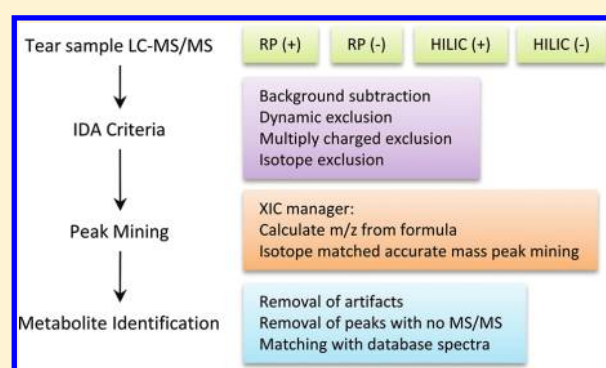


Characterization of The Human Tear Metabolome by LC–MS/MS

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ABSTRACT: The tear film overlying the epithelial cells of the eye's surface is vital to visual function, and its composition is reflective of ocular surface health. The ultrasmall volume of tears poses challenges in its analysis, contributing to the limited number of reports on the tear metabolome. In addition, using a standard clinical method of tear collection posed some confounding factors in metabonomic analysis. We sought to establish an analytical platform for the global characterization of human tear metabolites. Following information dependent acquisition (IDA) directed liquid chromatography–tandem mass spectrometry (LC–MS/MS), isotope pattern matched peak mining was performed using Extracted Ion Chromatogram (XIC) manager within the PeakView software. Sixty metabolites representing diverse compound classes were identified in human tears, most of which have not been previously reported. Selected metabolites were verified using pure standards. Unsupervised chemometric analysis showed good separation between tear samples and blanks (PC1 = 87%, $R^2 = 0.91$, $Q^2 = 0.87$). The results demonstrated the potential of our platform for untargeted metabonomic studies of eye diseases.

KEYWORDS: metabonomics, metabolic profiling, tear fluid, liquid chromatography mass spectrometry, isotope pattern, tear metabolites, biomarkers, chemometric, information dependent acquisition, metabolomics



INTRODUCTION

The tear film is a thin layer of fluid contained between the eyelids, covering the anterior surface of the eyeball. It provides lubrication, protection and nutrition to the ocular surface.¹ Its stability is critical for the optical quality of the cornea.² Tears, the extra-cellular fluid bathing the epithelial cells that form the ocular surface, consist of three components: aqueous, lipid and mucin.^{1,3} The aqueous layer is the largest in volume and contains dissolved electrolytes, proteins and metabolites. Being directly associated with the corneal surface, the tear film is an accessible source in studying ocular surface disorders. The global profiling of tear metabolites is of particular interest as metabonomics provides a top-down insight of the dynamic biochemical processes occurring in a biological system.⁴ In addition, the collection of tears is a noninvasive procedure that in turn facilitates large-scale clinical studies.

While global -omics platforms have been utilized in the study of the tear proteome^{5–7} and lipidome,^{8–10} the analysis of tear metabolites has been limited to targeted compound classes.^{11–24} The number of identified metabolites is also much lesser compared to other biological fluids. A detailed list with references is provided in Table 1.

To the best of our knowledge, there is no publication describing the global metabonomic analysis of tear fluid. This is possibly attributed to challenges in instrumental sensitivity, as the typical sample volume of tears is only 5–10 μ L.

The main analytical techniques adopted in global metabonomic analysis are NMR, GC–MS and LC–MS. Both NMR and GC–MS offer high reproducibility and straightforward identification of metabolites through extensive spectra libraries. In addition, NMR is nondestructive, requires minimal sample preparation and offers the benefit of obtaining quantitative data simultaneously. However, NMR is the least sensitive of the three techniques and may be biased toward the detection of high abundance metabolites.

One key advantage of GC–MS is its deconvolution function,²⁵ which facilitates spectral resolution of coeluting metabolites (not possible by visual inspection of the chromatogram). However, the technique may be prone to confounding factors²⁶ introduced through the sample derivatization process or thermal degradation of metabolites at elevated temperatures.

LC–MS has emerged as a complementary metabonomic technique addressing the limitations of NMR and GC–MS. It provides high sensitivity and the ability to detect a wide range of metabolites. Nevertheless, LC–MS is susceptible to retention time (RT) drift and matrix effects related to electrospray ionization (ESI). It is well established that adducts, dimers and in-source fragment product ions (collectively termed redundant signals) are

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Table 1. Tear Metabolites Identified in Literature

metabolite	ref	metabolite	ref
Alkanes and Alkenes		Carnitines	
Squalene	11	Acetylcarnitine	19
		Carnitine	19
		Propionylcarnitine	19
Amino Acids		Catecholamines and derivatives	
Alanine	12	Epinephrine	20
Arginine	12		
Aspartic acid	12	Hydroxy acids	
Asparagine	12	Ascorbic acid	13,14
Citrulline	12	Lactate	15
Cysteine	12–14	Histamine	21
Glutamic acid	12		
Glutamine	12	Keto-acids	
Glycine	12	Pyruvate	15
Histidine	12		
Isoleucine	12	Nucleotides	
Leucine	12	AP ₃ A	22
Lysine	12	AP ₄ A	22
Methionine	12	AP ₅ A	22
Ornithine	12		
Phenylalanine	12	Peptides	
Proline	12	Glutathione	13,14
Serine	12		
Taurine	12	Purines and derivatives	
Threonine	12	Uric acid	13,14,23
Tryptophan	12		
Tyrosine	12–14	Retinoids	
Valine	12	Retinol	24
Amino Ketones		Steroids and derivatives	
Urea	15,16	Cholesterol	11
Carbohydrates			
Glucose	15,17,18		

formed alongside $[M + H]^+/[M - H]^-$ parent ions. Unlike GC–MS where electron impact ionization is robust and reproducible, ESI is highly variable within the same instrument and between different instruments. Postacquisition data processing typically comprises of peak detection and alignment, followed by chemometric analysis to identify marker ions. Metabolites are identified through database searches, and their identities confirmed by comparison of RT and tandem mass spectra with pure standards.²⁷ Such downstream data-processing is time-consuming and constitutes the largest bottleneck in a metabolomics workflow.

In this paper, we report the first global characterization of the human tear metabolome by information dependent acquisition (IDA) directed liquid chromatography–tandem mass spectrometry (LC–MS/MS) coupled to a peak mining workflow built on isotope pattern matching.

MATERIALS AND METHODS

Chemicals

Adenosine diphosphate, allantoin, arginine, carnitine, citric acid, glutamine, panthenol, tyrosine, tryptophan, uric acid, xanthine and HPLC grade ammonium formate were purchased

from Sigma Aldrich (St. Louis, MO). HPLC grade acetonitrile, formic acid, methanol and water were purchased from Merck Chemicals (Darmstadt, Germany).

Sample Collection

Tears were collected from six healthy subjects using Schirmer strips⁵ without local anesthesia. All subjects had neither ocular complaints nor a history of contact lens usage. Written consent was obtained, and the study had the approval from the SingHealth Institutional Review Board. Twelve tear samples were obtained from the last subject over a period of 2 weeks between 9 a.m. to noon, with no more than one tear sample collected per eye per day. Samples were stored at -80°C prior to extraction.

Sample Preparation

Metabolites were extracted from Schirmer strips by vortex-mixing (1200 rpm) each strip in 400 μL of 9:1 MeOH/ H_2O for 15 min. Extracts were centrifuged for 10 min at 15 000 rcf and dried in a vacuum concentrator. Individual samples from the first five subjects were reconstituted in 30 μL of water. Samples from the last subject were divided into 2 pools of 6 extracts each. Samples from pools 1 and 2 were reconstituted in 50 μL of water and acetonitrile, respectively, after drying. Schirmer strips with no tears were subjected to the same extraction protocol to serve as blank controls.

IDA LC–MS/MS Analysis

Chromatographic separation was performed on a Prominence UFLC system (Shimadzu, Kyoto, Japan). The autosampler and column heater temperatures were maintained at 10 and 40°C , respectively. The injection volume was 10 μL for all analyses. The fraction of pooled tear samples injected was equivalent to the volume of a single tear sample.

Samples from the first five subjects and pool 1 were injected onto a T3 C₁₈ reversed phase (RP) column 2.1 $\times$ 100 mm, 3 μm (Waters, Milford, MA). The mobile phase was A, 0.1% formic acid in water and B, 0.1% formic acid in acetonitrile. The gradient profile was 2% B from 0 to 2 min, 15% B at 6 min, 50% B at 12 min, 95% B at 16–18.5 min, and 2% B at 19–25 min. The reconstituted sample from pool 2 was injected onto a ZIC hydrophilic interaction chromatography (HILIC) column 2.1 $\times$ 100 mm, 3.5 μm (Merck SeQuant AB, Umea, Sweden). The mobile phase was A, 2 mM NH₄COOH in water (pH 6.6) and B, 9:1 acetonitrile/2 mM aqueous NH₄COOH (pH 6.6). The gradient profile was 90% B at 0 min to 80% B at 8 min, 10% B from 15 to 18 min, 90% B at 19–24 min. The flow rate for all separations was 0.3 mL/min.

Each sample was analyzed in positive and negative ionization modes, using a hybrid quadrupole time-of-flight (Q-TOF) instrument—a TripleTOF 5600 fitted with a DuoSpray ion source (AB Sciex, Concord, Canada). Column effluent was directed to the ESI source. The source voltage was set to 5.0 kV for positive ionization and 4.0 kV for negative ionization mode. The declustering potential was 80 V and source temperature was 550°C for both modes. The curtain gas flow, nebulizer, and heater gas were set to 25, 45, and 55 arbitrary units.

The instrument was set to perform one TOF MS survey scan (150 ms) and 20 MS/MS scans (50 ms each) with a total duty cycle time of 1.2 s. The mass range of both scan types was 50–1000 m/z . Acquisition of MS/MS spectra was controlled by IDA function of the Analyst TF software (AB Sciex, Concord, Canada) with application of following parameters—dynamic background subtraction, charge monitoring to exclude multiply

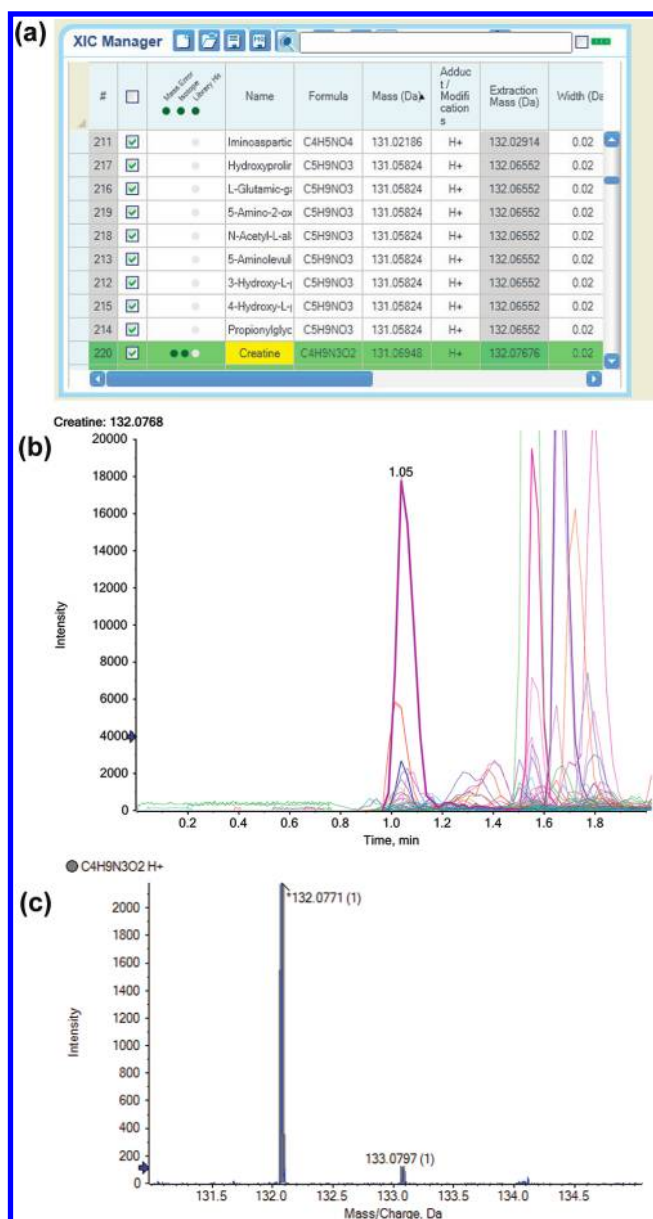


Figure 1. (a) Output of XIC Manager after isotope pattern matched peak mining was performed on RP (+) data file. In this example the putative metabolite creatine was selected for identification. The green circles indicate that the mass error and isotope pattern error are under 10 ppm and 20% respectively (user-set thresholds). (b) In addition to an output table, XIC manager also extracts ion chromatograms of all putative metabolites, and peaks could be visualized by clicking on the respective entries in the output table. Creatine has a RT of 1.05 min on the RP column. (c) TOF-MS spectra of putatively identified creatine. Regions highlighted in gray indicate a match to the theoretical isotope pattern.

charged ions and isotopes, and dynamic exclusion of former target ions for 5 s. Rolling collision energy was set whereby the software calculated the CE value to be applied as a function of m/z .

Mass accuracy was maintained by the use of an automated calibrant delivery system (AB Sciex, Concord, Canada) interfaced to the second inlet of the DuoSpray source. Calibrations were performed at the start of a workday or whenever ionization polarity was changed.

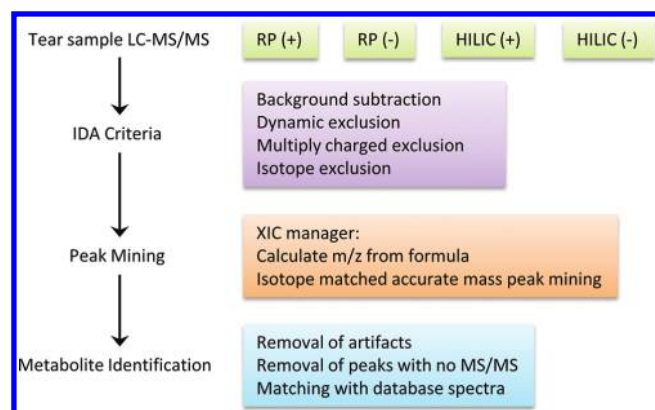


Figure 2. Schematic of the IDA directed LC-MS/MS and isotope pattern matched peak mining workflow.

For standardization, the four combinations of column chemistry and ionization modes polarities were termed as RP(+), RP(-), HILIC(+) and HILIC(-).

Data Processing

MarkerView (AB Sciex, Concord, Canada) was used to generate a peak table of m/z and RT for samples in the individual study using the following parameters. For peak detection: noise threshold of 50 counts, minimum chromatographic peak width of 3 scans, minimum spectra width of 10 mDa, background subtraction offset of 20 scans and subtraction multiplication factor of 1.2. For peak alignment: RT window of 0.7–10 min, RT tolerance of 0.3 min, mass tolerance of 12 ppm, presence of peaks in at least 3 samples and maximum number of peaks at 50 000. Principle component analysis (PCA) on aligned peak data was performed using SIMCA (Umetrics AB, Umea, Sweden) with Pareto scaling.

Metabolite Identification

Data files of pooled tear samples were subjected to isotope pattern matched peak mining using the Extracted Ion Chromatogram (XIC) manager add-on for PeakView (AB Sciex, Concord, Canada). Screen captures of the software interface illustrating its workings are provided in Figures 1a–c. From a list of metabolites derived from HMDB²⁸ (2884 entries, MW from 100 to 800), the software calculated an accurate mass list of monoisotopic parent ions, $[M + H]^+$ and $[M - H]^-$, derived from positive and negative ionization modes, respectively. The list of extraction masses were used subsequently to mine for peaks from the data files of the RP(+), RP(-), HILIC(+) and HILIC(-) analyses. The RT window was set to 0–10 min and 1–21 min for RP and HILIC analyses, respectively. The criteria of intensity counts over 200, S/N ratio greater than 3 and isotope pattern matching of 80% and higher were applied in all data mining experiments.

Entries with peak area values less than 1000 units were removed from the results list. The remaining entries were used as the search list in mining the data files of the blank controls, with the following modifications: the expected RT was further constrained to within 1 min of the peak found in pooled tears, and the isotope matching function disabled. Entries with peak area values in the pooled tear samples present at less than 5 times than that of the blank controls were removed.

Selected ion chromatograms were extracted for each m/z in the resultant data table to further screen for isobaric ions at

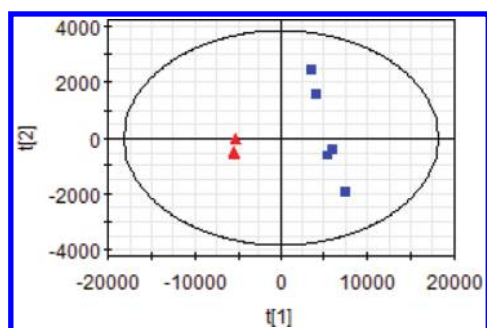


Figure 3. PCA scores plot of tear samples (blue squares, $n = 5$) and blank controls (red triangles, $n = 5$). PC1 constitutes 87% of the variation between the two sample groups. Model performance statistics: $R^2 = 0.91$, $Q^2 = 0.87$.

other RTs, as the XIC manager reported only the most intense peak across the chromatogram. MS/MS spectra of all putative identifications were retrieved and matched with entries in the Metlin,²⁹ Massbank³⁰ and HMDB²⁸ databases. Efforts were made to distinguish metabolites from other isobaric compounds whenever possible by virtue of differences in fragmentation pattern. Entries where no experimental MS/MS spectra were acquired and those with no MS/MS spectra available in the databases were not further pursued. A schematic of the workflow is given in Figure 2. The metabolites adenosine diphosphate, allantoin, arginine, carnitine, citric acid, glutamine, panthenol tyrosine, tryptophan, uric acid, xanthine were verified by analyzing pure standards using the same conditions.

RESULTS AND DISCUSSION

Differentiation of Tear Samples from Collection Matrix

A PCA scores plot of tear samples and blank controls obtained from a RP analysis in negative ionization mode is shown in Figure 3. The model was optimized at 2 principal components, with $R^2 = 0.91$ and $Q^2 = 0.87$. Tear samples were well separated from blank controls, with t_1 accounting for 87% of the variation. Similar results were also obtained in the positive ionization mode. While nonbiological samples were adopted as blank controls, our findings suggested the unique metabolotypes of human tears.

Metabolite Identification

Using isotope pattern matched peak mining, 60 metabolites were identified in human tears (Table 2). Most are endogenous and have not been reported in human tears thus far. Metabolites were reported together as a single entry when resolution of isobaric compounds was not possible. A diverse range of compound classes are represented (Table 2), thereby demonstrating good coverage of the analytical method. The RTs of metabolites found in each analytical mode are shown in Table S-1 (Supporting Information). Metabolites for verification were selected to cover a range of chemical structures, polarities and isotope distribution patterns. MS/MS spectra of two verified metabolites, xanthine and tryptophan, are provided in the Supporting Information as representative spectra (Figures S-2a, S-2b). Removal of entries present in tear samples that were less than 5 times the levels in blank controls greatly reduced the likelihood of mis-identifying artifacts as metabolites.

Analytical Strategy

Although NMR and GC–MS have been explored in the characterization of lesser studied metabolomes of saliva,³¹ amniotic fluid³² and cerebrospinal fluid (CSF),³³ there have been no reports on their use in characterizing the human tear metabolome. This might be attributed to the low volume of tear samples, about 10 μ L, being less abundant than other biofluids by at least 1 order of magnitude and thus posing a challenge in analytical sensitivity. In our paper, we adopted the sensitive LC–MS platform for profiling the tear metabolome.

Untargeted LC–MS metabolomic analysis typically operates using a “sniper” approach³⁴ — a profiling experiment is first performed where only high resolution and accurate MS1 is acquired. Following peak detection and RT alignment, chemometric analysis is performed to determine markers, out of which only a fraction could be assigned to be metabolites.^{35,36} Putative identifications are confirmed subsequently by (1) performing targeted fragmentation on reinjected samples and comparing the obtained MS/MS spectra with entries in databases, or (2) analyzing pure standards and comparing their RT and fragmentation pattern.^{37,38} An alternative workflow has been reported by Evans et al.³⁹ by scoring experimental data against an in-house MS/MS library constructed on an ion trap instrument. However, applicability of the method in a research environment is limited due to the large expense of cost and time in library construction.

By contrast, data acquisition in untargeted proteomic analyses using MS are often performed in a shotgun manner,⁴⁰ with profiling and IDA directed qualitative data obtained in a single analysis. This is enabled by the nonredundant nature of peptide ionization and the selection of only multiply charged species for fragmentation. In addition, the use of nano LC in proteomics further reduces the demand on sensitivity and duty cycle.

Unlike typical data processing in metabolomic analysis where data is subjected to peak detection and experimental masses are obtained, our workflow operates in a reverse manner whereby theoretical masses from the in-house library are used to extract isotope pattern matched peaks from experimental data. Although a recent publication reported the use of similar software for drug screening in conjunction with targeted analysis,⁴¹ there is no precedence of isotope pattern matched peak mining in global metabolomic analysis.

Mass accuracy and resolution form the foundation of our peak mining workflow and were measured to be <6 ppm (average 2.4 ppm) and over 35 000 at 400 m/z , respectively (Figure S-3, Supporting Information). The use of an orthogonal isotope pattern filter⁴² greatly reduced the probability of detecting background noise and redundant signals as a monoisotopic peak. The accuracy of isotope abundances is influenced by several factors such as ion intensity, relative abundance of isotope peak and competitive ionization.⁴³ Our workflow was based on the assumption that in spite of redundant ionization, parent ions occurred above detection limit and their isotope intensities were within 80% accuracy.

We have performed profiling of the tear metabolome with simultaneous qualitative fragmentation by utilizing a short duty cycle with a high number of product ion scans (20 in a cycle time of 1.2 s). This facilitated the resolution and selection of $[M + H]^+ / [M - H]^-$ species for fragmentation among redundant signals. In addition, IDA parameters maximized the instrument's productivity in selecting new-eluting metabolite signals for MS/MS.

Table 2. Metabolites Detected in Tears by IDA LC–MS/MS Peak Mining

Amino Acids	Aromatic Acids	Nucleotides
1-Methylhistidine/	Cinnamic acid	ADP ^a
3-Methylhistidine	<i>o</i> -Coumaric acid/	AMP
Arginine ^{a,b}	<i>m</i> -Coumaric acid/	CMP
Asymmetric dimethylarginine/	<i>p</i> -Coumaric acid	Cytidine diphosphate choline
Symmetric dimethylarginine		GMP
Citrulline ^b	Carbohydrates	IMP
Creatine	<i>N</i> -Acetylneuraminic acid	UDP
Glutamic acid ^b		UMP
Glutamine ^{a,b}	Carnitines	UDP- <i>N</i> -acetylglactosamine/
Homoarginine	Acetylcarnitine ^d	UDP- <i>N</i> -acetylglucosamine
Hydroxyproline	Carnitine ^{a,d}	
Phenylalanine ^b	Hexanoylcarnitine	Peptides
Proline ^b	Palmitoylcarnitine ^d	Oxidized glutathione
Pyroglutamic acid		
Serine ^b	Cyclic Amines	Phospholipids
Taurine ^b	Niacinamide	1-Palmitoyl- lysophosphatidylcholine
Threonine ^b		
Tryptophan ^{a,b}	Dicarboxylic Acids	Purines and derivatives
Tyrosine ^{a,c}	Fumaric acid/Maleic acid	Hypoxanthine
Urocanic acid		Theobromine
Valine ^b	Nucleosides	Uric acid ^{a,e}
Amino Alcohols	1-Methyladenosine	Xanthine ^a
Panthenol ^a	Adenosine	
Amino Ketones	Cytidine	Pyridoxals and derivatives
Allantoin ^a	Guanosine	4-Pyridoxic acid
Creatinine	Inosine	
	<i>S</i> -Adenosyl-homocysteine	Quaternary Amines
	<i>S</i> -Adenosyl-methionine	Acetylcholine
	Uridine	Glycerolphosphocholine
	Xanthosine	Phosphocholine
		Tricarboxylic Acids
		Citric acid ^a

^a Verified with standard. ^b Reported in ref 12. ^c Reported in refs 12–14. ^d Reported in ref 19. ^e Reported in refs 13, 14, and 23.

We attempted to maximize the coverage of the tear metabolome via the utilization of complementary column chemistries and ionization in both positive and negative polarities. The use of solely RP(+), RP(–), HILIC(+), or HILIC(–) would result in the identification of only 26, 25, 35, or 22 metabolites respectively. While HILIC is accepted to be complementary to RP in selectivity,⁴⁴ a total reversal in the elution order of metabolites should not be expected. For example, hypoxanthine elutes before inosine in both RP and HILIC.^{45,46} The ZIC HILIC particle is zwitterionic; thus, selectivity and retention are more strongly influenced by pH and ionic strength than for RP.

To date, only 41 metabolites have been identified in the tears of normal human subjects. Our approach has identified 16 of the previous reported metabolites and another 44 new metabolites in tears.

Limitations

No analytical platform is capable of resolving and detecting all the metabolites present within a sample. Thus, it was of no surprise that some previously reported tear metabolites, for

example, glucose and ascorbic acid, were not included in Table 2 due to background interfering signals.

Furthermore, the list of identified metabolites presented in Table 2 is by no means exhaustive. Metabolites displaying these attributes would possibly remain unreported: (1) strong adduct formation with inorganic ions, (2) present at a low concentration in tear samples, and (3) low molecular weight with small number of carbon atoms. For example, allantoin (C₄H₆N₄O₃) was only identified upon further investigation. It was initially unreported by XIC manager as the M+1 isotope peak (5% relative abundance) was below the instrument detection limit. A detailed retrospective evaluation of the workflow is provided in the Supporting Information.

CONCLUSION

We have identified 60 metabolites in human tears encompassing a wide range of compound classes using isotope pattern matched peak mining with XIC manager. The separation of tear samples from Schirmer strip blanks by PCA demonstrated the

applicability of the analytical platform in future untargeted metabolotyping of ocular diseases.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Characterization of The Human Tear Metabolome by LC/MS-MS

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The following are provided in the supporting information:

Retention time of identified metabolites with multiple analytical modes

Representative MS/MS spectra of verified metabolites

TOF MS resolution measured at m/z 400

A detailed retrospective evaluation of the workflow

SUPPORTING INFORMATION

Table S-1. Retention time of metabolites identified with multiple analytical modes

#	Formula	Mass (mono)	Name	Retention time			
				RP (+)	RP (-)	HILIC (+)	HILIC (-)
1	C ₁₁ H ₁₅ N ₅ O ₄	281.1124	1-Methyladenosine	1.67			
2a	C ₇ H ₁₁ N ₃ O ₂	169.0851	1-Methylhistidine ^a			10.8	
2b			3-Methylhistidine ^a				
3	C ₂₄ H ₅₀ NO ₇ P	495.3325	1-Palmitoyl- lysophosphatidylcholine			1.76	
4	C ₈ H ₉ NO ₄	183.0532	4-Pyridoxic acid		2.67		
5	C ₉ H ₁₇ NO ₄	203.1158	Acetylcarnitine	1.53		5.13	
6	C ₇ H ₁₆ NO ²⁺	146.1181	Acetylcholine			10.09	
7	C ₁₀ H ₁₃ N ₅ O ₄	267.0968	Adenosine	3.29	3.13	1.42	1.41
8a	C ₈ H ₁₈ N ₄ O ₂	202.1430	Asymmetric dimethylarginine ^b			16.26,	
8b			Symmetric dimethylarginine ^b			16.49	
9	C ₁₀ H ₁₅ N ₅ O ₁₀ P ₂	427.0294	ADP *		1.28		
10	C ₄ H ₆ N ₄ O ₃	158.0440	Allantoin ^{c, *}				2.43
11	C ₁₀ H ₁₄ N ₅ O ₇ P	347.0631	AMP	1.55	1.60	3.70	3.68
12	C ₆ H ₁₄ N ₄ O ₂	174.1117	Arginine *			17.08	
13	C ₇ H ₁₅ NO ₃	161.1052	Carnitine *	1.01		7.21	
14	C ₉ H ₈ O ₂	148.0524	Cinnamic acid				2.01
15a	C ₆ H ₈ O ₇	192.0270	Citric acid*		1.83		
16	C ₆ H ₁₃ N ₃ O ₃	175.0957	Citrulline			6.79	
17	C ₉ H ₁₄ N ₃ O ₈ P	323.0519	CMP		1.13		
18a	C ₉ H ₈ O ₃	164.0473	o-Coumaric acid ^a				
18b			m-Coumaric acid ^a	2.12		3.22	
18c			p-Coumaric acid ^a				
19	C ₄ H ₉ N ₃ O ₂	131.0695	Creatine	1.06		5.39	
20	C ₄ H ₇ N ₃ O	113.0589	Creatinine				1.78
21	C ₉ H ₁₃ N ₃ O ₅	244.0935	Cytidine	1.51			
22	C ₁₄ H ₂₆ N ₄ O ₁₁ P ₂	488.1073	Cytidine diphosphate choline			10.05	
23a	C ₄ H ₄ O ₄	116.0110	Fumaric acid ^a		1.18		
23b			Maleic acid ^a				
24	C ₅ H ₉ NO ₄	147.0532	Glutamic acid			5.56	5.42
25	C ₅ H ₁₀ N ₂ O ₃	146.0691	Glutamine *		1.03	5.69	5.66
26	C ₈ H ₂₀ NO ₆ P	257.1028	Glycerophosphocholine			8.13	
27	C ₁₀ H ₁₄ N ₅ O ₈ P	363.0580	GMP	1.65	1.73		
28	C ₁₀ H ₁₃ N ₅ O ₆	284.0917	Guanosine	3.79	3.76	2.35	2.35
29	C ₁₃ H ₂₅ NO ₄	259.1784	Hexanoylcarnitine			2.43	
30	C ₇ H ₁₆ N ₄ O ₂	188.1273	Homoarginine			16.75	

31	C ₅ H ₉ NO ₃	131.0582	Hydroxyproline			4.64	4.63
32	C ₅ H ₄ N ₄ O	136.0385	Hypoxanthine	1.80	1.80	1.55	
33	C ₁₀ H ₁₃ N ₄ O ₈ P	348.0471	IMP	1.70	1.80		
34	C ₁₀ H ₁₂ N ₄ O ₅	268.0808	Inosine	3.79	3.76	1.84	1.83
35	C ₁₁ H ₁₉ NO ₉	309.1060	N-Acetylneuraminic acid				4.58
36	C ₆ H ₆ N ₂ O	122.0480	Niacinamide	1.65			
37	C ₂₀ H ₃₂ N ₆ O ₁₂ S ₂	612.1520	Oxidized glutathione	2.15	2.29		
438	C ₂₃ H ₄₅ NO ₄	399.3349	Palmitoylcarnitine			1.64	
39	C ₉ H ₁₉ NO ₄	205.1314	Panthenol *	5.66	5.69	1.18	
40	C ₉ H ₁₁ NO ₂	165.0790	Phenylalanine	4.37	4.35	2.01	
41	C ₅ H ₁₄ NO ₄ P	183.0661	Phosphocholine	1.04			
42	C ₅ H ₉ NO ₂	115.0633	Proline				3.80
43	C ₅ H ₇ NO ₃	129.0426	Pyroglutamic acid			5.66	
44	C ₁₄ H ₂₀ N ₆ O ₅ S	384.1216	S-Adenosyl-homocysteine	2.17			
45	C ₁₅ H ₂₂ N ₆ O ₅ S	398.1372	S-Adenosyl-methionine			15.71	
46	C ₃ H ₇ NO ₃	105.0426	Serine			6.01	5.98
47	C ₂ H ₇ NO ₃ S	125.0147	Taurine		0.98	4.56	4.56
48	C ₇ H ₈ N ₄ O ₃	181.0647	Theobromine	5.98			
49	C ₄ H ₉ NO ₃	119.0582	Threonine				4.76
50	C ₁₁ H ₁₂ N ₂ O ₂	204.0899	Tryptophan *	6.65	6.68	2.28	2.28
51	C ₉ H ₁₁ NO ₃	181.0739	Tyrosine *	2.15	2.22		3.24
52	C ₉ H ₁₄ N ₂ O ₁₂ P ₂	404.0022	UDP			7.48	
53	C ₉ H ₁₃ N ₂ O ₉ P	324.0359	UMP		1.33		3.78
54	C ₅ H ₄ N ₄ O ₃	168.0283	Uric acid *	1.72	1.78	3.12	2.94
55	C ₉ H ₁₂ N ₂ O ₆	244.0695	Uridine	2.17	2.32		1.54
56a	C ₁₇ H ₂₇ N ₃ O ₁₇ P ₂	607.0816	UDP-N-acetylgalactosamine ^a			6.26	
56b			UDP-N-acetylglucosamine ^a				
57	C ₆ H ₆ N ₂ O ₂	138.0429	Urocanic acid	1.55	1.58	1.96	1.93
58	C ₅ H ₁₁ NO ₂	117.0790	Valine			3.11	
59	C ₅ H ₄ N ₄ O ₂	152.0334	Xanthine *	2.05	2.17		1.51
60	C ₁₀ H ₁₂ N ₄ O ₆	284.0757	Xanthosine		4.52		

a = chromatographically and spectrally unresolvable

b = partially resolved by chromatography, spectrally unresolvable

c = identified through manual search

* = verified with standard

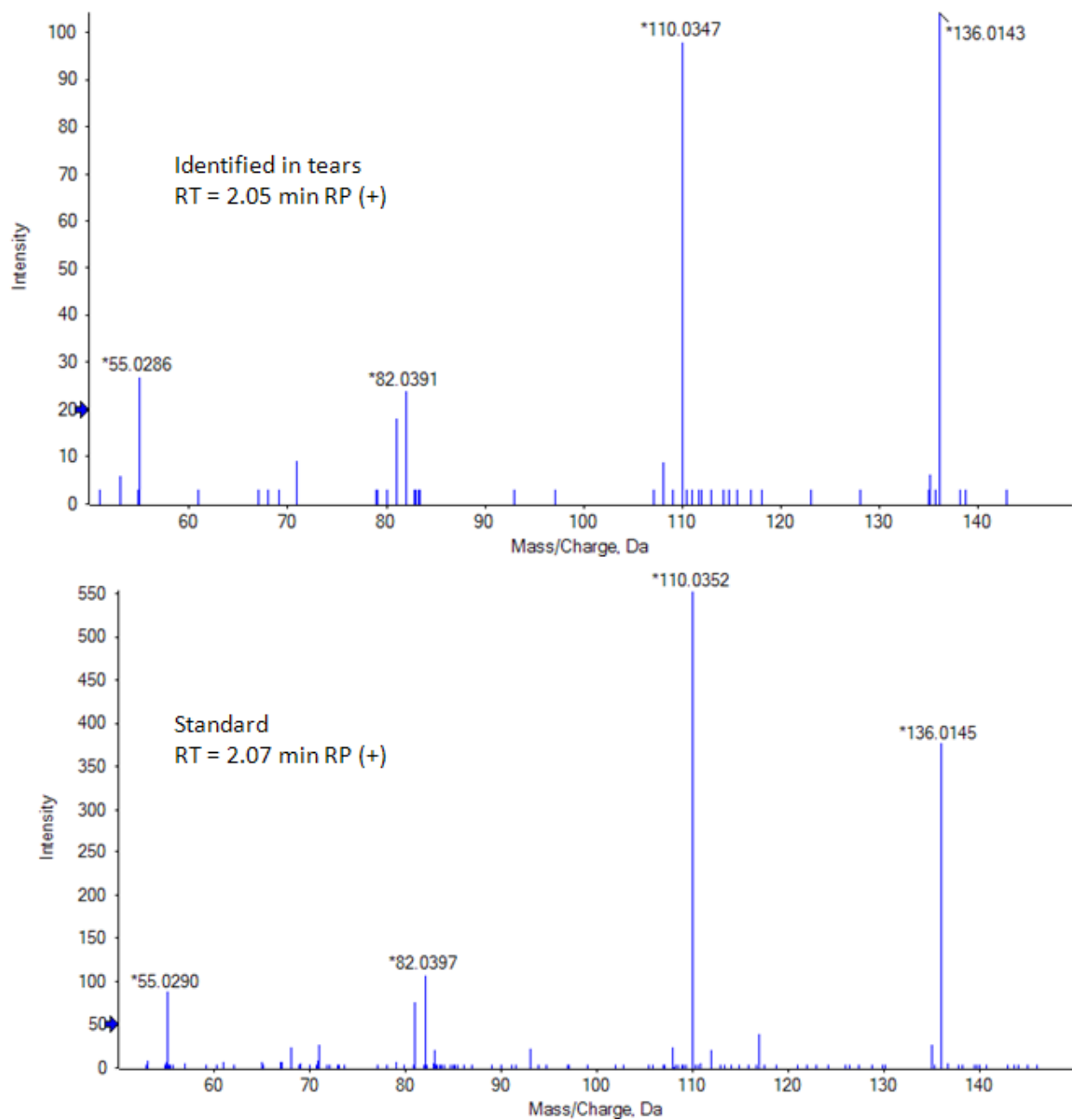


Figure S-1a. MS/MS spectra of xanthine identified in tears (top), verified against metabolite standard (bottom)

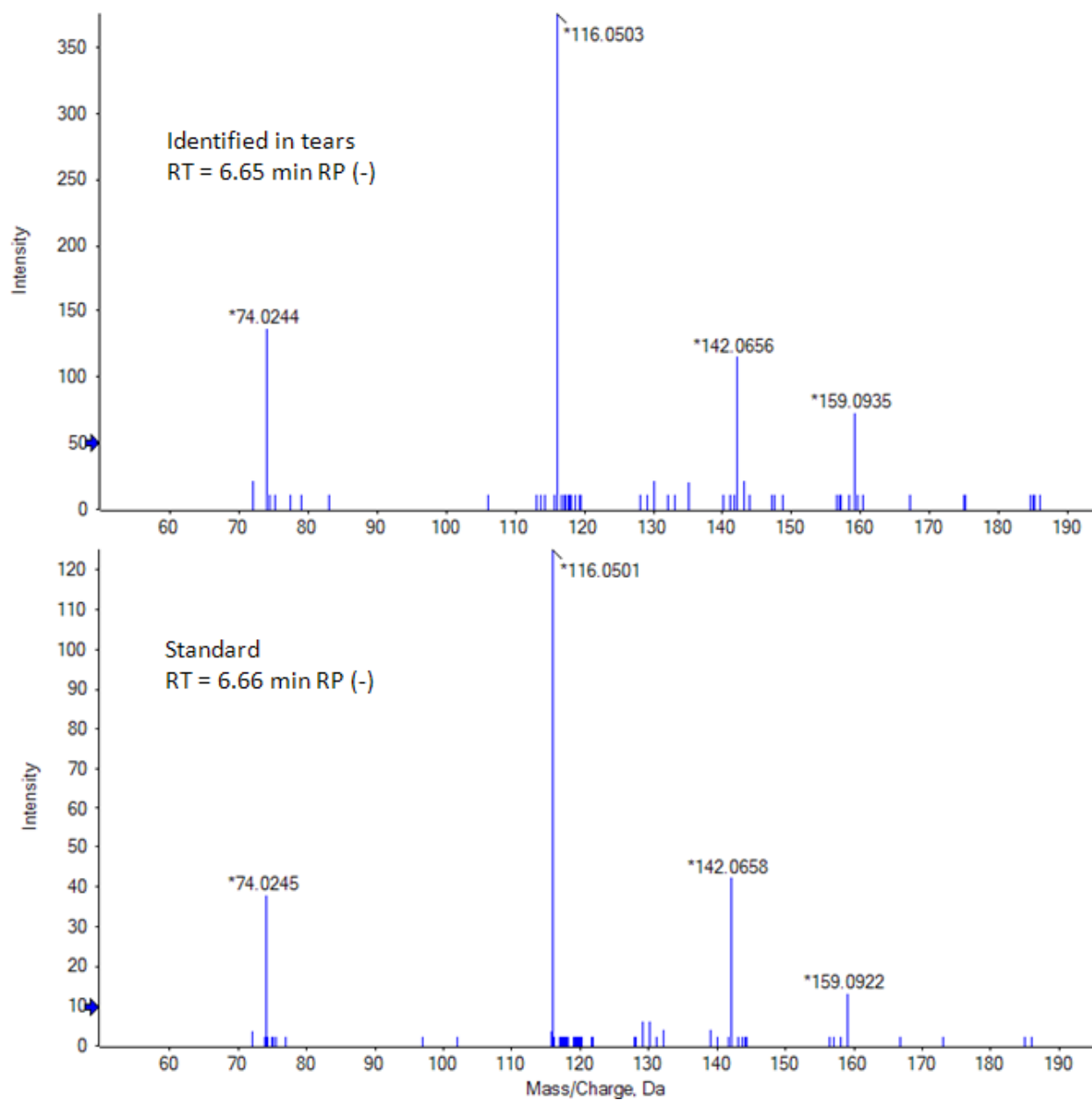


Figure S-1b. MS/MS spectra of tryptophan identified in tears (top), verified against metabolite standard (bottom)

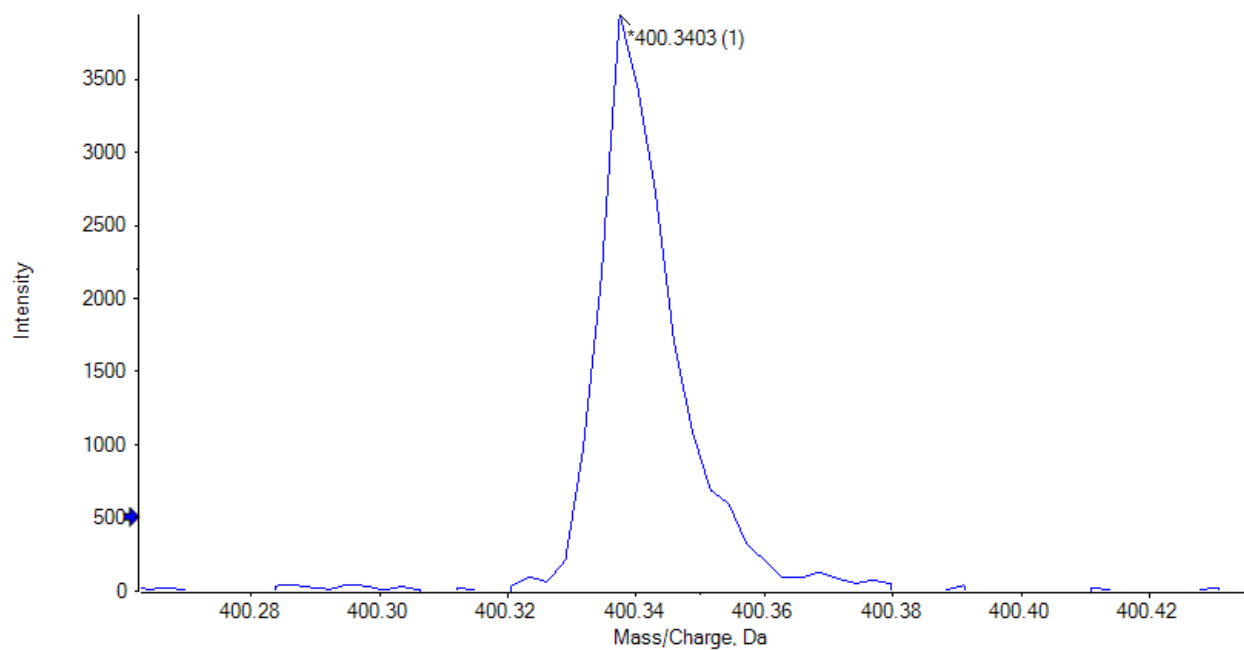


Figure S-2. TOF MS spectra of palmitoylcarnitine, $m/z = 400.3403$. Peak resolution = 36, 463

The metabolites verified by analyses of pure standards are: ADP, allantoin, arginine, carnitine, citric acid, glutamine, panthenol, tyrosine, tryptophan, uric acid and xanthine.

A retrospective evaluation of the workflow was performed using the subset of data from RP (+) run where metabolites of MW of 100 to 300 were searched for using XIC manager (Table S-1). After removing entries with no MS/MS spectra and duplicate m/z values, 36 unique extraction masses remained. Out of these, 20 metabolites were identified, and 3 entries failed to match with database spectra. The rest were found to be adducts or product ions ($n = 5$) and 8 remained as putative identifications with no mass spectra available for comparison. Thus, the false discovery rate of our workflow was less than 10% (3 out of 36). Using the same data subset, some issues encountered in the identification of metabolites and their resolutions are listed in Table S-2.

In the first example, a peak with m/z 282.0833 was detected at 3.76 min; with guanosine or 8-hydroxy-deoxyguanosine being possible identifications. By comparing the experimentally-derived MS/MS spectra to entries in Metlin, the peak was identified to be guanosine.

The next example illustrates in-source fragmentation: Inosine and hypoxanthine were both putatively identified at 3.79 min, with MS/MS spectra matching database entries. Inosine has been reported to fragment at source to form hypoxanthine. Actual hypoxanthine should elute at an earlier RT and extraction of m/z 137.0458 from the chromatogram revealed another peak at 1.80 min. MS/MS spectra confirmed its identity as hypoxanthine.

In the last example, N-succinyl-2-amino-6-ketopimelate (m/z 290.0871) was putatively identified at 3.29 min but a query of the compound on HMDB and KEGG indicated that it is not involved in primary metabolism in humans. As it displayed identical retention with adenosine and a difference of 22.9900 Da from the monoisotopic mass, this peak was identified as the sodium adduct of adenosine.

Table S-2. Evaluation of Workflow[#]

Starting entries	109
Entries with no MS/MS spectra	13
Duplicate m/z	59
Putative identifications	36
Identified metabolites	20
No match	3
Adducts and fragments	5
No spectra in databases	8
[#] based on data subset of MW 100 to 300 search in RP (+)	

Table S-3. Issues faced in metabolite identification and their resolution

Issue	Metabolite Name	Formula	H ⁺ adduct	RT	Assignment
Same formula and MW	Guanosine	C ₁₀ H ₁₃ N ₅ O ₅	282.0833	3.76	Guanosine
	8-Hydroxy-deoxyguanosine	C ₁₀ H ₁₃ N ₅ O ₅	282.0833	3.76	
In-source fragmentation	Hypoxanthine	C ₅ H ₄ N ₄ O	137.0458	1.80	Inosine fragments at source, giving a false contribution.
	Inosine	C ₁₀ H ₁₂ N ₄ O ₅	269.0881	3.79	Hypoxanthine is identified at 1.80 min
Adduct formation	Adenosine	C ₁₀ H ₁₃ N ₅ O ₄	268.1040	3.29	m/z 290.0856 is the sodium adduct of Adenosine.
	N-Succinyl-2-amino-6-ketopimelate	C ₁₁ H ₁₅ NO ₈	290.0871	3.29	