological matrices.

2.4 Alcohols, phenols and other hydroxyl-bearing compounds

Alcohols and phenols are generally neutral compounds across the range of pHs used in liquid chromatography. Some can be highly hydrophilic, making it difficult for them to be well

retained in reversed phase liquid chromatography. Their ionization efficiency can also be very poor, especially when utilizing ESI. Therefore, the integration of derivatization, with the developed bioanalytical methods, can increase chromatographic retention, ionization efficiency, and the ability to develop methods that provide optimal sensitivity and reproducibility. A schematic diagram for the reaction of common ISD reagents used to target alcohols, phenols and other hydroxyl-bearing compounds is shown in Fig. 2-3. ISD reagents for alcohols, phenols and other hydroxyl-bearing compounds are summarized in Table 2-2.

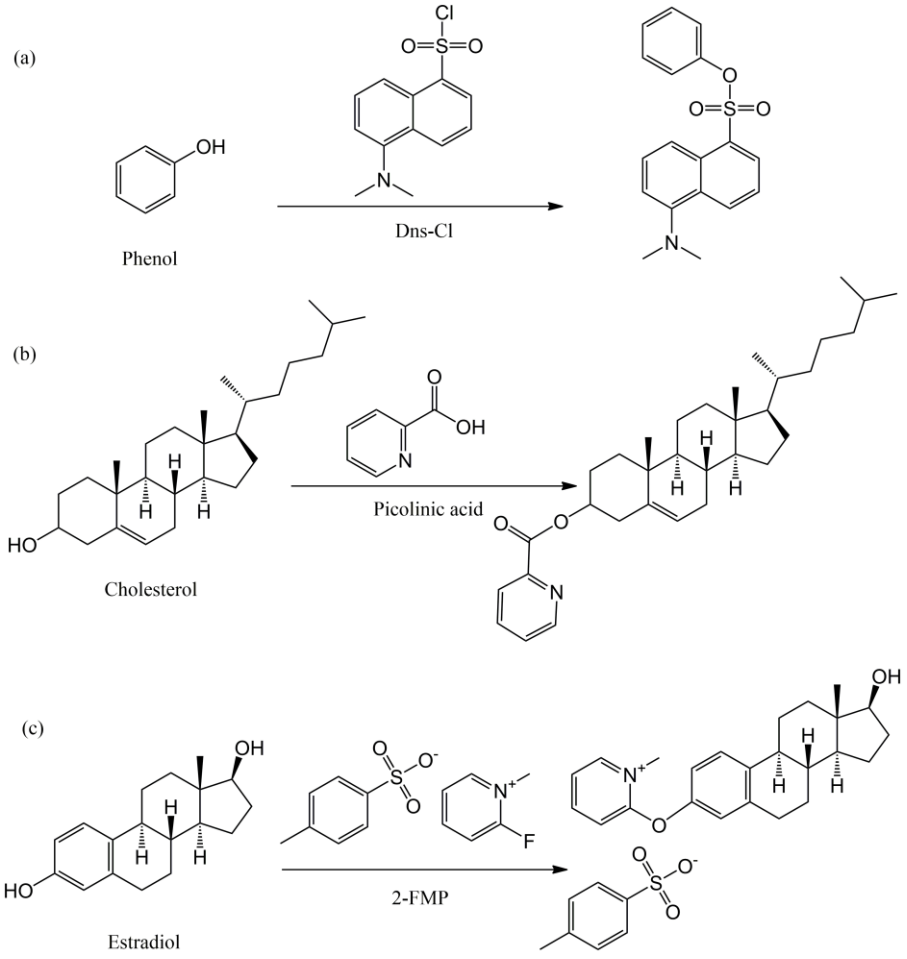

Figure 2-3. Example derivatization reactions for alcohols and phenols: (a) Dns-Cl; (b) Picolinic acid; and (c) 2-FMP.

Table 2-2. ISD reagents for alcohols and phenols

Analyte (s)	Bio-matrix	Derivatization reagent	Ionization source	Reference
Bisphenol A	Human saliva	Dansyl chloride	+ ESI	[38]
D- and *L*-2-Hydroxyglutarate Enantiomers	Human urine	Diacetyl-*L*-tartaric anhydride (DATAN)	- ESI	[44]
Neutral Sterols	Dried Human serum	Picolinic acid	+ ESI	[41]
Estrone, estradiols and estriol	Human and bovine serum	2-fluoro-1-methylpyridium p-toluenesulfonate (2-FMP)	+ ESI	[42]
Estrone, 17 α-estradiol, 17 β-estradiol, and estriol	Human cerebrospinal fluid	Dansyl chloride	Dual ionization source (positive mode)	[36]
Amine- and phenol-containing metabolites	Human cerebrospinal fluid	^{12}C-dansyl and ^{13}C-dansyl chloride	+ ESI	[39]
Amines and phenolic metabolites	Human urine	Triplex $^{12}C_4$-,$^{12}C_2{}^{13}C_2$-, and $^{13}C_4$-5-diethylamino-naphthalene-1-sulfonyl chloride (DensCl)	+ ESI	[40]

One of the most commonly used reagents is 5-dimethylamino-1-naphthalenesulfonyl chloride (dansyl chloride; Dns-Cl). Weber was the first one to design and use Dns-Cl in 1952 [33]. Dns-Cl was initially used to form a yellow-green fluorescent derivative with primary and secondary amines [34]. Therefore Dns-Cl derivatives can be detected by their fluorescence emission following normal phase or reversed-phase liquid chromatography [35]. Later on, Dns-Cl was adopted in LC-MS to improve both the chargeability and hydrophobicity of analytes, as it has a tertiary amino group as an ionizable moiety and an aromatic napthyl moiety as a hydrophobic moiety. Schug and coworkers published a method for the simultaneous detection of four native estrogens in human cerebrospinal fluid using Dns-Cl as the ISD reagent to study neuroprotective effects of estrogens and their diffusion across the blood–brain barrier [36]. They combined the ISD

with the restricted access media (RAM) [37] for on-line sample prep where biologically relevant sensitivities on the order of pg/mL were achieved. They also used Dns-Cl for bulk derivatization of bisphenol A in human saliva to reach pg/mL detection limit [38].

Dansylation was also adopted by another group for the qualitative metabolome analysis of amine- and phenol-containing metabolites in human cerebrospinal fluid (CSF). An average of 519 ^{13}C-/^{12}C- dansylated ion pairs were observed in a 25-min LC-MS run with the injection of an equivalent of 0.5 μL of the original CSF sample and 1132 unique ion pairs were detected in total from combined results of four different CSF samples [39]. Similarly, 5-diethylamino-naphthalene-1-sulfonyl chloride (Dens-Cl) was used to bulk derivatize the amine and phenol submetabolome in biological samples, by adding a hydrophobic moiety and a readily ionizable moiety [40]. Compared to Dns-Cl (dimethylamino), Dens-Cl has ethyl moieties appended to the amine functional unit, which can further enhance the ionization efficiency of derivatized analytes analyzed by ESI-MS.

Honda *et al*. derivatized sterols in dried serum into picolinyl esters using picolinic acid as an ISD reagent. The limit of detection of cholesterol picolinate was about 100 fg on-column, which was about 3,860 times more sensitive than that of native cholesterol [41]. Their developed method used only 1 μL of serum for the analysis, which is useful for the serological diagnosis of inherited disorders in cholesterol metabolism.

2-fluoro-1-methylpyridium p-toluenesulfonate (2-FMP) is another reagent that has been used for ISD of estrogens in human and bovine serum [42]. Dns-Cl was excluded for consideration in this application due to its pH-dependent charging; 2-FMP is another reagent that will impart a permanent positive charge onto the derivatized analyte. Both ISD using 2-FMP and weak cation exchange RAM-based trap-and-elute strategies were combined as an on-line pretreatment to reach

ultra-trace levels of detection for estrogens at low pg/mL concentration levels. 2-FMP was also used for the derivatization of cysteine during LC-MS analysis of biologically-active hydrophilic compounds related to a study of cysteine dioxygenase (CDO) activity [43]. This latter work demonstrated the use of 2-FMP for targeted derivatization of thiol-containing compounds; additional strategies for targeted ISD of thiol-containing compounds are covered later in this review.

For differentiation of enantiomers, diacetyl-*L*-tartaric anhydride (DATAN) was used as an ISD reagent to target *D*- and *L*-2- hydroxyglutarate enantiomers in human urine. Derivatization of the enantiomers using the chirally-pure reagent resulted in the formation of diastereomers, which could then be separated on an achiral column [44]. Such a strategy is very advantageous as current research aims to understand the role and activity of chiral biological molecules and the interplay between compounds originating from eukaryotic vs. prokaryotic organisms in biological systems.

2.5 Amines

Amines can generally be easily protonated under acidic conditions and thereby detected with reasonable sensitivity using positive ionization mode ESI-MS. However, detection sensitivity may not be high enough to enable some trace determination applications, due to background noise caused by matrix interferences, especially for low molecular weight amines, which might appear in the noisier low mass spectral range. Thus, chemical derivatization can provide an increase in molecular weight, which makes their detection possible in the high mass range, further from interfering matrix components. Other challenges for sensitive determination of amines include their polarity, basic character, and high water solubility [45]. Derivatization can make amine-containing molecules more hydrophobic, which can provide for better extraction efficiency, an

ability to use higher percentages of organic mobile phases for separation, and better resolution, due to increased retention on a reversed phase column. A schematic diagram for the reaction of the common ISD reagents used with amines is shown in Fig. 2-4. ISD reagents for amines are summarized in Table 2-3.

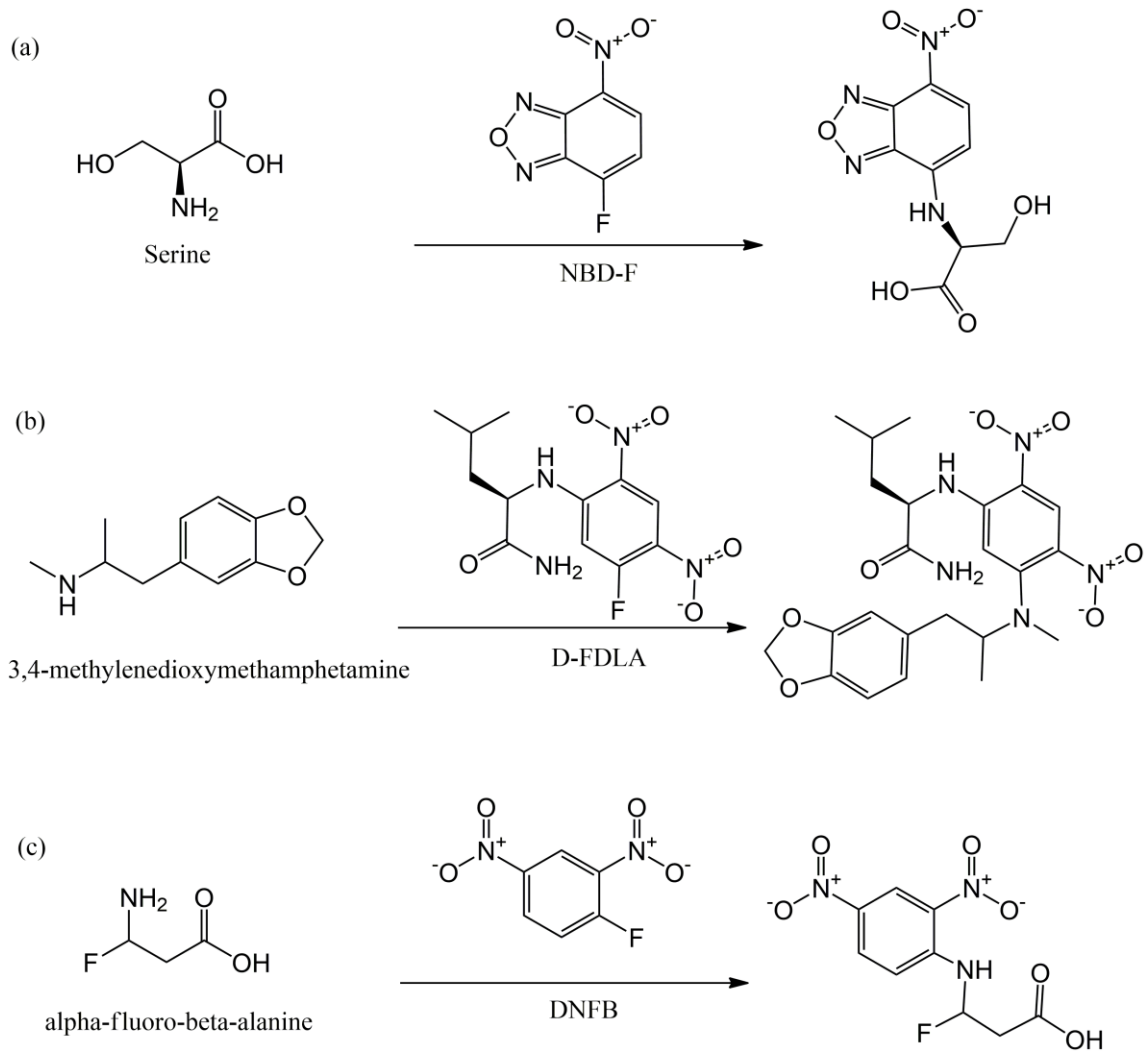

Figure 2-4. Example derivatization reactions for amines: (a) NBD-F; (b) D-FDLA; and (c) DNFB.

Dansyl chloride derivatization has been successfully applied to greatly enhance ionization efficiency of amines [46] or amines and phenols [39, 47, 48] in the positive ionization mode ESI

in several studies. Isobutyl chloroformate was utilized in dispersive derivatization liquid–liquid microextraction (DDLLME) of amino acids in human urine [49]. In comparison with LLE, DDLLLME requires shorter time and much less solvent. Detection limits of the amino acids were in the range of 0.05–0.1 ng/mL and sarcosine was chromatographically baseline resolved from alanine isomers, to allow its sensitive and accurate quantification in human urine. Cimlová *et al.* used isobutyl chloroformate to achieve a detection limit of 0.2 μM for the urinary dipeptide biomarker L-prolyl-4-L-hydroxyproline (PHP) in human urine [50]. The coupled action of isobutanol, isobutyl chloroformate, and the pyridine catalyst enabled the conversion of highly polar low molecular weight PHP into the N-isobutyloxycarbonyl isobutyl ester. This allowed its elution away from the column dead volume where interferences from highly polar components of urine matrix often elute.

A chiral derivatization method was adopted to measure enantiomers of 3,4-methylenedioxymethamphetamine (MDMA) and its metabolites (3,4-methylenedioxyamphetamine, 4-hydroxy-3-methoxymethamphetamine (HMMA), HMMA glucuronide, and HMMA sulfate in human urine (Fig. 2-5). This method utilized Marfey's reagent, Nα- (5-fluoro-2,4-dinitrophenyl)- D-leucinamide (D-FDLA). D-FDLA enabled direct derivatization of amino groups of target analytes in aqueous media, such as urine, without prior hydrolysis or extraction. The limits of detection of these analytes ranged from 0.01 to 0.03 μg/ml [51].

Derivatization with 7-fluoro-4-nitrobenzoxadiazole (NBD-F) has been successfully used to achieve both effective in-line pre-concentration and sensitive MS detection of the amino acids in the central nervous system and heart tissues of Aplysia californica. Limits of detection between 96 and 160 ng/mL were reached [52]. 4-bromobenzenesulfonyl chloride has been used for

Table 2-3. ISD reagents for amines

Analyte (s)	Bio-matrix	Derivatization reagent	Ionization source	Reference
Enantiomers 3,4-methylenedioxymethamphetamine and its metabolites (3,4-methylenedioxyamphetamine, 4-hydroxy-3-methoxymethamphetamine; HMMA), HMMA glucuronide, and HMMA sulfate	Human urine	Marfey's reagent, N^{α}- (5-fluoro-2,4-dinitrophenyl)- D-leucinamide (D-FDLA)	+ ESI	[51]
Amine- and phenol-containing metabolites	Human cerebrospinal fluid	^{12}C-dansyl and ^{13}C-dansyl chloride	+ ESI	[39]
Amines and phenolic metabolites	Human urine	Triplex $^{12}C_4$-,$^{12}C_2{}^{13}C_2$-, and $^{13}C_4$-5-diethylamino-naphthalene-1-sulfonyl chloride (DensCl)	+ ESI	[40]
D-Ser, *D*-Asp, and *D*-Glu. (*D, L* -amino acids)	Central nervous system and heart tissues of Aplysia Californica	7-fluoro-4-nitrobenzoxadiazole (NBD-F)	+ ESI	[52]
Gemcitabine and 2,2-difluoro-2-deoxyuridine	Human plasma	Dansyl chloride	Positive ionization mode Turbo Ion spray interface (TIS)	[46]
Metabolites containing primary amine, secondary amine, or phenolic hydroxyl group(s).	Human urine	^{13}Cdansyl chloride, along with ^{12}C-dansyl chloride	+ ESI	[47]
Glycine	Human cerebrospinal fluid	Edman's reagent (phenylisothiocyanate; [PITC])	+ ESI	[54]
Risperidone, 9-hydroxyrisperidone, monoamine and amino acid neurotransmitters	Human urine	Dns-Cl	+ ESI	[48]
α-fluoro-β-alanine	Human urine	Sanger's reagent, 2,4-dinitrofluorobenzene (DNFB)	- ESI	[55]
Histamine and its major metabolite tele-methylhistamine	Human cerebrospinal fluid	4-bromobenzenesulfonyl chloride	+ ESI	[53]
Sarcosine, alanine, leucine and proline	Human urine	Isobutyl chloroformate	+ ESI	[49]
L-prolyl-4-L-hydroxyproline (PHP)	Human Urine	Isobutyl chloroformate	+ ESI	[50]

derivatization of primary amines in human cerebrospinal fluid. The stronger isotopic signals with diagnostic duplicate peaks of the two isotopes of bromine and the isolation of the hydrophobic derivatives on a C18 column allowed the sensitive mass spectrometric detection of the bromine-containing derivatives [53]. A limit of detection of 12.5 pM for each derivatized amine was achieved by MS/MS analysis and positive ESI.

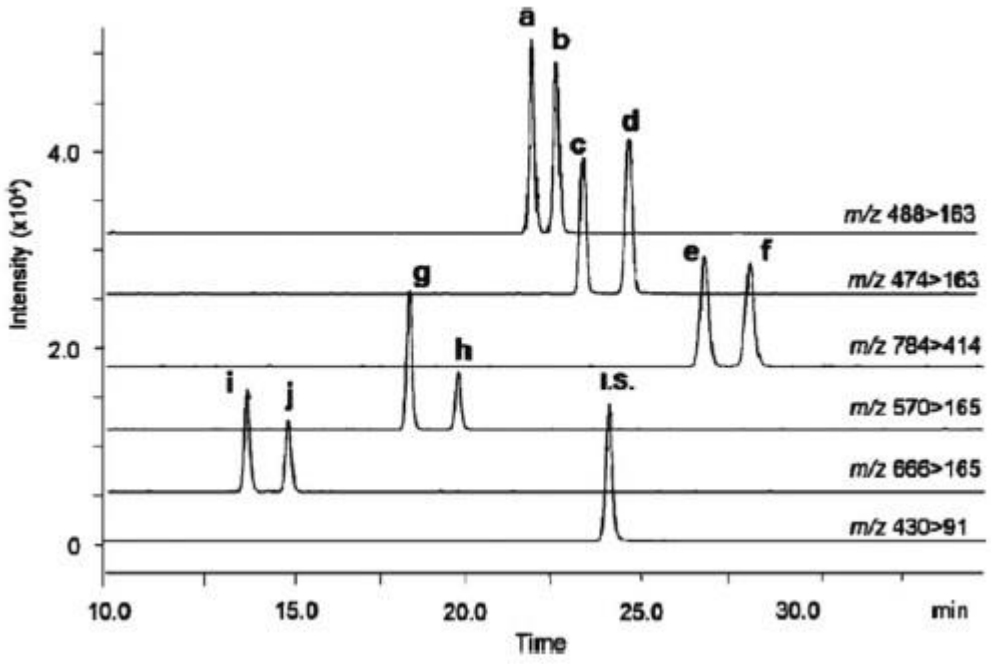

Figure 2-5. Multiple reaction monitoring (MRM) chromatograms of DDLA derivatives of MDMA and its metabolites added to drug-free human urine at 1 µg/mL for each one (2 µg/mL as the racemic mixture) Peaks: a (S)-MDMA, b (R)-MDMA, c (R)-MDA, d (S)-MDA, e (S)- HMMA, f (R)-HMMA, g (S)-HMMA-sul, h (R)-HMMA-sul, i (S)- HMMA-glu, j (R)-HMMA-glu. Reproduced with permission from Springer (copyright 2012) [51].

Wilson *et al.* adopted Edman's reagent (phenylisothiocyanate, [PITC]) for the quantification of glycine, an extremely hydrophilic amino acid, in human CSF [54]. The derivatization procedure was shown to have the advantage of being readily adaptable to a modern 96-well plate. The LC–MS/MS based bioanalytical analysis was able to be performed at room

temperature. The resulting phenylthiocarbamyl derivative (PTC-glycine) could be readily separated and quantified using reversed-phase LC-MS/MS, where a lower limit of quantitation of 50 ng/mL was achieved.

In another work, Sanger's reagent (2,4-dinitrofluorobenzene, [DNFB]) [55] was successfully used for the ISD of α-fluoro-β-alanine (FBAL). FBAL is the main metabolite of the antineoplastic drug 5-fluorouracil and the method was developed to target this analyte in human urine. The researchers used a pre-column FBAL derivatization followed by solid phase extraction sample clean-up to achieve a quantification limit of 1 μg/L.

2.6 Carboxylic acids

Matrix effects and background noise often interfere with the sensitivity required for the detection of carboxylic acids in complex biological matrices using negative ESI-MS. Furthermore, mobile phases used for separation of carboxylic acid-containing compounds (acidified to promote reversed phase retention) are not always compatible with negative ionization mode ESI-MS. Therefore, carboxylic acids may be derivatized to more hydrophobic non-ionic or basic derivatives to enable their more sensitive detection using positive ESI or APCI. A schematic diagram for the reaction of the common ISD reagents used for determination of carboxylic acids is shown in Fig. 2-6. ISD reagents for carboxylic acids are summarized in Table 2-4.

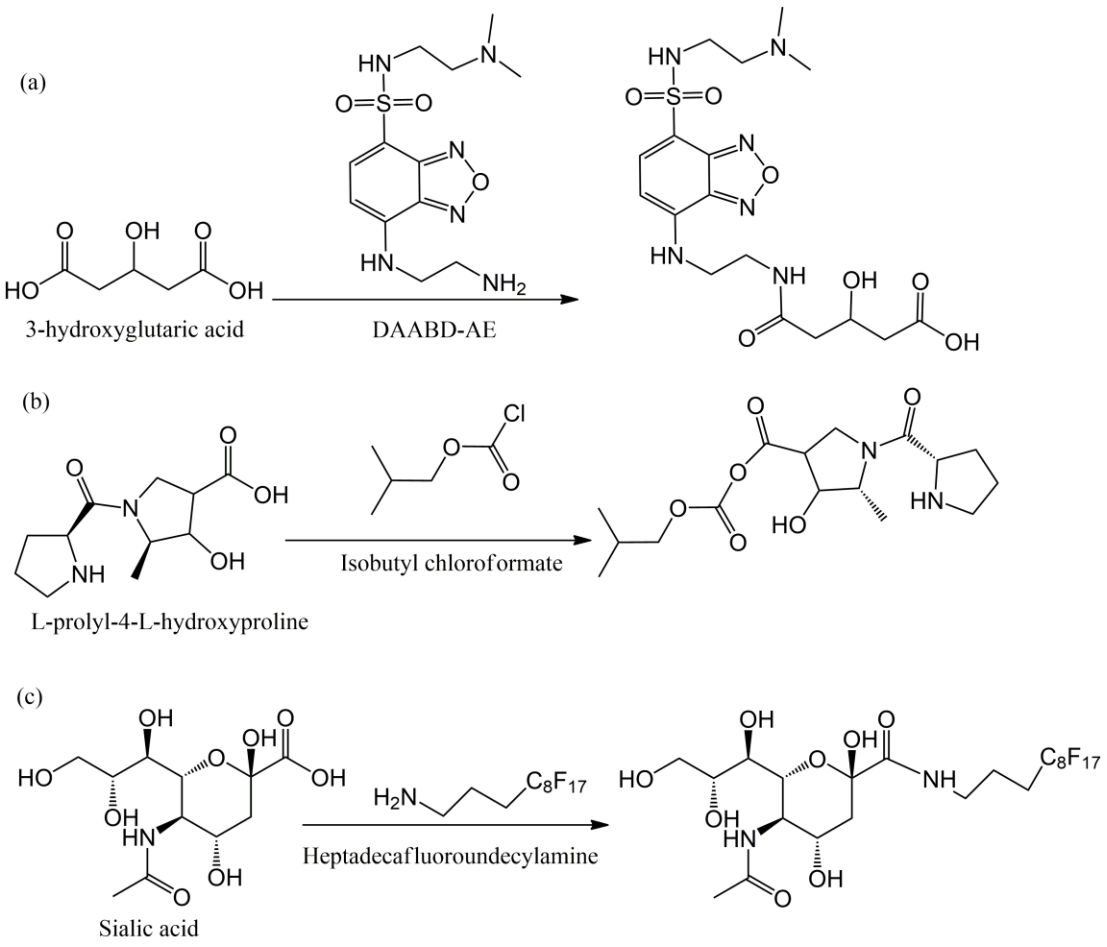

Figure 2-6. Example derivatization reactions for carboxylic acids: Use of (a) DAABD-AE; (b) Isobutyl chloroformate; and (c) Heptadecafluoroundecylamine.

Hayama *et al.* developed a fluorous derivatization technique, where the carboxylic groups in sialic acids were derivatized through amidation [56]. Amidation, using heptadecafluoroundecyl-amine, enabled a separation-oriented derivatization by attaching perfluoroalkyl groups to the sialic acids. Fluorous derivatives can be retained specifically on LC columns possessing fluorous bonded phases. Moreover, the hydrophobicity of fluorous derivatization reagents enhanced the ESI response. The detection limits of sialic acids were determined to be in the range of 60–750 amol on column when this strategy was used.

Table 2-4. ISD reagents for carboxylic acids

Analyte (s)	Bio-matrix	Derivatization reagent	Ionization source	Reference
Sialic acids (N-acetylneuraminic, N-glycolylneuraminic, and 2-keto-3-deoxy-D-glycero-D-galactonononic acid)	Human urine and fetuin (bovine serum) samples	Heptadecafluoroundecylamine,	+ ESI	[56]
L-prolyl-4-L-hydroxyproline	Human Urine	Isobutyl chloroformate	+ ESI	[50]
Glutaric and 3-Hydroxyglutaric acids	Human dried urine spots	4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethyl amino)-2,1,3-benzoxadiazole (DAABD-AE)	+ ESI	[58]
Glutaric and 3-hydroxyglutaric acids	Human urine	4-[2-(N,N-dimethyl amino) ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE)	+ ESI	[57]
Methylmalonic acid	Human serum	2,3,4,5,6-pentafluorobenzyl Bromide (PFBBr)	- APCI	[11]

Al-Dirbashi *et al.* used 4-[2-(N,N-dimethylamino) ethylaminosulfonyl]-7-(2-aminoethyl amino)-2,1,3-benzoxadiazole (DAABD-AE) for the benzofurazan-derivatization of glutaric and 3-hydroxyglutaric acids [57, 58]. This ISD was used to enable sensitive and positive ion detection of these organic acid markers in human urine. These biomarkers are useful for the diagnosis of glutaric aciduria type 1. The ISD reagent contains ionizable sites with high proton affinity to enhance the MS ionization efficiencies and a hydrophobic benzofurazan moiety to improve chromatographic separations.

Cimlová *et al.* performed ISD by the coupled action of isobutanol, isobutyl chloroformate and the pyridine catalyst followed by LLE for the sensitive determination of urinary biomarker prolyl-4-hydroxyproline [50]. This derivatization reaction offered two clear advantages. It occurred readily in aqueous media and the desired derivatization products were efficiently extracted from interfering ionic components into a water-immiscible organic phase. The lower

limit of quantitation was determined to 2 µM, while the limit of detection was 0.2 µM for the derivatized analyte, when this method was validated.

Alkylative extraction of methylmalonic acid in human serum using 2,3,4,5,6-pentafluorobenzyl Bromide (PFBBr) allowed the use of a sensitive negative ionization mode detection of APCI for methylmalonic acid in human serum [11]. This ISD allowed the researchers to overcome challenges of quantitation of methylmalonic acid caused by its low concentration in biological matrix, low molecular weight, and the presence of a more abundant endogenous isomer, succinic acid. Limits of detection and quantitation were 0.03 and 0.08 µmol/L, respectively, for a 50 µL serum sample.

2.7 Thiols

Thiol-containing compounds are easily oxidized during and after collection of biological samples. This can deleteriously impact their trace determination and requires special considerations for ex vivo stabilization. A common procedure adopted in bottom-up proteomics, after reducing disulfide bonds, is to cap cysteines with iodoacetamide to prevent their reunion or oxidation. [59]. Several derivatization agents have been reported for ISD of thiol-containing compounds. They have been shown to be successful for both stabilizing thiols and increasing ionization efficiency for enhanced MS sensitivity. A schematic diagram for the reaction of the common ISD reagents used with thiols is shown in Fig. 2-7. ISD reagents for thiols are summarized in Table 2-5.

In work by Suh *et al.*, sulfur-containing amino-acid metabolites were double derivatized in human blood with iodoacetamide (IAM) and isopropylchloroformate (IPCF), following blood draws to prevent ex vivo thiol oxidation and to esterify the amino and the carboxylic groups. Limits of detection of 0.25- 0.5 nM were achieved [60]. In another study, two ferrocene-based maleimide

reagents were used together to stabilize and to increase hydrophobicity of cysteine, glutathione, cysteinylglycine, N-acetylcysteine, homocysteine and their disulfides in human urine [61]. Additionally, methyl acrylate (MA) has been reported to be a useful alkylating reagent for *in situ* protection of thiols in biological fluids [62-64].

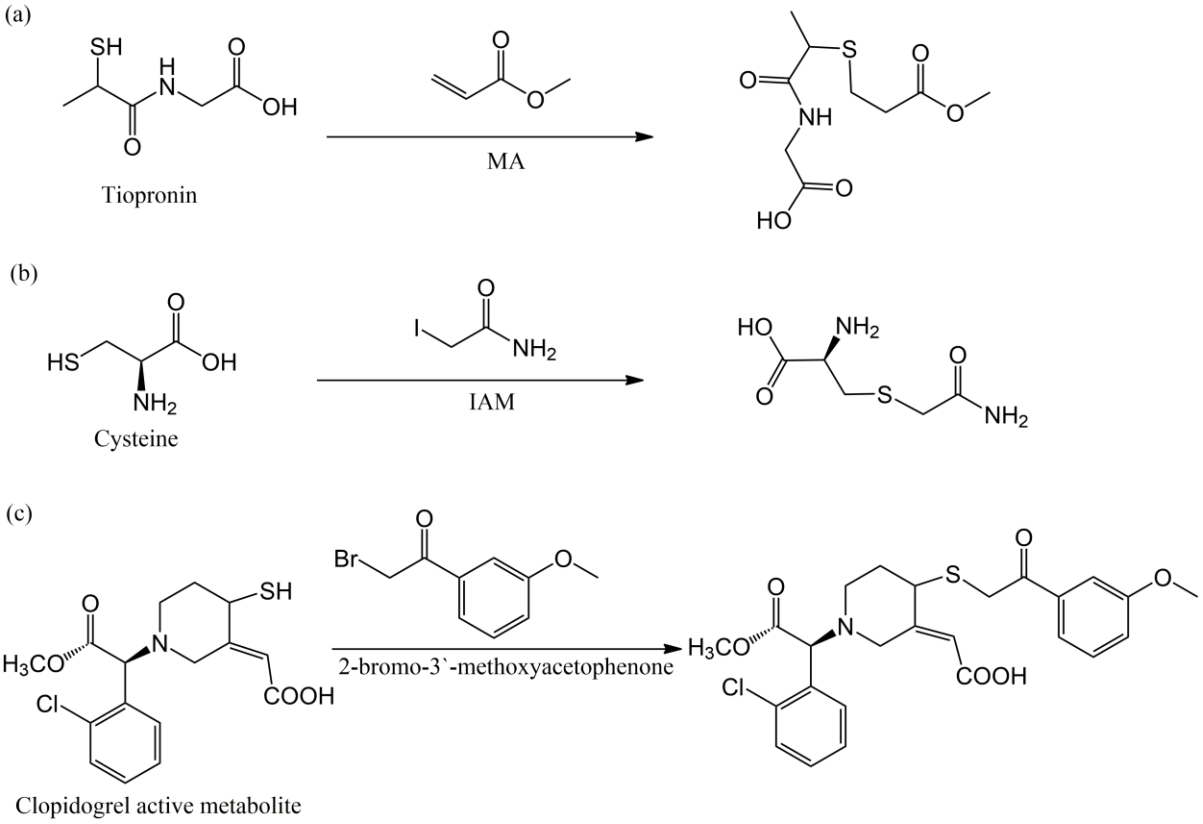

Figure 2-7. Example derivatization reactions for thiols: Use of (a) MA; (b) IAM; and (c) 2-bromo-3`-methoxyacetophenone.

2-bromo-3`-methoxyacetophenone was used for the ISD of active and inactive metabolites of prasugrel [65] and the active metabolite of clopidogrel [66]. This ISD alkylating reagent was used to stabilize the active thiol group immediately after blood collection to ensure the stability of

these metabolites during sample processing and storage. The LOQ for prasugrel and clopidogrel active metabolites was found to be 0.5 ng/mL.

Table 2-5. ISD reagents for thiols

Analyte (s)	Bio-matrix	Derivatization reagent	Ionization source	Reference
4-methoxy-2-methyl-2-butanethiol (volatile thiols)	Lipid matrices (e.g. Virgin olive oil)	Ebselen (an Se-N containing reagent)	+ ESI	[70]
Reduced forms of homocysteine, cysteine, glutathione, and cysteinylglycine and oxidized disulfides	Human blood	Double derivatization with IAM and IPCF	+ ESI	[60]
Cysteine, glutathione, cysteinylglycine, N-acetylcysteine, homocysteine, and their disulfides	Human urine	Two ferrocene-based maleimide reagents.	+ ESI	[61]
Thiol-containing active metabolite R-138727 of Prasugrel	Human plasma	2-bromo-3`-methoxyacetophenone	Positive Turbo Ionspray (+ESI)	[65]
Clopidogrel active metabolite	Human plasma	2-bromo-3`-methoxyacetophenone	+ ESI	[66]
Captopril	Human Plasma	p-bromo-phenacyl-bromide	+ ESI	[67]
Tiopronin and 2-mercaptopropionic acid	Rat blood	Methyl acrylate	- ESI	[62]
3,5-dinitrosalicylic acid hydrazide [nifursol metabolites]	Poultry muscle and liver tissue.	2-nitrobenzaldehyde	- ESI	[69]
Captopril	Human plasma	Monobromobimane	+ ESI	[68]
Total tiopronin	Human plasma	Methyl acrylate	- ESI	[64]
Gemopatrilat	Dog plasma	Methyl acrylate	+ APCI	[63]

Para-bromo-phenacyl-bromide was used for the determination of captopril, a competitive inhibitor of angiotensin-I converting enzyme, in human plasma [67]. This ISD reagent was effective for preventing captopril disulphide formation. This method was able to achieve a good mean recovery of 99.9% and a LOQ of 10 ng/mL. Monobromobimane was also used for the ISD

of captopril in human plasma to stabilize captopril and to improve its ionization yield [68]. A mean recovery of 96% and a LOQ of 2.5 ng/mL were achieved.

Mulder *et al.* used 2-nitrobenzaldehyde for the identification and quantitative determination of 3,5-dinitrosalicylic acid hydrazide (DSH). In this method, acid-catalysed hydrolysis of tissue-bound metabolites to free DSH was followed by ISD with 2-nitrobenzaldehyde to the corresponding nitrophenyl derivative NPDSH [69]. Then LLE with ethyl acetate was performed for extraction. Decision limit of 0.04 and 0.025 µg/kg in muscle and liver, respectively, were reported.

A one-step derivatization/extraction using ebselen as the ISD reagent was used for the determination of volatile thiols in lipid matrices [70]. The LOQ of the spiked thiols in the virgin olive oil matrix ranged from 0.05 to 0.5 ng/kg. The recovery ranged from 79% to 20% for derivatives with the highest and lowest polarity, respectively.

2.8 Conclusion

Integrating derivatization with LC-MS analysis either online or offline can be a very attractive and viable solution to enhance the capabilities of an analytical platform, especially to reach ultra-high detection sensitivities and specificities when analyzing complex biological samples. However, some loss of target analytes with lengthy and multistep sample preparation can occur. The incorporation of ISD to bulk derivatize analytes in their native biological matrices can help overcome this obstacle and achieve the desired goals. But challenges still exist when using ISD. Care must be taken to avoid any dilution or ion suppression from using high volumes of ISD, as well as to avoid formation of co-eluting or isobaric interferences. These effects can be minimized through the optimization of ISD reagent selection and derivatization reaction conditions. Combinations with appropriate online or offline sample extraction or pre-

concentration strategies can be considered post ISD, and in some cases, enhanced as a result of the additional step.

With a growing demand for performing routine analysis in core labs, now is the time to utilize streamlined sample preparation methods. These methods can achieve advantages of reduced operator burden and necessary skill required. Along with the rapid advancements in the capabilities of LC-MS, ISD should be taken into future considerations during early stages of method development. With the goal of either selecting or even synthesizing a new ISD reagent, specially designed for integration with LC-MS biological fluid analysis and with high specificity for the target analytes, one can reach new levels of sensitivities. As further emphasis is placed on the health effects of extremely low levels of various endogenous and exogenous chemical substances in the body, it will be necessary to consider such steps. Chosen appropriately, the extra step needed for ISD can be a minimal burden yet provide maximal benefit.

2.9 References

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CHAPTER THREE: EVALUATION OF EFFICIENCY AND TRAPPING CAPACITY OF RESTRICTED ACCESS MEDIA TRAP COLUMNS FOR ONLINE TRAPPING OF SMALL MOLECULES

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Evaluation of Efficiency and Trapping Capacity of Restricted Access Media Trap Columns for Online Trapping of Small Molecules

Yehia Z. Baghdady, Kevin A. Schug*

Department of Chemistry & Biochemistry, The University of Texas at Arlington, Arlington TX USA

*Correspondence to: 700 Planetarium Pl.; Box 19065; Arlington TX 76019-0065; (ph) 817-272-3541; (fax) 817-272-3808; (email) kschug@uta.edu

KEYWORDS: Breakthrough curve; on-line sample preparation; frontal analysis; frontal chromatography; solid phase extraction.

3.1 Abstract

Restricted access media are generally composed from multi-modal particles, which combine a size excluding outer surface and an inner-pore retention mechanism for small molecules. Such materials can be used for either online isolation and pre-concentration of target small molecules or removal of small molecule interferences from large macromolecules, such as proteins in complex biological matrices. Thus, they are considered as enhanced online solid phase extraction materials. We evaluated the efficiency and trapping capacity of different semi-permeable surface restricted access media columns (C18, C8, and C4 inner pores) for four model small molecule compounds (dopamine hydrochloride, acetaminophen, 4-hydroxybenzoic acid, and diethyl phthalate) having variable physicochemical properties. We further studied the effect of mobile phase flow rate (0.25, 0.5, 1 and 2 mL/min) and pH, using 98:2 0.5% acetic acid in water: methanol (pH 2.88) and 5 mM ammonium acetate in 98:2 water: methanol (pH 6.61) as mobile phases. Breakthrough curves generated using frontal analysis were analyzed to determine important chromatographic parameters specific for each of the studied compounds. Experimental determination of these parameters allowed selection of the most efficient trap column and the best loading mobile phase conditions for maximal solute enrichment and pre-concentration on restricted access media trap columns.

3.2 Introduction

Restricted access media (RAM) are materials designed to restrict the access of large biomolecules to inner pores, typically through the incorporation of a hydrophilic restrictive outer surface. Small molecules of interest are retained and captured on the retentive inner pores of a RAM bed. Trap columns incorporating RAM technology can be directly integrated with liquid chromatography (LC) to provide effective online sample preparation for complex biological samples. Such a strategy can minimize sample loss, provide means for pre-concentration of target analytes, reduce total analysis time, and enable simultaneous extraction of multiple analytes with minimal exposure to chemical contaminants, light, or oxygen [1, 2].

Different RAM formats include internal surface phase, semipermeable surface (SPS) phase (including polymeric and protein-based hybrids), and molecularly imprinted polymer RAM [1]. Figure 3-1 illustrates the differences between these phases. Despite the availability of many commercial RAM variants and their early initial development in the 1980s and 1990s by Pinkerton, Boos, and Regnier [3-5], their online integration for sample preparation has not been widely pursued.

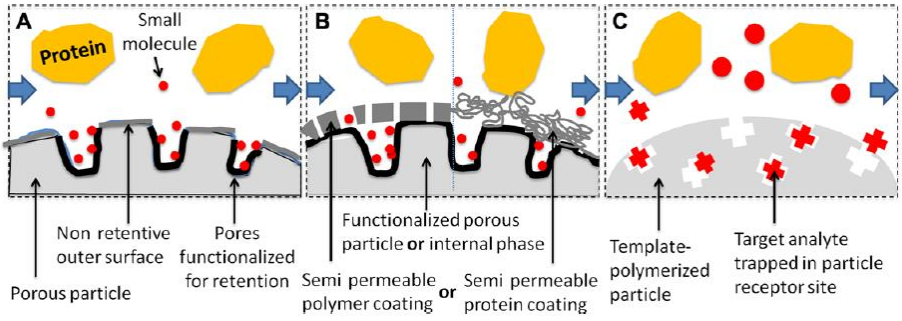


Figure 3-1. Different RAM formats include (A) Internal surface, (B) semi-permeable surface (including both polymeric and protein based hybrids) and (C) molecularly imprinted polymer phases. Reproduced with permission from John Wiley & Sons, Ltd. (copyright 2013) [1].

Schug and coworkers used *in situ* derivatization [6] with on-line RAM sample preparation for targeted analysis of small molecule analytes from biological fluids [1, 7-9]. The work of Walles *et al.* exemplifies the application of online biocompatible solid phase microextraction (SPME) RAM capillary for the direct in-tube extraction of drugs and metabolites from biological fluids [10]. Bentayeb and collaborators adopted online RAM ultra-high performance LC for the sensitive determination of bile acids in human serum [11]. However, one of the reasons that RAM have not been adopted widely into routine work flows is a lack of fundamental understanding of the optimal means for their incorporation in method development processes. A recent study was conducted to evaluate the efficiency of RAM trapping and matrix effects [12]. This study evaluated the trapping of four hydrophobic estrogen compounds. For optimization of the coupling of RAM with mass spectrometry for their sensitive determination, a gradient loading flow rate and C4 RAM were reported to be necessary.

To better understand and optimize RAM columns, the use of frontal chromatography is another alternative. In frontal chromatography, the sample is fed continuously onto the adsorbent stationary phase and the eluent signal is monitored with time. The resulting chromatogram has a sigmoid shape and is usually referred to as a breakthrough curve (BTC). This technique has been well established and widely used in different studies such as affinity chromatography [13] and thermodynamic measurements to measure adsorption isotherms on solid adsorbents [14]. Frontal analysis is a time and sample intensive process, but it provides direct access to important parameters that characterize the retentive nature of a stationary phase.

The aim of this work was to evaluate the trapping performance of a commercial RAM column series using frontal analysis. For this particular column series, the outer surfaces of silica particles were coated with a methyl cellulose polymer and the inner surface of pores were

chemically bonded with a reversed phase stationary phase (C18, C8, or C4 RAM in a semi-permeable surface format). Having a hydrophilic surface and a size exclusion function, these trap columns can be used for isolation and pre-concentration of small target molecules of interest [15]. Four model compounds having different physicochemical properties were selected. These compounds covered a wide range of hydrophobicity starting with highly hydrophilic dopamine hydrochloride (DPC), less hydrophilic acetaminophen (ACM), followed by 4-hydroxybenzoic acid (HBA) and ending with diethyl phthalate (DEP), which is highly hydrophobic. The evaluation parameters were efficiency of trapping or number of theoretical plates (N), breakthrough volume (V_b), retention volume (V_r) and equilibrium volume (V_e). When these parameters are optimized by investigating LC-relevant mobile phase pH and loading flow rates using a series of semi-permeable surface reversed phase RAM trap columns, a high efficiency on-line sample preparation procedure can be achieved that can effectively reduce sample preparation burden and improve method precision and sensitivity relative to traditional off-line methods.

3.3 Frontal Analysis Theory

In frontal analysis, a sample of analyte with concentration C_o is continually loaded onto a stationary phase until some of the analyte begins to appear in the effluent. Breakthrough occurs when the capacity of the sorbent has been reached. The response of the analyte in the effluent can be measured by any analyte-responsive detector. A plot of the analyte response, which incorporates a breakthrough event, as a function of time is a breakthrough curve (BTC). The ideal BTC has a sigmoid-shape and relevant chromatographic parameters can be determined from the curve as shown on the idealized diagram in Figure 3-2 [16, 17]. The values of these parameters depend on the kinetics of sorption, the retentiveness of the analyte in the given chromatographic system, and the capacity of the sorbent bed [17, 18]. The relative physicochemical properties of the analyte,

the sorbent bed, and the mobile phase (as well as the flow rate) are essential considerations that control these parameters.

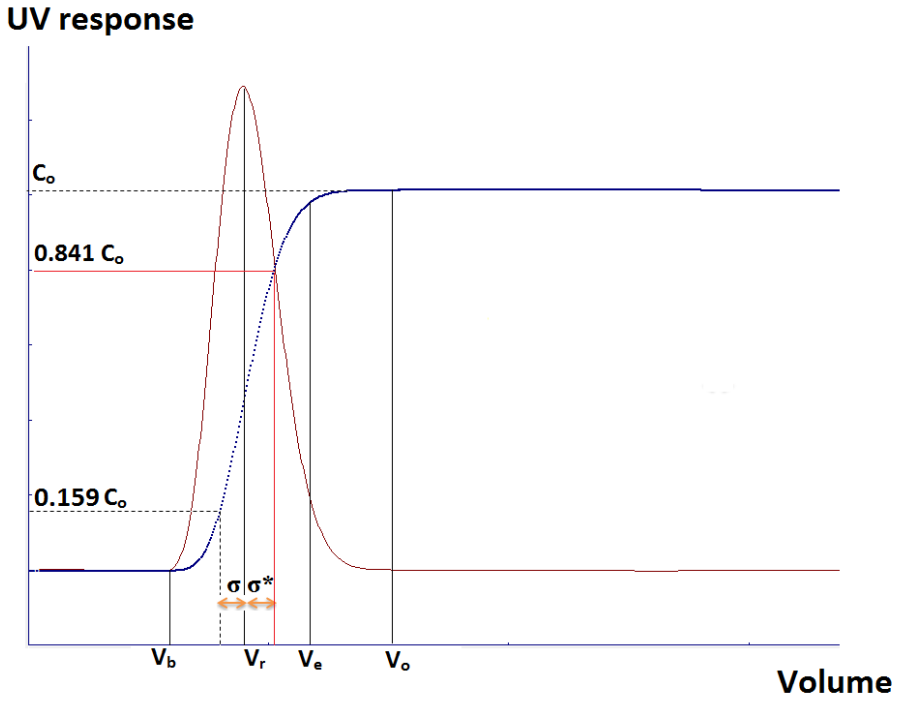


Figure 3-2. A typical breakthrough curve (blue) and its first derivative (D1) (red) showing the characteristic chromatographic parameters.

The retention volume of an analyte (V_r) corresponds to the inflection point of the BTC. This point can be ideally determined as the value corresponding to half the original concentration (0.5 C_o). This value can be more easily and accurately obtained from the maximum of the first derivative (D1) of the BTC. Breakthrough volume (V_b) is the volume of mobile phase that corresponds to 1% of maximum concentration of the analyte in the effluent (0.01 C_o). V_b defines the maximum volume of sample that can be loaded with an acceptable minimal loss of analyte of 1%. Thus V_b can be used to indicate the trapping capacity of the studied phase. Equilibrium volume (V_e) is the volume of the mobile phase that corresponds to 99% of maximum concentration of the

analyte in the effluent (0.99 C_o). V_e occurs when the saturation capacity of the phase is reached and the concentration of analyte in the effluent exiting is almost the same as that in the influent entering the trap column. Hence, V_e represents the minimum volume of sample that will achieve isolation of the maximum amount of sorbed analyte by the phase but with a lower overall recovery, because a fraction of the analyte is lost during the sorption process [19]. V_e can also represent the exhaustion volume of the sorbent bed. V_o is the volume of the solute, of concentration C_o, used to generate a BTC. Values of V_b and V_e can be obtained from the BTC using the following equations [20-22]:

$$V_b = V_r - 2\,\sigma \quad (1)$$

$$V_e = V_r + 2\,\sigma^* \quad (2)$$

Where σ and σ* are the standard deviations of the D1 curve. Since the BTC is usually asymmetric, these can be determined from the values corresponding to 0.159 and 0.841 of the maximum concentration of analyte in the effluent (C_o), respectively. σ represents the axial dispersion of analyte along the sorbent phase and is directly related to the efficiency of the trap column (N), which can be calculated from the BTC using the following equation:

$$N_{Frontal} = (V_r * V_{0.159})/ \sigma^2 \quad (3)$$

$$V_{0.159} = V_r - \sigma \quad (4)$$

N can also be calculated from the D1 of the BTC. This approximately Gaussian curve is similar to the curve obtained in a standard chromatographic elution involving a fixed finite injection volume. Hence, one can also calculate N using the following commonly used chromatographic equation:

$$N_{Gaussian} = 5.54\,(V_r/W_{0.5})^2 \quad (5)$$

3.4 Materials and methods

3.4.1 Chemicals

HPLC-grade water, methanol, and glacial acetic acid were obtained from J.T. Baker (Phillipsburg NJ, USA). Diethyl phthalate and 4-hydroxybenzoic acid were from Alfa Aesar (Ward Hill MA, USA). Acetaminophen, ammonium acetate, and dopamine hydrochloride were purchased from Sigma-Aldrich (St. Louis MO, USA). The structures, pKa, log P, and log D values for this analyte set are summarized in Table 3-1.

3.4.2 Apparatus

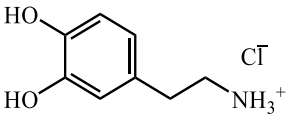
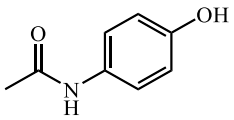
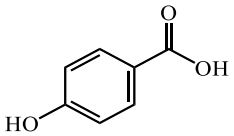
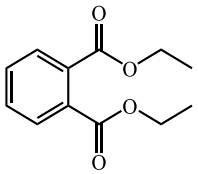
The experiments were performed on a Shimadzu LC system (Shimadzu Scientific Instruments, Inc., Columbia MD, USA), equipped with two pumps (LC-20AD) in a high-pressure mixing arrangement, an autosampler (SIL-20A HT), and a SPD-M20A PDA detector. Three Shim-pack MAYI-trap columns (Shimadzu) with different stationary phases were tested, including MAYI-ODS (G) (10 mm×4.6 mm, 50 μm dp, 12 nm pore size), MAYI-C8 (G) (10 mm×4.6 mm, 50 μm dp, 12 nm pore size) and MAYI-C4 (G) (10 mm×4.6 mm, 50 μm dp, 12 nm pore size).

3.4.3 Experimental conditions

Samples were dissolved directly in both 98:2 0.5% acetic acid (pH = 2.88) in water: methanol and 5 mM ammonium acetate (pH = 6.61) in 98:2 water: methanol mobile phases to the concentration of 50 μg/mL for HBA and 20 μg/mL for ACM and DPC. DEP, because of its difficult aqueous solubility, was dissolved in both 93:7 0.5% acetic acid in water: methanol mobile phase and 93:7 5 mM ammonium acetate in water: methanol mobile phase to a concentration of 100 μg/mL. Each analyte concentration was selected such that it did not immediately overload the RAM phase and provided a reasonable detector response in a reasonable time for ease of recording

the breakthrough curve. Using a UV-Vis photodiode array detector, detection wavelengths were set to 270.0, 254.0, 280.0, and 275.0 nm for HBA, ACM, DPC and DEP, respectively. Variable isocratic loading flow rates of 0.25 mL/min, 0.5 mL/min, 1 mL/min, and 2 mL/min of the mobile phase were investigated. Shimadzu's LCMS Solutions software was used for data collection and analysis. Analysis of parameters, extracted directly from BTC, was performed with Microsoft Excel.

Table 3-1. Physicochemical properties of model analytes.

Analyte	Structure	pKa*	Log D**		Log P for neutral form*
			pH 2.88	pH 6.61	
Dopamine hydrochloride		8.73, 10.67, 15.25	-2.98	-2.61	0.58
Acetaminophen		9.76	0.31	0.34	0.55
4-hydroxybenzoic acid		4.11, 9.69	1.41	-0.58	1.20
Diethyl phthalate		NA	2.7	2.7	2.35

*Calculated using ChemDraw.
Calculated using ACD/Labs.**

3.5 Results and discussion

Efficient online trapping of analytes is a critical step during sample preparation for trace analysis, especially for analytes present in highly complex matrices. For online solid phase extraction using RAM, optimization of loading conditions is possible to achieve the highest possible pre-concentration without occurrence of breakthrough. The retention of target analytes on the RAM trap columns should ideally be similar to, or lower than, that on the analytical column to limit band-broadening during desorption from the trap phase and prior to the subsequent analytical separation step. Thus, considering a reversed phase retention mechanism, the least retentive phase should be utilized where possible (e.g., a C4 trap); however, more hydrophilic compounds may require more retentive phases (e.g., C8 or C18 traps). Similarly, if the sample is loaded too rapidly, to wash out undesired matrix components, loaded analytes may not be given enough time to penetrate the pores resulting in a less trapped amount and band spreading. It is difficult to correct this band-broadening with subsequent chromatography. Thus, optimizing all these aspects is of great importance to achieve a high efficiency on-line sample preparation procedure with promise of high recoveries and effective enrichment of the desired analytes.

3.5.1 Effect of pH of the mobile phase

Modification of the pH of a loaded sample (and the loading mobile phase) is a common practice to enhance the retentive nature of an analyte on a reversed phase trapping system. When an analyte is partially or fully ionized, its affinity for an aliphatic stationary phase will be reduced. Depending on the functional nature of an analyte, different pH values for the loading may be desired in order to maximize retention. Here, we examine potential good and bad case scenarios in terms of pH for the loading of model analytes on the reversed phase RAM columns.

The effect of pH of mobile phase was studied by using two different pH modifiers, 0.5% acetic acid (pH = 2.88) or 5 mM ammonium acetate (pH = 6.61). This effect was evaluated by studying the resulting shape of the breakthrough curve, and measured values for trapping capacity (V_b), saturation capacity (V_e), and number of theoretical plates or efficiency ($N_{Gaussian}$ and $N_{Frontal}$). Each analyte and each RAM trap phase was evaluated in a systematic fashion.

To elucidate the behavior of trap columns under different pH, we first have to understand the nature of the RAM stationary phases. The outer surface of the silica particles is covered by methyl cellulose coating to exclude proteins and other undesired macromolecules. This polymer coating is hydrophilic and stable to pH range 3-11. Methylcellulose is nonionic and will not interact with other ionic species [23]. It is immobilized to the outer surface of silica by hydrogen bonding and Van der Waals effects [15, 24]. The inner surface of the pores is bonded with C4, C8, or C18 phases. All bonded phases were manufactured using mono-functional alkyl silanes and end capped. Values of carbon loading percentages, as provided by the manufacturer, are 17.6%, 10.8% and 7.8 % for C4, C8 and C18, respectively. We used the Berendsen–de Galan equation [25] to calculate the surface coverage or bonding density of the different RAM bonded phases. The calculated surface concentrations of bonded phases of C4, C8 and C18 RAM traps were 9.18, 2.88 and 0.98 μmole/m^2, respectively. Accordingly, the C4 phase has the highest bonding density of 9.18 μmol/m^2 which means that it has the least accessible residual silanols. However, the degree of shielding of underlying polar silanols will be higher with longer C18 chains [26]. Under the high aqueous conditions adopted in this work, collapse of long chain C18 bonded phase can occur where folding down of the alkyl bonded phase onto the surface can contribute to more shielding of the surface silanols. The predominant retention mechanism in this case would be adsorption on top of the collapsed bonded phase.

The reported number of silanol groups per surface area unit of unmodified silica range from 6 to 9 µmol/m^2 [27-30]. For a monolayer bonded phase, the longer the chain length, the more carbon would be present and more retention would be achieved. However, if a shorter chain bonded phase had a higher surface concentration resulting in more bonded carbon on the surface, then it could have the same or more retention than a C18 phase. Residual and accessible surface silanols can interact with basic and acidic analytes via hydrogen bonding, dipole/dipole and ion exchange mechanisms. These secondary silanol effects can cause increased retention, peak deformation, and excessive peak tailing, especially at an intermediate pH range where silanols are partially ionized. The accessibility of these residual silanol groups to solutes and solvents is increased in the monofunctional bonded phases, as in the case of the RAM phases studied here, compared to the trifunctional bonded phases.

To have an efficient online extraction, conditions leading to the observation of an ideal BTC shape (as shown in Figure 3-2) is important. It helps to easily identify and determine the different characteristic chromatographic parameters, such as breakthrough volume. Moreover, broadening of the concentration profile during loading conditions can negatively affect trapping efficiency, capacity, and subsequent elution by affecting N, V_b and V_r.

DPC has a primary amino group which can be well protonated and ionized at both pH 2.88 and 6.61 as indicated by the calculated negative log D values at these pH values of -2.98 and -2.61, respectively. The BTC obtained with pH 6.61 using C4 RAM phase and 0.25 mL/min flow rate showed an abnormal sigmoidal shape which can be more easily understood by examining the associated D1 chromatogram as shown in Figure 3-3A. The derivative chromatogram indicates that there was severe tailing which led to a small associated peak at its tail. This was improved by increasing the flow rate, which resulted in reduced tailing with no associated small peaks. A C18

RAM phase may be a better alternative for effective trapping of this hydrophilic analyte at pH 6.61, since excessively high loading flow rates should generally be avoided if maximum trapping efficiency is desired. Using the same conditions for the C18 RAM phase, even with the slowest flow rate of 0.25 mL/min (Figure 3-3B), much less tailing was observed. This suggests that C18 has better shielding for residual silanols and is the best choice for highly hydrophilic basic analytes. This effect of residual silanols was also confirmed by the use of the mobile phase with acetic acid (pH 2.88) where frontal analysis of DPC exhibited no tailing and a good BTC shape, as shown in Figure 3-3C. At this pH, the Log D value of DPC is comparable to the one in pH 6.61, but the residual silanols' effect can be greatly reduced by their protonation at this acidic pH.

HBA is an intermediate hydrophilic compound and is essentially neutral at pH 2.88 (pKa = 4.11, log D value of 1.41). Hence, a high V_b value was obtained for HBA due to the increased trapping of its neutral form. Moreover, a typical sigmoidal BTC with no tailing was observed at this pH with all studied trap columns such as the one shown in Figure 3-3D. However, at pH 6.61, the carboxylic acid group of HBA is ionized (log D = -0.58) and this resulted in a lower V_b value. At this essentially neutral pH, residual silanols can also be ionized and contribute to the obtained unusual BTC and its D1 of HBA using the C4 trap column, as shown in Figure 3-3E. Using the C18 RAM trap, this effect was reduced, but it did not fully resolve the obtained BTC shape (Figure 3-3F). This shows that an acidic pH is a necessity for effectively trapping these types of acidic compounds.

Both ACM and DEP showed typical BTC shapes in both pH conditions and the results are summarized in Table 3-2. All results were comparable using the two pH conditions which were in accordance with the similar log D values they have in both pH conditions. Accordingly, acetic acid mobile phase was the optimum loading mobile phase for all studied compounds.

Table 3-2. Effect of pH on the trapping parameters of ACM and DEP.

Analyte	Evaluation Parameters	Acetic acid mobile phase			NH_4Ac mobile phase		
		C4	C8	C18	C4	C8	C18
ACM	$N_{Gaussian}$	80.0 ± 10.5%	82.3 ± 2.6%	56.4 ± 3.0%	89.2 ± 1.2%	103.4 ± 12.5%	47.4 ± 11.1%
	$N_{Frontal}$	69.5 ± 6.0 %	67.6 ± 10.9%	34.7 ± 8.4%	60.1 ± 11.8 %	82.3 ± 4.6%	33.0 ± 9.9%
	Trapping capacity (V_b, mL)	1.39 ± 0.9%	1.26 ± 1.2%	1.19 ± 3.2%	1.51 ± 1.0%	1.59 ± 0.8%	1.42 ± 2.7%
	Saturation capacity (V_e, mL)	2.14 ± 1.3 %	1.98 ± 1.5%	2.23 ± 1.7%	2.43 ± 1.8 %	2.38 ± 1.3 %	2.72 ± 2.3 %
DEP	$N_{Gaussian}$	1062.9 ± 6.1%	723.6 ± 5.8%	NA	1122.5 ± 2.9%	689.3 ± 2.1%	NA
	$N_{Frontal}$	493.2 ± 5.6%	445.0 ± 11.9%	53.7 ± 6.0%	455.6 ± 5.4%	481.8 ± 0.8%	55.1 ± 1.5%
	Trapping capacity (V_b, mL)	50.88 ± 0.3%	52.08 ± 0.7%	45.25 ± 0.6%	55.38 ± 0.4%	56.25 ± 0.4%	47.50 ± 0.9%
	Saturation capacity (V_e, mL)	59.54 ± 0.7%	61.58 ± 0.6%	68.42 ± 0.6%	64.88 ± 0.3%	66.17 ± 0.6%	71.33 ± 0.5%

*All values are average of three determinations and measured at 0.25 mL/min.

3.5.2 The effect of different flow rates

An ideal trapping for any target analyte will require the highest possible trapping capacity (V_b) and the highest efficiency (N) values. A high V_b value will provide an increase in the amount trapped and a high N value will yield a steeper BTC. Having a steeper BTC chromatogram (i.e., a narrower sigmoid shape), the V_b capacity closely approaches the V_e capacity of the sorbent bed. This helps to increase the sorbent bed utilization per loading cycle and eventually increase the amount trapped onto the sorbent bed [31, 32].

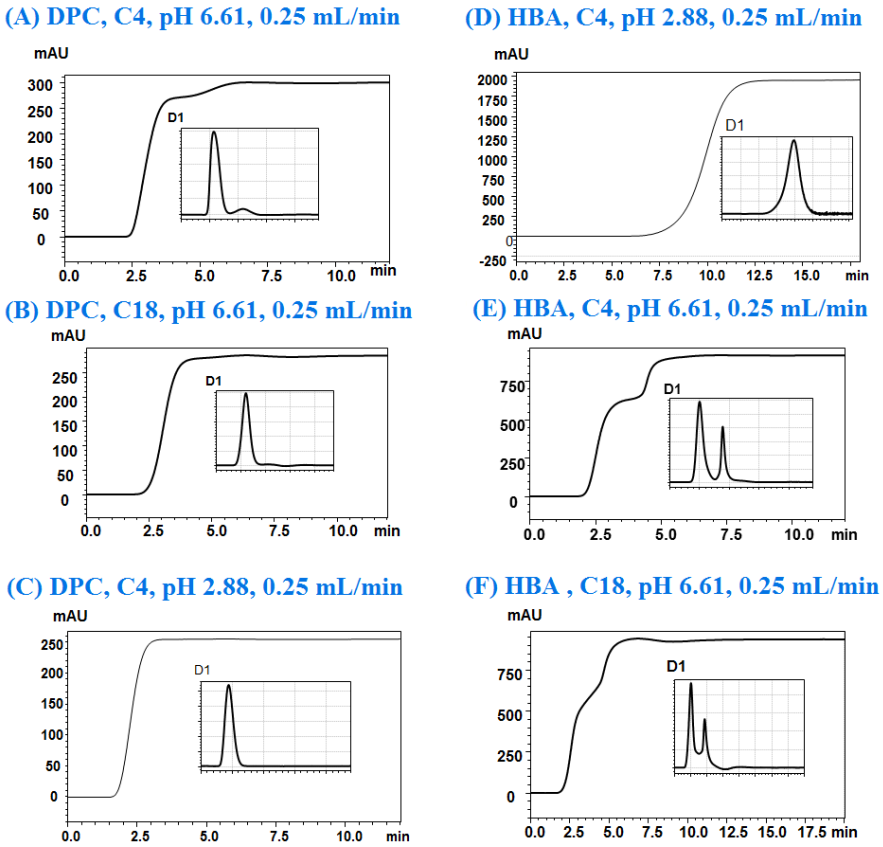


Figure 3-3. Breakthrough and first derivative (D1) curves using 0.25 mL/min flow rate for (A) dopamine hydrochloride, C4 trap, and pH 6.61, (B) dopamine hydrochloride, C18 trap and pH 6.61, (C) dopamine hydrochloride, C4 trap and pH 2.88, (D) 4-hydroxybenzoic acid, C4 trap and pH 2.88, (E) 4-hydroxybenzoic acid, C4 trap and pH 6.61, and (F) 4- hydroxybenzoic acid, C18 trap and pH 6.61.

Four different loading flow rates of 0.25, 0.5, 1, and 2 mL/min were investigated (data not shown). The 0.25 mL/min gave the best results in terms of measured values for N and V_b. Contrary to what was expected before running experiments, varying the loading flow rates did not affect both V_r and V_b to any significant degree for the studied compounds. This was due to the very small dimensions of the utilized sorbent beds and the presence of the outer hydrophilic polymer coating, which decreased the total available surface area for retention (only the inner pores are functionalized for reversed phase retention). Hence the residence time of the adsorbate in the trap was enough for complete contact and trapping even with the high flow rate of 2 mL/min. However, increasing the flow rate significantly decreased the efficiency of trapping (N) values for all studied compounds. This was evidenced by a less abrupt change from minimum to maximum absorbance values (i.e., a wider sigmoid shape) as the BTC curve developed at higher flow rates. Thus, it could be concluded that this broadening reflects the trapping of analytes over a wider mass-transfer zone at the head of the sorbent bed during loading with a high flow rate. This explains and confirms a previous conclusion in a publication by Papouskova *et al.*, which found that using a gradient loading flow rate with a slow initial loading flow rate, was the best for trapping estrogens to ameliorate observed tailing, which occurred during elution onto an analytical column when using high initial loading flow rates [12]. Using high flow rates eventually during loading is a necessity to ensure proper washing of RAM trap columns from any remaining unretained proteins and to shorten the portion of time needed for sample loading and washing. Better efficiency can be obtained by initiating analyte load at lower flow rates, and then increasing the flow rate after the compounds of interest have been trapped.

3.5.3 The Effect of different restricted access media phases

The choice of trap columns is similar in concept to the choice of analytical columns for separation of different analytes. Reducing the chain lengths of the bonded phases increases the polarity (or reduces the hydrophobicity) of the stationary phase. Typically, hydrophilic compounds suggest the use of more hydrophobic phases such as C18 for better trapping or retention while hydrophobic compounds may be more optimally retained (and subsequently released) using shorter chains such as C4. In general, for on-line sample preparation, the least retentive trapping phase should be used whenever possible to help facilitate elution of trapped compounds to the analytical columns, once this is desired, in a tight band [33].

Prior experiments indicated that the acetic acid mobile phase (pH 2.88) was the best loading mobile phase for all studied compounds. In subsequent comparisons of different stationary phases using this mobile phase, the C4 RAM trap gave the best N and V_b, even for the highly hydrophilic compounds. This finding was contrary to what one might predict for trapping of high hydrophilic compounds. The bonded C4 phase has the highest surface coverage (9.18 μmol/m^2) for the phases tested. The higher surface coverage leads to a greater number of interaction sites with analytes. At the same time, being of shorter alkyl chains, these interactions have reduced strength. This combination appears to provide overall optimum trapping interactions. The C8 trap gave very similar, and sometimes slightly better, results than C4 trap, but both the C4 and C8 traps provided significantly better performance than the C18 phase. Results are summarized in Table 3-3.

Table 3-3. Effect of RAM reversed phase on the trapping parameters

Evaluation Parameters*	DPC			ACM			HBA			DEP		
	C4	C8	C18	C4	C8	C18	C4	C8	C18	C4	C8	C18
$N_{Gaussian}$	48.6 ± 3.9%	49.4 ± 3.2%	52.5 ± 5.1%	80.0 ± 10.5%	82.3 ± 2.6%	56.4 ± 3.0%	152.0 ± 1.2%	149.0 ± 4.5%	44.7 ± 9.7%	1063.0 ± 6.1%	724.0 ± 5.8%	NA
$N_{Frontal}$	50.0 ± 2.4%	53.2 ± 6.0%	50.5 ± 16.4%	69.5 ± 6.0%	67.6 ± 10.9%	34.7 ± 8.4%	81.5 ± 10.1%	108.0 ± 11.4%	19.2 ± 5.5%	493.0 ± 5.6%	445.0 ± 11.9%	53.7 ± 6.0%
Trapping Capacity (V_b, mL)	0.42 ± 1.5%	0.42 ± 0.0%	0.45 ± 2.8%	1.39 ± 0.9%	1.26 ± 1.2%	1.19 ± 3.2%	1.94 ± 0.7%	2.14 ± 0.7%	1.87 ± 2.1%	50.90 ± 0.3%	52.10 ± 0.7%	45.20 ± 0.6%
Saturation Capacity (V_e, mL)	0.73 ± 0.9%	0.73 ± 0.0%	0.78 ± 0.7%	2.14 ± 1.3%	1.98 ± 1.5%	2.23 ± 1.7%	2.93 ± 0.9%	3.11 ± 0.5%	4.03 ± 0.4%	59.50 ± 0.7%	61.60 ± 0.6%	68.40 ± 0.6%

*All values are average of three determinations and measured using acetic acid mobile phase and 0.25 mL/min flow rate.

The most hydrophobic analyte tested, DEP, showed abnormal BTC shapes when loaded on the C18 trap. The first derivative transformation as shown in Figure 3-4A revealed a severely-fronted peak. This occurred to a less extent with the C8 trap and was mostly resolved with the C4 trap, Figure 3-4B. The time required to obtain a full BTC curve using a 0.25 mL/min flow rate and C18 trap was about 300 minutes and peak fronting remained even with the highest flow rate of 2 mL/min. This abnormal BTC behavior and fronting, associated particularly with the interaction between C18 and DEP, can be due to RAM column overloading [18, 34, 35]. This was probably

caused by the strong interaction between the C18 trap and the highly hydrophobic DEP resulting in early saturation for all available C18 chains and eventually overloading of RAM.

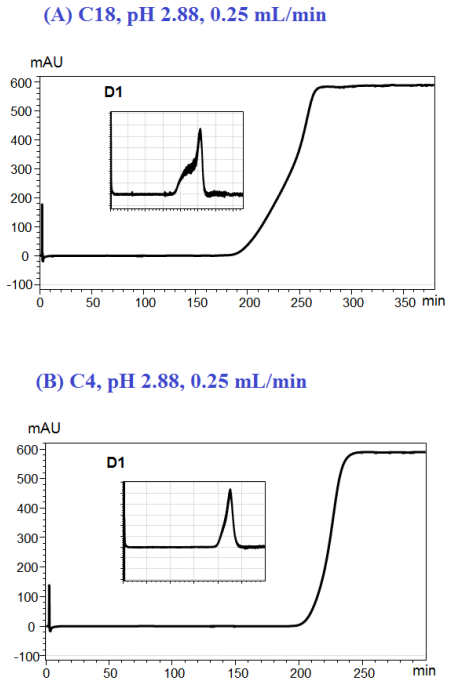


Figure 3-4. Breakthrough and first derivative (D1) curves for diethyl phthalate using 0.25 mL/min flow rate, pH 2.88 and using (A) C18 trap and (B) C4 trap.

3.6 Conclusion

Evaluation of the effect of different loading conditions to maximize the efficiency and pre-concentration behavior of different RAM reversed phases was performed in this work. Acidic conditions using acetic acid in the mobile phase gave the best trapping conditions for all studied compounds. The lowest flow rate of 0.25 mL/min was found to be optimal. C4 and C8 RAM traps (of the semi-permeable surface type provided by one particular manufacturer) were proven to be the best for trapping of both highly hydrophilic and highly hydrophobic studied compounds. The

C18 trap is preferred only for better shielding of residual silanol effects, if an acidic modifier is not to be used.

These results may serve well the method optimization stages during the online incorporation of these RAM phases in analytical applications for real and complex biological samples. However, further research may be needed to investigate the possible interferences from complex matrices, which could potentially alter the results shown here. For example, the presence of other small undesired adsorbates in the biological matrix can exhaust the RAM sorbent bed quickly and lead to earlier breakthrough and lower recovery for the target analyte. Moreover, elution of these trapped undesired adsorbates and their presence in the electrospray droplets along with the target analytes may cause significant ion suppression. In such a case, further optimization may be a necessity for a complete understanding and for achieving the desired outcomes. In such a case, frontal analysis may not be the preferred means. While frontal analysis could allow the systematic investigation of critical variables in the model study here, performing such studies with real matrices or even mimics thereof could be very time and resource intensive.

Even so, frontal analysis provided insight into underutilized materials for on-line sample preparation. Such investigations should lower the barrier to the use of RAM. When optimized, these materials can provide significant advantages over typical solid phase extraction materials, especially for automated biological sample analysis.

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CHAPTER FOUR: A NOVEL DIAGNOSTIC *IN SITU* DERIVATIZATION KIT FOR THE SIMULTANEOUS DETERMINATION OF 14 BIOMARKERS OF EXPOSURE TO BENZENE, TOLUENE, ETHYL BENZENE AND XYLENES IN HUMAN URINE BY ISOTOPE DILUTION LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY AND KIT OPTIMIZATION USING RESPONSE SURFACE METHODOLOGY

Baghdady, Y. Z.; Schug, K. A. A novel diagnostic *in situ* derivatization kit for the simultaneous determination of 14 biomarkers of exposure to benzene, toluene, ethyl benzene and xylenes in human urine by isotope dilution liquid chromatography tandem mass spectrometry and kit optimization using response surface methodology. *Anal. Chim. Acta* **2018**, *1036*, 195-203.[1]

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A Novel Diagnostic *In Situ* Derivatization Kit for the Simultaneous Determination of 14 Biomarkers of Exposure to Benzene, Toluene, Ethyl Benzene and Xylenes in Human Urine by Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry and Kit Optimization Using Response Surface Methodology

Yehia Z. Baghdady, Kevin A. Schug*

Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington TX 76019

*corresponding author: 700 Planetarium Pl., Box 19065, Arlington TX 76019-0065; (ph) 817-272-3541; (fax) 817-272-3808; (email) kschug@uta.edu

KEYWORDS: *In situ* derivatization; biomarkers; BTEX; central composite design; occupational exposure; metabolites

4.1 Abstract

Metabolite profiling can be used as a diagnostic measure for both short- and long-term co-exposure by individuals to benzene, toluene, ethylbenzene and xylenes (BTEX). A novel one pot derivatization *in situ* kit (OPDISK) was developed and optimized using a multivariate approach based on central composite design. The OPDISK was designed to simultaneously derivatize, in a urine sample matrix, a series of fourteen carboxylic acid and phenol-bearing urinary metabolites of BTEX to enhance their chromatographic analysis and sensitivity for detection by liquid chromatography – electrospray ionization – tandem mass spectrometry (LC-ESI-MS/MS). Using the reagent kit, the less responsive functional units on the molecules were converted to permanently positively-charged functional units. The kit was composed of three components, 2-fluoro-1-methylpyridinium p-toluenesulfonate (FMP), 3-carbinol-1-methylpyridinium iodide (CMP) and triethylamine (TEA) as a basic catalyst and, only after diluting a urine sample 20 fold with acetonitrile, was applied under mild conditions of room temperature and short reaction time of 20 minutes. The derivatized biomarkers were then directly analyzed using isotope dilution LC-ESI-MS/MS. The method was sensitive (limit of detection on column ranged from 1.4 pg to 3.1 ng), accurate (mean accuracy from 85% to 114%), and precise (mean coefficient of variation from 1% to 14%). The method results indicated a good linearity ($R^2 \geq 0.990$) for all metabolites. ClinChek® urine control samples were used successfully to demonstrate the accuracy of the method.

4.2 Introduction

Benzene, toluene, ethylbenzene, and xylenes (BTEX) are monoaromatic hydrocarbons, which are ubiquitous in the surrounding environment. They are extensively used as solvents, as raw materials for the synthesis of pharmaceuticals and many other organic compounds, and as vital components of many industrial products including gasoline, paints, pesticides, plastics, and glues. Humans can be easily exposed to BTEX compounds through inhalation, skin absorption, and/or ingestion of contaminated food and water [1]. Due to their high volatilities, BTEX vapors can easily contaminate air, water, and soil. BTEX exposure is not limited only to outdoor environments. Many studies have shown that indoor environments (e.g. furniture polishes, paintings, and household products) can provide high exposure levels [2, 3].

According to the International agency for research on cancer (IARC), benzene is a group 1 carcinogen to humans [4], where increased incidence of leukemia has been reported. Ethylbenzene is indicated to be a possible human carcinogen [5]. Toluene and xylenes are not carcinogenic based on the current evidence, but they are known to have neurotoxic effects [6]. Because of toxic and carcinogenic effects of BTEX, the American Conference of Governmental Industrial Hygienists (ACGIH) has recommended that urinary metabolites be used for the biological monitoring of occupational human exposure to BTEX [7]. These urinary metabolites are useful biomarkers of internal dose for occupational and environmental exposure as they reflect absorption by all routes (inhalation, dermal, and ingestion) and they have the advantages of long half-life, high specificity, low volatility, and the non-invasive sampling of urine.

These metabolites were reported in literature due to their strong correlation with exposure levels across a wide range of BTEX concentrations and include: t,t-muconic acid (MU), S-phenylmercapturic acid (PMA), phenol, and catechol for benzene; hippuric acid (HA), S-

benzylmercapturic acid (BMA), o-cresol, and p-cresol for toluene; mandelic acid (MA) and phenylglyoxylic acid (PGA) for ethylbenzene; and methylhippuric acids (MHA) for xylenes. Benzoic acid (BA) is not reported as a direct biomarker of exposure to BTEX but its intake as benzoate, which is a common food preservative, can affect urinary background levels of HA (glycine conjugate of benzoic acid) in the human population. Lord *et al.* summarized different interpretation scenarios for different BA/HA ratios, other than those caused by exposure to BTEX [8]. Hence, we added BA to our list of biomarkers to provide a method that can be adopted to obtain a complete diagnostic picture in toxicological studies. However, HA is not the only one of these metabolites which suffers from background levels in urine of non-exposed individuals. MU can be formed from dietary sorbic acid preservative [9]. BMA can be derived from benzyl alcohol which is present in many cosmetic products [10]. HA can be found in peaches, fruit juices, sodas, ketchup, and due to dietary intake of benzoate preservatives [11]. This necessitates simultaneous determination of multiple metabolites for each parent compound and not relying solely on a specific biomarker. Moreover, adopting a multiple biomarkers approach will help to achieve more accuracy and specificity for evidence-based decisions, to provide a pattern match for single or co-exposures, to show co-exposure interaction effects on delay, suppression, or enhancement of formation, and to capture the variability through the studied population.

Several analytical methods have been published for the determination of urinary metabolites of one or more of the BTEX compounds. This includes those based on paper and thin-layer chromatography [12, 13], gas chromatography-flame ionization detection (GC-FID) [14, 15], GC-MS [16], HPLC-UV [17-19], and HPLC-MS [20]. However, few studies reported the simultaneous determination of urinary metabolites derived from all BTEX compounds. Simultaneous determination of several metabolites would be particularly useful in cases of co-

exposures. Approaches which have adopted simultaneous determination include capillary electrophoresis [21], HPLC-UV [22, 23], HPLC-MS [24-26], GC-MS [27] and solid-phase microextraction followed by HPLC or GC-MS [28]. Despite being comprehensive in covering the determination of concurrent metabolites, these approaches have focused only on a representative metabolite or two for each of the BTEX compounds. Some of BTEX urinary metabolites were not incorporated in each of the reported simultaneous determination methods and most of the reported methods focused only on either the carboxylic or phenolic metabolites to target as biomarkers. Further, some of the aforementioned studies used some kind of biological sample pretreatment and clean-up steps such as liquid-liquid extraction, solid-phase extraction (SPE), and in case of GC, a derivatization procedure is usually employed for these polar metabolites. These traditional sample preparation methods are usually labor-intensive and can lead to sample loss, increased total analysis time, low recovery, and exposure to contaminants. This is most likely due to the lack of adequate specificity and/or their presence at very small concentrations in urine especially at low exposure levels to BTEX. Recent sample preparation methods, using restricted access media or online SPE columns for online trapping and pre-concentration, require additional hardware for automated column switching and expertise. Hence, a gap occurs in understanding the mechanism of toxicity and metabolite formation caused by single or co-exposure to BTEX compounds. Studies have shown that the relative proportions of urinary metabolites are affected by co-exposure as well as by the concentration, rate, and route of exposure. For instance, co-exposure to toluene and xylenes has been reported to reduce PMA in urine [29].

The goal of this work was to develop and optimize a novel combination of a one-pot derivatization *in situ* [30] kit (OPDISK) that can be directly integrated with LC-ESI-MS/MS for the simultaneous quantitation of 14 phenolic and carboxylic biomarkers of BTEX in human urine.

Our developed method accommodates small volumes of urine samples (50 µL) and can be fully automated and used as a single analytical platform to achieve the necessary short analysis time, high throughput, sensitivity, and specificity to qualify as a reliable broadly aimed tool for bio-monitoring in routine screening programs, large epidemiological, toxicological, and clinical studies, thus substituting a combination of analytical platforms for short and long term effects of single and multiple BTEX exposures. This will allow better understanding of the effects of exposure to BTEX compounds. Eventually, undertaking the necessary preventive measures and evidence based practices by public health practitioners and health authorities will minimize exposure and related harmful health effects.

4.3 Experimental

4.3.1 Reagents and chemicals

Triethylamine (TEA), phenol, 1,2-Dihydroxybenzene, o-cresol, p-cresol, trans, trans-muconic acid (MU), hippuric acid (HA), 2-methylhippuric acid (2MHA), 3-methylhippuric acid (3MHA), 4-methylhippuric acid (4MHA), phenylglyoxylic acid (PGA), DL-mandelic acid (MA), iodomethane, 3-pyridinemethanol, and Surine™ negative urine control were purchased from Sigma–Aldrich (St. Louis, MO). DL-phenylmercaprturic acids (PMA), DL-benzylmercapturic acid (BMA), 2-Fluoro-1-methylpyridinium p-toluenesulfonate (FMP) and LC-MS grade formic acid were purchased from TCI America (Tokyo, Japan). Phenylglyoxylic Acid-d5 was purchased from Toronto research chemicals. N-Acetyl-S-benzyl-2,3,4,5,6-d5-DL-cysteine, trans,trans-Muconic-d4 acid, 1,2-Dihydroxybenzene-d6, N-Benzoyl-d5-glycine, N-(2-Methyl-d3-benzoyl-d4)glycine, N-(3-Methyl-d3-benzoyl-d4)glycine, DL-Mandelic-2,3,4,5,6-d5 Acid, N-Acetyl-S-phenyl-d5-DL-cysteine, o-Cresol-d7, p-Cresol-d7, Phenol-2,4,6-d3, Benzoic-d5 Acid were purchased from CDN Isotopes (Quebec, Canada). Pooled normal human urine was purchased from

Innovative Research (Novi, MI). ClinChek® urine control was purchased from Recipe (Munich, Germany). LC-MS grade water, methanol and acetonitrile were purchased from Honeywell Burdick & Jackson International (Muskegon, MI).

4.3.2 Synthesis of 3-carbinol-1-methylpyridinium iodide

3-carbinol-1-methylpyridinium iodide (CMP) was synthesized in our lab adopting the scheme reported by Yang *et al.* [31]. Briefly, five-fold excess of methyl iodide (50 mmol) was mixed and shaken with 3-pyridine methanol (10 mmol) at room temperature for 1 h. The crystals formed were then washed with cold acetone and dried.

4.3.3 Stock solutions and validation

Stock solutions of phenol, MA, catechol, o-cresol, p-cresol were prepared in acetonitrile at a concentration of 2-3 mg mL^{-1}. A standard mixture stock solution of HA (4 mg mL^{-1}), 2MHA, 3MHA and 4MHA (each 2 mg mL^{-1}), PGA (0.8 mg mL^{-1}) and BA (0.1 mg mL^{-1}) were prepared in an 8:2 acetonitrile: water mixture. Individual stocks of labeled and unlabeled PMA and BMA were prepared at 1 mg mL^{-1} in 9:1 acetonitrile: water. MU labeled and unlabeled standards were prepared in 1:1 acetonitrile: water at 0.5 mg mL^{-1}. The remaining deuterated analogs were prepared in acetonitrile at 1 mg mL^{-1}. A working solution of deuterated internal standards (ISD) was prepared from individual deuterated analog stocks. A series of working solutions of analytes were prepared from individual stocks by successive dilutions with acetonitrile and the calibration standards were then prepared by spiking 50 µL pooled human urine with 50 µL working solutions of the desired concentrations. Quality control (QC) samples were prepared in quintuplicate at low, medium, and high concentration levels, different from those concentrations used for the calibration points. LabSolutions software (version v.5.80, Shimadzu) was used for peak integration, calibration, and quantification. Peak area ratios of analyte/ISD were plotted versus the

concentration ratios of analyte/ISD and unknown concentrations were then calculated from the generated calibration curve equations.

4.3.4 Optimization of derivatization kit variables

The most important parameters affecting the performance of the OPDISK were selected based on the literature [30] and preliminary experiments. These variables were narrowed to comprise concentration of FMP, concentration of CMP, and volume of TEA as being critical for optimization. Central composite design (CCD) was used to optimize the values of these three factors and to achieve the best response using Surine™ samples spiked with a standard mixture. The CCD is divided into three groups of design points, namely a two-level factorial design points for estimating first order and two-factor interactions, star points for estimating quadratic effects, and replicates of center points for estimating pure error and to tie blocks [32, 33]. The other variables (reaction temperature and time), which could influence the response, were individually optimized through additional experiments. The optimum reaction time and temperature were found to be 20 min at room temperature. These conditions were adopted and the ensuing CCD included 20 experiments in five levels for three factors and consisted of six replicates of center points as listed in Table 4-1. All injections were made in triplicate (n = 3) and in a random order to provide protection against bias from variables which change over time.

4.3.5 In Situ Derivatization-Direct injection workflow

A 50 µL aliquot of centrifuged urine was mixed with an equal volume of standards (STD) in autosampler glass vials followed by the addition of 25 µl of ISD. 200 µL of FMP (28 mg mL^{-1}), 200 µL of CMP (32 mg mL^{-1}) and 6 µL of TEA were then successively added to the reaction vial. The derivatization reaction was briefly vortexed and then allowed to proceed for 20 min at room temperature. After 20 min, 469 µL of 1% formic acid in water was added to the reaction

Table 4-1. Experimental design matrix in the central composite design for optimizing the derivatization conditions

Std. order	Block	Run order	FMP Concentration (mg mL^{-1})	CMP Conconcentration (mg mL^{-1})	Triethylamine volume (µL)
10	Day 1	1	19.0	21.0	13.0
2	Day 1	2	28.0	10.0	6.0
5	Day 1	3	10.0	10.0	20.0
3	Day 1	4	10.0	32.0	6.0
8	Day 1	5	28.0	32.0	20.0
11	Day 1	6	19.0	21.0	13.0
7	Day 1	7	10.0	32.0	20.0
9	Day 1	8	19.0	21.0	13.0
12	Day 1	9	19.0	21.0	13.0
4	Day 1	10	28.0	32.0	6.0
1	Day 1	11	10.0	10.0	6.0
6	Day 1	12	28.0	10.0	20.0
16	Day 2	13	19.0	39.5	13.0
19	Day 2	14	19.0	21.0	13.0
18	Day 2	15	19.0	21.0	24.8
17	Day 2	16	19.0	21.0	1.2
13	Day 2	17	3.9	21.0	13.0
14	Day 2	18	34.1	21.0	13.0
15	Day 2	19	19.0	2.5	13.0
20	Day 2	20	19.0	21.0	13.0

mixture to quench the derivatization reaction. The autosampler vial was then transferred to the autosampler for LC-MS/MS analysis. For unknown urine samples, the same procedure was adopted except no standards were spiked and instead, 50 µL of 95:5 acetonitrile: water was added.

4.3.6 Instrumentation

An upgraded Shimadzu LCMS-8030 (Shimadzu Scientific Instruments, Inc., Columbia, MD) was employed for the LC-MS/MS analysis. The triple quadrupole was operated using positive ionization ESI and scheduled multiple reaction monitoring (SMRM) modes. The mass

spectrometer conditions were as follows: Interface voltage, 4.5 kV; nebulizer gas, nitrogen at 3 L min^{-1}; heat block temperature, 300℃; desolvation line (DL) temperature, 200℃; drying gas, nitrogen at 13 L min^{-1}; and collision gas, argon at 230 kPa. The MRM events used for quantitation, confirmation, and internal standards are summarized in Table 4-2.

Table 4-2. MRM transitions for derivatized metabolites and their deuterated analogs.

Parent Compound	Metabolites	Quantifier MRM, m/z Transition (CE*, V)	Qualifier MRM transitions, m/z	Internal Standard MRM transition, m/z (CE*, V)
Benzene	Muconic acid	177>107 (-19)	177>92, 177>122	179>107 (-20)
	Phenylmercapturic acid	345>235 (-22)	345>107, 345>124	350>235 (-22)
	Phenol	186>110 (-24)	186>77, 186>51	189>111 (-26)
	Catechol	202>110 (-27)	202>93, 202>78	207>111 (-27)
Toluene	Benzoic acid	228>107 (-28)	228>105, 228>92	233>107 (-29)
	Hippuric acid	285>124 (-23)	285>107, 285>92	290>124 (-25)
	Benzylmercapturic acid	359>235 (-22)	359>107, 359>124	364>107 (-45)
	o-Cresol	200>110 (-24)	200>91, 200>65	207>98 (-28)
	p-Cresol	200>110 (-25)	200>91, 200>65	207>111 (-28)
Ethylbenzene	Mandelic acid	258>124 (-25)	258>107, 258>92	263>124 (-25)
	Phenylglyoxylic acid	256>107 (-22)	256>92, 256>106	261>107 (-25)
Xylenes	2-methylhippuric acid	299>107 (-32)	299>124, 299>92	306>107 (-32)
	3-&4-methylhippuric acids	299>124 (-25)	299>107, 299>92	306>107 (-33)

*CE: collision energy

LC was performed using a binary solvent delivery system (LC-20AD XR, Shimadzu) and autosampler (SIL-20ACXR, Shimadzu). The mobile phase consisted of 0.1% formic acid in water as solvent A and 0.1% formic acid in methanol as solvent B. Gradient elution started initially with 0% B for 1.5 min, then increasing linearly from 50% B to 54% B over 5.5 min, followed by a 0.5 min hold at 100% B and finally equilibrating at 0% B for 5 min. A flow rate of 0.3 mL min^{-1} was used and increased to 0.4 mL min^{-1} during the time interval from 7 to 10 min. The column oven temperature was set to 9℃. Chromatographic separations were performed using a Force™ Biphenyl (Restek Corporation, Bellefonte, PA) (2.7 um dp; 100 × 2.1 mm) column (biphenyl

bonded phase on a fully porous silica particle, 100 Å). Sample injection volume was 8 µL. Total LC run time was 12.5 min including the washing and equilibration steps.

4.4 Results and discussion

Low molecular weight polar metabolites usually suffer more background noise in the low mass spectral range. Hence, derivatization can result in an increase in their molecular weight to enable their detection in the high mass spectral range, further from low molecular weight matrix interferences. Moreover, phenols have very low ionization efficiencies when detected using LC-ESI-MS, as they are neutral at the usual pH conditions used for LC-MS analysis. Carboxylic acids are usually detected in the negative ESI mode which is known to be less sensitive than positive ESI. Charge reversal derivatization of carboxylic acids to enable their detection using (+) ESI was reported to increase their sensitivity by 10- to 20-fold [34] and by 2500-fold in another work [31]. Other challenges for sensitive determination of carboxylic acids include their acidic character and high water solubility which usually necessitates a compromise between increasing their chromatographic retention and resolution using acidic pH [35] and increasing their electrospray ionization efficiencies using a basic pH [36].

The fourteen targeted biomarkers adopted in this study have various functionalities but all share either a carboxylic or phenolic functionality. Hence, to simultaneously increase their ionization efficiencies, we adopted a strategy to develop a charge reversal derivatization kit. The OPDISK is composed of FMP, a charged alcohol CMP, and a basic catalyst TEA. FMP plays a dual role. It imparts a permanent (unaffected by pH) positive charge to phenols by forming charged ethers. At the same time, it activates the carboxylic acid functionalities for their charged ester formation with the charged alcohol. The formed N-methylpyridyl ether ions $[M+92]^+$ for phenols and 3-carbinol-N-methylpyridyl ester ions $[M+106]^+$ for all carboxylic acids, except muconic acid

$[M+212]^{++}$ (two derivatized carboxylic groups), are then amenable to sensitive detection by LC-ESI-MS/MS in the positive ionization mode. This derivatization reaction is a one-pot rapid reaction, which occurs at room temperature and does not require any post-derivatization extraction or cleaning for high throughput analysis. However, depending on the purpose of study and complexity of sample matrix, the OPDISK can also allow online integration of weak cation exchange restricted access media (RAM) for selective trapping and sample cleanup [37].

Although all derivatized biomarkers are polar and cationic, adopting hydrophilic interaction liquid chromatography (HILIC) mode using either silica or pentafluorophenyl chemistry (data not shown) was proven to be inefficient to achieve baseline resolution of important isomers under study (o- and p-cresols; 2-, 3-, and 4-MHA). Moreover, elution occurred at relatively high aqueous content in the mobile phase which counteracted the usual gained advantage of high sensitivity using an organic-rich HILIC mode. The final adopted LC method in this study used a biphenyl column chemistry in reversed phase mode, to take advantage of cation-π interactions, and a low oven temperature of 9℃ to add additional shape selectivity, which led to baseline resolution of critical positional isomers (except 3- and 4-MHA), as well as a lack of matrix interfences for analytes of interest, as shown in Fig. 4-1. These conditions also achieved a high sensitivity by eluting all biomarkers in reasonable high organic content (50 - 54% methanol).

The CCD experimental design was divided into two blocks to remove effects of unknown or uninterested variables on response which can vary from day to day. Four individual responses, representative of the fourteen biomarkers, were chosen to evaluate the derivatization yield efficiency. These responses were the peak areas of MU, 3-MHA + 4-MHA, PMA, and catechol. Design-Expert® software version 10 (Stat-Ease, Minneapolis, USA) was used to perform experimental design and the statistical analysis.

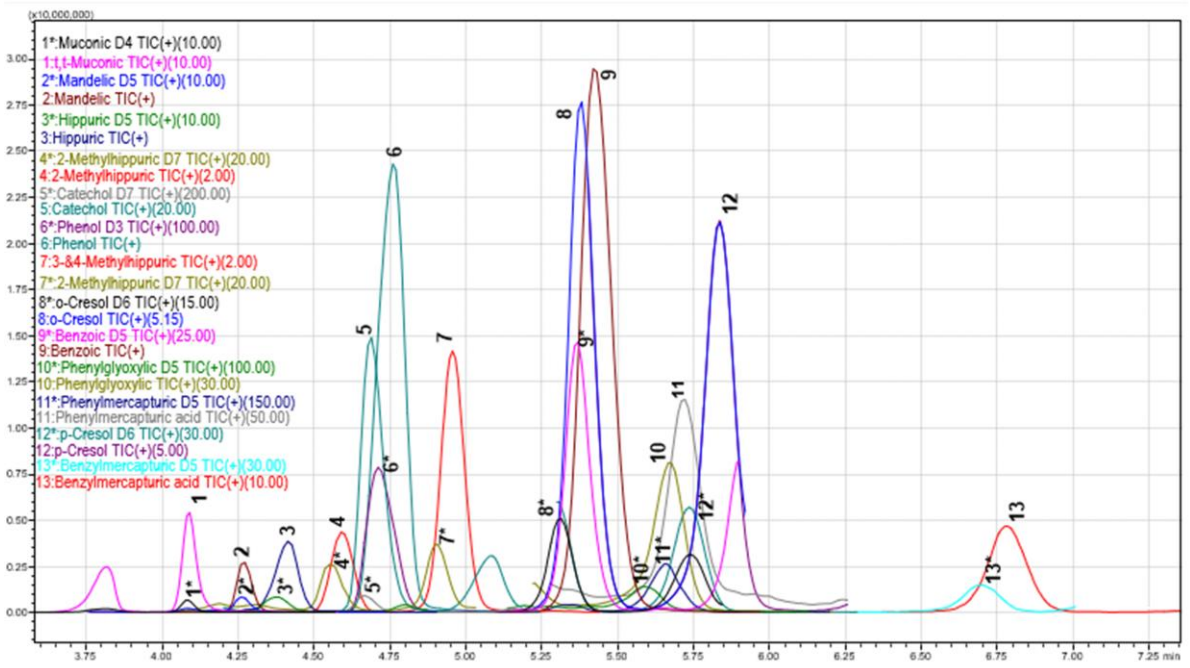

Figure 4-1. MRM chromatogram of a derivatized high QC urine sample spiked with 14 BTEX metabolites (analyte concentrations are given in Table 4-7).

To obtain the most convenient models, the significance of the regression models was evaluated by applying the analysis of variance (ANOVA) and eliminating the statistically insignificant parameters (p-value larger than 0.05), as shown in Tables 4-3 and 4-4. The lack-of-fit (LOF) values for all models were not significant relative to the pure error as indicated by their p-values. Final predictive polynomial equations for the chosen models were calculated and listed in Table 4-5 in terms of coded values. The coefficients for the significant factors selected in each polynomial model are shown based on coded factors (-1 to +1 for the low versus high levels) to show a direct comparison of the relative impact of different factors on the predicted response by each model for each response. Response transformations were applied for the responses of catechol and MU to improve their predictive models and to stabilize variance across the whole range of responses.

Table 4-3. ANOVA for Response Surface Model for Catechol & Phenylmercapturic Acid.

	Catechol						Phenylmercapturic Acid					
Source	**Sum of Squares**	**df** [a]	**Mean Square**	**F Value** [b]	**p-value** [c]		**Sum of Squares**	**df** [a]	**Mean Square**	**F Value** [b]	**p-value** [c]	
Block	930.7	1	930.7				2.11E+05	1	2.11E+05			
Model	3.86E+06	9	4.28E+05	103.6	< 0.0001	significant	6.33E+07	9	7.04E+06	28.44	< 0.0001	significant
Residual	37211.5	9	4134.6				2.23E+06	9	2.47E+05			
Lack of Fit	28270.5	5	5654.1	2.5	0.1947	not significant	9.39E+05	5	1.88E+05	0.58	0.7179	not significant
Pure Error	8941.0	4	2235.2				1.29E+06	4	3.22E+05			
Cor Total	3.89E+06	19					6.58E+07	19				

[a] Degrees of freedom.
[b] Test for comparing model variance with residual (error) variance.
[c] Probability of seeing the observed F-value if the null hypothesis is true.

Table 4-4. ANOVA for Response Surface Model for Muconic and 3-&4-Methylhippuric Acids.

	t,t-Muconic Acid						3-&4-methylhippuric Acids					
Source	**Sum of Squares**	**df** [a]	**Mean Square**	**F Value** [b]	**p-value** [c]		**Sum of Squares**	**df** [a]	**Mean Square**	**F Value** [b]	**p-value** [c]	
Block	0.03	1	0.03				1.23E+05	1	1.23E+05			
Model	3.46	8	0.43	19.68	< 0.0001	significant	3.08E+08	10	3.08E+07	29.56	< 0.0001	significant
Residual	0.22	10	0.022				8.34E+06	8	1.04E+06			
Lack of Fit	0.18	6	0.03	3.09	0.1472	not significant	4.86E+06	4	1.21E+06	1.39	0.3775	not significant
Pure Error	0.039	4	9.74E-03				3.48E+06	4	8.71E+05			
Cor Total	3.7	19					3.17E+08	19				

[a] Degrees of freedom.
[b] Test for comparing model variance with residual (error) variance.
[c] Probability of seeing the observed F-value if the null hypothesis is true.

Table 4-5. Predictive coded polynomial models.

Response	Intercept	A*	B**	C***	AB	AC	BC	A^2	B^2	C^2	A^2B	A^2C
Square root (Catechol)	1163.84	-326.27	119.01	356.57	-6.35	95.35	-48.93	51.81	1.35	-147.00		
Phenylmercapturic acid	2665.15	83.43	1711.09	-1004.59	368.29	76.88	-767.13	-355.87	159.51	272.83		
Log10 (Muconic acid)	4.50	0.27	0.17	-0.26	0.10	-0.12		0.11	-0.14	0.16		
3-&4-Methylhippuric acids	6195.70	135.74	2645.55	-3016.33	1454.19	-109.23	-1151.44	-705.72		1482.30	1348.35	1482.97

*A: FMP concentration (mg mL^{-1})
** B: CMP concentration (mg mL^{-1})
***C: TEA volume (µL)

Three-dimensional response surface plots for each one of the four target responses were generated in Fig. 4-2 to find regions of maximum yields. As can be easily noted, since the factor levels required to achieve optimum response values for carboxylic metabolites do not correspond with those required for catechol as a representative of phenolic metabolites, then a compromise among the responses should be achieved. The desirability function can be used to achieve the best compromise when dealing with multiple responses [33]. The optimum derivatization conditions in terms of desirability score were achieved at the following values: 28 mg mL^{-1} FMP, 32 mg mL^{-1} CMP and 6 µL TEA. Finally, these optimum conditions were adopted to carry out model confirmation for each metabolite as demonstrated in Table 4-6.

Table 4-6. Confirmation results for predictive models

Response	Predicted Mean	N[a]	95% PI[b] low	Data Mean	95% PI[b] high
Catechol	209714.0	3	94627.5	208532.0	359043.0
Phenylmercapturic acid	6599.3	3	5471.5	7627.1	7727.1
Muconic acid	374833.0	3	173637.0	309420.0	722600.0
Methylhippuric acids	15350.1	3	12882.5	17295.1	17817.7

[a] N: Number of replicates, each injected three times.
[b] PI: prediction interval.

4.4.1 Analytical performance

To ensure that the optimized method was suitable for application in routine urine analysis, analytical performance parameters, such as calibration model, linearity, limits of detection (LODs), precision (RSD%), accuracy, and stability of derivatized urine samples were evaluated and determined. Isotope dilution mass spectrometry calibration was chosen for quantitation, and the validity of the novel method was demonstrated by analysis of ClinChek® Urine controls

(available as two different spiked levels for each analyte) for seven of the biomarkers under study as shown in Fig. 4-3.

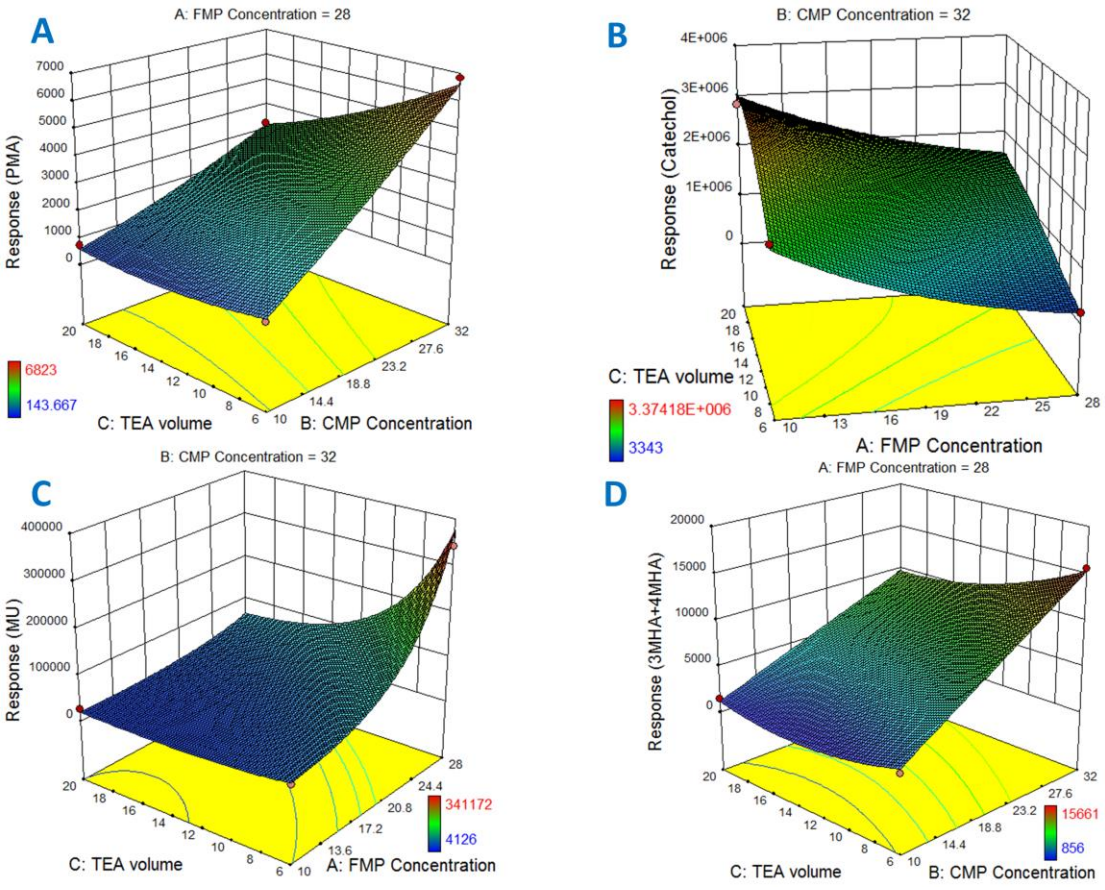

Figure 4-2. 3D surface plots showing the interacting effects of two derivatization parameters (other parameter constant at its optimum level) on derivatization yield of phenylmercapturic acid (A), catechol (B), muconic acid (C) and 3-&4-methylhippuric acids (D).

At least six non-zero standard solutions were analyzed to obtain calibration curves with linear regression. A characteristic quantifier ion of each analyte was chosen, and its authenticity was verified with two qualifier ions. The response of the quantifier ion was normalized to the response of the corresponding deuterated analogs. The LODs were calculated experimentally as the smallest concentration of each analyte which gave a signal to noise ratio of 3, and that when

diluted by 2- to 3-fold, the signal disappeared. We did not calculate LODs for analytes which have minimum background levels since these will not indicate the actual LODs and their zero concentration responses were included for plotting their calibration curves. Excellent linear responses were achieved for all analytes ($R^2 \geq 0.990$) where either a quadratic fit or a linear fit was employed with or without weighting of 1/c. LODs were calculated both as native concentration of biomarkers in urine and as the mass loaded on-column (after applying a dilution factor of 20) to show the actual performance concerning the sensitivity of the method. Accuracy at the three QC concentration levels ranged from 85 to 114% and %RSD were less than 14% for all biomarkers.

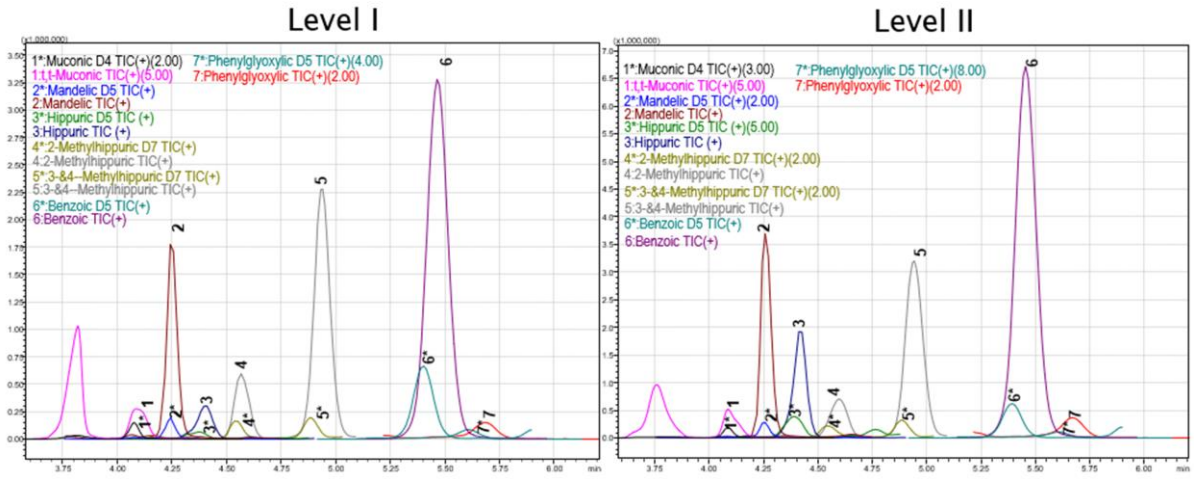

Figure 4-3. MRM chromatograms of derivatized ClinChek® level I and II urine samples.

The proposed method was successfully applied to the analysis of the ClinChek® urine control samples, as shown in Table 4-7. The stability of the derivatized urine samples was verified by the good linearity and accuracy obtained from calibration curves, where the triplicate measurements, of each calibration point, were obtained over time by the repeated injections of each calibration standard after the end of each calibration sequence. The stability was further

Table 4-7. Method validation and application to ClinChek® urine controls.

Analyte	Calibration range µg mL^{-1} (R^2)	LOD in sample (µg mL^{-1})	LOD on column (pg)	Spiked Concentration (µg mL^{-1})	Measured Concentration (µg mL^{-1}) (Accuracy % ± RSD%)	ClinChek® Level I Reference concentration range (µg mL^{-1})	ClinChek® Level I Measured values (Mean concentration ± SD)	ClinChek® Level II Reference concentration range (µg mL^{-1})	ClinChek® Level II Measured values (Mean concentration ± SD)
MU	0-25 (0.997)			20.0 3.6 1.3	106 ± 7 97 ± 10 98 ± 11	0.7-1.2	1.0 ± 0.1	2.4-3.6	2.8 ± 0.1
MA	9.4-500 (0.990)	1.6	640	480.0 85.7 30.0	91 ± 4 105 ± 10 113 ± 10	123-185	159 ± 16	430-645	458 ± 11
HA	0-1996 (0.999)			1596.8 285.1 99.8	112 ± 5 110 ± 6 105 ± 9	345-467	351 ± 21	1470-1990	1796 ± 70
2MHA	15.6-1000 (1.000)	7.8	3120	800.0 142.9 50.0	114 ± 4 91 ± 14 106 ± 11	138-207	167 ± 6	246-332	301 ± 18
3MHA+4MHA	5.3-2032 (0.998)	5.3	2120	1625.6 101.6 33.9	109 ± 2 113 ± 6 85 ± 12	456-683	508 ± 20	814-1101	907 ± 71
BA	0-50 (0.998)			40.0 7.1 0.8	103 ± 2 106 ± 1 100 ± 1				
PGA	12.5-400 (0.994)	3.1	1240	320.0 57.1 20.0	111 ± 6 101 ± 11 100 ± 10	47.8-71.7	52 ± 5	144-215	147 ± 39
PMA	1.6-100 (0.995)	0.8	320	80.0 14.3 5.0	107 ± 7 103 ± 3 99 ± 11				
BMA	0.5-60 (1.000)	0.2	80	48.0 8.6 1.0	102 ± 7 98 ± 6 97 ± 4				
Phenol	0-60			48.0 3.0 1.0	97 ± 3 93 ± 4 97 ± 5				
Catechol	0.3-134 (0.999)	0.1	40	107.2 19.1 2.2	95 ± 8 103 ± 5 90 ± 12				
o-cresol	0.01-4 (0.991)	0.0035	1.4	3.2 0.6 0.1	97 ± 3 99 ± 3 103 ± 10				
p-cresol	0-4 (0.996)			3.2 0.2 0.1	97 ± 3 109 ± 6 101 ± 6				

studied over a period of 24 hours at room temperature and in the autosampler at 4℃ by injecting high QC samples in triplicate. The LC-MS/MS results showed that the derivatized urine samples were stable with < 5% change in their concentration.

4.5 Conclusion

A novel charge-reversal-one-pot derivatization kit was developed, optimized, and successfully applied to simultaneously and directly derivatize all biomarkers in human urine over a wide concentration range. The entire method analysis time was less than 35 minutes at room temperature and multiple samples can be processed in parallel. The entire proposed method can be easily fully automated and applied to the routine high throughput analysis of very small volumes of urine samples (50 µL). Detection limits can additionally be improved simply by using a narrower I.D. column (e.g. 1mm I.D.), multiple injection loading scheme and/or a more sensitive instrument (e.g. next generation triple quadrupole systems). Further, we successfully applied the OPDISK to the analysis of commercial ClinChek® urine control samples.

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4.6 References

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CHAPTER FIVE: HIGH PH MASS SPECTROMETRY-COMPATIBLE REVERSED PHASE LIQUID CHROMATOGRAPHY FOR ALTERED SELECTIVITY IN SEPARATIONS OF INTACT PROTEINS

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High pH Mass Spectrometry-Compatible Reversed Phase Liquid Chromatography for Altered Selectivity in Separations of Intact Proteins

Yehia Z. Baghdady, Kevin A. Schug*

Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington TX 76019

*corresponding author: 700 Planetarium Pl., Box 19065, Arlington TX 76019-0065; (ph) 817-272-3541; (fax) 817-272-3808; (email) kschug@uta.edu

KEYWORDS: High pH; reversed phase liquid chromatography; intact proteins; top-down proteomics; altered selectivity

5.1 Abstract

Intact proteins are increasingly being recognized as potential biomarkers and biotherapeutic agents for cancer and other serious diseases. Low pH reversed phase plays an important role in both single and multidimensional protein separations for resolving complex protein samples prior to mass spectrometric detection. In this work, we evaluated the use of high pH reversed phase liquid chromatography as an alternative chromatographic separation to gain different selectivity while maintaining the high resolving power and MS compatibility of reversed phase separations. The altered selectivity gained by high pH reversed phase liquid chromatography can further help to separate unresolved protein peaks or to increase peak capacity and resolving power of a multidimensional setup for complex biological samples. Hence, we evaluated the use of different MS-friendly buffers, ion pairing reagents, and stationary phases (silica- and polymer-based) at alkaline pH for intact protein separations. The best chromatographic separation, with complementary selectivity to low pH reversed phase, was achieved using triethylammonium bicarbonate at pH 10 and hybrid silica particles.

5.2 Introduction

Different chromatographic modes have been used to address the challenges associated with characterization and/or quantitation of complex proteome samples in top-down proteomics. The high resolving power of reversed phase liquid chromatography (RPLC) and its direct coupling to mass spectrometry detection (MS) via electrospray ionization (ESI) enabled it to be the most widely used tool for analysis and purification of intact proteins [1]. However, many chromatographic challenges remain for intact protein separations, including poor recoveries, carryover (ghost peaks), and wide and distorted peaks. Typical mobile phase conditions used for RPLC of intact proteins usually involve the use of low pH and trifluoroacetic acid (TFA) as an ion pairing additive [2]. The low pH helps to suppress silanophilic interactions and TFA provides better peak shapes and recovery due to its solubilizing and intermediate ion pairing properties. However, due to the inherent complexities and the wide dynamic range of proteins in biological samples, some proteins co-elute at low pH and peak capacities are usually insufficient using one dimension.

Proteins are less sensitive to changes in the RPLC bonded phases than are small molecules [3]. The chromatographic surface (footprint) of protein is the one which plays the main role in determining its retention behavior. These large three-dimensional structures are amphoteric in nature with different isoelectric points (pI) and thus a different mobile phase pH can significantly affect protein retention and elution order. Nevertheless, high purity silica-based columns with wide pores commonly used for RPLC of proteins are not suitable for use at high pH; typically, pH > 8 dissolves silica and shortens column lifetime. Few columns with stationary phases tailored for protein separation at high pH are available commercially. Moreover, using such pH-resistant stationary phases, such as polymeric-based columns, failed to give satisfactory results concerning

peak widths, shapes and recovery of proteins as reported in an early study in 1988 [4]. Another challenge would be to find a suitable high pH volatile buffer capable of producing acceptable protein chromatography and producing conditions compatible with direct MS detection. These challenges have led to a paucity of reported data for high pH RPLC of intact proteins. Very few studies in the literature reported using high pH RPLC for intact protein separation [5-7] and only one article used a MS-compatible high pH buffer as the first dimension in offline 2D-LC [5].

In this study, we investigated different high pH MS-compatible chromatographic conditions, including: different buffers lacking ion pairing properties, such as ammonium formate (AF), ammonium bicarbonate (ABC), and ammonium trifluoroacetate (ATFA); ion pairing reagents with different counter anions, including triethylammonium bicarbonate (TEAB), triethyl ammonium acetate (TEAA), and triethylammonium formate (TEAF); and different HPLC packing supports, namely hybrid silica-based and polystyrene divinylbenzene (PS-DVB) supports. Although these types of packings are resistant to alkaline pH, most reported applications still favor low pH elution conditions to achieve resolution of proteins [8]. To the best of our knowledge, this is the first article qualitatively evaluating different MS-friendly high pH chromatographic conditions for orthogonal RPLC of intact proteins. It further represents the first report of using triethylammonium bicarbonate (TEAB) as a volatile ion pairing reagent to enable good separations of intact proteins at high pH, especially displaying orthogonal selectivity to low pH separations.

5.3 Experimental

5.3.1 Chemicals and stationary phases

Cytochrome C from equine heart (CCE), cytochrome C from bovine heart (CCB), lysozyme from chicken egg white (LZ), holo-transferrin bovine (HT), bovine serum albumin (BSA), α-lactalbumin from bovine milk type III (LA), myoglobin from equine skeletal muscles

(MY), β-lactoglobulin B from bovine milk (LG), β-casein from bovine milk (CS), carbonic anhydrase from bovine erythrocytes (CA), triethylammonium bicarbonate (TEAB) buffer (1.0 M, pH 8.5), ammonium formate (AF), ammonium trifluoroacetate (ATFA), and LC-MS grade formic acid (FA) were obtained from Sigma–Aldrich (St. Louis, MO). LC-MS grade triethylamine (TEA) and trifluoroacetic acid (TFA) were purchased through VWR (Missouri, TX). LC-MS grade ammonium hydroxide, acetic acid, and ammonium bicarbonate were obtained from Honeywell (Morris Plains, NJ, USA). LC-MS grade water and acetonitrile were purchased from Honeywell Burdick & Jackson International (Muskegon, MI).

Individual protein standards were prepared by dissolving 1.5 mg of each of HT and BSA in LC-MS grade water and 1 mg of CS in 0.05% TEA. All other protein standards were prepared by dissolving 1 mg of each one in LC-MS grade water. Silica-based hybrid columns were XBridge BEH C4 Column 300Å, 3.5 µm, 2.1 mm i.d. with column lengths of 50 and 150 mm (Waters, Milford, MA). The polymer-based PS-DVB was PLRP-S, 300Å, 3 µm, 2.1 mm i.d. and 50 mm length (Agilent Technologies, Santa Clara, CA).

5.3.2 Instrumentation and chromatographic conditions

A LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA), consisting of two LC-30AD pumps, DGU-20A5R degassers, a CTO-20AC column oven, a SPD-M30A photodiode array UV-VIS detector (PDA) and a SIL-30AC autosampler was utilized for performing the chromatographic separations of intact proteins. The LC system was interfaced to a triple quadrupole MS through a heated electrospray ionization (ESI) source. The LC-MS data was visualized and analyzed by Shimadzu LabSolutions software (version 5.80). After optimal chromatographic conditions were determined using the PDA detector, the triple quadrupole was operated in the single ion monitoring mode (SIM) of the

standard proteins for further experiments. For the negative ESI mode, the SIM channels were m/z 1029 for CCE, m/z 1018 for CCB, m/z 1787 for LZ, m/z 1732 for HT, m/z1661 for BSA, m/z 1771 for LA, m/z 1410 for CS, m/z 1381 for CA, m/z 1058 for MY and m/z 1405 for LG. While for the positive ESI mode the SIM channels were m/z 884 for CCE, m/z 1020 for CCB, m/z 1302 for LZ, m/z 1817 for HT, m/z 1385 for BSA and m/z 1419 for LA, m/z 943 for MY, m/z 1407 for LG, m/z 1044 for CS and m/z 908 for CA. The MS detector settings were an interface temperature at 200 °C, an interface voltage at 4 kV (positive ionization mode) and -3 kV (negative ionization mode), a desolvation line temperature at 250 °C, a heat block temperature at 400 °C, a nebulizer gas flow of nitrogen at 1.5 L/min, a heat gas flow of nitrogen at 10 L/min, and a drying gas flow of nitrogen at 8 L/min.

The gradient range was kept constant for data collection and comparison from 18% -54% ACN (after a brief initial hold at 18% ACN). The gradient time (t_G) was also adjusted in proportion to the column length to keep the gradient slope constant. Hence, the t_G was 20 min for the 150 mm length and 6.5 min for the 50 mm length columns. A flow rate of 0.2 mL/min, a sample injection volume of 10 µL (150 mm column) and 4 µL (50 mm column), and a column oven temperature of 45 °C were used in all experiments unless noted otherwise.

5.4 Results and discussion

Protein probes with a broad pI (acidic, neutral, and basic) and molecular masses ranging from 12 to 81 kDa were injected individually to confirm identity and to record their peak shapes without overlap among the tested different high pH chromatographic conditions. Table 5-1 shows the 10 protein standards used in this work with their different pI and molecular masses. Different high pH modifiers and column packings were screened to find the optimum high pH RPLC conditions. The objectives of this study were to achieve a different elution order (i.e. orthogonal

selectivity) than the one obtained at low pH while maintaining the good peak shapes and good recovery obtained by using low pH conditions. Hence, eight proteins were used in all initial screening experiments for probing altered selectivity at high pH using PDA detection at 280 nm. Once the optimum conditions were identified, two more proteins were added to the protein analyte set to confirm selectivity difference among a wider variety of proteins including structural isomers such as CCB and CCE which differ only by 3 amino acid residues in their 12 kDa sequence. Notably, MY, throughout this work, gave two peaks using PDA detection but only one corresponded to the protein part. The other peak of heme, the non-protein part of MY, was recognized based on the detection of 616 m/z (unprotonated iron (III)-heme bearing a single positive charge) [9] using ESI-MS in the positive mode. MY peak was identified in each chromatogram and labeled accordingly.

Table 5-1. Molecular masses and isoelectric points of protein standards used in this study.

Protein Probe	Molecular Mass (kDa)	Isoelectric Point
Cytochrome C Equine (CCE)	12	10.0 - 10.5
Cytochrome C Bovine (CCB)	12	10.0 - 10.5
Lysozyme (LZ)	14	11.4
Holo-transferrin (HT)	76-81	5.9
Bovine serum albumin (BSA)	66	4.9
Lactalbumin (LA)	14	4.5
Myoglobin (MY)	17	7.1
Lactoglobulin (LG)	18	4.9
Casein (CS)	24	5.1
Carbonic anhydrase (CA)	29	6.6

5.4.1 Effect of pH

XBridge™ BEH C4 300 Å stationary phase was used to evaluate the effect of high pH only on chromatographic separation excluding the effect of ion pairing reagents or buffer salts. This column can tolerate high pH up to pH 12 due to the hybrid silica-based particles which contain both inorganic (silica) and organic (organosiloxanes) components. Hence, this column is

characterized by fewer surface active silanols than conventional type B silica. To exclude any selectivity differences due to the nature of these hybrid particles, separation of protein standards was first evaluated using two different acidic conditions of 0.05% TFA + 0.1 % FA (pH 2) and 0.5% FA (pH 2.2) as shown in Figures 5-1A and B, respectively. The order of elution for the injected proteins followed what has been reported using traditional high purity silica and PS-DVB packings [2, 10-12]. Upon exchanging of TFA with FA as acidic modifier, a comparable performance was obtained with a minor selectivity difference as demonstrated by the reversal of elution order of MY and LA. This shows the benefit of using this low silica activity column for conditions which favor less ionic suppression caused by TFA addition.

In comparison with acidic modifiers, using basic modifiers only such as 0.1 % ammonium hydroxide (pH 10.7) and a column oven temperature of 30 °C resulted in the elution of six proteins in the dead volume, Figure 5-1C. The other two basic proteins CCB and LZ did not return any peaks (i.e., they were not recovered). We attributed these results to the anionic silanol sites on the surface of hybrid packings with ion-exclusion effects for anionic carboxyl sites of proteins. CCB and LZ were not recovered, probably due to denaturation exposing hydrophobic residues and/or irreversible adsorption of their basic groups to the ionized surface silanols at this high pH. Since all other neutral and acidic proteins were eluted in the dead volume, we think that this finding can be advantageous to potentially substitute sample preparation steps for online segregation of proteins from samples having acidic and neutral proteins and small molecules, which exhibit reasonable retention at high pH conditions.

A solution of 1% TEA (72 mM, pH 11.8), which is the highest recommended ionic strength to be used with hybrid silica particles according to the manufacturer, was also tested as the basic pH modifier, as shown in Figure 5-1D. In this case, TEA probably acted as a sacrificial base to

mask the ion-exclusion effect caused by the negatively charged surface silanols. This condition was successful to give the proteins some retention; however, all tested proteins still eluted early in the gradient with broad and/or multiple peaks resulting in a poor chromatogram. LZ was still not recovered using 1% TEA. At pH 11.8, TEA (pKa 10.8) would not be protonated and ionized to the degree required to provide sufficient ion pairing effects and thus, higher retention and resolution of proteins.

5.4.2 Effect of high pH buffers and ionic strength

Because of the amphoteric nature of proteins, both pH and ionic strength of mobile phase play important roles in determining the retention behavior of proteins. Figure 5-2 shows the effect of volatile ammonium buffer salts with different counter anions used at high pH conditions. AF was used at 20 mM concentration and high pH ranging from 8.5 to 10 and at 10 mM and pH 10, as shown in Figures 5-2A-D, respectively. The best compromise of good peak shapes, recovery of all injected proteins including highly basic protein LZ, and an orthogonal selectivity to low pH RPLC was achieved using 20 mM AF at pH 10. Nevertheless, using pH 10 gave very broad peaks for some proteins (CCB, CA and LZ) and thus more overlapping of retention zones. Both 20 mM ABC at pH 8.5 (Figure 5-2E) and 20 mM ATFA at pH 10 (Figure 5-2F) failed to achieve better performance regarding recovery of all proteins (e.g., LZ was not recovered). Notably, HT gave multiple peaks under all investigated high pH conditions discussed here and below, which are believed to be various forms of transferrin with different amounts of iron [6, 13].

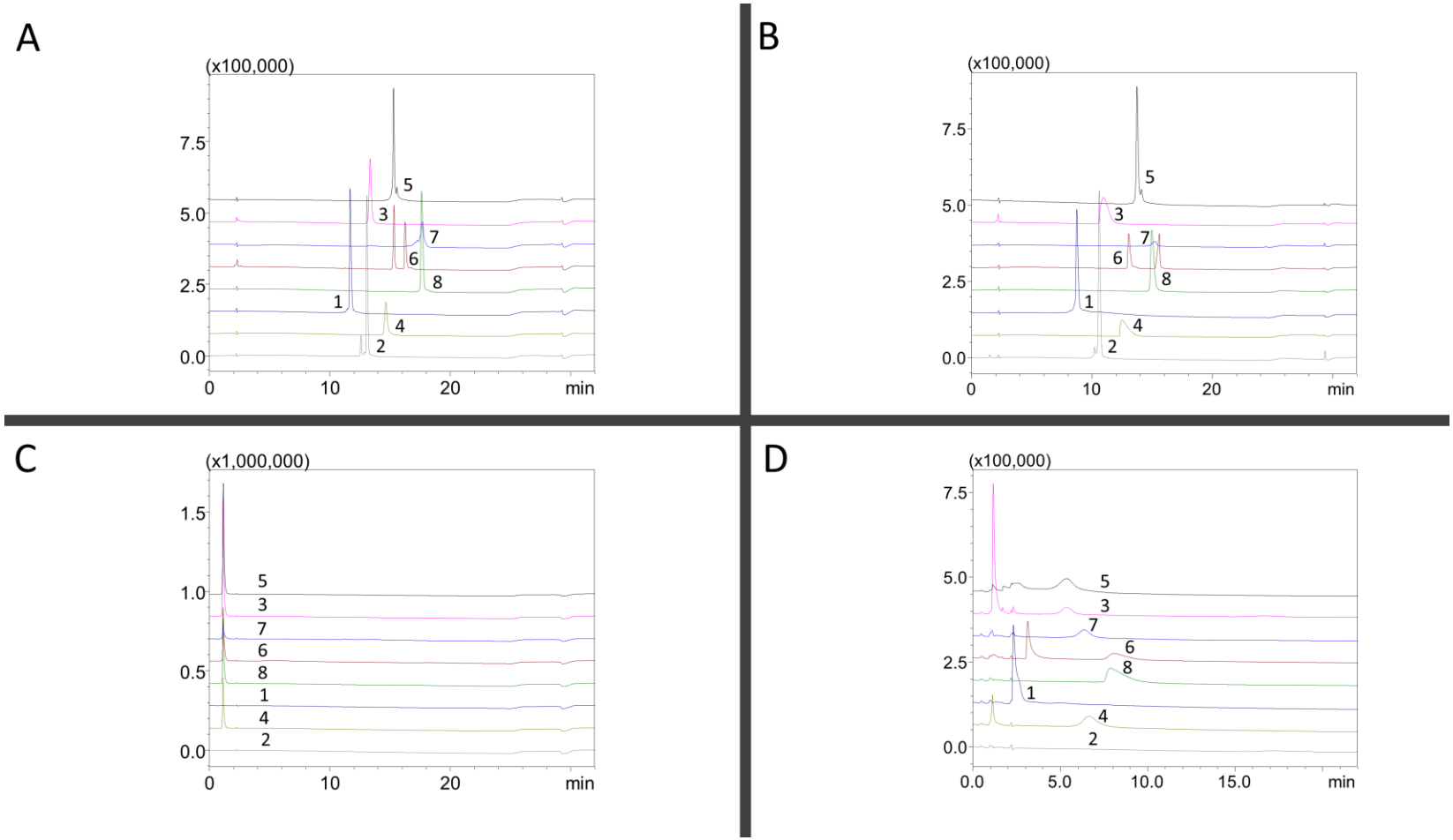

Figure 5-1. PDA chromatograms of protein standards injected on 150 mm XBridge BEH C_4 1. Cytochrome C bovine, 2. Lysozyme, 3. holo-transferrin, 4. bovine serum albumin, 5. α-lactalbumin, 6. Myoglobin, 7. Casein and 8. Carbonic anhydrase obtained using A. 0.05% TFA + 0.1 % FA, B. 0.5 % FA, C. 0.1% NH_4OH and D. 1% TEA.

5.4.3 Effect of ion pairing reagents and ionic strength

Ion pairing reagents can be very useful to enhance retention and polarity differences of ionized protein analytes. Acidic hydrophobic ion pairing reagents, such as TEA, improve differences in acidic polarity at high pH mobile phase. Other advantages of using TEA include its good solubility in organic solvents used in RPLC, and the better masking of the ion-exclusion with acidic proteins, or silanophilic interactions with highly basic proteins. Hence, we evaluated the use of TEA with different ionic strengths and counter anions. First, we used TEAB buffer, pH 8.5, with ionic strengths ranged from 18 mM (0.25%) to 72 mM (1%) concentration. Representative chromatograms for these conditions are shown in Figures 5-3A-C. 72 mM TEAB was the only one successful to achieve recovery of all proteins, including LZ. Since, we were limited by this ionic strength as the highest manufacturer's recommended concentration, we had to use a lower ionic strength titrated with TEA to increase the pH value to pH 10. Hence, we then used 36 mM TEAB + 18 mM TEA (0.25%) at pH 10 (i.e. ~54 mM TEA), as shown in Figure 5-3D. This was successful to yield the same chromatographic performance but with better orthogonality due to shifting of LZ peak to the end of retention order. Finally, we compared this performance to the one obtained by 54 mM TEA titrated with acetic acid to pH 10 (Figure 5-3E) and 54 mM TEA titrated to pH 10 with formic acid (Figure 5-3F). Surprisingly, unlike ammonium buffer salts, where formate was the best counter anion, the bicarbonate anion was the only one capable of yielding good recovery for all proteins including basic proteins CCB and LZ. Accordingly, we adopted 36 mM TEAB + 0.25% TEA (pH 10) as the best high pH buffer and ion-pairing reagent for further experiments.

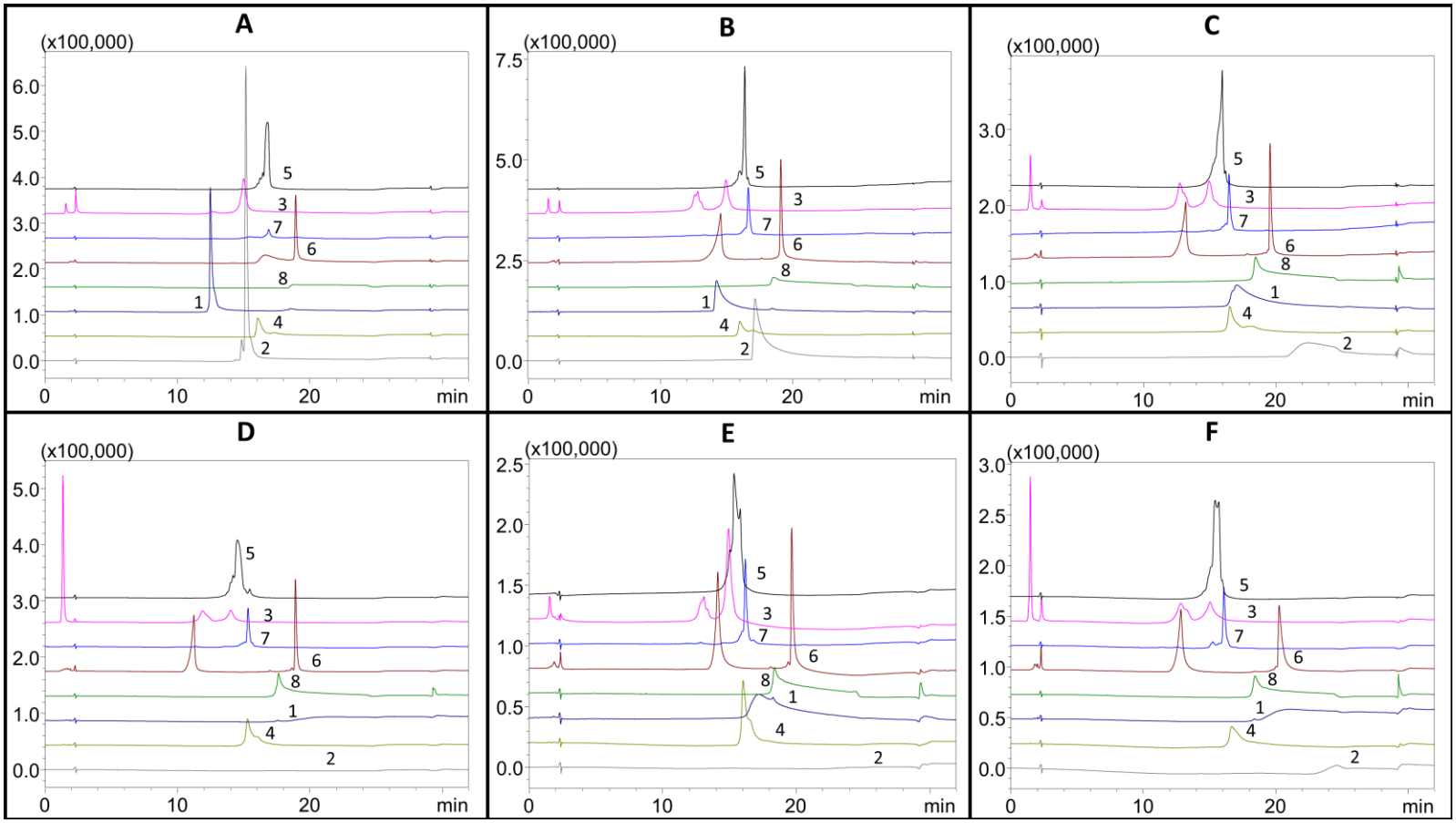

Figure 5-2. PDA chromatograms of protein standards injected on 150 mm XBridge BEH C_4 1. Cytochrome C bovine, 2. Lysozyme, 3. holo-transferrin, 4. bovine serum albumin, 5. α-lactalbumin, 6. Myoglobin, 7. Casein and 8. Carbonic anhydrase obtained using A. 20 mM ammonium formate, pH 8.5, B. 20 mM ammonium formate, pH 9.5, C. 20 mM ammonium formate, pH 10, D. 10 mM ammonium formate pH 10, E. 20 mM ammonium bicarbonate pH 8.5 and F. 20 mM ammonium trifluoroacetate, pH 10.

5.4.4 Effect of column length

Proteins are characterized by steep elution isotherms, which essentially manifest as an on/off retention mechanism. Because of this, unlike small molecules, column length does not have significant contribution to the resolution of protein peaks unless a longer gradient is used [14, 15]. We evaluated the same stationary phase packing of XBridge BEH C4, but with a shorter column length of 50 mm. The gradient time and injection volumes were then decreased and adjusted in proportion to the column length to have a fair comparison, for the eight proteins in common, with the previous longer column. Because of the MS-compatibility of our selected optimum high pH buffer, we used this time SIM detection of proteins in either positive mode for acidic conditions using 0.1% FA + 0.05% TFA (pH 2) or negative mode for basic conditions of 36 mM TEAB + 0.25% TEA (pH 10) for this evaluation. Representative chromatograms are shown in Figures 5-4A and B. Table 5-2 summarizes the results of using our optimum selected high pH buffer using both shorter and longer hybrid silica columns at low and high pH conditions. The elution time was normalized to the percentage of pure organic (% ACN) for direct comparison. The longer column (150 mm length) gave slightly wider elution window (+3.6%) under basic conditions compared to that of acidic conditions, while the shorter column (50 mm length) gave slightly narrower elution window (-4.4%) under basic conditions. The elution order, on the other hand, differed significantly by the pH switch using both shorter and longer columns. The column length solely did not affect the retention order under acidic pH, while under basic pH, the LZ peak shifted to be at the end of the retention window using the longer column. A shallower gradient on the shorter column (24% ACN - 44% ACN in 19.5 min) at pH 10 was successful to yield increased resolution of the protein peaks, as shown in Figure 5-4C. This shallower gradient further resulted in an increased orthogonal selectivity as illustrated by shifting of the LZ peak to the end of elution

window and reversal of the elution order of CS and BSA. This confirmed the nice performance of the high pH buffer with a shallower gradient.

Table 5-2. Elution window and order for injected proteins using different chromatographic conditions

Chromatographic Conditions[a]	Elution Window[b] (% ACN)	Elution Order[c]
pH 2, 150 mm (Figure 5-1A)	34.2% - 48.6%	2, 3, 4, 5, 6, 7, 9+10
pH 10, 150 mm (Figure 5-3D)	32.4% - 50.4%	4, 4, 4, 2, 4, 6, 5+9, 10, 7, 3
pH 2, 50 mm (Figure 5-4A)	37.4%-54%	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
pH 10, 50 mm (Figure 5-4B)	36.3%-48.5%	4, 4, 4, 2, 1, 4, 8, 6, 5, 9, 10+3, 7
pH 10, 50 mm, longer t_G (Figure 5-4C)	29.2%-40.5%	4, 4, 2, 1, 4, 8, 6, 9, 5, 10, 7, 3

[a] Chromatographic conditions are the same as in corresponding Figures.
[b] Elution window excludes HT peaks eluting in the void volume.
[c] Peak numbers are the same as in Figure 5-4.

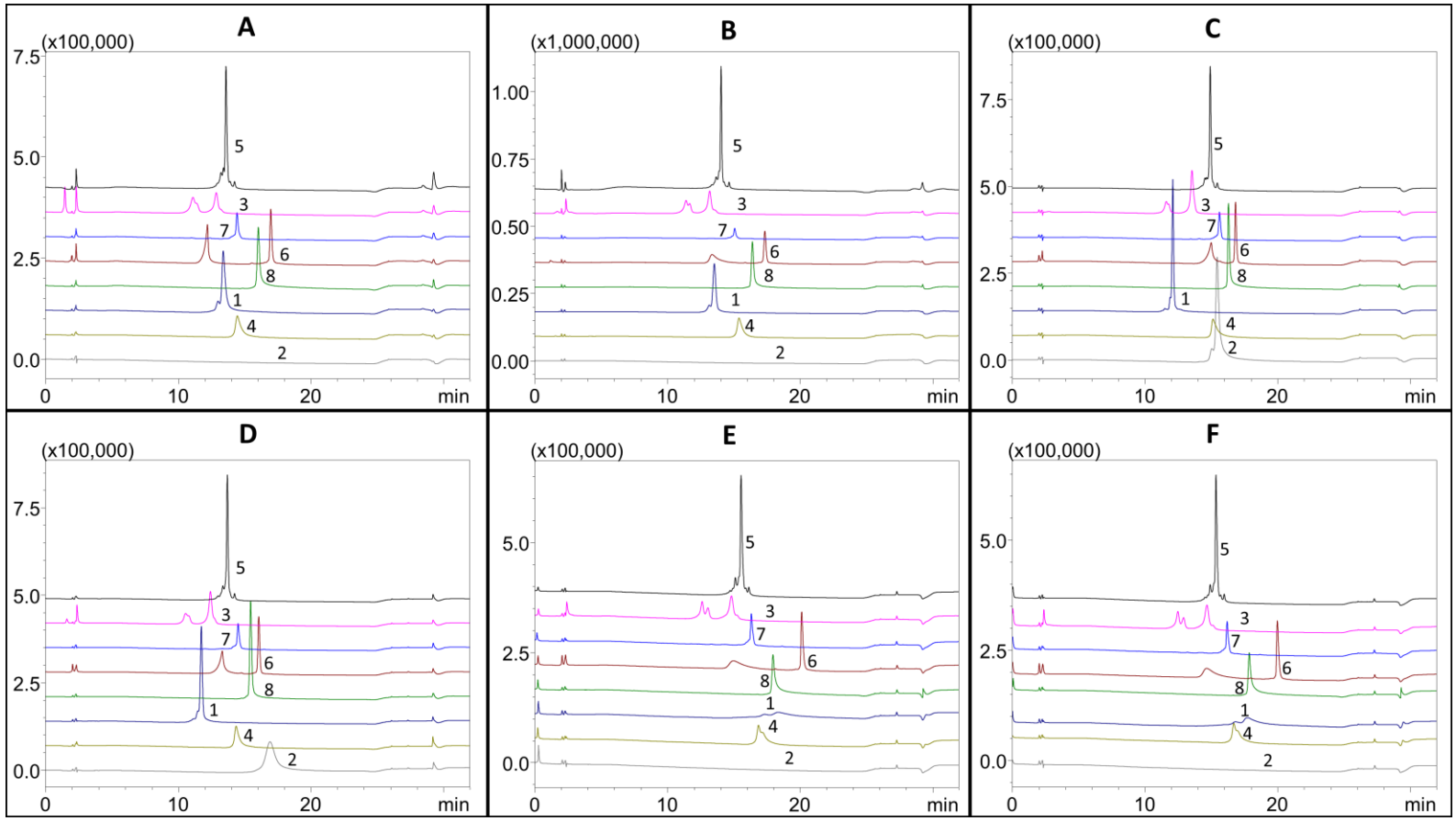

Figure 5-3. PDA chromatograms of protein standards injected on 150 mm XBridge BEH C_4 1. Cytochrome C bovine, 2. Lysozyme, 3. holo-transferrin, 4. bovine serum albumin, 5. α-lactalbumin, 6. Myoglobin, 7. Casein and 8. Carbonic anhydrase obtained using A. 18 mM triethylammonium bicarbonate, pH 8.5, B. 36 mM triethylammonium bicarbonate, pH 8.5, C. 72 mM triethylammonium bicarbonate, pH 8.5, D. 36 mM triethylammonium bicarbonate + 0.25% TEA, pH 10, E. 54 mM Triethylamine, pH 10 with acetic acid and F. 54 mM triethylamine, pH 10 with formic acid.

5.4.5 Effect of packing materials

Polymer-based PS-DVB packings feature chemical stability over a wide pH range of 1 – 14 and long column lifetime. However, continuous changes in pore diameters during gradient elution, due to the increased wetting and the subsequent swelling and shrinking of the organic polymeric matrix, can lead to changes in mass transfer and poor chromatography [16]. PLRP-S column packing has no bonded phase and its surface is inherently hydrophobic owing to the large number of aromatic rings in its structure. Hence, protein samples can tail or be strongly adsorbed unless there is a competing electron rich additive (e.g. TEA) and/or organic modifier, such as ACN, present.

As has been previously reported, the elution order of proteins on PS-DVB columns is generally similar to that of silica-based columns using acidic conditions and TFA as an acidic ion pairing reagent [11]. Accordingly, we first evaluated the PLRP-S column (50 mm length) performance using 0.1% FA and 0.05% TFA (Figure 5-5A) and compared it to the one obtained using the short XBridge column (Figure 5-4A). The acidic pH RPLC performance was very similar between the hybrid silica- and the polymer-based packings. However, the alkaline pH RPLC performance using 20 mM AF at pH 9.5 (Figure 5-5B) and the adjusted chromatographic conditions, to be in proportion to column length of PLRP-S, resulted in poor chromatography. Very poor recovery (no peaks for LZ, BSA and HT), bad peak shapes (MY and CCB), higher retention (appearance of all peaks near the end of gradient), and appearance of additional protein peaks at the end of each run during column re-equilibration for some proteins were the results of this poor performance. This higher retention at high pH can be due to the highly hydrophobic polymeric matrix and the higher surface area. PLRP-S has a significantly higher surface area (384 m^2/g) compared to that of XBridge BEH C4 (95 m^2/g), according to their manufacturer's

specifications. Because of the higher retention, a steeper gradient (32% - 76% ACN in 6.5 min) was also attempted on the PLRP-S column using 72 mM TEAB at pH 8.5, but the performance was still unsatisfactory and incomparable to hybrid silica-based polymers, as shown in Figure 5-5C.

5.5 Conclusion

In this study, we screened different high pH volatile buffers and ion pairing reagents by using two different types of high pH resistant RPLC packing materials. The optimal MS-compatible high pH buffer, for the ten protein standards used in this work, was found to be triethylammonium bicarbonate using ionic strength of 36 mM and adjusted to pH 10 with 0.25% TEA. Qualitative evaluation showed that the hybrid silica particles are the best suited for these high pH conditions while at low pH both polymeric and silica-based packing had very similar performance. Further, protein peaks resolved and eluted from high pH RPLC using TEAB buffer can be subjected directly to proteolytic digestion in a combined top-down/bottom-up approach. The high orthogonality obtained with this high pH RPLC configuration lends itself to its online coupling to low pH reversed phase for a comprehensive two-dimensional liquid chromatography (RPLC x RPLC) in top-down proteomics. This RPLC x RPLC for intact proteins is currently under investigation in our lab.

Acknowledgements

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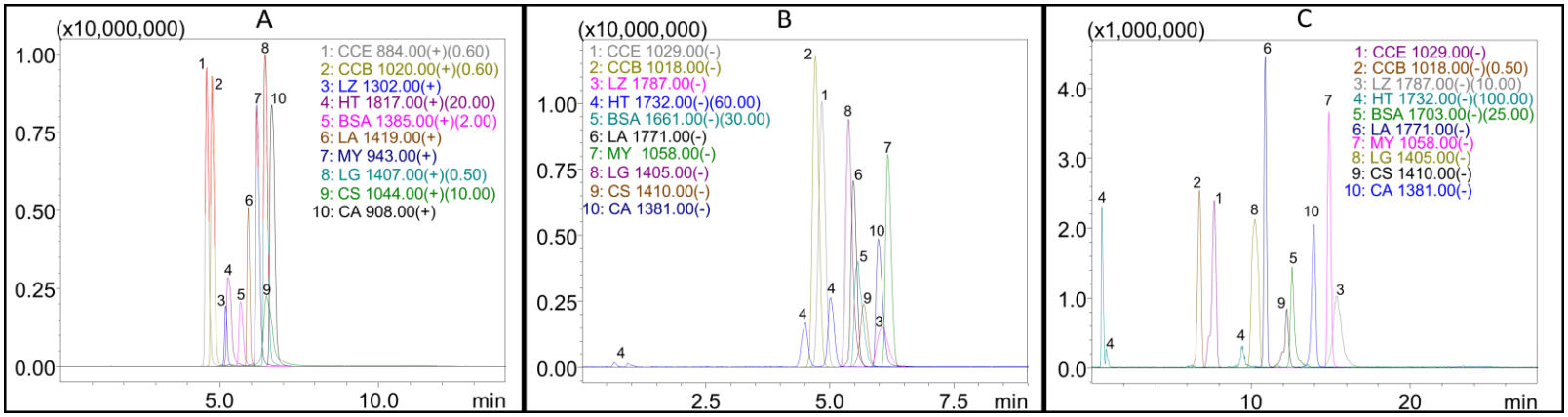

Figure 5-4. SIM chromatograms of protein standards injected on 150 mm XBridge BEH C_4 using A. 0.05% TFA + 0.1 % FA, pH 2, B. 36 mM triethylammonium bicarbonate + 0.25% TEA, pH 10 and C. 36 mM triethylammonium bicarbonate + 0.25% TEA using a shallower gradient as described in the results and discussion section.

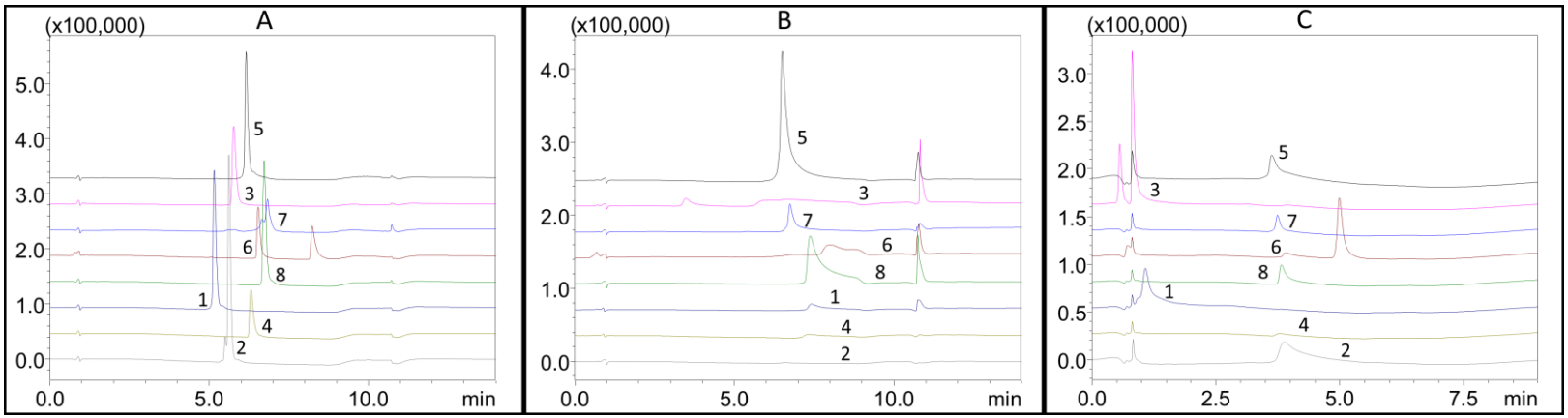

Figure 5-5. PDA chromatograms of protein standards injected on 50 mm PLRP-S column 1. Cytochrome C bovine, 2. Lysozyme, 3. holo-transferrin, 4. bovine serum albumin, 5. α-lactalbumin, 6. Myoglobin, 7. Casein and 8. Carbonic anhydrase obtained using A. 0.05% TFA + 0.1 % FA, B. 20 mM ammonium formate, pH 9.5, C. 72 mM triethylammonium bicarbonate, pH 8.5 using a steeper gradient as described in the results and discussion section.

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CHAPTER SIX: AN ONLINE COMPREHENSIVE HIGH PH REVERSED PHASE X LOW PH REVERSED PHASE FOR PROOF-OF-CONCEPT TWO-DIMENSIONAL SEPARATION OF INTACT PROTEINS IN TOP-DOWN PROTEOMICS

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An Online Comprehensive High pH Reversed Phase x Low pH Reversed Phase for Proof-Of-Concept Two-Dimensional Separation of Intact Proteins in Top-Down Proteomics

Yehia Z. Baghdady, Kevin A. Schug*

Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington TX 76019

*corresponding author: 700 Planetarium Pl., Box 19065, Arlington TX 76019-0065; (ph) 817-272-3541; (fax) 817-272-3808; (email) kschug@uta.edu

KEYWORDS: Comprehensive chromatography; LC x LC method development; high pH separations; triple quadrupole mass spectrometry; peak capacity

6.1 Abstract

A proof-of-concept study is presented on the use of comprehensive two-dimensional liquid chromatography mass spectrometry (LC x LC-MS) for the separation of intact protein mixtures using different mobile phase pH in each dimension. This system utilizes mass spectrometry (MS) friendly pH modifiers for the online coupling of a high pH reversed phase liquid chromatography (HPH-RPLC) in the first dimension (^{1}D) followed by a low pH reversed phase liquid chromatography (LPH-RPLC) in the second dimension (^{2}D). Owing to the ionic nature of proteins, the use of a different mobile phase pH was successful to provide altered selectivity between the two dimensions, even for closely related protein variants, such as cytochrome c variants from bovine and equine. Ultrafast sub-minute gradient separation of proteins in the second dimension was successful to minimize analysis time, while maintaining high peak capacity. Unlike peptides, the elution order of studied proteins did not follow their isoelectric points, where acidic proteins would be expected to be more retained at low pH (and basic proteins at high pH). The steep elution isotherms (on-off retention mechanism) of proteins and the very steep gradients utilized in the second-dimension column succeeded to overcome pH and organic solvent content mismatch. The utility of the system was demonstrated with a mixture of protein standards and an *Escherichia coli* protein lysate. The effective peak capacity of the entire LC x LC-MS analysis was 2268 with a peak production rate of 37.8 peaks per minute for the 60 minute gradient run.

6.2 Introduction

Proteins are of central importance as new biomarkers and potential therapeutic agents for cancer and other serious medical conditions. Top-down proteomics is concerned with the analysis of proteins at the intact level which imposes various challenges for their characterization and quantitation [1]. Recent advances in intact protein quantitation are exemplified by the use of triple quadrupole mass spectrometry which has been the work horse for the determination of small molecules in biological fluids [2, 3]. This usually requires a highly efficient front-end separation technique tailored for intact proteins and with high resolving power to help achieve more identifications and/or lower levels of quantitation when coupled online with mass spectrometric detection [4]. Despite the current availability of high resolution and high-performance mass spectrometers, the integration of online multidimensional methods with high resolution mass spectrometry detection (MS) and high throughput proteome coverage is still in its infancy.

Comprehensive two-dimensional liquid chromatography (LC x LC) has the potential to achieve the highest peak capacity that can be obtained in a short analysis time through a high throughput and fully automated platform [5]. This has motivated several research groups to investigate and to apply such platforms for intact protein separations using different combinations to achieve additional orthogonal selectivity and large peak capacities. Bushey and Jorgenson were the first to demonstrate LC x LC for a protein sample using cation exchange chromatography (CEX) in the first (^{1}D) and size exclusion chromatography (SEC) in the second dimension (^{2}D) [6]. This publication was followed by several others, which used LC x LC for intact proteins in different configurations, including SEC x RPLC [7], CEX x RPLC [8-10], anion exchange chromatography (AEX) x eight parallel RPLC [11], AEX x twelve parallel RPLC [12] and AEX x three parallel RPLC [13]. As indicated by these publications, RPLC is the dominant ^{2}D LC mode

in all these comprehensive combinations. This can be attributed to the very rapid equilibration of RPLC (as short as one column volume), high separation efficiency, wide applicability to a variety of samples, availability of many different stationary phases, and compatibility with MS. On the other hand, all the other chromatographic approaches used in the ^{1}D for intact proteins did not use RPLC and used instead another LC mode. Each of these ^{1}D alternative separation modes suffers from drawbacks, such as limited resolution and efficiency for SEC, and MS-incompatibility and the need for online desalting for IEX. Hence, another highly efficient ^{1}D separation mode with a reasonable altered selectivity is highly desirable in such online combinations for the top-down proteomic field. Both hydrophilic interaction chromatography (HILIC) and hydrophobic interaction chromatography (HIC) are interesting candidates for ^{1}D, but HILIC requires protein samples to be soluble in organic rich solvents, which is problematic for many proteins, and HIC requires either high concentrations of non-volatile salts, or volatile MS-compatible salts, which provide inadequate retention on conventional HIC stationary phases [14].

A high resolution and efficient ^{1}D LC mode with altered selectivity is then highly desirable for LC x LC-MS. Alkaline pH can be used as an additional separation method to gain orthogonal selectivity while maintaining the high resolving power and the MS compatibility of reversed phase mode [15]. However, to the best of our knowledge, none has reported the hyphenation of a comprehensive online high pH reversed phase (HPH-RPLC) x low pH reversed phase (LPH-RPLC) to mass spectrometry (MS) for the online separation and analysis of intact proteins. Only offline coupling of high pH and low pH was recently reported for the two-dimensional liquid chromatography (2D-LC) of intact proteins [16] but offline approaches usually suffer from being time consuming, labor intensive, and very low throughput. They are also susceptible to sample contamination, sample loss, and sample degradation. Even so, online coupling (HPH-RPLC x

LPH-RPLC) also poses technical challenges which need to be addressed to attain a successful LC x LC platform using such a strategy. These challenges include pH and elution strength mismatch between two dimensions, under sampling of ^{1}D, ultrafast separations in ^{2}D, suitable modulation interface, MS compatible buffers, and flow splitting for online integration with MS [17].

The online platform described here is the first report of online comprehensive HPH-RPLC x LPH-RPLC utilizing mass spectrometric detection for intact protein analysis. MS adds a third online dimension to this 2D-LC system, which can yield useful online molecular weight and tandem mass spectrometry information for top-down proteomics. With this system, it is possible to collect the separated intact protein analytes by using a fraction collector after the flow splitter, which is used before MS, for further offline proteolysis if desired. Two ^{2}D gradient regimes were further evaluated to increase the two-dimensional surface coverage. Moreover, online dilution with a lower strength and acidic mobile phase (0.5% formic acid in water) was compared to the direct injection of different collected ^{1}D effluent volumes (25, 50 and 90 µL).

6.3 Experimental Section

6.3.1 Chemicals and Materials.

Cytochrome C from bovine heart (CCB) (12 kDa), cytochrome C from equine heart (CCE) (12 kDa), carbonic anhydrase from bovine erythrocytes (CA) (29 kDa), α-lactalbumin from bovine milk type III (LA) (14 kDa), β-casein from bovine milk (CS) (24 kDa), myoglobin from equine skeletal muscles (MY) (17 kDa), β-lactoglobulin B from bovine milk (LG) (18 kDa), triethylammonium bicarbonate (TEAB) buffer (1.0 M, pH 8.5), and LC-MS grade formic acid (FA) were obtained from Sigma–Aldrich (St. Louis, MO). *Escherichia coli* (*E. coli*) protein sample was purchased from Bio-Rad (Hercules, California). LC-MS grade triethylamine (TEA) and trifluoroacetic acid (TFA) were purchased through VWR (Missouri, TX). LC-MS grade water and

acetonitrile were purchased from Honeywell Burdick & Jackson International (Muskegon, MI). A mixture solution of the seven standard proteins was prepared by dissolving 0.5 mg of each of CCB, CCE, CA, and MY, and 1 mg of each of CS, LA, and LG in 1 mL of 0.05% TEA. The *E. coli* protein powder (2.7 mg) was dissolved in 1.5 mL water.

6.3.2 Instrumentation and Chromatographic Conditions.

Comprehensive 2D-LC analyses were performed on a Nexera-e liquid chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA), consisting of five LC-30AD pumps, DGU-20A5R degassers, a CTO-20AC column oven, a SIL-30AC autosampler and a two high pressure two-position, six-port switching valves (model FCV-32 AH, Shimadzu) placed inside the column oven and equipped with two 100-μL stainless steel sample loops. The two-dimensional LC system was interfaced to a flow splitter (Analytical Scientific Instruments, CA) and the split eluate (0.4 mL/min) directly entered a Shimadzu LCMS-8050 triple quadrupole MS, through an electrospray ionization (ESI) source. A schematic diagram of the comprehensive 2D-LC valve and plumbing arrangement is shown in Figure 6-1. The LC × LC- MS system and the symmetric switching valves (first-in-first-out) were controlled by Shimadzu LabSolutions software (version 5.80). The LC × LC data were visualized and analyzed using ChromSquare version 2.2 software (Chromaleont, Messina, Italy). The triple quadrupole was operated in either the single ion monitoring mode (SIM) for the mixture of seven standard proteins (m/z 1020 for CCB, m/z 1766 for CCE, m/z 1828 for LG, m/z 1576 for LA, m/z 925 for CS, m/z 1038 for CA, and m/z 1696 for MY) with a 30 msec dwell time for each SIM channel, or in the scan mode for the *E. coli* total proteome sample with a total single scan time of 0.4 sec for scanning m/z 600 to m/z 2000. The MS detector settings included an interface temperature set at 300 °C, an interface voltage at 4 kV (positive ionization mode), a desolvation line temperature at 250 °C, a heat block

temperature at 400 °C, a nebulizer gas flow of nitrogen at 2.5 L/min, a heat gas flow of nitrogen at 10 L/min, and a drying gas flow of nitrogen at 10 L/min.

The first-dimension separation was performed on an Acquity UPLC BEH C4 column (150 mm x 1 mm, 1.7 µm, 300 Å) (Waters, USA). The mobile phases were (A) 36 mM TEAB + 0.25% TEA in water, pH 10 and (B) 36 mM TEAB + 0.25% TEA in 80% acetonitrile: 20% water. A flow rate of 0.05 mL/min was used. Sample injection volume was 5 µL for the standard mixture of proteins and 50 µL for the *E. coli* proteins. The solvent gradient conditions used for protein probes and *E. coli* proteins is shown in Table 6-1. The second-dimension chromatography was carried out on a Halo protein C4 column (30 mm × 3 mm, 2.7 µm, 1000 Å) (Advanced Materials Technology, USA), using 0.2% FA + 0.025% TFA in water, pH 2 as mobile phase C, and 0.2% FA + 0.025% TFA in acetonitrile as mobile phase D. The ^{2}D flow rate was 2.5 mL/min and the gradient conditions used was a so-called segmented gradient, as shown in Table 6-1. The temperature of the oven containing both the ^{1}D and the ^{2}D columns was held at 55 °C.

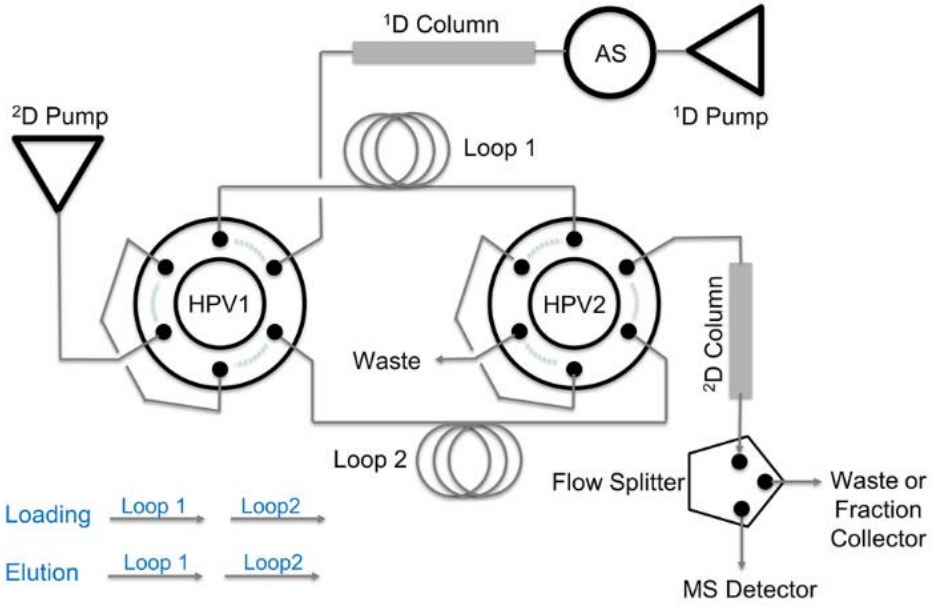

Figure 6-1. Schematic diagram of the comprehensive 2D-LC system used in this work.

Table 6-1. Gradient conditions of LC x LC method for separation of protein probes and the *Escherichia coli* proteome.

Sample	First dimension (^{1}D)	Second dimension (^{2}D)
Standard protein mixture	35-50% B in 60 min	Segment 1 (0-20 min): 25-50% D in 0.7 min
		Segment 2 (20-40 min): 30-55% D in 0.7 min
		Segment 3 (40-60 min): 35-60% D in 0.7 min
Escherichia coli proteome	30-55% B in 60 min	Segment 1 (0-20 min): 15-55% D in 0.7 min
		Segment 2 (20-40 min): 25-60% D in 0.7 min
		Segment 3 (40-60 min): 30-70% D in 0.7 min

6.4 Results and Discussion

Unlike small molecules, proteins have diverse three-dimensional structures, with a heterogenous distribution of functional groups (hydrophobic, acidic, and basic) on their surface that will be involved in the chromatographic process. Multiple interactions, with the residual silanols and/or the alkyl bonded phases used in reversed phase chromatography (RPLC), are then experienced by the protein analytes and good peak shapes results from absence of secondary retention effects or conformational interconversions [18, 19]. These multi-point interactions of the chromatographic foot-print surface of proteins can be advantageous, though, to gain altered selectivity through using only a different pH in RPLC. Such an altered selectivity can be a great fast tool in proteomics workflows to resolve coeluted protein peaks and/or to create a powerful online LC x LC platform for top-down proteomics.

6.4.1 Optimization of HPH-RPLC x LPH-RPLC Conditions

A mixture of seven protein probes covering a reasonably broad spectrum of protein properties were used in this work to test and optimize performance of LC x LC. These large biomolecule probes cover a broad isoelectric point range to evaluate the altered selectivity for

acidic (LG, LA and CS), neutral (CA and MY), and basic proteins (CCB and CCE). For the high pH RPLC in this work, we selected TEAB buffered at pH 10 as the high pH MS-compatible buffer in the ^{1}D, since it offers many advantages over the other common high pH volatile buffers (e.g., ammonium bicarbonate and ammonium formate). TEAB provides an ion pairing effect for acidic residues of proteins, a masking effect for residual silanols to reduce undesirable silanophilic interactions, a significantly higher solubility in organic solvents used in RPLC, and greater column life time at high pH (being an organic buffer [20]).

For the ^{2}D, we used 0.2% FA and 0.025% TFA to achieve a low pH of 2. These acidic modifiers together provided the optimum acidic conditions for having a good and an ultrafast ^{2}D chromatography without the severe ion suppression effect caused by a higher TFA concentration for MS detection. Ultrafast ^{2}D separations are more challenging for proteins than they are for small molecules. These challenges become even more drastic in LC x LC, due to the large injection volumes of ^{1}D effluent typically used. The high Van Deemter C-term, caused by low diffusivities and the slow sorption kinetics of proteins, significantly reduce column efficiency at high flow rates. Hence, to achieve a sub-minute separation for proteins, we used a high temperature of 55 °C and a column packed with small 2.7 um-diameter fused core particles (1000 Å pore size) in the ^{2}D column to reduce diffusion pathlengths and enable rapid bulk transfer of mobile phase within the pores.

Figure 6-2A shows the successful coupling of HPH-RPLC x LPH-RPLC for the seven protein probes in this study. The online coupling of the two RPLC systems is achieved by two six-port valves with a full symmetrical path for two storage loops (Figure 6-1). Orthogonal selectivity was successfully demonstrated between the two dimensions by the different retention order obtained in each dimension. The elution order of proteins in the ^{1}D (pH 10) was CCB followed by

CCE, LG, LA, CS, CA, and MY. On the other hand, the elution order in the ^{2}D (pH 2) was CCE followed by CCB, LA, MY, LG, CS, and CA. Unlike for peptides [21] the spread of proteins across the two dimensions with different pH, did not follow their pI values. Acidic proteins were expected to be more retained at low pH, while basic proteins should be more retained at high pH. Neutral proteins (CA and MY) also showed a different elution order and an altered selectivity with pH. These results emphasize the importance of other parameters in predicting the retention behavior of proteins, such as the different conformations of proteins that can be induced by different pH, and the chromatographic footprint of each protein in proximity to the stationary phase.

We then used the segmented gradient (SG) strategy in the ^{2}D, to achieve a better distribution of protein peaks across the two-dimensional (2D) separation space (Figure 6-2B), relative to the full in fraction (FIF) gradient shown in Figure 6-2A. Having the gradient range of SG in the ^{2}D divided into three segments, as illustrated in Table 6-1, instead of the same constant gradient segment (25% D to 60% D) used by the FIF strategy for the analysis of each ^{1}D effluent fraction, achieved greater surface coverage of the available 2D analysis space. Hence, we adopted this SG of ^{2}D for further optimization. The zoom in ^{2}D chromatogram of Figure 6-2B shows the benefit of orthogonal selectivity for providing a sub-minute baseline resolution of two coeluted proteins (CCE and LG) from ^{1}D. Since the same C4 sorbent was used in both LC dimensions, the orthogonality is mostly due to the difference in pH of mobile phase.

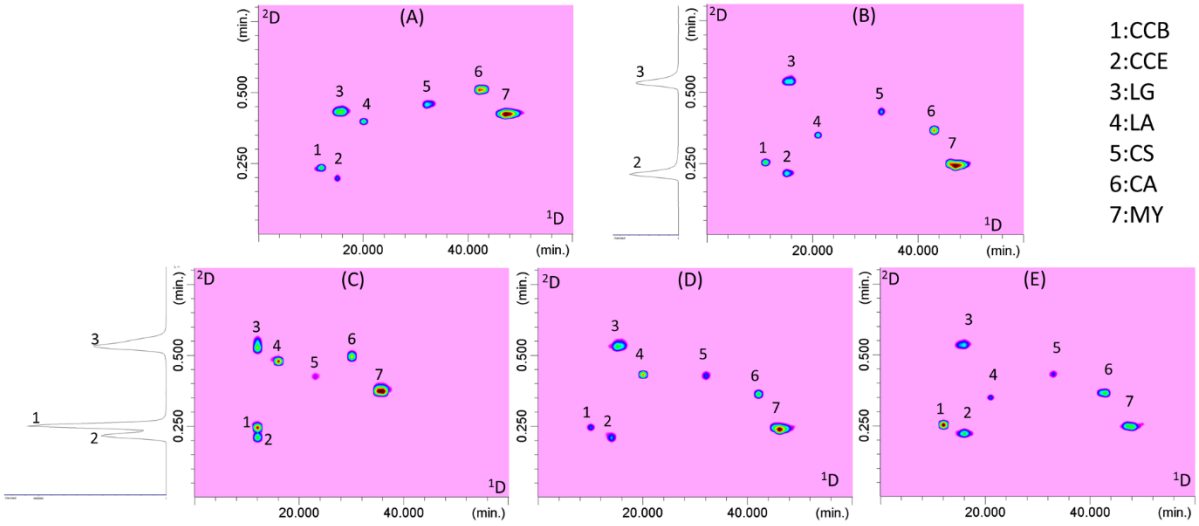

Figure 6-2. 2D contour plots of protein standards acquired with 2D-LC-MS method using full in fraction gradient (A), segment gradient (B), 0.025 mL/min first-dimension flow rate (C), 0.09 mL/min flow rate first-dimension flow rate (D) and online dilution of the 0.05 mL/min first-dimension effluent with a 0.04 mL/min of 0.5% formic acid in water (E). Zoom-in chromatograms of 16 min (B) and 12 min (C) cuts to show the sub-minute second dimension separation of coeluted proteins. Peaks 1-7 are cytochrome c bovine (CCB), cytochrome c equine (CCE), β-lactoglobulin B (LG), α-lactalbumin (LA), β-Casein (CS), carbonic anhydrase (CA), and myoglobin (MY), respectively.

Sampling time is a very important parameter in LC x LC since it affects the injection volume into the ^{2}D dimension and the number of ^{1}D effluent fractions to be transferred for each peak from the ^{1}D separation. A compromise must be made to avoid the decrease in ^{1}n due to severe under sampling, and the decrease in ^{2}n due to the steep gradient in ^{2}D. Hence, the optimum sampling time corresponds to between two and three fractions per peak in ^{1}D [22, 23]. We used a sampling time of one minute and two passive loops each had a 100 µL volume. Three different flow rates of ^{1}D (0.025, 0.05 and 0.09 mL/min) were then used to evaluate the effect of different injection volumes of ^{1}D effluent fractions on the subsequent ^{2}D chromatography and peak shapes. Figures 6-2B, C, and D represent the LC x LC chromatograms generated by using 0.05, 0.025 and 0.09 mL/min, respectively, corresponding to a loop filling percentage of 50%, 25% and 90%, respectively. No distortion, splitting, or breakthrough of peaks was observed for any of the tested

loop filling percentages. The steeper ^{1}D gradient from the lower flow rate of 0.025 mL/min resulted in a coelution of three of the proteins (CCE, CCB, and LG) in the ^{1}D injected fraction. However, as illustrated by the ^{2}D chromatogram of Figure 6-2C, the three peaks were still distinguished with a sufficient resolution (R_s=1), including the structurally similar proteins, CCB and CCE, which differ only by three amino acids in their 12 kDa sequence.

These results confirmed the successful online coupling of the two dimensions, and the ability to overcome any pH or organic solvent strength mismatch, even with injection volumes up to 90 µL (representing about 60% of ^{2}D column dead volume). We attribute these findings to the steep elution isotherms of the proteins (so called, on/off retention) [18]. They have an inherently higher sensitivity to slight changes in the organic percentage of eluent leading to a greater gradient peak compression with the steep gradients used in ^{2}D. Thus, the protein would initially be refocused at the ^{2}D column inlet, due to the initial lower ^{2}D eluent strength used to push proteins from the sampling loops of the interface valves, before it starts to migrate when the eluent gradient reaches a composition which promotes elution. This provided ample time for the injected protein band inside the second-dimension column to overcome pH and organic solvent composition mismatch.

Our interpretations are also in agreement with the findings reported by Sarrut and coworkers [24]. They highlighted that very steep gradients typically used in ^{2}D, enabled the injection of very large volumes of up to 70% of the column dead volume without any additional band broadening for peptides in RPLC x RPLC. It is also possible that the higher pressure experienced by proteins in ^{2}D (1000 psi higher) caused a higher retention and refocusing for these large biopolymers [25, 26]. Further, we tested the use of online dilution of the ^{1}D effluent (0.05 mL/min) with 0.04 mL/min of 0.5% FA in water, using an extra isocratic pump and a tee

connection, to reduce its eluotropic strength and pH before injection into the ^{2}D column. Online dilution was reported by Stoll, *et al.* [27] to overcome pH and elution strength mismatch between two RP dimensions for small molecules . However with large biomolecules, as illustrated in Figure 6-2E, ^{2}D retention times for the seven proteins did not change and the only significant difference was found to be a three-fold higher signal intensity for CCB, which may be attributed to the higher ESI ionization efficiencies caused by the higher ionic strength of formic acid, introduced by online dilution, for CCB which is a basic protein. Moreover, no breakthrough peaks of the injected proteins were observed without online dilution or with the injection of larger loop volumes (90 µl) into the ^{2}D. This demonstrated the high flexibility of this online 2D-LC system to tolerate large injection volumes with no need for online dilution or any other forms of online preconcentration (e.g. trap columns).

6.4.2 Corrected Peak Capacity

Experimental peak capacities (n_c) for each dimension were calculated according to eq 1.

$$n_c = 1 + \frac{t_G}{w} \quad (1)$$

where t_G is the gradient time and w is the average 4σ peak width. The comprehensive 2DLC predicted peak capacity ($n_{c,2D}$) was then calculated according to eq 2

$$n_{c,2D} = {}^{1}n_c \times {}^{2}n_c \quad (2)$$

In eq 2, the so-called product rule [28], $^{1}n_c$ is the ^{1}D peak capacity and $^{2}n_c$ is the ^{2}D peak capacity. This equation provides the maximum theoretical peak capacity under ideal conditions and overestimates the true experimental peak capacity of the system. In our experiments, the ^{1}D peak capacity for the 60 min gradient was 36 (^{1}w = 1.71 min) and ^{2}D peak capacity was 18.5 (^{2}w =0.04

min) and thus, the predicted multiplicative peak capacity was 666. To account for undersampling of the ^{1}D, we used the Davis-Stoll-Carr factor [29, 30]:

$$< \beta > = \sqrt{1 + 3.35(\frac{t_s}{{}^1w})^2} \quad (3)$$

where $< \beta >$ is the peak broadening factor due to remixing of ^{1}D effluent, t_s is the modulation time and ^{1}w represents the average ^{1}D peak width. Therefore, dividing our predicted peak capacity by 1.46 (value of $< \beta >$) would give us a corrected total peak capacity of 456 for the 60 min gradient used in our experiments.

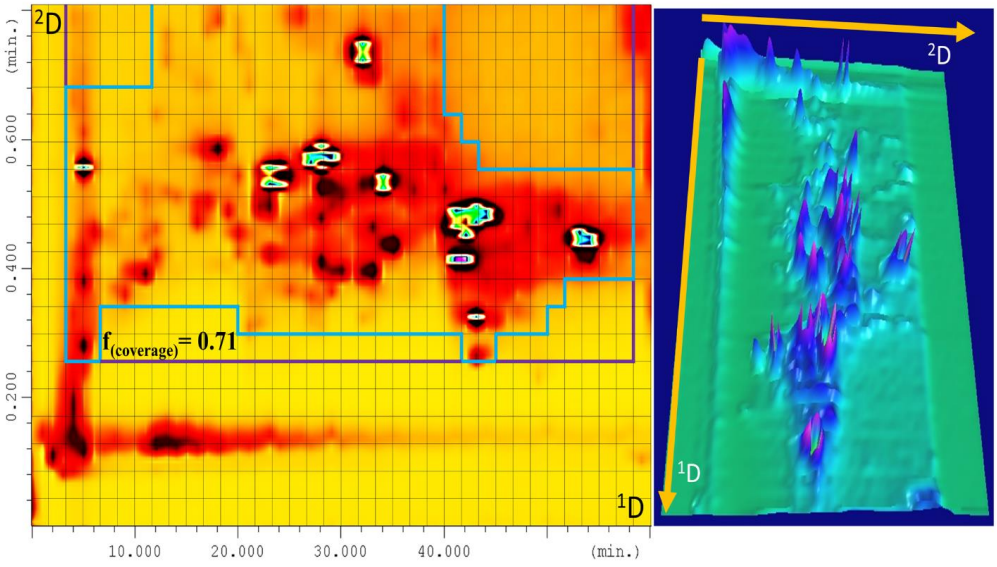

Figure 6-3. Contour and three-dimensional (3D) plots of the *Escherichia coli* proteome. A grid is drawn on the contour plot in proportion to the peak capacity of each axis to show the fraction of surface occupied by protein peaks.

6.4.3 Application of the Comprehensive 2D-LC-MS System

In this study, the separation of a commercial proteome sample of *E. coli* was used to carry out a proof-of-principle experiment, and to further demonstrate the capacity of the HPH-RPLC x LPH-RPLC-MS platform. In this case, the chromatographic conditions were very similar to those used in the analysis of protein standards; only now, the 60 min reversed phase gradient started at

30% instead of 35% and ended at 55% instead of 50%. We selected 0.05 mL/min as the ^{1}D flow rate (50% loop filling) to avoid loss of analytes in the ^{1}D effluent that can be caused by the higher velocity in the center of the parabolic flow profile. Figure 6-3 shows the 2D contour plot and 3D plot for the total proteome of *E. coli*. To calculate the surface coverage for the two-dimensional separation space, we utilized the Stoll-modified-Gilar method [31]. A grid is drawn on the 2D space to divide it in proportion to the relative peak capacity of each axis. The fractional surface coverage ($f_{(coverage)}$) is then calculated by counting number of bins occupied by chromatographic peaks of *E. coli* proteins versus unoccupied ones. The $f_{(coverage)}$ was 0.71 (Figure 6-3) owing to the nice orthogonality of the HPH-RPLC x LPH-RPLC system. Hence, the calculated peak capacity of 456 can be further corrected to account for incomplete filling of the 2D separation space by:

$$n^{*}_{c,2D} = \frac{{}^{1}n_c \times {}^{2}n_c \, x \, f_{cov}}{<\beta>} \qquad (4)$$

Using eq 4, the effective peak capacity ($n^{*}_{c,2D}$) was calculated to be 324. For these experiments, our full scan mass spectra ranged in m/z from 600 to 2000. Typical peak widths for a triple quadrupole mass spectrometer are 0.75–1 Da. Thus, a conservative estimate for the peak capacity of the mass spectrometer is 1400 for small molecules. But, for proteins, with their signals distributed over multiple charge states, a conservative estimate is 7. Therefore, the maximum peak capacity, while running a 60 min gradient, for the entire LC x LC-MS online platform was 324 × 7 = 2268.

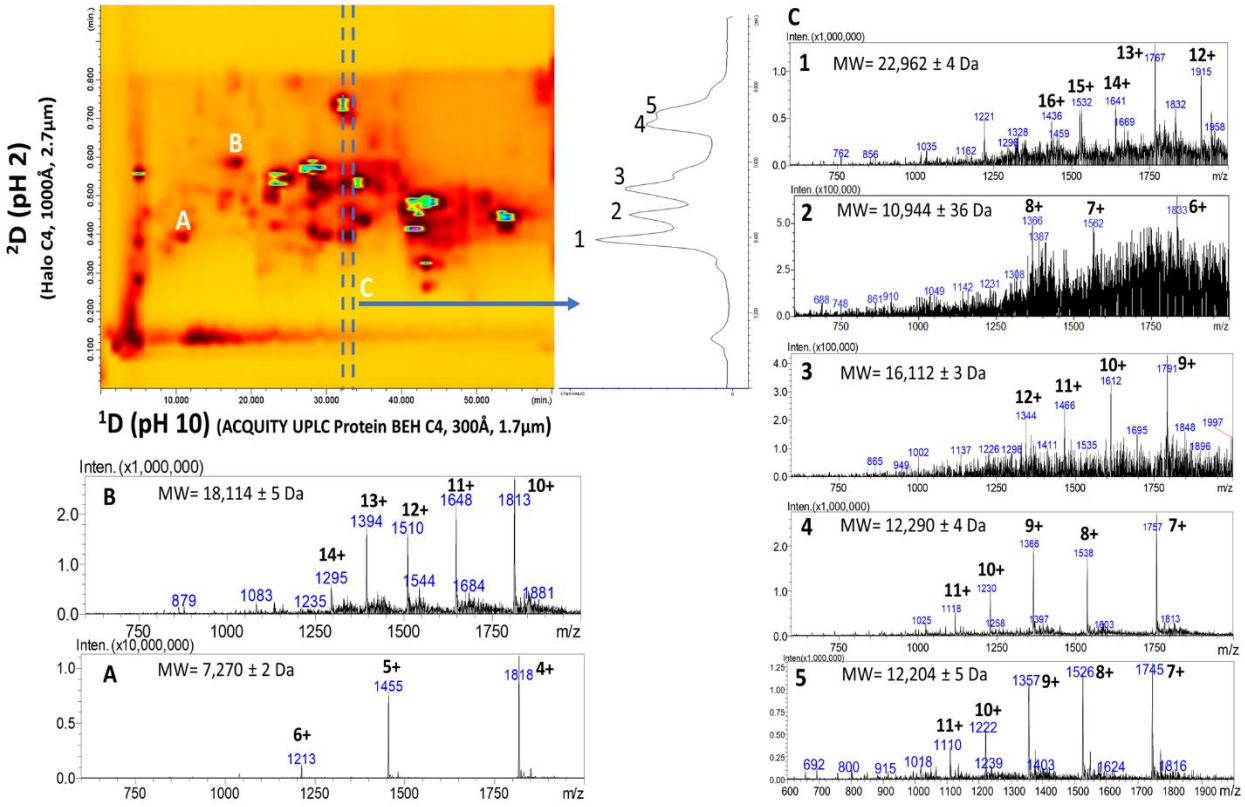

Figure 6-4. Mass spectra from peaks of 2D contour plot of *Escherichia coli* protein sample at 11 min (A), 18 min (B) and 33 min (C) cuts.

To show ESI-MS charge states of proteins more clearly, mass spectra from 11 min (A) and 18 min (B) cuts are presented in Figures 6-4A and B. The TIC chromatogram of the 33 min fraction in Figure 6-4C shows five apparently distinguished peaks during the gradient time of 42 s. This demonstrates the excellent separation power of this comprehensive 2D-LC-MS system. The mass spectra of these peaks showed charge state distributions of proteins that are consistent with well separated individual proteins. The molecular weight obtained by the deconvolution of these mass spectra, as shown in Figure 6-4, can be used to search online protein databases for matching of the measured molecular masses to proteins of *E. coli*.

6.5 Concluding Remarks

In this study, we have demonstrated that switching pH, using MS-compatible buffers, between the first and second dimensions is effective for RPLC x RPLC of intact proteins. Very good 2D space coverage and orthogonal selectivity were achieved by combining fully porous BEH 300 Å C4 (1 mm x 150 mm, 1.7 um) in the first dimension and fused-core 1000 Å C4 (3 mm x 30 mm, 2.7 um) in the second dimension. The HPH-RPLC x LPH-RPLC proved to be applicable for top-down proteome analysis by hyphenation to online MS detection. Additionally, separated proteins can be recovered intact because of the 6:1 flow-split before the mass spectrometer. This would allow for offline proteolysis in a combined top-down/bottom-up proteome platform.

The current proof-of-concept setup can be further optimized to improve proteome-wide identification by adopting a longer ^{1}D gradient and enhancing the ^{2}D performance with the use of a smaller particle size stationary phase and/or a multiple parallel array of ^{2}D columns. Additionally, reducing the modulation time, using a separate column oven for each dimension with a higher temperature for ^{2}D, using a high resolution and a high scan sensitivity MS instrument, and/or having an efficient dedicated software could be used as well to achieve higher performance of the LC x LC platform, if desired. Not only would this enhance the resolving power and the effective peak capacity of the system, but it would also enhance the sensitivity of this system to detect low abundance proteoforms by making the peaks narrower and less dilute. This method has the potential to improve front-end online protein fractionation and to replace current methods of intact protein separation which include time consuming, sample inefficient, MS-incompatible, and/or offline two-dimensional separation followed by MS data acquisition.

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CHAPTER SEVEN: SUMMARY AND FUTURE WORK

LC-MS has matured enough to be the most common analytical platform for analysis of biomarkers and biotherapeutics. However, challenges for accurate quantitation and discovery of new biological molecules of interest in complex matrices has also increased the demand for more powerful state-of-the-art instruments with streamlined sample preparation and capable of reaching very low detection limits. Accordingly, developing novel methods to streamline sample preparation and to enhance the resolving power and detection sensitivity are of utmost importance. Hence the goal of this work was to develop and optimize novel methods for multiple analytical research areas including online sample preparation, *in situ* derivatization, one- and two-dimensional separations for both small and large bio-molecules.

Restricted access media (RAM) columns were used to evaluate the optimum conditions for maximizing their performance for online isolation and enrichment of small molecules in complex matrices. The best loading conditions were achieved by using acidic conditions, low flow rates and C4 or C8 for small analytes with various physicochemical properties. Using these optimized trapping conditions for these underutilized RAM columns should result in a higher throughput, through fully automated analysis, and a better pre-concentration performance over traditional solid phase extraction formats.

To reach ultra-high detection sensitivities especially for target analytes with less responsive functional groups, derivatization may be the only way to go. Nevertheless, derivatization can also result in more tedious and time-consuming sample preparation steps which are more prone to errors and result in more uncertainty for the determination of these analytes. Hence, we developed a novel one-pot charge reversal derivatization kit capable of simultaneously bulk derivatizing phenolic and carboxylic functionalities of biomarkers in their native biological matrices into

permanently positively charged quaternary nitrogen derivatives. We found significant increases in their ionization efficiencies using electrospray ionization mass spectrometry but at the expense of poor chromatography caused by the higher polarity of derivatives. Biphenyl stationary phase, methanol as the organic modifier (to enhance π-π and cation-π interaction) and very low column oven temperature (to add additional shape selectivity) were more successful than HILIC to yield good chromatogram with a baseline resolution of isomers and isobaric interferences. Multivariate analysis was further used to find the optimum operating region of this kit and isotope dilution mass spectrometry was then successfully used for their quantitative determination.

Advances in stationary phases with new morphologies and new chromatographic modes, such as hydrophobic interaction chromatography, has enabled purification, characterization and analysis of large intact proteins. Nevertheless, proteins are inherently complex and usually present at a wide dynamic range in biological matrices. This necessitates the development of complimentary and orthogonal front-end separation methods that can be integrated online to mass spectrometry. Thus, we developed a novel high pH reversed phase liquid chromatography (HPH-RPLC) using volatile triethylammonium bicarbonate buffer (TEAB) at pH 10 and hybrid silica-based packings. The excellent chromatographic performance of this TEAB buffer at high pH should direct future efforts to develop an online top-down/bottom-up approach. This HPH-RPLC configuration successfully achieved the required orthogonal selectivity to low pH reversed phase chromatography while maintaining the high resolution and MS-compatibility of reversed phase. We further used it as the first dimension in online comprehensive two-dimensional liquid chromatography mass spectrometry to be used as a very powerful platform for top-down proteomics.

These results may serve well the foundation of future efforts for developing a powerful and a universal online multidimensional platform capable of routine high throughput analysis of small and large biomolecules in the same biological sample. Such a platform could benefit from a fully automated *in situ* derivatization followed by enrichment and online segregation of small and large biomolecules using RAM columns. These segregated analytes could then be transferred online into two- or multi-dimensional separation columns ending with an extra dimension of a highly sensitive mass spectrometer for ultra-high specificity, sensitivity and resolving power.

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