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Highly sensitive and selective analysis of widely targeted metabolomics using gas chromatography/triple-quadrupole mass spectrometry

Hiroshi Tsugawa,[1,2] Yuki Tsujimoto,[1] Kuniyo Sugitate,[3] Norihiro Sakui,[3] Shin Nishiumi,[4] Takeshi Bamba,[1] and Eiichiro Fukusaki[1,*]

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan,[1] RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan,[2] Agilent Technologies Japan, Ltd., 9-1 Takakura-cho, Hachioji-shi, Tokyo 192-8510, Japan,[3] and Division of Gastroenterology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-Cho, Chu-o-ku, Kobe, Hyogo 650-0017, Japan[4]

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In metabolomics studies, gas chromatography coupled with time-of-flight or quadrupole mass spectrometry has frequently been used for the non-targeted analysis of hydrophilic metabolites. However, because the analytical platform employs the deconvolution method to extract single-metabolite information from co-eluted peaks and background noise, the extracted peak is artificial product depending on the mathematical parameters and is not completely compatible with the pure component obtained by analyzing a standard compound. Moreover, it has insufficient ability for quantitative metabolomics. Therefore, highly sensitive and selective methods capable of pure peak extraction without any complicated mathematical techniques are needed. For this purpose, we have developed a novel analytical method using gas chromatography coupled with triple-quadrupole mass spectrometry (GC–QqQ/MS). We developed a selected reaction monitoring (SRM) method to analyze the trimethylsilyl derivatives of 110 metabolites, using electron ionization. This methodology enables us to utilize two complementary techniques—non-targeted and widely targeted metabolomics in the same sample preparation protocol, which would facilitate the formulation or verification of novel hypotheses in biological sciences. The GC–QqQ/MS analysis can accurately identify a metabolite using multichannel SRM transitions and intensity ratios in the analysis of living organisms. In addition, our methodology offers a wide dynamic range, high sensitivity, and highly reproducible metabolite profiles, which will contribute to the biomarker discoveries and quality evaluations in biology, medicine, and food sciences.

[**Key words:** Gas chromatography coupled with triple-quadrupole mass spectrometry; Widely targeted metabolomics; Selected reaction monitoring; Multichannel selected reaction monitoring transitions; Sensitivity]

Metabolomics aims to identify and quantify the levels of all metabolites, and has been widely applied in many research areas such as biology, medicine, and food science (1–4). In metabolomics studies, gas chromatography coupled with mass spectrometry (GC/MS) has frequently been used to analyze low-molecular-weight metabolites because of the high equipment stability and availability of user-friendly tools for data analysis (5,6). In particular, researchers have used GC/MS for non-targeted analysis, which analyzes all peaks detected by scanning a wide *m*/*z* range (6). As a result of this scan-mode analysis, a great deal of metabolite information has been obtained, enabling fruitful investigations in biological studies.

However, non-targeted GC/MS analysis uses a mathematical method, namely deconvolution, to extract single-peak information from co-eluted peaks and background noise (7–9). Although the deconvolution method gives a component including the retention time and MS information for identifying and quantifying a metabolite, the extracted component is not completely compatible with the pure component obtained by analyzing a standard compound. Moreover, it is a complicated task to select a unique *m*/*z* for the precise quantification of a peak that is discriminable from co-eluted peaks and background noise. The main problem is that the exported result varies depending on the deconvolution parameters. In addition, the sensitivity and dynamic range are not satisfactory in scan-mode analysis.

Recently, a widely targeted analysis method complementary to conventional non-targeted analysis, which detects many focused metabolites in a single run, has been developed using single-ion monitoring (SIM) in quadrupole MS (10). The SIM method allows for highly sensitive analysis of metabolites by reducing the number of unwanted *m*/*z* values from among the large number of *m*/*z* values, resulting in improved deconvolution accuracy for pure peak acquisition. However, the quantitative issue in metabolomics has not been solved yet, because the extracted peak is still an artificial (mathematical) product. In addition, its dynamic range is insufficient. Consequently, highly sensitive and selective analytical methods for obtaining pure peak information without any complicated mathematical techniques are needed in order to precisely quantify the metabolites and thereby expedite the biological investigations, biomarker discoveries, and quality estimations.

* Corresponding author. Tel./fax: +81 6 6879 7424.
E-mail address: fukusaki@bio.eng.osaka-u.ac.jp (E. Fukusaki).

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Selected reaction monitoring (SRM) analysis using GC coupled with triple-quadrupole mass spectrometry (GC−QqQ/MS) is an important technique that can be used to achieve this goal. SRM analyses can easily quantify metabolites because the peak for a single metabolite can be easily distinguished from the co-eluted peaks and background noise on the basis of precursor (MS1) and product (MS2) ion-pairs. The SRM mode also offers high sensitivity and a wide dynamic range compared to single-quadrupole MS (11). Several SRM-based techniques using GC−QqQ/MS have been developed based on chemical ionization or methyl chloroformate derivatization methods (12,13). Although these methods could mostly obtain the molecular ion (suitable as the precursor ion); it is difficult to use them practically, because only a few databases and commercial derivatization reagents are available. In contrast, trimethylsilyl (TMS)-derivatization and electron ionization (EI) methods have frequently been used as robust techniques in many metabolomics studies. Researchers can easily access retention time/spectral databases of chemicals in library, and buy a derivatization kit for experiments. Furthermore, it would be beneficial to construct two complementary techniques (e.g., non-targeted and widely targeted) that could be employed prior to MS analysis. Nevertheless, a widely targeted SRM method using a combination of TMS-derivatizing reagent and EI has not been reported yet. This is because it is a tedious and complicated task to optimize the SRM conditions for the following reasons: (i) an automatic SRM optimization method using a flow injection or infusion mode that can be used in atmospheric-pressure-ionization MS is unavailable; (ii) TMS derivatives rarely generate a molecular ion in the EI method; and (iii) almost all the fragment ions are derived from the TMS derivatives without structural information, which makes it difficult to identify the typical transition to a metabolite (14,15).

To address these challenges, we have developed a practical GC−QqQ/MS system for a highly sensitive and selective targeted metabolite profiling of TMS-derivatized compounds using EI method. First, multichannel SRM transitions and intensity ratios were used to accurately identify the metabolites. Next, the SRM conditions were optimized for semiautomatic data preprocessing and statistical analysis. The developed method includes 325 transitions for 110 metabolites analyzed in the time-segmented SRM mode. We validated the linear range and limit of detection (LOD) using standard mixtures. Moreover, we used the method for metabolic profiling of two living organisms, namely *Saccharomyces cerevisiae* and mouse plasma. Our SRM method library, which offers higher sensitivity and wider dynamic range for metabolites, is available in Table S1.

MATERIALS AND METHODS

Construction scheme of SRM method for GC−QqQ/MS analysis Fig. 1 illustrates the construction workflow and overview of GC−QqQ/MS-based widely targeted metabolomics. First, 110 metabolites were selected for the GC−QqQ/MS-based metabolic profiling. These metabolites (compounds) have been detected in earlier GC/MS metabolomics studies (10,16,17). The metabolites included low-molecular-weight hydrophilic metabolites such as amino acids, sugars, organic acids, and amine compounds (see Table S1).

Next, SRM methods were constructed for individual standard metabolites. Unlike atmospheric-pressure-ionization MS, GC−QqQ/MS is not suitable for auto-optimization of SRM. Even though auto-optimization could be performed in some cases, this method is not recommended because the high-intensity fragment ions generated from TMS groups do not give any useful information on compound structure. The typical precursor−product ion-pairs need to be manually determined. Therefore, optimization experiments were performed by individually analyzing a standard compound as per the following procedure. A standard compound (15 μL, 1 mM) was dried using a vacuum-centrifuge dryer. Methyloximation derivatization was performed by dissolving methoxyamine hydrochloride in pyridine (100 μL, 20 mg/mL), and the resulting mixture was incubated at 30°C for 90 min. Trimethylsilylation derivatization was performed by adding 50 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and the mixture was incubated at 37°C for 30 min. A 1 μL aliquot of the sample was injected into GC−QqQ/MS in split mode (25/1, v/v).

Further, the appropriately derivatized standard compound was analyzed by scan-mode analysis from *m*/*z* 85 to 600, and the retention time and mass spectra were obtained. EI method rarely generates the molecular ion of a metabolite. Therefore, two to three precursor−product ion-pairs as SRM transitions for each metabolite were evaluated for reducing the risk of false positive identification (18,19). After the scan-mode analysis, three or four *m*/*z* values were selected as the candidates for the precursor ions. The peaks from the derivatizing reagent (e.g., *m*/*z* 147 and 174) were obviously excluded in the precursor ion selection. Next, the product-ion scan analyses of the selected precursor ions were performed in order to obtain unique product ions at collision energies of 2 and 12 V. Consequently, two to three precursor−product ion-pairs were identified. The peaks from the derivatizing reagent (e.g., *m*/*z* 73 and 147) were also excluded in the product-ion selection. After these selections (precursor−product ion-pair candidates), the SRM analysis of the standard compound was performed for optimizing collision energies. The transitions optimized at collision energies of 2 and 12 V were re-estimated at 2, 4, 6, 8, and 10 V, and 12, 14, 16, 18, and 20 V, respectively.

Finally, the time-segmented SRM method was optimized using a mixture of standard compounds. The cycle time was set at a minimum of 3 cycles/s in each time segment for accurate peak top assessment. In addition, the dwell time was set at over 10 ms for more sensitive analysis.

GC−QqQ/MS analysis The GC−QqQ/MS system was an Agilent 7890A series GC system coupled with an Agilent 7000B QqQMS (Agilent Technologies Inc., USA). The columns were J&W Scientific HP-5MS (30 m × 0.25 mm, i.d. 0.25 μm, d.f., Agilent Technologies Inc.). The front inlet temperature was 230°C. The helium gas flow rate through the column was 1 mL/min. The column temperature was held isothermally at 60°C for 1 min and then raised at a rate of 10°C/min to 210°C, at a rate of 5°C/min to 230°C, and at a rate of 15°C/min to 325°C, and held isothermally at 325°C for 5 min. The transfer line and the ion-source temperature were 250°C and 230°C, respectively. To correct the retention time, retention-time-locked methods using ribitol (retention time: 15.37 min) as the internal standard were used (20). The collision gas was nitrogen. Peak detection and calculation of peak intensity were performed using Agilent Mass Hunter quantitative software. Both the SRM- and scan-mode analyses were performed to compare their metabolite profile results between these two modes of operation. The scan range was set at *m*/*z* 85−600. The scan rate was set at 5 cycles/s. The peak detection and identification were performed by AMDIS (21) and Aloutput (6) softwares.

Method validation We evaluated the repeatability, linearity, and LOD of each metabolite detected by the optimized method. We prepared the standard mixture at 15 concentrations: 100 nM, 200 nM, 500 nM, 1 μM, 2 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM, 200 μM, 500 μM, 1 mM, 2 mM, and 5 mM. We added 150 μL of 500 mM ribitol as an internal standard to 150 μL of the prepared samples and dried them in a vacuum-centrifuge dryer and a freeze dryer. The derivatization process was the same as that for the optimization of the SRM method. This calibration result was used for the quantification of living organism analyses.

Preparation of *S. cerevisiae* sample The *S. cerevisiae* sample was prepared according to a previous report (16). *S. cerevisiae* strain BY4742 (*MATα leu2Δ0 lys2Δ0 ura3Δ0 his3Δ1*) was used in this experiment. To obtain a single-colony isolate, glycerol-stocked cells were streaked onto a yeast extract peptone dextrose medium (YPD, 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) agar plate and incubated at 30°C for 2 days. A single-colony was inoculated into 3 mL of YPD liquid medium and grown at 30°C for 16 h. The culture was diluted with fresh medium to $OD_{600} = 0.1–0.2$, and cultivation was continued in baffled flasks up to $OD_{600} = 1.0$. The flasks were rotated at 200 rpm on a gyratory shaker. The BY4742 strain was grown in six independent flasks.

The cells were harvested by centrifugation (4000 ×*g*, 3 min). The cell pellet was washed with Milli-Q water (Millipore, Tokyo, Japan) and centrifuged again at 4000 ×*g*, 3 min. The supernatant was removed, and the pellet was frozen in liquid nitrogen. The cells were lyophilized and stored at −80°C until they were used.

The dried cells (10 mg) were homogenized and disrupted using MM 301 mixer mills (Retsch, Haan, Germany) at 20 Hz for 1 min. The cells were mixed with 1 mL of a solvent mixture ($MeOH/H_2O/CHCl_3 = 2.5/1/1$, v/v/v) containing 60 μL of 0.2 mg/mL ribitol (internal standard) dissolved in distilled water. The mixtures were incubated at 1200 rpm for 30 min at 37°C and then centrifuged at 16,000 ×*g* for 3 min at 4°C. A 900 μL aliquot of the supernatant was transferred to a clean 1.5 mL Eppendorf tube, and 400 μL of distilled water were added to the tube. After mixing, the solutions were centrifuged at 16,000 ×*g* for 3 min at 4°C, and the supernatant (800 μL) was dispensed into a clear tube, and then dried in a vacuum-centrifuge dryer and a freeze dryer.

For oximation, 100 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) were added and the mixture was incubated at 30°C for 90 min. For trimethylsilylation, 50 μL of MSTFA were added and the mixture was incubated at 37°C for 30 min. The mixture was centrifuged at 16,000 ×*g* for 5 min at 4°C, and then a 1 μL aliquot of the supernatant was injected into the GC/QqQMS in split mode (15/1, v/v).

Preparation of mouse plasma sample The preparation of the mouse plasma sample was performed according to a previous report (17). Eight-week-old C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). Five mice were sacrificed under anesthesia, and subsequently the blood was collected. The obtained blood was immediately treated with sodium heparin, centrifuged at 3000 ×*g* for 10 min at 4°C, and then the plasma was collected as the supernatant.