Untargeted Metabolic Profiling of Oral Cancer Cells Using Capillary Ion Chromatography Coupled with an Orbitrap Mass Spectrometer

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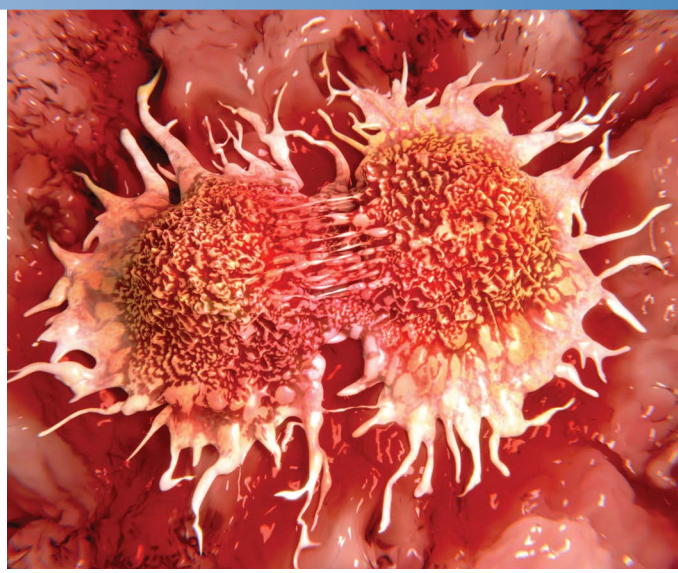
Application Note 1137

Key Words
Dionex ICS-4000 System, Q Exactive, Mass Spectrometry (MS), Metabolomics, SIEVE, Dionex IonPac AS11HC-4 µm Column

Goal
Demonstrate the resolution and sensitivity provided by capillary ion chromatography (IC) coupled with high-resolution, accurate-mass (HRAM) Orbitrap mass-analyzer-based detection when applied to untargeted profiling of anionic polar metabolites in oral cancer cells.



Introduction
Untargeted metabolomics profiling attempts to identify, quantify, and pathway-map the metabolites present in a biological system. Because metabolites are the end products of processes occurring in cells, tissues, and organs, metabolomics provides a snapshot of a biological system not possible with proteomics or genomics.

Despite their usefulness, metabolomics studies present multiple analytical challenges. In particular, the samples are very complex, containing a diverse set of compounds over a wide concentration range. Further, many metabolites are small, polar molecules that are difficult to separate by traditional techniques such as reversed-phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) (Figure 1). Due to its nonpolar stationary phase, RPLC yields poor selectivity and poor reproducibility of ionic or very polar compounds. With multimodal separation capability, HILIC offers an improvement,[1,2] but suffers from poor retention time (RT) precision when analyzing water-based samples. RT precision is important for metabolite identification and confirmation.

Capillary electrophoresis (CE) is another alternative technique, but CE is less widely used because of its lower sensitivity, capacity, and RT precision compared to HILIC.[3,4] Gas chromatography (GC) is also used, but requires derivatization prior to detection because many important metabolites, such as those produced in cellular energy metabolism, are non-volatile. Additionally, derivatization reagents are less desirable to use because of their cost and toxicity.

IC has emerged as a superior technique for the determination of charged, polar compounds that are difficult to separate using other methods. Often associated with determinations of small inorganic ions, IC is widely used to identify compounds at trace levels in complex matrices in environmental, pharmaceutical, and food and beverage applications.

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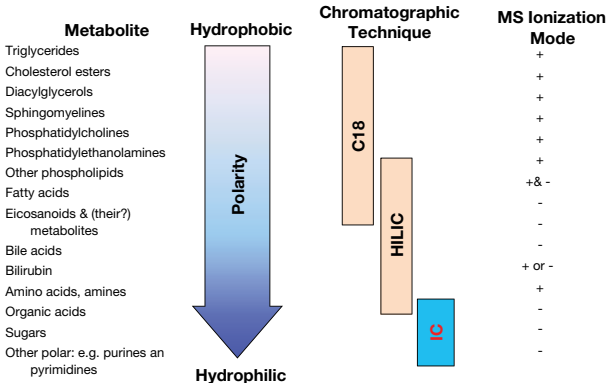


Figure 1. The diverse physical properties of metabolites require a range of separation techniques. IC is able to separate very polar metabolites. Most metabolites can be ionized and detected using MS.

MS and MS/MS are the preferred metabolite detection techniques of ionized compounds due to their ability to detect and identify a wide range of underivatized, thermally labile metabolites.[5,6] Because HILIC uses solvents compatible with MS, it is increasingly used to study complex aqueous metabolomes. However, HILIC methods require buffers to ionize the compounds but these buffers produce high background levels that interfere with MS measurement and reduce MS sensitivity.

IC provides superior selectivity resulting in superior separation capability and additionally delivers analytes in their ionized form. However, the typical IC eluent (potassium hydroxide solution) is highly corrosive and conductive and thus unsuitable for direct injection into a mass spectrometer. To allow compatibility with MS, eluent suppression technology provides online desalting and conversion of strong base and strong acid eluents into pure water. While IC coupled to MS has been applied to targeted screening and quantification of metabolites such as carbohydrates, organic acids, sugar phosphates, and nucleotides in biological samples,[7,8] its application to untargeted metabolite profiling, particularly at capillary flow rates (5–30 µL/min), is less well reported.

This application note examines application of the Thermo Scientific™ Dionex™ ICS-4000 Capillary High-Pressure™ Ion Chromatography HPIC™ System coupled with the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer to untargeted metabolic profiling of oral squamous cell carcinoma (OSCC) metastasis in cell lysates. The metabolite profiles of oral cancer stem-like cells (CSCs) are compared with non-stem cancer cells (NSCCs). Three separation methods (capillary IC, HILIC, and RP ultra-high-pressure LC (RP-UHPLC)) are evaluated for their resolution, impact on limit of detection, and overlap of detected polar anionic metabolites. Inter-day reproducibility results are also shown for IC. Complementary research, *"Metabolomic Profiling of Anionic Metabolites in Head and Neck Cancer Cells by Capillary Ion Chromatography with Orbitrap Mass Spectrometry,"*[9] provides supporting information and additional evaluation of capillary HILIC and capillary RPLC techniques.

Oral cancer (OSCC) is a type of head and neck cancer (head and neck squamous cell carcinoma (HNSCC)). Because OSCC is often discovered late in its development and has a high risk of producing tumors, the death rate is high.[10] In 2013, OSCC resulted in 135,000 deaths worldwide up from 84,000 deaths in 1990.[11] Five-year survival rates in the United States are 63%[11] and have not significantly improved over time.[10]

Mass spectrometry has been previously applied to metabolite profiling of tissue and body fluid samples from HNSCC patients.[13,14] These studies have led to the discovery of biomarkers potentially applicable to disease detection and treatment monitoring. In these studies, LC-HRAM profiling of saliva samples from patients with oral cancer or oral pre-cancer was found to have value in distinguishing oral cancer from the healthy control or precancerous conditions.

Experimental

Standard Solutions for Calibration and Method Evaluation

One-ppm (1 µg/mL) stock solutions of 42 polar metabolite standards (Sigma-Aldrich®, St. Louis, MO) were prepared in water and stored at –20 °C. A combined standard was prepared from the stock solutions and diluted in series to the desired calibration concentrations.

The Thermo Scientific™ Pierce™ Amino Acid Standard H (P/N 20088) mixture of 18 amino acids, at 2.5 µmol/mL each in 0.1 M HCl, was used to evaluate correct implementation of the separation methods.

The standard mixtures were diluted with water or water/organic solvent as required by the evaluated chromatography methods.

Sample Preparation and Extraction

Cells cultured as described in the literature references[9,15] were sourced from the School of Dentistry and Jonsson Comprehensive Cancer Center, University of California, Los Angeles. Three OSCC cell lines, UMSCC1, UMSCC5, and cancer stem-like cells (CSC), and corresponding wild-type controls with biological replicates were harvested and counted. Cellular metabolites were extracted using a liquid nitrogen snap-freezing method with methanol/water according to Lorenz et al.[16] The extracts were transferred to micro centrifuge tubes and pelleted at 4 °C for 3 min at 16,100x g. Supernatants were transferred to new micro centrifuge tubes for IC-, HILIC-, and RP-UHPLC- HRAM analysis. All experiments were performed in triplicate.

Chromatography

In this application note, three separation methods are compared using the same Q Exactive mass spectrometer system.

- Capillary IC running at 25 µL/min (plus 10 µL/min makeup flow)
- HILIC running at 250 µL/min
- RP-UHPLC running at 450 µL/min

Chromatographic parameters are provided in Table 1. The complementary research also included evaluation of capillary HILIC running at 25 µL/min and capillary LC running at 40 µL/min.[9] The results of those separations are not presented here because the separations were poorer and mass spectrometer responses ten-fold lower for the capillary flow methods compared to the RP-UHPLC and HILIC methods listed below.

Table 1. Chromatographic conditions

	Capillary IC	HILIC	RP-UHPLC
Instrument	Dionex ICS-4000 Capillary HPIC system with eluent generation	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC HPG system	Dionex UltiMate 3000 RSLC HPG system
Columns	Thermo Scientific™ Dionex™ IonPac™ AS11-HC-4 µm column, 0.4 × 250 mm, (P/N 078031)	SeQuant®- ZIC®-HILIC column, 2.1 × 150 mm, 5 µm	Thermo Scientific™ Hypersil GOLD™ C8 column, 2.1 × 150 mm, 1.9 µm (P/N 25202-152130)
Eluent / Mobile phase	KOH gradient delivered by Thermo Scientific™ Dionex™ EGC-KOH capillary cartridge	A: 20 mM ammonium acetate, pH 9.9 B: acetonitrile	A: 1 mM acetic acid in water B: 1 mM acetic acid in methanol
Gradient	2 mM KOH (-5–0 min), 2–12 mM (0–13.5 min), 12–20 mM (13.5–22.5 min), 20–70 mM (22.5–31.5 min), 70 nM (31.5–37.5 min), 70–2 mM (37.5–37.6 min), 2 mM (37.6–45 min to equilibrate)	90–30% B (0–15 min), 30% B (15–18 min), 30–80% B (18–19 min), 90% B, (19–27 min to equilibrate)	0.5–50% B (0–5.5 min), 50–98% B (5.5–6 min), 98% B (6–12 min), 98–0.5% B (12–13 min), 0.5% B (13–15 min to equilibrate)
Flow rate	25 µL/min supplemented post-column with 10 µL/min make-up flow of methanol or 2 mM HOAc in methanol	250 µL/min	450 µL/min
Run time	45 min	27 min	15 min
Injection volume	2 µL	5 µL	5 µL
Column temperature	35 °C	25 °C	55 °C

Capillary IC

Capillary IC analyses were performed using a Dionex ICS-4000 Capillary HPIC System consisting of a capillary pump, an eluent generator KOH cartridge (EGC KOH), and a Thermo Scientific™ Dionex™ IC Cube™ module with a Thermo Scientific™ Dionex™ ACES™ 300 Anion Capillary Electrolytic Suppressor and conductivity detector (CD) (Figure 2). Suppression technology enables coupling of IC to MS by converting the potassium hydroxide gradient to pure water (Figure 3). A shallow gradient for the capillary IC method was selected to maximize resolution of the numerous unknown components. A low void volume mixing tee (Idex, P/N P-890) was used to minimize peak spreading between the capillary IC and the Q Exactive MS. Faster IC gradients can be achieved as demonstrated using the 2 mm i.d. format column in AN 622.[17,18]

As shown in Figure 2, the suppressor was operated in external-water mode with ultrapure water (EMD Millipore®, Billerica, MA). Ultrapure water used as suppressor regenerant was delivered by an external Thermo Scientific Dionex AXP auxiliary pump at a flow rate of 40 µL/min. After being desalted by the suppressor and passing though the CD detector, the column effluent was directed to a zero-volume mixing tee where the eluent stream was combined with methanol containing 2 mM acetic acid to aid electrospray desolvation. The acidic methanol solution was delivered to the mixing tee at 10 µL/min via an external Dionex AXP-MS pump. The combined eluent-solvent passed through a grounding union before entering the mass spectrometer (Figure 2). Later experiments found that the acetic acid was not needed in the methanol makeup solution.

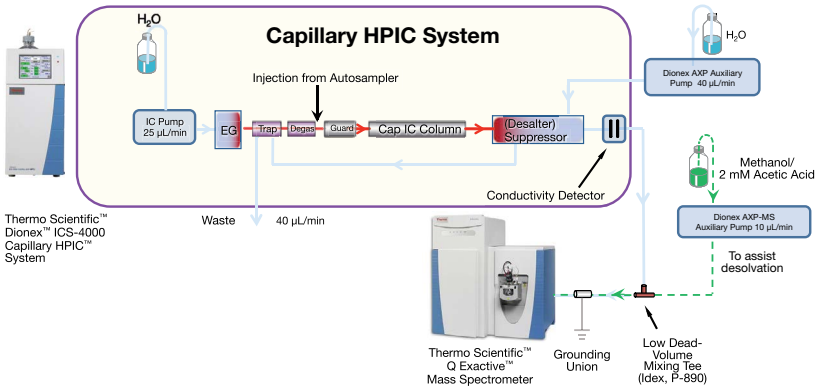


Figure 2. Diagram of the Dionex ICS-4000 capillary HPIC system with the Q Exactive mass spectrometer.

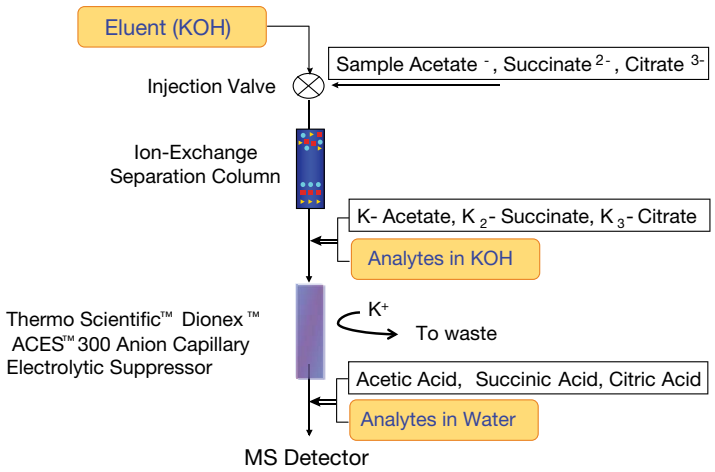


Figure 3. Suppression technology converts highly caustic mobile phase to pure water and the potassium salt of the analyte to its acid form. Continuous online desalting of both the eluent and analytes makes it possible to connect the Dionex ICS-4000 capillary HPIC system to a mass spectrometer without concern for high salt concentration. The technology also reduces chemical noise and increases method sensitivity.

Mass Spectrometry

A Q Exactive hybrid quadrupole-Orbitrap mass spectrometer operated in negative ion electrospray ionization (ESI) mode was used for metabolite detection. The Q Exactive mass spectrometer produces HRAM data, which reduce isobaric misidentifications and increase confidence in compound identifications. Mass spectrometer ion source settings were optimized with respect to the method flow rate (Table 2). Full scan and data-dependent MS/MS parameters are provided in Table 3.

Table 2. Mass spectrometer ion source parameters.

	Capillary Flow IC	Higher-Flow HILIC and RP-UHPLC
Ionization mode	Negative	Negative
Spray voltage	-2.8 kV	-3.2 kV
Transfer capillary temperature	325 °C	325 °C
S-lens voltage	50 V	50 V
Heater temperature	125 °C	350 °C
Sheath gas flow	26 arbitrary units N_2	45 arbitrary units N_2
Aux gas flow	2 arbitrary units N_2	8 arbitrary units N_2

Table 3. Full scan and data-dependent MS/MS parameters.

Full scan mode	
Mass range	67-1000 *m/z*
Automatic gain control (AGC) target	1 x 10^6 ions
Resolution	70,000
Maximum ion injection time (IT)	50 ms
Data-dependent MS/MS mode	
MS/MS resolution	17,500
AGC target	1 x 10^5 ions
Maximum IT	100
Isolation window	1.5 amu
Normalized collision energy	35 %
Stepped collision energy	± 50 %
Under fill ratio	1.0 %
Apex trigger	(Peak width/2 - peak width/4) to (peak width/2 + peak width/4)

Data Analysis

Background subtraction, component detection, peak alignment, and differential analyses comparing the disease state to the controls were performed using Thermo Scientific™ SIEVE™ Software for Differential Analysis version 2.1. The ChemSpider™ chemical structure database, Kyoto Encyclopedia of Genes and Genomes (KEGG®), and METLIN® Metabolite and Tandem MS databases were used to produce statistical results, putative metabolite identifications, and metabolite pathways. Metabolites of interest were searched in METLIN using the observed *m/z* with a mass error constraint of 3 ppm. Raw data were converted to mzXML™ open data format using ProteoWizard™ and analyzed by XCMS® Online and metaXCMS for second-order analysis of untargeted metabolomics data.

Results and Discussion

Validation of Separation Methods

To validate correct implementation of the RP-UHPLC and HILIC methods, a 1000-fold diluted Thermo Scientific™ Pierce™ Amino Acid Standard H mixture (5 picomoles injected) was analyzed. The results showed near baseline resolution and intensity, reflecting the expected performance of RP-UHPLC and HILIC.

Analysis of Metabolite Standards

During the preliminary evaluations of the 42 metabolite standards, exceptional results for the TCA and glycolysis cycle metabolites were achieved using capillary IC with HRAM Orbitrap mass spectrometer-based detection. As these metabolites are traditionally difficult to analyze, the remainder of the experiments were focused on the 21 polar metabolites (Table 4). The separation and responses obtained by IC-HRAM analysis of the 21 polar metabolites at 600 ppb and 60 ppt, and by HILIC-HRAM analysis at 600 ppb are shown in Figure 4. The results of the RP-UHPLC-HRAM analysis of the polar metabolites were generally poor and are therefore not shown.

Table 4. LODs of representative metabolite standards for capillary IC-HRAM method.

Peak #	Metabolite Name	Formula	M-H	On column (fmol)	LOD * (nM)
1	D-Glucose	$C_6H_{12}O_6$	179.0561	0.17	0.3
2	Mevalonate	$C_6H_{12}O_4$	147.0663	2.0	0.1
3	Lactate	$C_3H_6O_3$	89.0244	3.4	0.1
4	Uridine	$C_9H_{12}N_2O_6$	243.0623	1.2	0.25
5	α-D-Glucose 1-phosphate	$C_6H_{13}O_9P$	259.0224	1.2	0.2
6	α-D-Glucose-6-phosphate	$C_6H_{13}O_9P$	259.0224	1.2	0.2
7	D-Fructose 6-phosphate	$C_6H_{13}O_9P$	259.0224	1.2	0.2
8	Adenosine 3'-5'-cyclic monophosphate (cAMP)	$C_{10}H_{12}N_5O_6P$	328.0452	0.91	0.2
9	Tartrate	$C_4H_6O_6$	149.0092	2.0	0.5
10	2-Oxoglutarate	$C_5H_6O_5$	145.0142	2.1	0.2
11	Adenosine 5'-monophosphate (AMP)	$C_{10}H_{14}N_5O_7P$	346.0558	0.87	0.1
12	2-phosphoglycerate	$C_3H_7O_7P$	184.9857	1.6	0.3
13	Citrate	$C_6H_8O_7$	191.0197	1.6	0.2
14	Isocitrate	$C_6H_8O_7$	191.0197	1.6	0.05
15	*cis*-Aconitate	$C_6H_6O_6$	173.0092	1.7	0.2
16	*trans*-Aconitate	$C_6H_6O_6$	173.0092	1.7	0.2
17	Phosphoenolpyruvate	$C_3H_5O_6P$	166.9751	1.8	0.2
18	D-Fructose-1,6-diphosphate	$C_6H_{14}O_{12}P_2$	338.9888	0.88	0.1
19	D-Fructose-2,6-diphosphate	$C_6H_{14}O_{12}P_2$	338.9888	0.88	0.1
20	Dihydroxy acetone-phosphate	$C_3H_7O_6P$	168.9908	1.8	0.04
21	Inosine 5'-monophosphate	$C_{10}H_{13}N_4O_8P$	347.0398	0.87	0.1

* S/N = 3

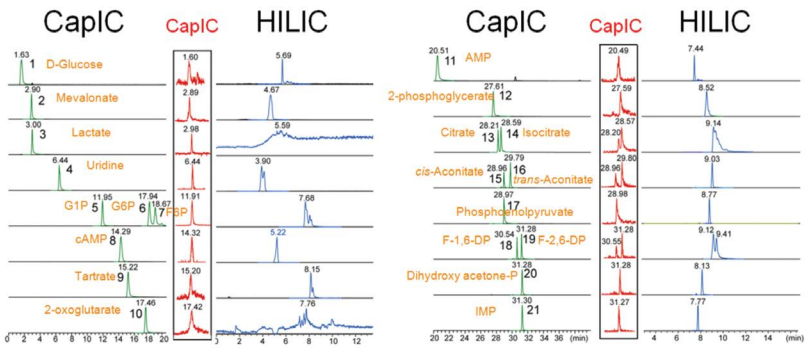


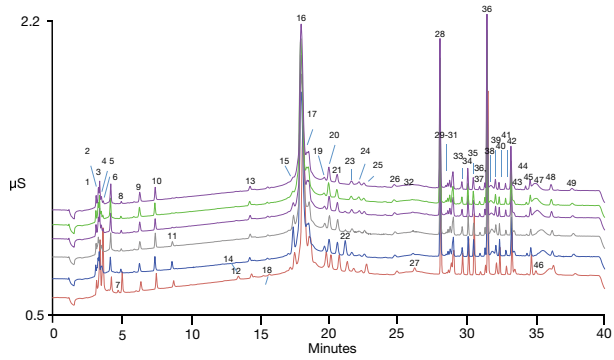
Figure 4. Separation and detection of 21 polar metabolites by capillary IC-HRAM analysis at (left) 600 ppb (left) and 60 ppt (middle), and by HILIC-HRAM analysis at 600 ppb (left).[9]

Compared to the other methods evaluated, the Dionex ICS-4000 Capillary HPIC System coupled with the Q Exactive mass spectrometer provided superior separation and detection sensitivity for the polar metabolite standards. Metabolites at 600 ppb (0.5–2.5 picomole on column) were detected with signal-to-noise ratio (S/N) of approximately 1000. Twenty-one metabolites were detected at 60 ppt (0.2–3.4 femtomole on column) with a S/N of 3 to 20. Shown in Table 4, limits of detection (LOD) for these metabolites ranged from 0.05 to 0.5 nmol/L. LOD concentrations were lower than those commonly reported for RP LC-HRAM methods. The mass LODs (0.2 to 3.4 femtomole) obtained are 1000-fold lower than those reported for metabolites when using CE-MS.[19]

Isomeric compounds including α-D-glucose-1-phosphate, α-D-glucose-6-phosphate, and D-fructose-6-phosphate (peaks 5, 6 and 7); citrate and isocitrate (peaks 13 and 14); *trans*- and *cis*-aconitate (peaks 15 and 16); and fructose-1,6-phosphate and fructose-2,6-phosphate (peaks 18 and 19) were baseline resolved (Figure 4). Despite lowering the concentrations 10,000-fold, resolution did not suffer. The Thermo Scientific™ Reagent-Free™ Ion Chromatography (RFIC™) system using electrolytically generated eluent provided precise and accurate eluent delivery online as demonstrated by the high RT reproducibility with RT shifts <0.04 min. In contrast, the RT reproducibilities (n=8) were lower using HILIC and RPLC, 0.1–0.5% for intraday and as high as ~1–2% interday for HILIC separations.

HILIC showed good separation for most of the metabolites analyzed (Figure 4). However, compared to capillary IC, certain isomers such as sugar phosphates and *cis*- and *trans*-aconitate were poorly resolved. The analytical sensitivity of capillary IC-MS-MS was generally 10–100 times better than the HILIC-MS-MS method.

To evaluate the inter-day reproducibility of capillary IC, analysis of the 42 metabolites was repeated six times over six days (Figure 5). The RSDs of intensity and RT were within 8%, an acceptable range for relative quantitation in metabolomics studies.



1. Lactate
2. Shikimate
3. Acetoacetate
4. Acetate
5. Formate
6. Pyruvate
7. Chloride
8. Uridine
9. Unknown
10. Chloride
11. Nitrite
12. Glucose 1-phosphate
13. cAMP
14. Methylmalonate
15. Succinate
16. Carbonate
17. Tartrate / Malonate
18. Fumarate
19. 2-Oxoglutarate
20. Fructose 6-phosphate
21. Maleate
22. D-Glucose 6-phosphate
23. Ribose 5-phosphate
24. Adenosine 5'-monophosphate
25. Unknown
26. Sulfate
27. 2-phosphoglycerate
28. Phosphate
29. Unknown
30. Citrate
31. Isocitrate
32. *cis*-Aconitate and Phosphoenolpyruvate
33. trans- Aconitate
34. Ribulose 5-phosphate
35. D-Fructose 1,6 diphosphate
36. D-Fructose 2,6 diphosphate
37. IMP/ Dihydroxyacetone phosphate/ CTP/Adenosine 5'-diphosphate
38-40. Unknown
41. PRPP
42-43. ATP/ dGTP/GTP
44-45. Unknown
46. GDP
47-49. Unknown

Figure 5. Reproducibility of capillary IC (with conductivity detection) analyses of 5 ppb metabolite standards over six days. RSDs of intensity were 5.5%, 7.8%, and 6.0%. RSDs of RT were 6.5%, 8%, and 7.2 %, respectively, for three inorganic ions chloride, carbonate and phosphate.

Metabolic Profiling of Cell Lysates

Prior to analyzing the cell lysate samples, a solvent blank was analyzed in full scan mode to obtain blank files for background subtraction using SIEVE software and to produce a mass (*m/z*) exclusion list for data-dependent MS/MS experiments. A pooled sample was used during method development and served as the quality control sample. Sample injections were randomized to eliminate systematic bias. To monitor system reliability with respect to intensity and RT, the samples were spiked with 0.5 µmol/L deuterated hippuric acid (hippuric acid-d5), and the pooled sample was injected every five runs.

The *m/z* list created in the analysis of the metabolites standards was used to extract the peaks obtained from the capillary IC-HRAM, HILIC-HRAM, and RP UHPLC-HRAM analyses of the UMSCC1 samples.

As shown in Figure 6, the capillary IC-HRAM, HILIC-HRAM, and RP UHPLC-HRAM analyses detected 65, 38, and 29 components, respectively. A total of 26 peaks were detected by all three methods. Only one compound, acetyl-CoA, was detected exclusively by HILIC-MS-MS. The authors conjecture that the acetyl-CoA molecules may require higher eluent concentration than currently possible by the eluent generator cartridge or that CoA molecules may not be sufficiently ionized for efficient acidification by the suppressor. (The raw data is shown in Appendix A.)

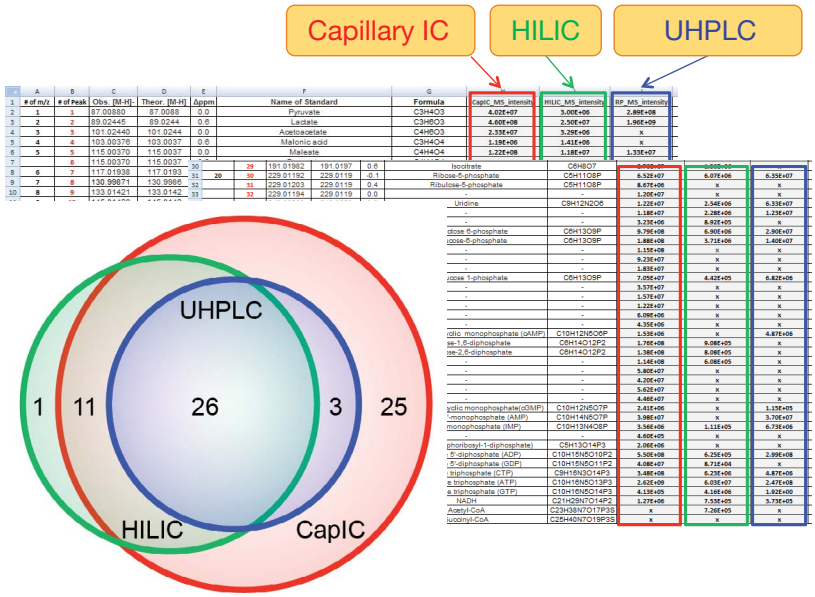


Figure 6. Overlap of components found in UMSCC1 cell samples by separation method.

Figure 7 shows the separation of eleven mono-phosphate sugar isomers corresponding to *m/z* 259.0224 by the capillary IC-HRAM, HILIC-HRAM and RP UHPLC-HRAM methods. A METLIN search of the capillary IC-HRAM data using a mass accuracy constraint of 3 ppm for the adduct ions of [M – H]– and [M + Cl – H]$^-$ returned 33 hits, all metabolites sharing the formula in the format [M – H]$^-$ $C_6H_{13}O_9P$. Hits included the major and positional isomers of monophosphate conjugating with various sugars such as glucose, fructose, galactose, mannose, and *myo*-inositol. Overall, the capillary IC-HRAM method detected the largest number of sugar phosphate variants. HILIC resolved only three peaks, hence many isomeric species were either missing or coeluted. RP-UHPLC-HRAM and HILIC-HRAM had significant signal loss, 15% and 67% respectively, as compared to the highest peak response by IC-HRAM.

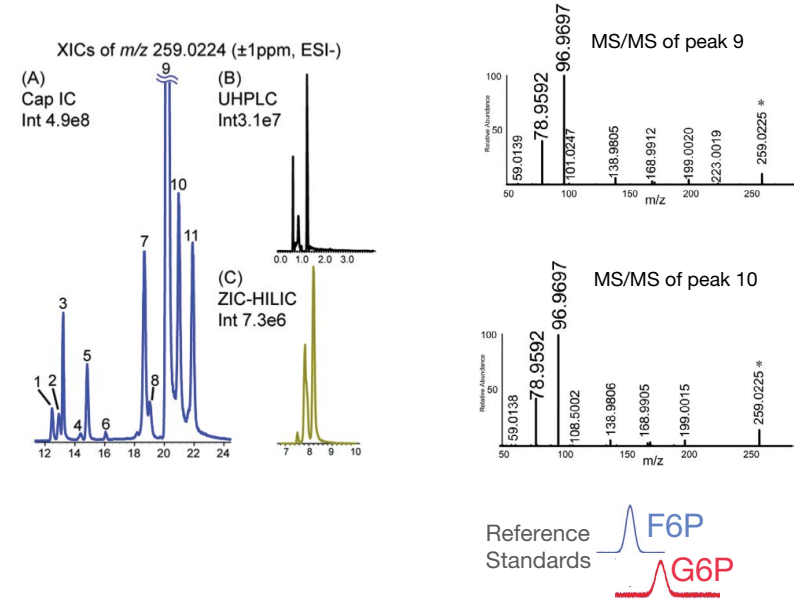


Figure 7. Separation of eleven mono-phosphate sugar isomers corresponding to m/z 259.0224 by capillary IC, UHPLC and HILIC methods (left) with MS/MS spectra of peaks 9 and 10 (right). The MS/MS spectra of the isomers represented by peaks 9 and 10 are nearly identical; hence IC is needed to separate them.

Metabolite identification is a key step in biological interpretation, but identification presents two major challenges. First, there is no comprehensive spectral library available containing the MS/MS spectra needed for searching and identification. Of Human Metabolome Database™ (HMDB), MassBank, NIST®, and METLIN, only METLIN provided reasonable coverage for the sugar monophosphates. Ten sugar monophosphate MS/MS spectra collected in both positive and negative ion ESI modes were in the METLIN database. The major MS-MS fragments acquired and their relative abundance matched the library spectra despite the different separation modes and instrumentation used. The data generated here were acquired using higher energy collisional dissociation (HCD) within an Orbitrap mass analyzer. In contrast the METLIN database entries were acquired using collision-induced dissociation (CID) within a Q-TOF mass analyzer.

Metabolite Identification is also challenging when many isomeric species are present in a single sample, as exemplified by the sugar monophosphates detected here: D-glucose 6-phosphate, D-mannose 6-phosphate, α-D-galactose 1-phosphate, and α-D-glucose 1-phosphate. To help identify the eleven sugar monophosphate peaks shown in Figure 7, high-resolution MS/MS spectra at *m/z* 259.0224 were collected from the pooled samples. Shown on the lower right of Figure 7, the MS/MS spectra of the isomers represented by peaks 9 and 10 are nearly identical. To address this challenge, the RTs of the peaks identified in the cell samples were compared to the RTs of the standards determined using capillary IC. Using a combination of RT matches and MS/MS spectral comparisons of standards and samples, five monophosphate sugar isomers were identified. Capillary IC provided the necessary chromatographic resolution to make confident assignments based on RT. Other metabolites were tentatively identified using MS/MS spectral pattern matching with the METLIN database.

Differential Analysis, Pathway Mapping, and Meta-analysis

SIEVE software version 2.1 was used to perform a pairwise comparison of UMSCC1 to UMSSC1 cells in which SOX11 was knocked down (SOX11-KD). SOX11 is a transcription factor believed to provide an important role in stem cell development and cancer cell progression. Using its component extraction algorithm that combines multiple ions, including monoisotopic peaks, isotopes, adducts, and neutral losses, SIEVE software detected 1160 components (features) in the data produced by the IC-HRAM method. To refine the target list to focus on the most altered pathways, it was filtered to 270 components using a threshold *p*-value <0.05 and ratio >2.

The masses of the 270 components were searched using KEGG, resulting in displays of the matching metabolic pathways. In the UMSCC1 versus the UMSCC1-KD data set, SIEVE software found 71 pathways containing between two and 36 metabolites. Each pathway included the component identification (#), component name, and related information such as maximum charge to facilitate data interpretation. In the matching pathways, glycolysis and tricarboxylic acid (TCA) were altered by the siRNA-mediated knockdown of SOX11. The complementary research paper[9] presents the glycolysis and tricarboxylic acid (TCA) pathways and the peak area differences between CSC and NSCCS samples. The capillary IC-HRAM method achieved nearly complete coverage of glycolysis with the accuracy (RSD < 8%) needed to monitor biologically relevant changes.

Because the metabolites were in common across all of the sample sets (CSC/NSCC, UMSSC1/UMSSC1-KD, UMSSC5/UMSSC5-KD) and of interest, a second order meta-analysis was performed to evaluate their differences. First, the three datasets were individually reanalyzed using XCMS Online, which detected 11377, 13302, and 10532 aligned features for UMSSC1, UMSCC5 and CSC sample groups, respectively. Then these results were combined and analyzed by metaXCMS. A total of 218 components were identified as being common to all three sample sets.

Conclusion

Three methods were evaluated for application to untargeted metabolite profiling of oral cancer cells. The Dionex ICS-4000 capillary HPIC system coupled with the Q Exactive mass spectrometer provided better separation of anionic polar metabolites with better detection sensitivity than the RP-UHPLC-HRAM and HILIC-HRAM methods. Sensitivity of the capillary IC-HRAM method was generally 10–100 times better than the HILIC-HRAM method. The results for anionic polar metabolites using the RP-UHPLC-HRAM method were generally poor as expected. The inter-day reproducibility of capillary IC was demonstrated to be acceptable for metabolomics studies.

The outstanding resolution of capillary IC enabled separations of isomeric polar metabolites and isobaric metabolites with identical MS/MS spectra and identification based on RT matches with standard compounds. When applied to metabolic profiling of cell lysate samples using SIEVE software, capillary IC-MS detected significantly more peaks than HILIC-MS and RP-UHPLC-MS. In particular, capillary IC-MS detected a larger number of sugar phosphate variants. However, one compound, acetyl-CoA, was detected only by HILIC-MS. Enhanced separation and detection of polar anionic metabolites establish capillary IC-HRAM analysis as a technique which complements HILIC-HRAM and RP-UHPLC-HRAM analyses for metabolomics applications.

Differential analysis of capillary IC-MS data obtained from three OSCC cell lines, UMSCC1, UMSCC5, cancer stem-like cells (CSC), along with pathway mapping and meta-analysis revealed significant changes in both glycolysis and TCA energy metabolism pathways. Pathway analysis indicated that the sugar phosphates in oral CSCs and NSCCs had more significant changes than other intermediates in the glycolysis pathway.

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Appendix A: Compounds from Figure 6

# of m/z	# of Peak	Obs. [M-H]-	Theor. [M-H]	Δppm	Name of Standard	Formula	CapIC_MS Intensity	HILIC_MS Intensity	RP_MS Intensity
1	**1**	87.00880	87.0088	0.0	Pyruvate	C3H4O3	4.02E+07	3.00E+06	2.89E+08
2	**2**	89.02445	89.0244	0.6	Lactate	C3H6O3	4.60E+08	2.50E+07	1.96E+09
3	**3**	101.02440	101.0244	0.0	Acetoacetate	C4H6O3	2.33E+07	3.29E+06	x
4	**4**	103.00376	103.0037	0.6	Malonic acid	C3H4O4	1.19E+06	1.41E+06	x
5	**5**	115.00370	115.0037	0.0	Maleate	C4H4O4	1.22E+08	1.18E+07	1.33E+07
	6	115.00370	115.0037	0.0	Fumarate	C4H4O4	4.80E+07	x	x
6	**7**	117.01938	117.0193	0.7	Succinic acid	C4H6O4	1.66E+08	8.05E+06	3.44E+08
7	**8**	130.99871	130.9986	0.8	Oxalacetate	C4H4O5	4.70E+05	1.90E+05	x
8	**9**	133.01421	133.0142	0.1	Malate	C4H6O5	1.19E+09	1.48E+08	1.31E+08
9	**10**	145.01426	145.0142	0.4	2-oxoglutarate	C5H6O5	2.01E+08	3.90E+07	9.26E+07
10	**11**	147.06626	147.0663	-0.3	Mevalonate	C6H12O4	1.05E+06	2.80E+06	8.47E+06
11	**12**	149.00913	149.0092	-0.5	Tartrate	C4H6O6	2.31E+06	9.15E+05	3.13E+06
12	**13**	149.04556	149.0455	0.4	D-arabinose	C5H10O5	1.28E+06	9.05E+05	2.31E+06
13	**14**	166.97514	166.9751	0.2	Phosphoenolpyruvate	C3H5O6P	1.56E+07	2.77E+06	3.87E+06
14	**15**	168.99072	168.9908	-0.5	Dihydroxy acetone-P	C3H7O6P	1.80E+08	7.22E+06	x
	16	168.99072	168.9908	-0.5	-	C3H7O6P	1.34E+08	x	x
	17	168.99070	168.9908	-0.6	-	-	2.34E+07	x	x
	18	168.99080	168.9908	0.0	-	-	7.90E+07	x	x
	19	168.99080	168.9908	0.0	-	-	1.80E+07	x	x
15	**20**	173.00910	173.0091	0.0	cis-Aconitate	C6H6O6	5.00E+07	3.45E+07	1.62E+07
	21	173.00919	173.0091	0.5	trans-Aconitate	C6H6O6	1.30E+07	x	x
	22	173.00919	173.0091	0.5	-	-	4.31E+06	x	x
16	**23**	173.04550	173.0455	0.0	Shikimate	C7H10O5	5.21E+05	x	x
17	**24**	179.05615	179.0562	0.0	α-D-Glucose	C6H12O6	1.08E+08	4.07E+06	2.60E+07
	25	179.05615	179.0562	0.0	-	C6H12O7	8.20E+06	1.20E+06	2.00E+07
18	**26**	184.98564	184.9857	-0.3	2-phosphoglycerate	C3H7O7P	1.96E+08	7.35E+05	x
	27	184.98573	184.9857	0.2		C3H7O7P	1.96E+08	x	x
19	**28**	191.01978	191.0197	0.3	Citrate	C6H8O7	2.71E+09	1.31E+08	1.53E+08
	29	191.01982	191.0197	0.6	Isocitrate	C6H8O7	8.70E+07	1.30E+06	x
20	**30**	229.01192	229.0119	-0.1	Ribose-5-phosphate	C5H11O8P	6.52E+07	6.07E+06	6.35E+07
	31	229.01203	229.0119	0.4	Ribulose-5-phosphate	C5H11O8P	8.67E+06	x	x
	32	229.01194	229.0119	0.0	-	-	1.20E+07	x	x
21	**33**	243.06238	243.0623	0.3	Uridine	C9H12N2O6	1.22E+07	2.54E+06	6.33E+07
	34	243.06243	243.0623	0.5	-	-	1.18E+07	2.28E+06	1.23E+07
	35	243.06235	243.0623	0.2	-	-	3.23E+06	8.92E+05	x
22	**36**	259.02250	259.0224	0.4	β-D-Fructose 6-phosphate	C6H13O9P	9.79E+08	6.90E+06	2.90E+07
	37	259.02250	259.0224	0.4	α-D-glucose-6-phosphate	C6H13O9P	1.88E+08	3.71E+06	1.40E+07
	38	259.02253	259.0224	0.5	-	-	1.15E+08	x	x
	39	259.02251	259.0224	0.4	-	-	9.23E+07	x	x
	40	259.02258	259.0224	0.7	-	-	1.83E+07	x	x
	41	259.02252	259.0224	0.5	α-D-Glucose 1-phosphate	C6H13O9P	7.05E+07	4.42E+05	6.82E+06
	42	259.02260	259.0224	0.8	-	-	3.57E+07	x	x
	43	259.02264	259.0224	0.9	-	-	1.57E+07	x	x
	44	259.02261	259.0224	0.8	-	-	1.22E+07	x	x
	45	259.02261	259.0224	0.8	-	-	6.09E+06	x	x
	46	259.02266	259.0224	1.0	-	-	4.35E+06	x	x

Appendix A: Compounds from Figure 6 *continued*

23	**47**	328.04520	328.0452	0.0	Adenosine 3'-5'-cyclic monophosphate (cAMP)	C10H12N5O6P	1.53E+06	x	4.87E+06
24	**48**	338.98895	338.9888	0.4	D-Fructose-1,6-diphosphate	C6H14O12P2	1.76E+08	9.08E+05	x
	49	338.98897	338.9888	0.5	D-Fructose-2,6-diphosphate	C6H14O12P2	1.38E+08	8.08E+05	x
	50	338.98897	338.9888	0.5	-	-	1.14E+08	6.08E+05	x
	51	338.98897	338.9888	0.5	-	-	5.80E+07	x	x
	52	338.98900	338.9888	0.6	-	-	4.20E+07	x	x
	53	338.98898	338.9888	0.5	-	-	5.62E+07	x	x
	54	338.98905	338.9888	0.7	-	-	4.46E+07	x	x
25	**55**	344.04049	344.0402	0.8	Guanosine 3',5'-cyclic monophosphate(cGMP)	C10H12N5O7P	2.41E+06	x	1.15E+05
26	**56**	346.05611	346.0558	0.9	Adenosine 5'-monophosphate (AMP)	C10H14N5O7P	3.98E+07	x	3.70E+07
27	**57**	347.04007	347.0398	0.8	Inosine 5'-monophosphate (IMP)	C10H13N4O8P	3.56E+06	1.11E+05	6.73E+06
	58	347.04004	347.0398	0.7	-	-	4.60E+05	x	x
28	**59**	388.94482	388.9445	0.8	PRPP (5-phosphoribosyl-1-diphosphate)	C5H13O14P3	2.06E+06	x	x
29	**60**	426.02240	426.0221	0.7	Adenosine 5'-diphosphate (ADP)	C10H15N5O10P2	5.50E+08	6.25E+05	2.99E+08
30	**61**	442.01727	442.0170	0.6	Guanosine 5'-diphosphate (GDP)	C10H15N5O11P2	4.08E+07	8.71E+04	x
31	**62**	481.97745	481.9772	0.5	Cytidine triphosphate (CTP)	C9H16N3O14P3	3.48E+08	6.23E+06	4.87E+06
32	**63**	505.98865	505.9887	-0.1	Adenosine triphosphate (ATP)	C10H16N5O13P3	2.62E+09	6.03E+07	2.47E+08
33	**64**	521.98328	521.9834	-0.2	Guanosine triphosphate (GTP)	C10H16N5O14P3	4.13E+05	4.16E+06	1.92E+00
34	**65**	664.11792	664.1175	0.6	NADH	C21H29N7O14P2	1.27E+06	7.53E+05	3.73E+05
35	**66**	-	808.1184	-	Acetyl-CoA	C23H38N7O17P3S	x	7.26E+05	x
36	**67**	-	866.1240	-	Succinyl-CoA	C25H40N7O19P3S	x	x	x
							65 Peaks	38 Peaks	29 Peaks

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