Ion-pairing reversed-phase liquid chromatography fractionation in combination with isotope labeling reversed-phase liquid chromatography–mass spectrometry for comprehensive metabolome profiling

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ABSTRACT

We report a novel two-dimensional (2D) separation strategy aimed at improving the detectability of liquid chromatography mass spectrometry (LC–MS) for metabolome analysis. It is based on the use of ion-pairing (IP) reversed-phase (RP) LC as the first dimension separation to fractionate the metabolites, followed by isotope labeling of individual fractions using dansylation chemistry to alter the physio-chemical properties of the metabolites. The labeled metabolites having different hydrophobicity from their unlabeled counterparts are then separated and analyzed by on-line RPLC Fourier-transform ion-cyclotron resonance mass spectrometry (FTICR-MS). This off-line 2D-LC–MS strategy offers significant improvement over the one-dimensional (1D) RPLC MS technique in terms of the number of detectable metabolites. As an example, in the analysis of a human urine sample, 3564 ^{13}C-/^{12}C-dansylated ion pairs or metabolites were detected from seven IP RPLC fractions, compared to 1218 metabolites found in 1D-RPLC–MS. Using a library of 220 amine- and phenol-containing metabolite standards, 167 metabolites were positively identified based on retention time and accurate mass matches, which was about 2.5 times the number metabolites identified by 1D-RPLC–MS analysis of the same urine sample.

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1. Introduction

Liquid chromatography combined with mass spectrometry (LC–MS) has become an important technique for metabolome profiling due to its high sensitivity and specificity. However, one of the major analytical challenges in current LC–MS-based metabolome analysis is the difficulty to generate a very comprehensive profile of a metabolome sample due to great diversity in chemical and physical properties of metabolites and a wide range of concentrations of different metabolites. Due to matrix and ion suppression effects often encountered in MS detection, highly efficient metabolite separation becomes critical for improving the overall detectability of LC–MS in metabolome analysis.

Multi-dimensional separation of metabolites provides a means of reducing sample complexity for MS analysis [1–3]. A variety of combinations of LC separation mechanisms including size-exclusion, ion exchange or reversed phase (RP) have been used in two-dimensional (2D) LC [1–3]. In an ideal situation, all the analytes in a complex sample will be differentially retained by two orthogonal mechanisms in 2D-LC, and each mechanism is considered an independent separation dimension [2,4]. One constraint in select-

ing the separation modes for LC–MS is related to the compatibility of the LC separation with MS detection. RPLC is commonly used for online LC–MS due to its high resolving power and readiness to combine with MS. For metabolome analysis, ion exchange-reversed phase (IEC-RP) [5] and size exclusion-reversed phase (SEC-RP) [6] mass spectrometry have been reported. Hydrophilic interaction liquid chromatography (HILIC) has also been used for online LC–MS [7–10]. Although the resolving power of HILIC is not as good as RPLC, it provides the opportunity of combining with other separation mode, such as strong cation exchange (SCX), for 2D-LC–MS [7]. The combination of HILIC and RPLC for metabolome analysis has also been reported [11–14].

Because of relatively low resolving power of IEC, SEC or HILIC, the peak capacity of 2D-LC involving one of these modes would be lower than a RPLC × RPLC, assuming the RPLC × RPLC mode is capable of offering true orthogonality. However, only limited differences in selectivity of the RP stationary phase are achievable using different chemistries or mobile phases. For example, peptides can be separated and fractionated at low pH mobile phases in the 1st dimension RPLC and a certain degree of orthogonal separation can be done by the 2nd dimension RPLC at a relatively higher pH [15]. There are also reports of using diagonal chromatography [16] to create some orthogonality between two RPLC dimensions for peptide or protein separation; modifications of peptides or proteins after the 1st dimension separation (e.g., oxidation of certain amino acid

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side chains) can result in changes in retention properties, thereby allowing altered separation in the 2nd dimension [17].

In this work, we report a 2D-LC strategy in which the orthogonality of the separation is achieved by altering the hydrophobicity of the analytes prior to the 2nd dimension RPLC separation. Thus, stationary phases of differing chemistries are not used for each dimension. The hydrophobicity of targeted analytes is altered by a chemical derivatization, dansylation, thus the derivatized metabolites can be analyzed by MS with much enhanced sensitivity, compared to the un-derivatized counterparts.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma–Aldrich Canada (Markham, ON, Canada) except those otherwise noted. The isotopic compound, $^{13}C_2$-dimethyl sulfate, used to synthesize the isotope tagged dansylation reagent (^{13}C-danysl chloride) was purchased from Cambridge Isotope Laboratories (Cambridge, MA, USA). LC–MS grade water, methanol and acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Edmonton, AB, Canada). Urine samples were collected from a healthy individual and processed by adding 50% (v/v) LC–MS grade acetonitrile, then stored at $-20\,^{\circ}C$.

2.2. Labeling reaction

The synthesis of ^{13}C-dansyl chloride as the labeling reagent and dansylation labeling reaction have been described elsewhere [18]. Briefly, two aliquots were taken from an individual fraction collected from the 1st dimension separation for isotope labeling. Each aliquot was mixed with an equal volume of sodium carbonate/sodium bicarbonate buffer (0.5 mol/L, pH 9.4) in reaction vials. About a 4-fold molar ratio excess ^{12}C-dansyl chloride solution in acetonitrile (20 mg/mL) (for light labeling) or ^{13}C-dansyl chloride in acetonitrile (20 mg/mL) (for heavy labeling) was then added, and the reaction was allowed to proceed for 60 min at $60\,^{\circ}C$ with shaking at 150 rpm. After 60 min, methylamine (0.5 mol/L) was added to the reaction mixture to consume the excess dansyl chloride and quench the dansylation reaction. After an additional 30 min of $60\,^{\circ}C$ incubation, the ^{13}C-labeled mixture was combined with its ^{12}C-labeled counterpart for LC–FTICR–MS analysis. Note that the 4-fold molar excess of the reagent used was estimated based on the peak intensity of methylamine. The remaining methylamine in the labeled sample was the difference of the amount added and the amount consumed to react with the excess amount of the labeling reagent.

2.3. Ion-pairing RPLC

Fig. 1 shows the workflow of the 2D-LC–FTICR–MS experiment. An Agilent 1100 series quaternary HPLC system (Agilent, Palo Alto, CA), and an Agilent Zorbax Rx-C18 column (9.4 mm × 250 mm, 5 μm particle size) were used in the 1st dimension separation. Mobile phase A was 12 mM heptafluorobutyric acid (HFBA) and mobile phase B was 100% acetonitrile. 254 nm was chosen as the UV detector wavelength. The gradient elution profile was as follows: $t=0$, 0% B; $t=8$ min, 10% B; $t=20$ min, 30% B; $t=23$ min, 95% B; $t=23.5$ min, 0% B; $t=50$ min, 0% B. The flow rate was 5 mL/min, and sample injection volumes were 800 μL.

2.4. LC–FTICR–MS

An Agilent 1100 series binary system (Agilent, Palo Alto, CA) and an Agilent reversed-phase Eclipse plus C_{18} column
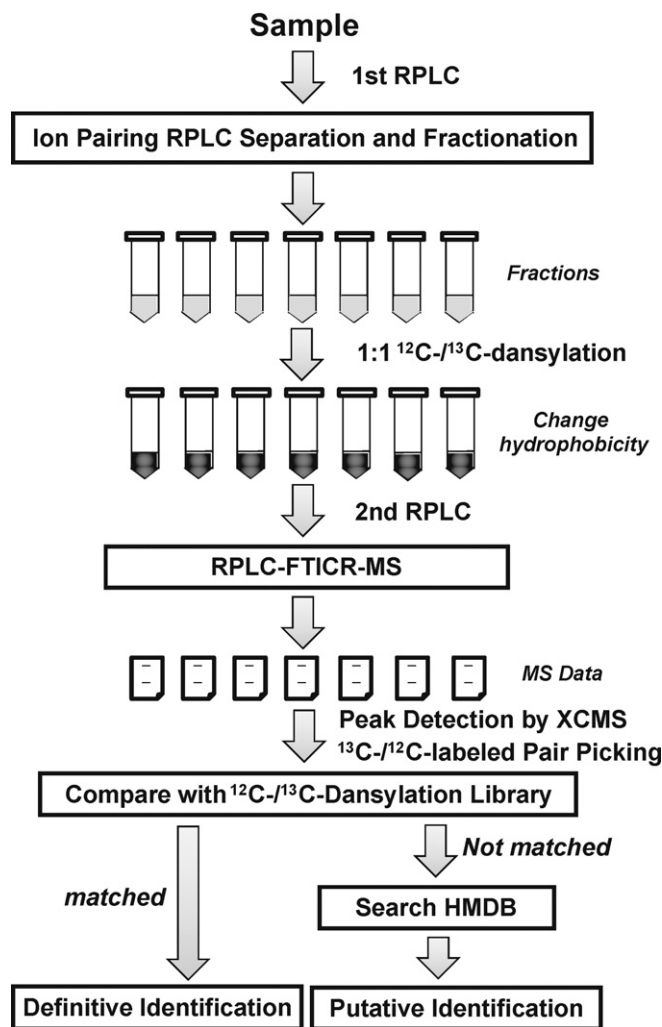

Fig. 1. Schematic of the 2D-RPLC–FTICR–MS workflow for metabolome analysis.

(2.1 mm × 100 mm, 1.8 μm particle size, 95 Å pore size) were used for online LC–MS. LC solvent A was 0.1% (v/v) LC–MS grade formic acid in 5% (v/v) LC–MS grade ACN, and solvent B was 0.1% (v/v) LC–MS grade formic acid in LC–MS grade acetonitrile. The gradient elution profile was as follows: $t=0$ min, 20% B; $t=3.0$ min, 35% B; $t=16$ min, 65% B; $t=18.6$ min, 95% B; $t=21$ min, 95% B; $t=21.3$ min, 98% B; $t=23.0$ min, 98% B; $t=24.0$ min, 20% B. The flow rate was 150 μL/min. The flow from RPLC was split 1:3 and a 50 μL/min flow was loaded to the electrospray ionization (ESI) source of a Bruker 9.4 Tesla Apex-Qe Fourier transform ion-cyclotron resonance (FTICR) mass spectrometer (Bruker, Billerica, MA, USA), while the rest of the flow was delivered to waste. All MS spectra were obtained in the positive ion mode.

3. Results and discussion

A large proportion of human metabolites in biofluids are highly polar compounds, and conventional RPLC often lacks the capability to adequately retain and separate ionic, polar metabolites. Recently HILIC has been used in bioanalytical applications, but the chromatographic efficiency is typically not as good as the RP separation [10]. An alternative approach to improve the retention of polar metabolites and maintain high separation efficiency is to use ion-pairing (IP) RPLC in which cationic species, such as amines or amino acids at low pH, are reported to be successfully separated using long chain perfluorocarboxylic acids, such as heptafluorobutyric acid (HFBA)
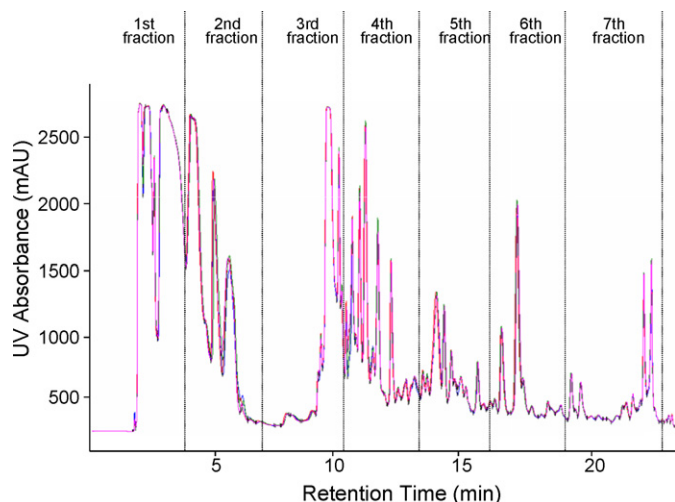
Fig. 2. Superimposed ion pairing RPLC–UV chromatograms from four consecutive injections of a human urine sample. Seven fractions were collected for RPLC–FTICR-MS analysis.

as the volatile ion pairing reagent [19–27]. In IP RPLC, the cationic species form an ion-pair with the negatively charged ion-pairing reagent in the mobile phase to become electrically neutral. Using HFBA the resulting hydrophobic character of the ion-pair leads to a greater affinity for the stationary reversed-phase and thus, results in greater retention of cationic polar compounds in the RPLC column. As a result, ion pairing RPLC can be used to generate selective retention of cationic polar metabolites and elute the anionic polar metabolites at or near void volume.

We observed that a long column equilibration is necessary to maintain reproducible chromatographic separation in IP RPLC. Systems containing 12 mM heptafluorobutyric acid required at least 25 column volumes to be well equilibrated. Fig. 2 shows the superimposed RPLC–UV chromatograms of a human urine sample from four consecutive injections. All the major chromatographic peaks are perfectly superimposed in both retention times and intensities. This high reproducibility of IP RPLC ensures the integrity of the fraction collections for multiple LC injections, if a large quantity of analytes are required for the 2nd dimension separation and analysis. This is also important for quantitative metabolome profiling where replicate runs are required [18].

It is well known that a high ionic strength of the mobile phase, and particularly when using many ion-pairing reagents, causes discharges and severe ion suppression in ESI [28]. It has been reported that, with commonly used volatile ion-pairing reagents, such as perfluoroheptanoic acid, ESI signal intensity decreased about 30–80%, compared with the use of formic acid [21]. This problem of ion suppression on ESI from high concentrations of ion pairing reagents did not present a problem in this work, because heptafluorobutyric acid was eluted in the RPLC void volume of our 2nd dimension LC–MS analysis. However, it is still preferable to use volatile ion-pairing reagents, such as HFBA, as a non-volatile ion-pairing reagent may form crystals and pose a problem by contaminating the MS interface.

In contrast to most current 2D-LC separations that employ two columns with two different stationary phases with orthogonal retention mechanisms, in this work, we generate the orthogonality of RPLC × RPLC by altering the hydrophobicity of analytes through a chemical derivatization prior to the 2nd dimension separation on a RPLC column (see Fig. 1). The major components of cationic metabolites, such as amines and amino acids, and polar phenolic compounds in a given sample are separated by IP RPLC in the 1st dimension separation. Their hydrophobicity can be significantly altered by a simple and robust dansylation derivatization proce-

dure. Dansylation is a well-studied derivatization chemistry that targets primary amines, secondary amines and phenolic hydroxyls [18,29–34]. Tertiary amines and alkyl hydroxyls cannot be dansylated. The large hydrophobic dansylation tag attached to the metabolites changes their hydrophobicity, and thus, their retention in the 2nd dimension RPLC. The degree to which the hydrophobicity changes is mainly dependent on the number of dansylation tag(s) added on, while the structures of the polar cationic compounds have less impact on their hydrophobicity and their resulting retention on the 2nd dimension RPLC. In general, these dansylated compounds elute according to the number of incorporated tags with more tags resulting in longer retention. We note that one limitation of the current approach of using IP RPLC is that the anionic metabolites will not be efficiently separated in the first dimension separation. However, we expect that not many amine-containing metabolites are present in anionic forms in a urine sample. Some anionic phenolic compounds may not be separated in IP RPLC.

Seven fractions were collected from the 1st dimension IP RPLC as shown in Fig. 2. Aliquots of individual IP RPLC fractions were differentially dansylated by ^{13}C-dansyl chloride and ^{12}C-dansyl chloride, and then combined in 1:1 equal molar ratio. The use of differential isotope labeling allows easy picking of the metabolite peaks in the mass spectra (see below). The top trace of Fig. 3A shows a representative base-peak ion chromatogram obtained from a labeled fraction (fraction #1 in Fig. 2). For comparison, the lower trace of Fig. 3A shows the ion chromatogram of the unlabeled fraction. A representative mass spectrum showing a pair of peaks is shown in Fig. 3B. As Fig. 3A shows, many chromatographic peaks were observed over the entire gradient elution window after labeling. Compared to the 1st dimension separation, a significant increase in separation space was achieved in the 2nd dimension, indicating that some orthogonality has been attained between the two RPLC's. In addition, much stronger signals were obtained from the dansylated fraction, which is consistent with the notion that dansylation generally improves the detection sensitivity by 1–3 orders of magnitude for the reasons already discussed in our previous report [18].

Based on accurate masses and retention time information of the ion pairs detected, both definitive and putative (or tentative)
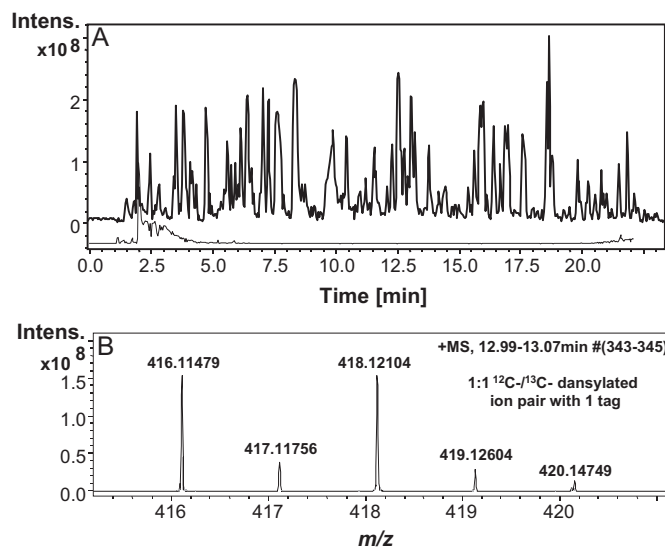

Fig. 3. (A) Base-peak ion chromatograms of the dansylation-labeled fraction #1 (top trace) and the unlabeled fraction #1 (bottom trace). (B) A representative mass spectrum showing a pair of ion peaks obtained at a particular retention time. Accurate mass match against the Human Metabolome Database indicates that the ion pairs were likely from homovanillic acid or other isomers (see Supplemental Table S4 for putative identification of metabolites detected in this work).
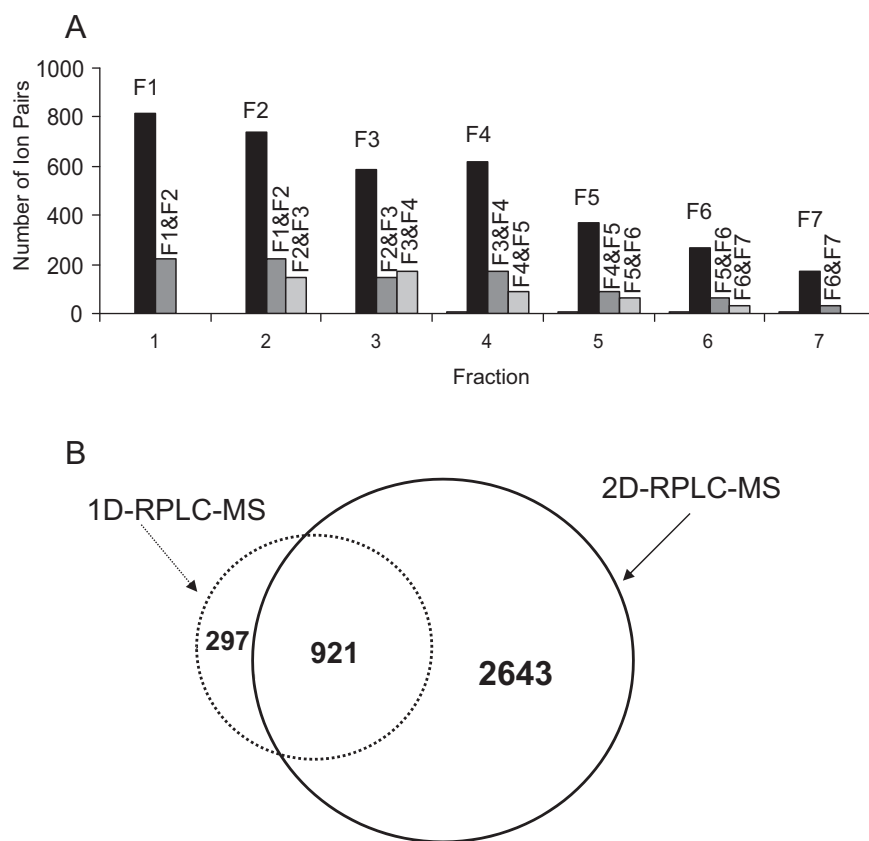
Fig. 4. (A) Distribution of the number of ion pairs detected by RPLC–FTICR-MS from the 7 fractions collected by IP RPLC of a human urine sample; the numbers of the unique and common ion pairs detected in neighboring fraction(s) are shown. (B) Comparison of the number of ion pairs detected by 2D-RPLC–FTICR-MS and 1D-RPLC–FTICR-MS.

metabolite identification were carried out from RPLC–FTICR-MS analysis of the seven IP RPLC fractions. Using the differential isotope labeling method, metabolite peaks can be readily detected by examining the masses and intensities of the peak pairs. Error in mass difference was the mass error between the measured mass difference and theoretical mass difference for ^{13}C-/^{12}C-dansylation ion pairs. The theoretical mass difference for one dansylation tag is 2.00671. Two parts per million (2 ppm) for error in mass difference was used as a key criterion to assign the ^{13}C-/^{12}C-dansylation ion pairs. High mass accuracy FTICR-MS measurement ensures confident assignment of the true ^{13}C-/^{12}C-ion pairs. Note that non-reactive metabolites, background ions, instrumental and electronic noises will not have characteristic mass differences as ^{13}C-/^{12}C-ion pairs. In addition, ^{13}C-/^{12}C-dansylation ion pairs do not show isotopic chromatographic separation in RPLC, i.e., ^{13}C-/^{12}C-ion pairs will be shown in the same spectrum. In our workflow shown in Fig. 1, a software program based on a peak analysis script, XCMS [35], was written to pick up the ^{13}C-/^{12}C-ion pairs. The program eliminated isotopic peaks, common adduct ions, multiply charged ions and multimers. Only the protonated ion pairs were exported to an Excel table for further analysis. If the ion pairs were detected as multiply charged ions, not the usual singly charged ions, only one form of the multiply charged ions was retained and exported to Excel.

Fig. 4A shows the distribution of the number of ion pairs or metabolites detected from individual IP RPLC fractions. The number of ion pairs detected was 1042, 1111, 902, 877, 523, 366, and 211 from fractions 1 to 7, respectively. The number of common ion pairs found in adjacent fractions is also indicated in Fig. 4A. As Fig. 4A shows, there are many unique ion pairs detected in each fraction. Supplemental Table S1 lists the ion pairs from the combined results of seven ^{13}C-/^{12}C-dansylated IP RPLC fractions.

In total, 3564 ion pairs were detected, which is almost three-fold of ion pairs detected from 1D-RPLC–FTICR-MS of the same urine sample after dansylation (i.e., 1218 ion pairs). Fig. 4B shows the comparison of the number of ion pairs detected from off-line 2D-RPLC–FTICR-MS and 1D-RPLC–FTICR-MS. A major portion of the ion pairs found in the 1D experiment were detected in 2D-LC–MS. A small number of ion pairs uniquely found in the 1D experiment reflects the complexity of the urine metabolome. These unique metabolite were preferentially ionized in 1D-LC–MS while they might be suppressed during the 2D experiment; the extent of ion suppression is dependent on the chemical composition of the elute at a given MS detection window. Reduced complexity results in overall reduction of ion suppression. However, this does not mean ion suppression is reduced evenly for all the ions. In fact, some ions may be more suppressed due to the increase in signals from other otherwise suppressed ions. It is also plausible that some of the missing metabolites in 2D-LC–MS were lost during the fraction collection and chemical labeling processes. Further optimization of these processes, such as the use of different solvents to rinse the collection vial more thoroughly during fraction transfer, is needed to avoid sample loss. Nevertheless, the 2D-LC–MS experiment clearly increases the metabolome coverage significantly, compared to 1D-LC–MS. We note that 2D-LC separation should benefit the detection of low abundance ions in FTICR-MS. Since the number of ions trapped in the FTICR cell is limited, reducing the number of co-eluting metabolites by using 2D-LC should facilitate the trapping of the low abundance ions from the mixture, thereby increasing the detectability of these ions.

To provide definitive identification of the metabolites in the urine sample, we have constructed a dansylation compound library consisting of 220 authentic standards of amine- or phenol-containing metabolites (see Supplemental Table S2 for the list).

Table 1
List of metabolites identified by 2D-RPLC–FTICR-MS from a human urine sample along with those identified by 1D-RPLC–FTICR-MS in bold and underlined.

Compound name	Compound name	Compound name
Phospho-tyrosine	5-Hydroxylmethyluracil	**Lysine**
Hydroxylamine	5-Aminopentanoic acid	3-Hydroxybenzoic acid
Hydrochlorothiazide	2-Aminoisobutyric acid	Vanillic acid
Phospho-serine	2-Aminobutyric acid	Isoferulic acid
Phosphoethanolamine	**Sarcosine**	**4-Hydroxybenzoic acid**
Glucosamine	Pyridoxine	Aniline
Taurine	**Proline**	**Histidine**
Saccharopine	**Methylamine**	Desaminotyrosine
Phospho-threonine	Aminocaproic acid	3-Hydroxyanthranilic acid
3-Methylhistidine	**Valine**	Benzylamine
1-Methylhistidine	Salicyluric acid	Tryptamine
Carnosine	**Methionine**	m-Coumaric acid
Hypotaurine	**3-Hydroxyl-picolinic acid**	Trans-ferulic acid
Arginine	Gly–Trp	Ephedrine
Guanidine	3-Nitrotyrosine	2-Aminooctanoic acid
Asparagine	**Tryptophan**	Pyridoxamine
Homoarginine	Kynurenine	5-Hydroxyltryptophan
Histamine		**1,3-Diaminopropane**
Glutamine	Phenylephrine	**Tyrosinamide**
Citrulline	**2-Phenylglycine**	1,2-Diaminopropane
1-Methylhistamine	3-Aminobenzoic acid	**1,4-Diaminobutane**
3-Methylhistamine	3-Aminosalicylic acid	o-Tyrosine
3-Sn-phosphatidylethanolamine	Ethylamine	Thyroxine
Aspartic acid amide	Diaminopimelic acid	
Methylguanidine	Vanillylmandelic acid	**Cadaverine**
Homoserine	**Pipecolic acid**	**Tyrosine**
Adenosine	**Phenylalanine**	**Metoprolol**
Methionine sulfoxide	Hydroxyphenylacetylglycine	**Phenol**
Homocitrulline	Acetyl-tyrosine	4-Nitrophenol
Serine	Leu–Pro	**Cysteamine**
Glutamic acid	**3-Hydroxymandelic acid**	16b-Hydroxyestradiol
Aspartic acid	**Isoleucine**	4,9-Dioxa-1,12-dodecanediamine
Diglycine	L-Cystathionine	**Octopamine**
4-Hydroxy-proline	**Leucine**	p-Cresol
Aminoadipic acid	5-Hydroxylysine	Protocatechuic acid
Threonine	**Cystine**	Gentisic acid
Folic acid		o-Cresol
Dopamine	4-Hydroxy-3-methoxyphenyllactic acid	Serotonin
Iminodiacetic acid	**Phenylethanolamine**	Caffeic acid
Diethanolamine	**Hydroxyphenyllactic acid**	Metanephrine
Ethanolamine	**5-HIAA**	Piperazine
Epinephrine	**Dimethylamine**	Thyronine
Glycine	**2,4-Diaminobutyric acid**	Phenylephrine or Synephrine
Glycylproline	**Homocystine**	**Tyramine**
Beta-alanine	Salicylic acid	**Spermidine**
Tyrosine methylester	**Ornithine**	Xanthurenic acid
Alanine	Methyl-phenylalanine	Estradiol
γ-Aminobutyric acid	**5-Methoxysalicylic acid**	3-Isopropylphenol
Aminolevulinic acid	3- or 4-hydroxyphenylacetic acid	Pyrocatechol
Procaine	**Homovanillic acid**	Estrone
Pantothenic acid	5-Methoxytryptamine	Norepinephrine
p-Aminohippuric acid	Syringic acid	**Thymol**
Salbutamol	**Homocarnosine**	Hydroquinone
Hypoxanthine	**3-Cresotinic acid**	Deoxyepinephrine
Isoguanine	**Carnosine**	Desipramine
3-Aminoisobutyric acid	**Gentisic acid**	

Definitive identification was done by matching the accurate mass and retention time of the ^{13}C-/^{12}C-dansylation ion pairs detected in the urine sample to those of the ^{13}C-/^{12}C-dansylated standards. In total, 167 ion pairs were found to match the accurate mass and retention time of the authentic ^{13}C-/^{12}C-dansylated standards. Table 1 lists the compound names of the 167 metabolites definitively identified from the human urine sample from this work, according to their order of elution in RPLC–MS. The corresponding 167 ^{13}C-/^{12}C-ion pairs with their retention time and accurate mass are shown in Supplemental Table S3. Note that in Supplemental Table S3 ethylamine was detected in several fractions; this compound was an impurity in methylamine used to consume the excess amount of the isotope reagent. The number of definitively identified

metabolites from the 2D-LC–MS experiment is about 2.5 times the number of metabolites definitively identified by 1D-LC–MS analysis of the same urine sample after ^{13}C-/^{12}C-dansylation (i.e., 68 metabolites as listed in bold and underlined in Table 1).

All the ion pairs that were not matched to the ^{13}C-/^{12}C-labeled authentic standard library were used to search against the Human Metabolome Database (HMDB) for putative identification [36]. The HMDB database search was based on matching the accurate mass of the measured ions (^{12}C-dansyltion ions minus the dansyl group) against the accurate masses of 10,364 human metabolites listed in the database. The search criterion for accurate mass match was ±3 mDa. The search result is shown in Supplemental Table S4. For the 3381 unique ion pairs listed in Supplemental Table S4, 53%

of the ion pairs do not match with any metabolites in the HMDB database and 47% of pairs (1586 pairs) match with one or more putative metabolite (18% of pairs match to one putative metabolite). Many of the matches are likely coincidental, as an accurate mass can generate a number of possible molecular formula and, even with one formula, many possible chemical structures can be postulated. Clearly definitive identification of many ion pairs detected from the 2D-LC–MS experiment remains to be an analytical challenge. One strategy of averting this problem is to carry out relative quantification of the metabolomes of a number of comparative samples (e.g., diseased vs. controlled) first, followed by statistics analysis, such as principal component analysis (PCA), to discover one or a few putative biomarkers. After the discovery of the putative biomarkers, major efforts are then devoted to the identification of these metabolites using techniques such as tandem MS, NMR and synthesis of standard compounds. Definitive identification of the biomarkers is important for both studying the disease biology and obtaining regulatory approval for clinical applications.

4. Conclusions

We have developed a new off-line 2D-LC strategy combined with FTICR-MS for analyzing a large number of amine- and phenol-containing metabolites in a complex biological sample. Cationic and polar species in a biological sample were successfully separated and fractionated by ion-pairing RPLC. Many of these species contained amine moiety which could be easily derivatized by dansyl chloride. The RP chromatographic retention behavior of the polar amines, amino acids, phenols, etc., were altered to an extent after dansylation derivatization such that they could be well retained and separated with high efficiency in the 2nd RPLC interfaced to FTICR-MS. We have shown that this 2D-RPLC–MS technique was able to detect 3564 metabolites in a human urine sample, compared to 1218 metabolites detected in 1D-RPLC–MS. While this strategy was demonstrated for the analysis of amine- and phenol-containing metabolites using dansylation derivatization, it should be generally applicable to other derivatization chemistries where derivatized metabolites have vastly different hydrophobicity than their un-derivatized counterparts (e.g., p-dimethylaminophenacyl labeling for carboxylic acid-containing metabolites [37]).

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.04.024.

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