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Targeted metabolomics method using IC Orbitrap with high resolution mass spectrometry

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Goal

In this application note, the analysis method of ion chromatography/Fourier transform mass spectrometry (IC Thermo Scientific™ Q Exactive™ system) was developed to establish a versatile method of detecting polar metabolites (particularly anionic metabolites), the isolation and analysis of which are complicated despite their importance in vivo. A number of different water-soluble compound standards were used, and the retention time and MS/MS spectra for more than 350 types of compounds that can be analyzed with this system were acquired to construct a metabolite library. Additionally, using cultured animal cells and human serum samples, optimization of pretreatment for the IC Q Exactive system was studied.

Introduction

In the principal metabolic pathways which support life such as the glycolytic pathway, pentose phosphate pathway, citric acid cycle, and nucleic acid metabolism, most of the intermediate metabolites are highly polar anionic compounds. Therefore, accurate profiling and comparison of these compounds may greatly contribute to the understanding of living systems. Various methods have been used previously to measure anionic metabolites. CE-MS[1,2], ion-pair LC-MS[3] and HILIC LC-MS[4] are the main approaches, and each has advantages and disadvantages.

CE-MS has high separation performance, being capable of measuring a wide range of anionic metabolites. However, compared to the approach using LC, the measurement throughput time and reproducibility of electrophoresis are lower. For these reasons, CE-MS requires acquisition of high level skills for stable operation[5]. In addition, because the sample loading volumes should be 30 nL or less, ingenuity is needed for pretreatment.

On the other hand, ion-pair LC-MS has relatively higher sensitivity without requiring special techniques, enabling high throughput analysis. However, the effects of ion-pairing reagents on a mass spectrometer cannot be ignored, and it is essential to use a mass spectrometer specialized for ion-pairing reagents. HILIC LC-MS is an approach without ion-pairing reagents and so does not require a specialized machine, but, due to insufficient separation of many existing isomers, the range of measurable anionic metabolites becomes narrower. Due to the above, it is desirable to develop an analytical approach that has a resolving power capable of separating and detecting a wider range of anions, high sensitivity to detect trace amounts of metabolites that have physiological activities, a throughput capable of acquiring data from various biological samples, and is rugged.

In this application note, IC was used for the separation part. IC was performed with an anion-exchange column (Thermo Scientific™ Dionex™ IonPac AS11-HC-4µm) and non-volatile potassium hydroxide as the eluent for IC under a concentration gradient (10–100 mM). Generally, salt contamination is a concern when IC is connected directly to MS. However, using an electrolytically regenerated suppressor that removes nonvolatile salt to make the eluent water enables routine analysis with the IC-MS system. A high-resolution and accurate mass (HRAM) Q Exactive mass spectrometer was used as a detector in order to acquire a clear and highly accurate profile.

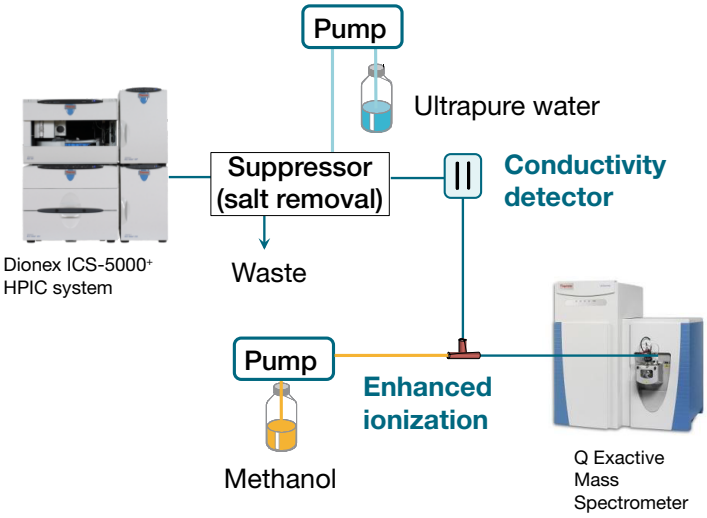


Figure 1. IC Q Exactive system

IC Q Exactive system measurement conditions

A) Ion Chromatography (IC) conditions	
Equipment	Thermo Scientific™ Dionex™ ICS-5000+ HPIC system
IC analytical column	Dionex IonPac AS11-HC-4µm, 2 × 250 mm (P/N 4078035)
IC guard column	Dionex IonPac AS11HC-9µm, 2 × 50 mm (P/N 078036)
Trap column	Thermo Scientific™ Dionex™ CR-ATC™ 500 Trap column (P/N 075550)
Column temperature	30 °C
Eluent	KOH, Thermo Scientific™ Dionex™ EGC 500 KOH Potassium Hydroxide Eluent Generator Cartridge (P/N 075778)
Gradient	10 mM (0 min) – 100 mM (24 min) – 100 mM (27 min) – 10 mM (27.1 min) – 10 mM (30 min)
Suppressor	Thermo Scientific™ Dionex™ AERS™ 500e Electrolytic Suppressor, 2 mm, 75 mA, external water mode (P/N 302662)
Flow rate	0.3 mL/min
Make-up	0.1 mL/min (MeOH with 1 mM ammonium acetate)
Injection volume	2 µL
B) Q Exactive mass spectrometer conditions	
Equipment	Thermo Scientific Q Exactive mass spectrometer
Ionization method	Positive/Negative ESI
Spray voltage	3.5 kV / -2.0 kV
Vaporizer temperature	400 °C
Sheath gas	60 arb
Aux gas	10 arb
Ion transfer tube temperature	250 °C
S-Lens level	50
[Full MS]	
Resolution	70,000
Mass width	*m/z* 70–1050
[Data dependent-MS²]	
Resolution	17,500
TopN	5
Isolation window	*m/z* 1.5
Intensity threshold	5,000
Normalized collision energy	10, 30, 45

Sample preparation

The standard products used to construct an IC Q Exactive metabolite library were prepared in ultrapure water to a final concentration of 50 µM. HeLa cells cultured in a 10 cm plate (1×10^6–1×10^7) were washed twice with 10 mL of each wash solvent (ultrapure water, 150 mM ammonium acetate solution, 150 mM ammonium carbonate solution, 150 mM PBS, all at 4°C) after the culture medium in the plate was removed. Subsequently, the metabolism was quenched by the addition of 1 mL cold methanol (-30°C), and the cells were scraped. The resulting cell suspension was collected in a 2 mL tube. Subsequently, 400 µL chloroform was added and extraction was performed. After centrifugation, 700 µL supernatant was collected. After ultrapure water (400 µL) and chloroform (300 µL) were added, two-phase fractionation was performed by centrifugation, and the upper phase (water/methanol) was collected. Then, the sample was completely dried with a centrifugal evaporator. The sample was then re-dissolved in 50 µL ultrapure water and placed on the analytical system.

For 20 µL serum, 1 mL mixed solvent (methanol : chloroform : water = 2.5 : 1 : 1) was added, and extraction was performed as done with the cell samples. After two-phase fractionation, the upper phase was collected. After that, the fraction of polar proteins was removed using a 5,000 molecular weight cutoff ultrafiltration membrane. The resulting filtrate was dried at reduced pressure, and re-dissolved in 50 µL ultrapure water to be used as a sample.

Results and discussion

Construction of IC Q Exactive metabolite library

Using the IC Q Exactive system, the conditions for IC separation were optimized, and MS and MS/MS spectral libraries were constructed (Figure 2). First, the separation conditions for IC analysis were studied using 600 types of hydrophilic metabolite standard products that are anionic, cationic, or amphoteric. As a result, 264 types in positive ion mode and 336 types in negative ion mode, a total of 359 types of precursor ions, were confirmed (common: 241 types, positive ion only: 23 types, negative ion only: 95 types). Subsequently, MS/MS spectra were acquired for the confirmed precursor ions, and their information was stored in Library Manager 2.0 (Figure 3).

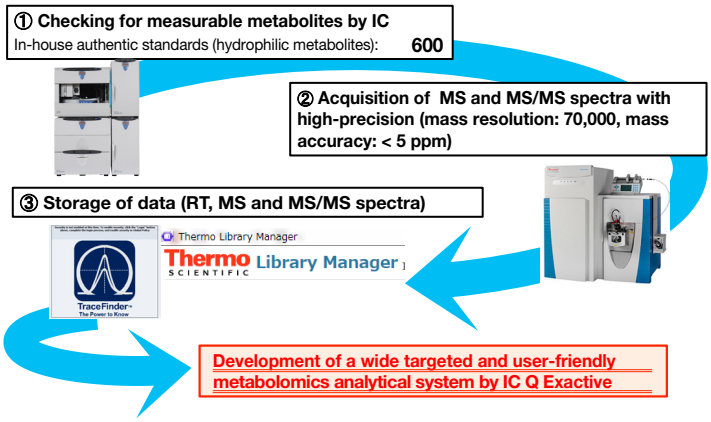
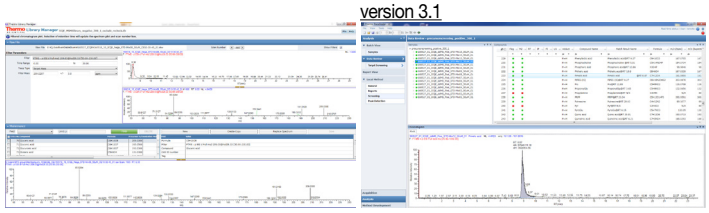


Figure 2. IC Q Exactive development workflow

Library Manager 2.0

Thermo Scientific™ TraceFinder™ Software, version 3.1



	In-house database
Positive-ion	264
Negative-ion	336
Total Compounds	**359**

Figure 3 . IC Q Exactive metabolite library

Assessment of IC separation performance in targeted metabolome analysis

Among 359 types of metabolites successfully separated and analyzed using IC Q Exactive, coenzymes such as ATP were included, which were difficult to analyze by the conventional approaches such as CE-MS, ion-pair LC-MS and HILIC LC-MS.

These metabolites are highly polar compounds containing two or more phosphate groups (logP < –6) and strong metal chelators. Therefore, it is suggested that, in the conventional approaches, absorption of compounds with phosphate groups onto the wetted metal part of the apparatus such as stainless steel tubing occurs, leading to peak tailing and reduced sensitivity and reproducibility.

On the other hand, it is now clear that the IC Q Exactive system enables separation and analysis of such coenzymes, keeping sharp peak shapes (Figure 4). It is presumably because coenzymes, which are highly polar anionic metabolites, are suitable for the separation characteristics of IC, “separation by interaction between ion exchangers and ion types (ion valency, ionic radius and ion hydrophobicity”,[4] and also adsorption of compounds with phosphate groups onto metals, do not occur as all flow paths in the IC apparatus are made of PEEK resin (metal-free).

In addition, one of the evaluation indexes for metabolome analysis is “isomer separation”. It is known that many structural isomers and stereoisomers exist in vivo and have specific functions. Usually these isomers cannot be distinguished with a mass spectrometer, and chromatographic separation is required. Various structural isomers of hexose monophosphate, a typical isomer in principal metabolism, exist depending on the type of sugar (glucose, fructose, and mannose) and the phosphate binding position. The study revealed that the approach using IC Q Exactive has high reproducibility with cell extract samples as well, and also higher separation performance compared to the conventional approaches (Figure 4). Furthermore, because trace amounts of secondary messengers such as cAMP were also detected from cell extracts, this approach can be considered as a highly sensitive analysis system (Figure 4).

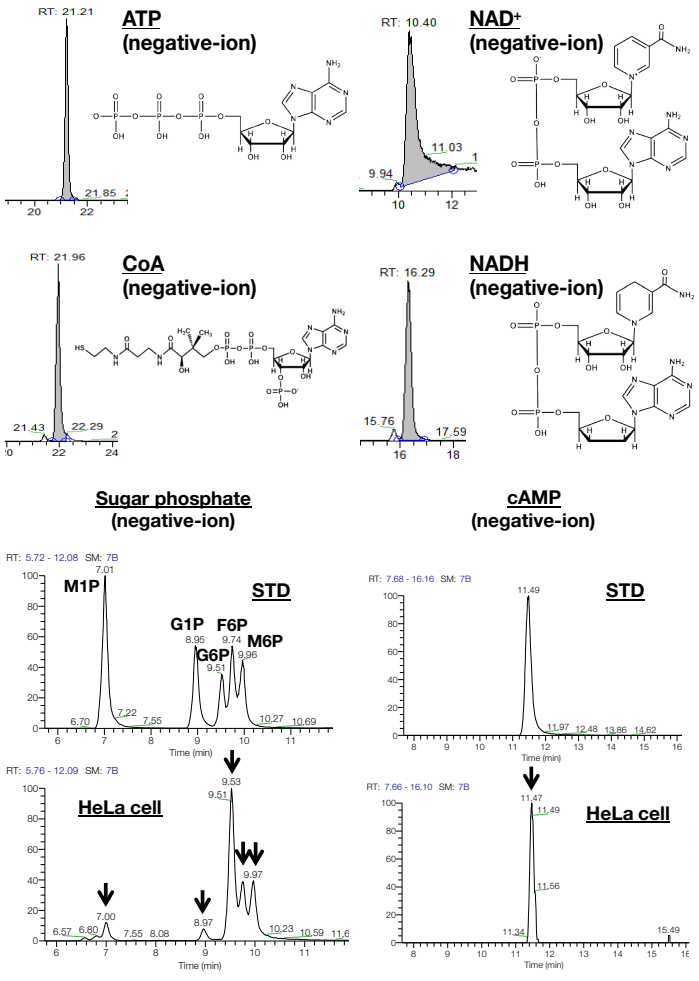


Figure 4 . Example analysis results for anionic metabolites

Most of the metabolites measured using this analysis system were, of course, anionic metabolites. However, some amphoteric metabolites such as intermediates in the amino acid metabolic pathway can also be measured. Thus, MS measurement in positive and negative ion modes enables measurement of a wide variety of metabolites in one sample injection. Most cationic metabolites seem to be undetectable because they cannot be retained in the column (anion exchanger) used in this analysis, and also removed by the electrolysis regeneration suppressor.

The study demonstrated that this analysis system is a practical analytical approach that is capable of high-sensitivity measurement with high reproducibility of retention times and peak areas of intermediates in the principal metabolic pathways (Figure 5) such as the glycolytic pathway, pentose phosphate pathway, citric acid cycles and nucleic acid metabolism.

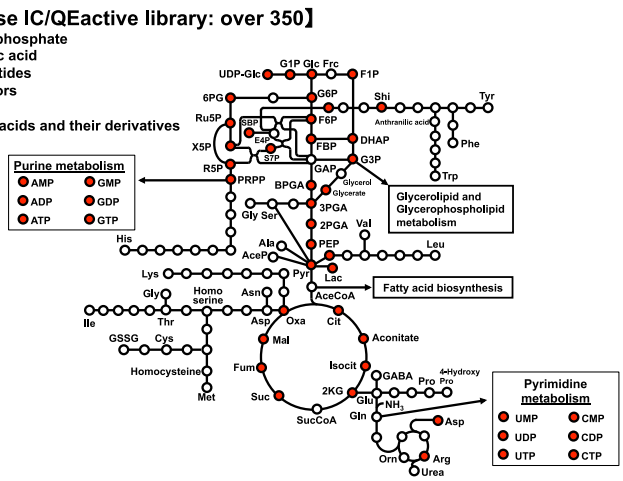


Figure 5. Metabolites that can be analyzed with IC Q Exactive

Effects of salts contained in samples

Although the IC-MS system uses potassium hydroxide (non-volatile salt) as the eluent, measurement is now possible by removal of potassium ions due to the action of the ion exchange resin and ion exchange membrane by connecting an electrolytically regenerated suppressor after column separation. Based on this principle, we have reached the idea that this approach may be applied to the analysis of samples containing non-volatile salts, which was the greatest disadvantage in MS analysis (unlike peptides and lipid molecules, many hydrophilic metabolites are highly polar, and there is no way to separate them from inorganic salts contained in samples). In fact, when performing analysis on HeLa cell extracts rinsed using PBS that is used as a cell wash solution in biochemistry experiments, etc., we found that sodium ions contained in PBS were removed by the suppressor, which enabled highly sensitive and highly reproducible metabolite measurement. As for animal cells, because amino acids and serum-derived metabolites are contained in the culture media in addition to glucose, it is essential to perform cell washing for accurate measurement of the amounts of metabolites in the cell. In the previous metabolome analysis, washing was performed mainly by using cold water or ammonium acetate solution (volatile salts), etc. However, the effects of cell wash solutions on metabolomics data had not been discussed. There were great differences in the measurement values of each metabolite between cells washed with PBS, and cells washed with cold water or an aqueous solution containing volatile salts (Figure 6). Based on these results, IC-HRAMS is the only approach that can accurately measure the amounts of anionic metabolites in the cells.

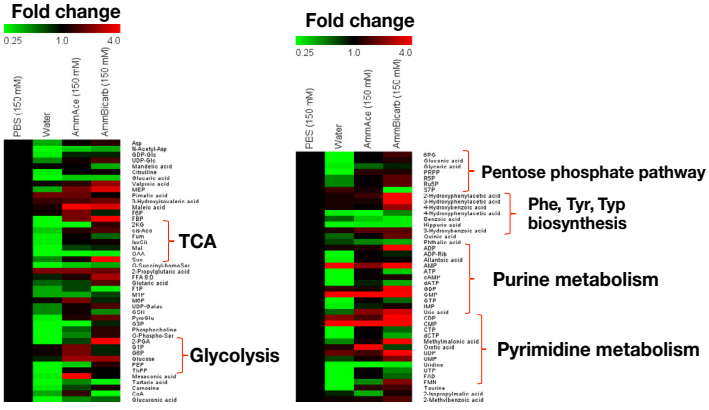


Figure 6. Effects of cell wash solutions on metabolomics data

Analysis of blood samples

Extraction was performed according to the general protocol for metabolome extraction, and the prepared serum samples were placed in the IC Q Exactive system. As a result, the electrolytically regenerated suppressor became unusable after three analyses. We searched for the cause, and found that the problem was contamination with polar proteins contained in samples extracted from blood. As a result, it was possible to perform stable analysis by removing polar proteins contained in the water/methanol extraction fraction using a 5,000 molecular weight cutoff ultrafiltration membrane (Figure 7). Measurement was performed using the serum extract that had undergone this optimized pretreatment, resulting in the detection of 111 hydrophilic metabolites.

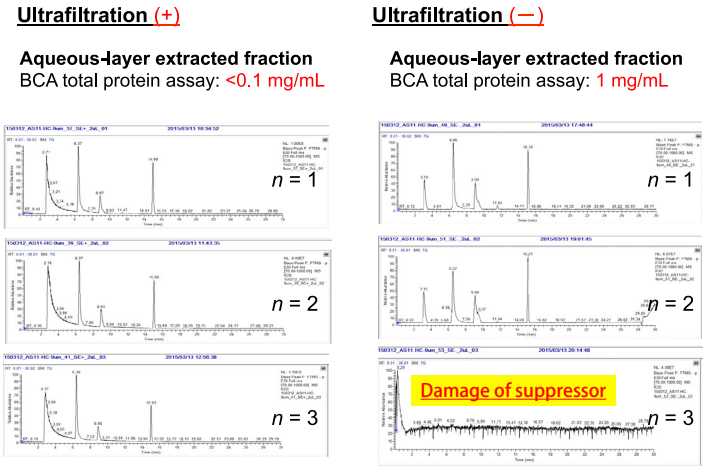


Figure 7. Effects of salts contained in samples

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Summary

Many scientists acknowledge the importance of metabolome analysis, however, as few as 100 types of hydrophilic metabolites in total have been identified by existing technologies. Therefore, progress in measurement techniques and sample preparation is essential to further breakthroughs in metabolome research. In addition, the principal metabolic pathways (glycolytic pathway, pentose phosphate pathway, citric acid cycle, and nucleic acid metabolism) are involved in energy generation, cell maintenance, and repair processes for living organisms, and are also the most important pathways in order to understand the metabolism of cancer, etc.

The characteristics of the new approach of metabolome analysis with the IC Q Exactive system are that it is capable of comprehensive and high-sensitivity measurement of intermediates in principal metabolic pathways, and that it also has high reproducibility of retention times and peak area in analysis of biological samples. Because IC-MS is capable of analysis with high reproducibility, time spent on data analysis such as peak alignment and peak identification is reduced, and the accuracy of identification of chemical compounds is improved.

Moreover, the IC Q Exactive system works well on separation and analysis of biological samples containing non-volatile salts, and can acquire more accurate metabolic information in vivo. On the other hand, for samples containing high amounts of polar proteins such as blood, we found that the removal of proteins during pretreatment is essential.

We expect that IC-MS analysis technology will be further developed in the future, and utilized as a revolutionary analytical tool leading to medicine and drug discovery on the basis of metabolic research, clinical diagnosis, and the knowledge gained from these findings.

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