MP 493

# Development of MRM-based detection of the plant metabolome using liquid chromatography/tandem mass spectrometry

Satoshi Yamaki[1], Junichi Masuda[1], Yoshihiro Hayakawa[1], Muneo Sato[2] , Mami Okamoto[2], Yuji Sawada[2] , Masami Yokota Hirai[2]

1 Shimadzu Corporation. 1, Nishinokyo-Kuwabaracho Nakagyo-ku, Kyoto 604–8511, Japan 2 RIKEN CSRS. 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

## 1. Introduction

Multiplexed metabolite screen using MRM-MS has become a powerful strategy to bring information of biological state. In the field of plant research, it is hoped that the metabolomics is used for developing new varieties of plants to get a valuable feature, for understanding for the stress-response from environmental conditions and so on. Liquid chromatography-mass spectrometry is one of a widely used tool for metabolome analysis, and it have an advantage of widely applicable properties, to analyze high molecular weight, thermolabile and polar compounds without any derivatization.

In this study, we attempted to develop a LC-MRM-based approach to determine metabolite profiles including both primary and secondary metabolites aiming at high throughput and comprehensive methods using triple quadrupole MS acquisition.

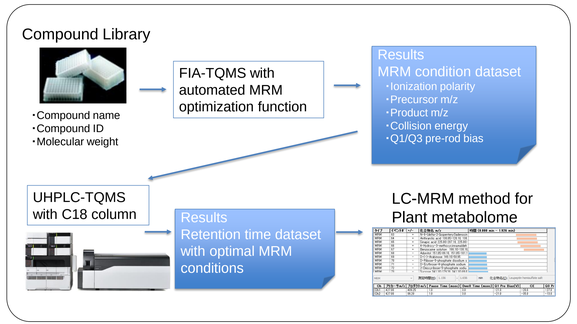



Figure 1 Developmental scheme of the LC-MRM-based metabolome platform.

## 2. Methods and Materials

The library of commercially available compounds were used for the optimization of multiple-reaction monitoring (MRM) conditions and the confirmation of LC retention time. Authentic standards of endogenous metabolite were dissolved in each adequate solvent and diluted with water, then transfer to deep 96-well plates.

In order to develop the large scale analytical method, automated MRM parameter optimization were carried out by flow injection analysis (FIA) of polar metabolites with a function of the LabSolutions LCMS control software. The parameter optimization performed precursor ion *m/z* search with polarity switching, collision energy adjustment, product ion *m/z* selection of top 3 channels and voltage adjustment of the Q1/Q3 pre-rod, at least 5 injections per compound. All ultra-high performance liquid chromatograph (UHPLC) units were used a Shimadzu Nexera X2 system with a solvent system. A maximum of six microtiter plates of 384/96-well can be used by an autosampler SIL-30ACMP at one time. The rapid MRM measurement was carried out on a Shimadzu LCMS-8050 triple quadrupole mass spectrometer with the capability of simultaneously acquiring 555 MRM channels per second equipped with a heating electrospray source.

## 3. Results and Discussion

### 3-1. Optimization of MRM conditions for compounds

Automated MRM parameter optimization by FIA were conducted triplicate (n=3) for each compound to determine the optimum ionization polarity of targets followed by MRM transition optimization using LabSolutions software. PEEK tubing (800 mm x 0.13 mm I.D.) and PEEKsil tubing (500 mm x 50 um I.D.) was installed between autosampler and electrospray unit. Precursor ion search were applied by full scan acquisition of both positive and negative mode, from combination of molecular weight information and adducts setting (this time three candidate; $[M]^+$, $[M+H]^+$, $[M-H]^-$) with the threshold of minimum intensity 20,000.

As a consequence, most compounds were detected as the protonated ion in positive ion and de-protonated ion in negative ion mode. We have had totally 1470 events, over 4,000 set of MRM parameters (channels) from 288 compounds (three plates of 96-well microtiter plate) within about 7 days.

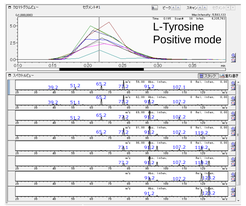



Figure 2 Product ion scan data of 9 different collision energy by single injection of FIA.

**Flow injection analysis conditions (Nexera X2 system)**

Sample concentration: 2.5 umol/L in water
Mobile phase A: 0.1% formic acid water
B: 0.1% formic acid acetonitrile
Flow rate: 0.15 mL/min
Time program: B conc.80%, stop 0.4 min
Injection vol.: 1.5 uL (loop injection)

**MS conditions (LCMS-8050)**

Ionization: ESI, Positive/Negative

### 3-2. Confirmation of optimal MRM transitions for compounds

LC-MRM analyses of 288 compounds, amino acid, organic acid, nucleic acid, sugar, flavonoid and others were conducted to confirm the optimal MRM transitions and optimum voltage of target compounds using the MRM parameters from FIA. C18 column with 1.0 mm I.D. were applied to detect peak and to confirm the retention time of each compounds using short 2 min gradient elution (Figure 3).

MRM optimization was succeeded in 89.2 of percentage in three of 96-well plate batch (257 compounds). 128 compounds such as flavonoids, amino acids (Table 1), could get MRM information of both polarity of positive and negative by this protocol, and as a result, there was an advantage that the choice of the measurement increases.

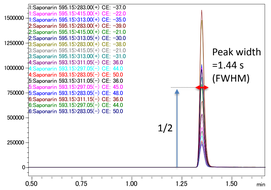



Figure 3 LC-MRM chromatograms of Saponarin (flavone glucoside) with 18 different MRM parameters obtained by FIA.

**UHPLC conditions (Nexera X2 system)**

Column: Reversed-phase C18, 50 mmL. x 1.0 mmI.D.
Sample concentration: 0.25 umol/L in water
Mobile phase A: 0.1% formic acid water
B: 0.1% formic acid acetonitrile
Flow rate: 0.24 mL/min
Time program: B conc.0.1%(0-0.25min)-9%(0.4min)-17%(0.8min)-99.9%(1.9-2.1min)-0.1%(2.11-3min)
Injection vol.: 1.5 uL (loop injection)
Column temperature: 30 C

**MS conditions (LCMS-8050)**

Ionization: ESI, Positive/Negative MRM mode
Loop time: maximum 0.250 s

Table 1 Compounds list of MRM optimization that detected both polarity (a part was indicated)

| Name | RT(min) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| L−Cystine | 0.173 | Adenine | 0.319 | 2'−Deoxyinosine | 1.233 | kaempferol−3−O−glucoside | 1.436 |
| L−Histidine | 0.175 | Uracil | 0.32 | 3−Hydroxyanthranilic acid | 1.253 | Hesperidin | 1.439 |
| L−Carnosine | 0.177 | Uridine−5'−monophosphate | 0.327 | Zeatin−9−glucoside | 1.273 | isorhamnetin−3−O−glucoside | 1.442 |
| Gly−Gly | 0.179 | Guanine | 0.33 | DL−Dihydrozeatin | 1.28 | apigenin−7−O−glucoside | 1.447 |
| L−Aspartic acid | 0.18 | urocanic acid | 0.37 | Pelargonin chloride | 1.293 | Quercetin−3−O−glucose−6''−acetate | 1.447 |
| 2−Aminoethylphosphonic acid | 0.181 | pyridoxal hydrochrolide | 0.37 | N−Formyl−L−methionine | 1.3 | luteolin−4'−O−glucoside | 1.448 |
| L−Asparagine | 0.182 | O−Succinyl−L−homoserine | 0.377 | Malvin chloride | 1.3 | Vanillin | 1.449 |
| L−Serine | 0.182 | Guanosine−5'−diphosphate−D−mannose | 0.4 | trans−Zeatin−riboside | 1.302 | Neohesperidin | 1.449 |
| L−Homocarnosine | 0.183 | Guanosine−5'−diphosphoglucose | 0.4 | Keracyanin Chloride | 1.306 | Spiraeoside | 1.452 |
| Hypotaurine | 0.187 | pyridoxal−5'−phosphate monohydrate | 0.426 | L−Tryptophane | 1.311 | naringenin−7−O−glucoside | 1.455 |
| L−Glutamine | 0.187 | 2'−Deoxycytidine | 0.433 | Procyanidin B1 | 1.312 | (+−)−Taxifolin | 1.455 |
| L−allo−threonine | 0.187 | Hypoxanthine | 0.449 | Chlorogenic acid Hemihydrate | 1.329 | Syringaldehyde | 1.457 |
| Pyridoxamine dihydrochloride | 0.189 | Inosine−5'−monophosphate | 0.49 | Procyanidin B2 | 1.34 | m−Hydroxycinnamic acid | 1.467 |
| L−saccharopine | 0.19 | Xanthine | 0.555 | Puerarin | 1.341 | Phloridzin | 1.471 |
| Dulcitol | 0.19 | Methylmalonic acid | 0.56 | Flavanomarein | 1.347 | rosmarinic acid | 1.476 |
| L−Glutamic acid | 0.191 | L−Tyrosine | 0.579 | Robinin | 1.349 | Neohesperidin dihydrochalcone | 1.496 |
| delta−Aminolevulinic acid | 0.193 | 2'−Deoxyadenosine−5'−monophosphate | 0.622 | Saponarin | 1.351 | 2−Hydroxycinnamic acid | 1.504 |
| D−(+)−Arabitol | 0.195 | Uridine | 0.651 | Pimelic acid | 1.355 | trans−3,5−Dimethoxy−4−hydroxycinnamaldehyde | 1.512 |
| L−Methionine sulfone | 0.199 | Cytidine−3',5'−cyclicmonophosphate | 0.662 | Homoorietin | 1.359 | 4−Hydroxy−3−methoxycinnamaldehyde | 1.52 |
| Argininosuccinic acid disodium salt | 0.2 | Thymine | 0.736 | 6−(gamma,gamma−Dimethylallylamino)purine | 1.365 | 2',6'−Dihydroxy−4'−Methoxychalcone−4'−O−Neohesperidoside | 1.538 |
| N−acetyl−D−mannosamine | 0.201 | Citraconic Acid | 0.759 | luteolin−3',7−di−O−glucoside | 1.366 | Poncirin | 1.538 |
| D−Ala−D−ala | 0.202 | Thymidine−5'−monophosphate | 0.841 | Leucylleucyltyrosine | 1.367 | Kaempferol−7−O−alpha−L−rhamnoside | 1.54 |
| N−acetylneuraminic acid | 0.208 | 6−Hydroxynicotinic Acid | 0.872 | Caffeic acid | 1.368 | Sebacic acid | 1.556 |
| Creatine,anhydrous | 0.209 | (+)−2'−Deoxyuridine | 0.982 | Peltatoside | 1.369 | Abscisic acid | 1.561 |
| 3−Guanidinopropionic acid | 0.216 | S−Adenosyl−L−homocysteine | 1.011 | Neoericiocitrin | 1.394 | Eriodictyol | 1.566 |
| D−(−)−Quinic acid | 0.22 | Adenosine | 1.11 | Hyperoside | 1.405 | Sissotrin | 1.572 |
| S−Carboxymethyl−L−cysteine | 0.221 | L−(−)−Phenylalanine | 1.174 | N−6'−(delta−2−Isopentenyl)adenosinehemihydrate | 1.41 | Naringenin | 1.622 |
| L−Homocystine | 0.241 | Guanosine | 1.205 | Leupeptin hemisulfate salt | 1.41 | Genistein | 1.625 |
| Carbamoyl−DL−aspartic acid | 0.262 | Inosine | 1.205 | Suberic acid | 1.422 | (+−)−Jasmonic acid | 1.633 |
| Nicotinic acid mono nucleotide | 0.285 | Adenosine−3',5'−cyclicmonophosphate | 1.224 | Rhoifolin | 1.426 | Hesperetin | 1.636 |
| N−Acetylglycine | 0.291 | Guanosine−3',5'−cyclic monophosphate | 1.227 | Anthranilic acid | 1.432 | Gibberellin A4 | 1.672 |
| L−Methionine | 0.316 | 2'−Deoxyguanosine monohydrate | 1.228 | 4−Coumaric acid | 1.434 | D−Glucosyl−beta1−1''−D−erythoro−Sphingosine | 1.676 |

### 3-3. Analytical performance confirmation

Amino acid standard mixture were applied to confirm the performance of the LC-MRM platform. 25 MRM transitions of single time segment including internal standard channel were applied in a single run by maximum loop time 0.275 s (dwell time 10 ms, pause time 1 ms) under positive ion mode using 2 min gradient. Wako amino acid standard mixture type-B and type-AN II were mixed and diluted by water to the concentration of 2.5 nM - 2.5 uM. Chromatogram of 25 nM mixture, correlation curves and the relative coefficient were shown in Figure 4. Reproducibility of n=10 analysis was shown in Table 2.

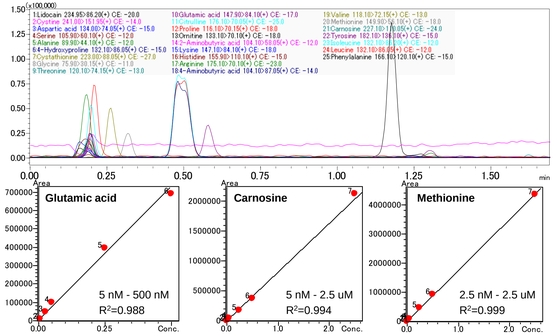
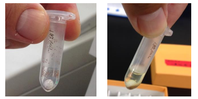



Figure 4 MRM chromatogram and response linearity of amino acids.

### 3-4. Plant metabolome analysis for relative comparison

To find out the optimal transition number and optimal dwell time of each MRM transition, we performed the detection and reproducible test using the plant seed extracts. Approximately 500 seeds of Arabidopsis thaliana Col-0 were put in a polypropylene tube with ceramic bead and added 500 uL of 80% methanol, 0.1% formic acid water, and processed it for 2 minutes in Shake Master Neo shaker (BMS Co., Tokyo), and were broken to extract the metabolite. After centrifugation, supernatants were diluted 100 times by 0.1% formic acid water, then applied to LC-MRM analysis with 25 MRM transitions of 10 ms dwell time.



In the result estimated by data of amino acids, this method detected a representative metabolites at good reproducibility of peak area under n=15 analysis (RSD 1.3〜14.2%, Table 2) within 45 min. Further improvement of the throughput of the analysis is expected by setting the time segment method (called MRM synchronization).

Table 2 Reproducibility of amino acids in seed extracts and standard solution

| | Col_0 (0.1mL/seed) , n=15 | | | ※Amino acid STD 25 nM, n=10 | | |
|---|---|---|---|---|---|---|
| | Peak area (average) | Area %RSD | Retention time %RSD | Peak area (average) | Area %RSD | Retention time %RSD |
| Cystine | n.d. | ----- | ----- | 17716 | 6.09 | 0.80 |
| Aspartic acid | 128761 | 3.65 | 0.30 | 15456 | 11.61 | 0.71 |
| Serine | 53561 | 3.50 | 0.35 | 6462 | 14.21 | 0.82 |
| Alanine | 97705 | 2.68 | 0.24 | 28494 | 4.53 | 0.45 |
| 4-Hydroxyproline | 7251 | 11.70 | 0.65 | 37446 | 9.12 | 0.38 |
| Cystathionine | n.d. | ----- | ----- | 27377 | 5.10 | 0.68 |
| Glycine | 3028 | 14.16 | 1.06 | 1032 | 17.74 | 2.05 |
| Threonine | 71413 | 2.86 | 0.25 | 14992 | 5.15 | 0.62 |
| Glutamic acid | 1734034 | 1.52 | 0.25 | 48383 | 7.84 | 0.43 |
| Citrulline | 65054 | 3.32 | 0.40 | 89092 | 3.80 | 0.27 |
| Proline | 2253407 | 2.50 | 0.21 | 118701 | 2.49 | 0.20 |
| Ornitine | 27973 | 4.98 | 0.57 | 25972 | 4.17 | 0.87 |
| 2-Aminobutyric acid | 151583 | 5.60 | 0.33 | n.d. | ----- | ----- |
| Lysine | 307830 | 2.35 | 0.32 | 39051 | 4.82 | 0.79 |
| Histidine | 47038 | 3.59 | 1.93 | 51055 | 4.43 | 1.00 |
| Arginine | 783096 | 2.03 | 0.28 | 125062 | 5.59 | 0.45 |
| 4-Aminobutyric acid | 29329 | 3.60 | 0.47 | 19856 | 5.87 | 0.38 |
| Valine | 480412 | 2.31 | 0.21 | 90346 | 3.31 | 0.37 |
| Methionine | 29855 | 4.76 | 0.35 | 45180 | 3.95 | 0.27 |
| Carnosine | n.d. | ----- | ----- | 19744 | 10.84 | 0.42 |
| Tyrosine | 89687 | 3.78 | 0.20 | 68764 | 3.47 | 0.22 |
| Isoleucine | 626285 | 1.30 | 0.19 | 295858 | 1.40 | 0.33 |
| Leucine | 582955 | 1.45 | 0.10 | 272801 | 1.65 | 1.94 |
| Phenylalanine | 1072199 | 1.62 | 0.15 | 292476 | 2.71 | 0.25 |
| average | 411545.52 | 3.96 | 0.42 | 76144.17 | 6.08 | 0.64 |

※except for cystathionine and 2-aminobutylic acid, 12.5 nM.

## 4. Conclusions

- A LC-MRM-based analytical workflow is presented as high-throughput and robust measurement for plant metabolome.
- We performed the optimization of approximately 300 compounds and obtained retention time information for each compound by the 50 mm length column with 2 min gradient elution.
- The results demonstrate that simultaneous MRM analysis with ultrafast MRM acquisition instrument improves the throughput for analysis of metabolites in plant sample.
- Targeted approach with UHPLC-triple quadrupole MS instruments enables robustness, high sensitivity and specificity in the femto mol range and a broad dynamic range. LC/MS is the complementary technique to GC/MS that is well established metabolomics platform.

## 5. Reference

・Y. Sawada et. al., *Plant Cell Physiol.* 50(1): 37-47 (2009).

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