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Comparison of sequential derivatization with concurrent methods for GC/MS-based metabolomics

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The gas chromatography/mass spectrometry (GC/MS)-based metabolomics requires a two-step derivatization procedure consisting of oximation and silylation. However, due to the incomplete derivatization and degeneration of the metabolites, good repeatability is difficult to obtain during the batch derivatization, as the time between completing the derivatization process and GC analysis differs from sample to sample. In this research, we successfully obtained good repeatability for the peak areas of 52 selected metabolites by sequential derivatization and interval injection, in which the oximation and silylation times were maintained at constant values. In addition, the derivatization times and amount of reagents employed were varied to confirm that the optimal derivatization conditions differed for the various metabolites. In conventional batch derivatization, six metabolites, viz. glutamine, glutamic acid, histidine, alanine, asparagine, and tryptophan, exhibited fluctuations in their peak areas. Indeed, we found that for all six metabolites these differences originated from the silylation process, while the variations for glutamine and glutamic acid were related to the oximation process.

[**Key words:** Metabolomics; Metabolome; Gas chromatography/mass spectrometry; Derivatization; Trimethylsilylation; Methoximation]

Metabolomics is the study of biochemical processes that involve metabolites, and its use in the comprehensive analysis of metabolites has been used in a range of fields, including bioengineering, food science, medicine, and medical science (1–6). In recent years, gas chromatography coupled with mass spectrometry (GC/MS) has been commonly employed in metabolomics to study low-molecular-weight metabolites due to its good repeatability and reproducibility, large peak capacity and quantity of information provided per unit time, and ability to identify candidates using existing libraries. To comprehensively measure polar molecules such as amino acids, organic acids, sugars, and nucleic acid bases, two-step derivatization that combines oximation and silylation has been employed in a number of cases (2–5,7). The purpose of oximation is to protect the keto group and prevent cyclization of the sugar moiety, thereby avoiding the appearance of multiple peaks in the chromatogram. Moreover, oximation plays a role in preventing the decarboxylation of α-keto acids (8,9). Silylation is a classical derivatization method employed to introduce a silyl group to a metabolite by replacing active hydrogen atoms (e.g., of hydroxyl groups, carboxyl groups, and amino groups) to generate stable, more volatile, and less polar metabolites (10).

However, it has been reported that good repeatability is difficult to achieve during batch derivatization, particularly for amino acids, as the time between the completion of the derivatization reaction and the GC analysis differs from sample to sample (11–13). This can result in significant variations in peak areas due to differences in both the derivatization efficiencies and the stabilities of the resulting derivatives. Even when the derivatization times for the oximation and silylation stages are kept constant, the coexistence times of the metabolites and reagents prior to GC injection differ among samples allowing, the metabolites to undergo various reactions and transformations prior to injection. For example, the extent of trimethylsilyl (TMS) incorporation into amino acids varies between a sample introduced into the GC immediately after derivatization and a sample allowed to stand at room temperature for a certain period of time prior to analysis. In the case of alanine, incomplete trimethylsilylation leads to the presence of two chromatographic peaks (i.e., Ala_2TMS and Ala_3TMS), where the intensity of the Ala_3TMS signal increases, while that of Ala_2TMS decreases upon increasing the time between derivatization and GC analysis (12,14). Indeed, such variations cause significant issues in metabolomics research when the analysis of large numbers of samples is required. This has led to the development of systematized methods for automated derivatization and sample introduction into the GC system (15–18). However, no studies focusing on individual metabolite variations have been reported to date, although the repeatability of conventional batch derivatization and sequential derivatization methods has been discussed.

Herein, we compare three different methods, namely batch derivatization (both oximation and silylation), batch oximation and sequential silylation, and sequential derivatization (both oximation and silylation). For this purpose, a range of amino acids, organic acids, sugars, and nucleic acid bases were employed as examples of key metabolites in metabolomics research. In addition, we

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examined the differences in derivatization times and the quantities of derivatization reagents employed using an intelligent GC autosampler to maintain constant derivatization and GC injection times for all samples. Furthermore, we determined which the factors responsible for deviations in peak areas deviation in the conventional batch derivatization process.

MATERIALS AND METHODS

Samples and chemicals The standard mixture employed herein for the GC/MS metabolomics studies contained 52 metabolites, and was obtained from GL Sciences Inc. (Tokyo, Japan). More specifically, this mixture contained 4-aminobutyric acid, alanine, asparagine, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, putrescine, serine, threonine, tryptophan, valine, aspartic acid, tyrosine, citric acid, isocitric acid, fumaric acid, glycolic acid, malic acid, succinic acid, aconitic acid, α-ketoglutaric acid, pyruvic acid, phosphoric acid, palmitic acid, stearic acid, xanthine, adenine, cytosine, guanine, inosine, thymine, uracil, caffeine, ergosterol, glycerol, fructose, glucose, inositol, β-lactose, maltose, raffinose, sucrose, and trehalose at concentrations of 10 mmol/L. The sake sample was purchased from a local market in Saitama, Japan.

Methoxyamine HCl (97%) and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA, >96%) were obtained from GL Sciences Inc. (Tokyo, Japan). Heptanoic acid, decanoic acid, eicosanoic acid (fatty acids), methyl eicosanoate (fatty acid ester), and pyridine (analytical grade) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Hexane and methanol (analytical grade) were purchased from Kishida Chemical Co., Ltd. (Tokyo, Japan).

Sample preparation for derivatization For the sequential derivatization, a 0.3-mL glass screw-top vial (Chromacol, Cheshire, United Kingdom) equipped with a magnetic screw cap (La-Pha-Pack GmbH, Langerwehe, Germany) was employed to dispense the derivatization reagents with a syringe. Then, the sample vial was transported to each station, specifically, a vortex mixer, an agitator, and a sample tray. In the batch derivatization procedure, a 1.5-mL polypropylene tube (Eppendorf, Hamburg, Germany) was employed.

The respective metabolite mixture (50 μL, 10 mmol/L) was added to methanol (5 mL) and the resulting diluted metabolite solution (50 μL, 0.1 mmol/L) was transferred into the appropriate tube or vial, and dried using a centrifugal concentrator at 2000 rpm over 120 min (Spin Dryer Lite VC-36R, Freeze Trap VA-500R, Taitec Co. Ltd., Saitama, Japan). Similarly, a portion of the sake sample (10 μL) was transferred into the appropriate tube or vial, and methanol (50 μL) was added prior to drying according to the aforementioned procedure, without any extraction pretreatment.

Preparation of the derivatization reagent The fatty acids and the fatty acid methyl ester were treated with the derivatization reagent to confirm the repeatability of dispensing by hand (i.e., with a pipette) and using an autosampler (i.e., with a syringe). More specifically, solutions of heptanoic, decanoic, and eicosanoic acids in hexane (50 μL, 20 μg/mL) were added to a solution of methoxyamine HCl in pyridine (5 mL of 20 mg/mL, dehydrated using 4 Å molecular sieve pellets). Similarly, a solution of methyl eicosanoate in hexane (30 μL, 3000 μg/mL) was added to MSTFA (3 mL).

The experimenter could not visually confirm the volume of the derivatization reagent that was added to each sample using an autosampler. Therefore, we decided to include the fatty acids and the fatty acid methyl ester in the derivatization reagent.

Batch derivatization and injection into the GC For the subsequent methoximation derivatization, a solution of methoxyamine HCl in pyridine (80 μL, 20 mg/mL) was added to the sample tube and the mixture was incubated at 37°C for 90 min at 1500 rpm using a MBR-022UP plate incubator (Taitec Co., Ltd.). Similarly, for the silylation, MSTFA (40 μL) was added to the sample tube and the mixture was incubated at 37°C for 30 min at 1500 rpm (MBR-022UP). After this time, an aliquot of the supernatant (60 μL) was transferred into a 0.2-mL glass vial (La-Pha-Pack) and placed on a PAL RTC GC autosampler (CTC Analysis, Zwingen, Switzerland). In this case, the autosampler was employed for sample injection only, and not for sample derivatization.

Sequential derivatization and GC injection Sequential derivatization was performed using the PAL RTC GC autosampler (CTC Analysis), which consisted of a syringe exchangeable park station (equipped with a 100 μL syringe for liquid handling and a 10 μL syringe for GC injection), a vortex mixer, an agitator, a sample tray holder, and a wash solvent module. After drying the sample in a 0.3 mL glass vial (Chromacol), derivatization was carried out as follows. For methoximation, a solution of methoxyamine HCl in pyridine (80 μL, 20 mg/mL) was added to the sample vial and the mixture was incubated at 37°C for 90 min at 250 rpm. For silylation, MSTFA (40 μL) was added to the sample vial and the mixture was incubated at 37°C for 30 min at 250 rpm. In this case, the GC autosampler was employed for the two-step process, which involved in-time derivatization and on-line GC injection of the samples.

As this GC autosampler was also suitable for use in the one-step in-time derivatization process (i.e., sample silylation and on-line GC injection), methoximation was carried out by batch derivatization, while silylation was carried out by sequential derivatization.

GC/MS analysis The GC-MS system employed herein was a Shimadzu 2010 series GC instrument coupled with a Shimadzu QP-2010 Ultra mass spectrometer (Shimadzu, Kyoto, Japan) and a GL Sciences InertCap 5MS/NP column (0.25 mm I.D. × 30 m, df = 0.25 μm). For the GC-MS analysis, a portion of each sample (1 μL) was introduced into a split/splitless injector (split ratio 25:1). Helium was used as the carrier gas at a linear velocity of 39 cm/s, and the inlet temperature was set to 230°C. The column oven temperature was initially set to 80°C for 2 min, then changed to 330°C at a rate of 15°C/min, and finally maintained at this temperature for 9 min. An electron ionization source was employed at 70 eV and 250°C, and the temperature of the MS transfer line was set to 250°C. A scan range and scan rate of *m/z* 85–500 and 3333 units/s, respectively, were employed. Peak identification was performed using an in-house library, the National Institute of Standards and Technology (NIST) standard reference database (2011), and MassBank high quality mass spectral database (http://www.massbank.jp/). Peak detection and calculation of the peak intensities were performed using Shimadzu GC/MS solution software. The retention times, retention indices, and quantitative ions of the 52 analytes are shown in Table S1.

Investigation of the derivatization conditions For the methoximation and silylation procedures, we employed 80 μL of a 20 mg/mL solution of methoxyamine HCl in pyridine and 40 μL of MSTFA, which was used commonly in batch derivatization. Then, we examined the effects of derivatization temperature and time on the reactions. For methoximation, the derivatization temperature was set to 37°C or 70°C, and the derivatization time was set to 30, 60, or 90 min. For these experiments, the silylation conditions were fixed to 30 min at each temperature. For silylation, the derivatization temperatures used were 37°C and 70°C, while the derivatization times were set to 15, 30, 60, and 90 min. For these experiments, the methoximation conditions were fixed to 90 min at 30°C or 30 min at 70°C.

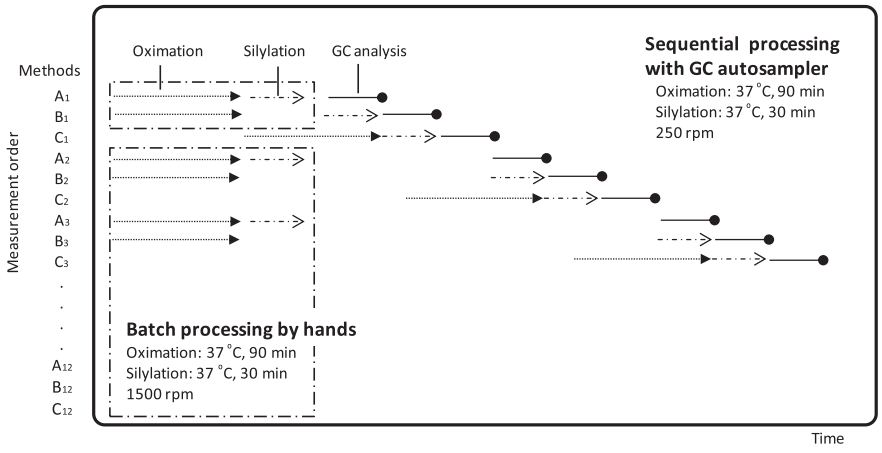

FIG. 1. Timing of different steps for all three methods. A*n*, batch derivatization; B*n*, batch oximation and sequential silylation; C*n*, sequential derivatization; $n = 12$.

TABLE 1. Time course changing of metabolites in the standard mixture comparing with the three methods A, B, C and MSTFA quantity (n=12).

Compound	RT (min)	Methoxyamine HCl in pyridine 80 μL, MSTFA 40 μL								Methoxyamine HCl in pyridine 40 μL, MSTFA 80 μL							
		A: Batch deriv.			B: Batch methoximation and sequential silylation			C: Sequential deriv.		A: Batch deriv.			B: Batch methoximation and sequential silylation			C: Sequential deriv.	
		Peak area av.	Δ_A (%/h)	RSD (%)	Peak area av.	Δ_B (%/h)	RSD (%)	Peak area av.	RSD (%)	Peak area av.	Δ_A (%/h)	RSD (%)	Peak area av.	Δ_B (%/h)	RSD (%)	Peak area av.	RSD (%)
Heptanoic acid_1TMS	6.295	1.8×10^5	0.08	3.3	1.7×10^5	0.44	6.0	1.8×10^5	4.9	7.1×10^4	0.28	3.1	7.0×10^4	0.18	2.7	7.1×10^4	1.7
Decanoic acid_1TMS	9.091	1.9×10^5	0.20	4.9	1.8×10^5	0.44	6.7	1.8×10^5	5.0	7.4×10^4	0.22	3.0	7.2×10^4	0.00	3.1	7.4×10^4	1.7
Eicosanoic acid_1TMS	15.764	1.3×10^5	0.29	3.6	1.2×10^5	0.11	3.6	1.2×10^5	5.1	5.4×10^4	0.33	5.6	5.1×10^4	−0.05	4.0	5.2×10^4	3.6
Methyl eicosanoate	15.139	2.4×10^4	0.45	5.4	2.2×10^4	0.03	4.3	2.2×10^4	4.4	4.7×10^4	0.20	2.4	4.5×10^4	0.17	3.1	4.4×10^4	3.4
Pyruvic acid _1methoxim_1TMS	4.990	9.3×10^3	0.15	5.3	1.1×10^4	1.00	9.2	7.4×10^3	10.0	8.3×10^3	−0.01	4.9	9.5×10^3	0.32	5.1	6.4×10^3	9.3
Glycolic acid_2TMS	5.297	2.2×10^4	0.19	4.3	2.1×10^4	0.52	7.5	2.2×10^4	6.4	1.7×10^4	0.34	4.4	1.7×10^4	−0.20	4.9	1.7×10^4	3.6
Alanine_2TMS	5.627	1.4×10^5	−1.11	24.8	2.2×10^5	1.24	15.6	2.3×10^5	24.3	2.5×10^5	−0.39	3.1	2.7×10^5	−0.04	3.0	2.6×10^5	2.5
Glycine_2TMS	5.630	1.3×10^5	−0.49	17.8	N.D.			N.D.		8.2×10^3	2.14	20.6	N.D.			N.D.	
Leucine_1TMS	6.180	1.1×10^5	0.28	12.9	9.8×10^4	−0.25	15.3	9.2×10^4	21.7	3.0×10^4	−0.81	20.7	2.3×10^4	1.41	22.0	2.4×10^4	21.8
Isoleucine_1TMS	6.416	1.0×10^5	0.08	14.1	9.1×10^4	−0.46	17.9	8.6×10^4	23.5	2.9×10^4	−1.11	19.1	2.2×10^4	0.41	17.7	2.4×10^4	20.2
Valine_2TMS	6.871	1.6×10^5	−0.19	11.8	1.7×10^5	0.70	10.1	1.8×10^5	12.7	1.8×10^5	0.26	4.4	1.9×10^5	−0.15	3.8	1.8×10^5	5.0
Serine_2TMS	7.267	5.8×10^4	−0.18	9.5	5.6×10^4	−0.45	17.7	5.1×10^4	22.6	2.1×10^4	−0.29	6.8	1.6×10^4	−0.12	6.4	1.6×10^4	8.6
Leucine_2TMS	7.436	1.3×10^5	−0.01	15.2	1.5×10^5	1.47	17.5	1.7×10^5	21.1	1.9×10^5	0.22	5.4	2.0×10^5	−0.23	4.8	1.9×10^5	6.1
Phosphoric acid_3TMS	7.455	2.5×10^5	0.09	4.2	2.4×10^5	0.61	8.1	2.4×10^5	4.8	1.8×10^5	−0.01	3.0	1.8×10^5	−0.19	3.6	1.7×10^5	3.3
Glycerol_3TMS	7.462	1.4×10^5	0.19	4.7	1.3×10^5	0.68	6.9	1.3×10^5	5.4	9.9×10^4	0.03	2.9	9.8×10^4	−0.03	2.9	9.1×10^4	2.6
Threonine_2TMS	7.651	6.0×10^4	0.50	12.1	5.3×10^4	−0.83	22.0	4.8×10^4	24.6	1.9×10^4	−0.48	8.3	1.6×10^4	0.14	5.3	1.5×10^4	8.3
Isoleucine_2TMS	7.659	3.4×10^4	0.34	11.1	3.6×10^4	1.48	13.3	3.8×10^4	15.6	3.9×10^4	0.36	5.3	4.0×10^4	−0.15	4.4	3.9×10^4	6.0
Proline_2TMS	7.702	8.9×10^4	−0.56	27.7	1.2×10^5	2.47	40.8	1.5×10^5	37.2	2.1×10^5	0.13	6.4	2.3×10^5	−0.21	6.0	2.2×10^5	6.4
Succinic acid_2TMS	7.791	3.0×10^4	0.38	4.4	2.8×10^4	0.53	6.9	2.9×10^4	5.8	2.3×10^4	−0.25	5.1	2.4×10^4	0.03	3.2	2.3×10^4	5.3
Glycine_3TMS	7.801	1.8×10^5	0.60	6.5	2.0×10^5	1.03	10.5	2.1×10^5	6.4	2.0×10^5	0.07	1.8	1.9×10^5	−0.09	3.0	1.9×10^5	3.2
Uracil_2TMS	8.076	1.0×10^5	0.44	9.9	9.6×10^4	0.75	17.3	1.1×10^5	13.7	1.0×10^5	−0.02	2.6	1.0×10^5	−0.08	4.7	9.8×10^4	3.5
Fumaric acid_2TMS	8.094	1.7×10^5	0.09	3.3	1.6×10^5	0.42	7.5	1.6×10^5	5.9	1.3×10^5	0.12	2.5	1.2×10^5	−0.10	4.9	1.2×10^5	2.8
Serine_3TMS	8.300	6.3×10^4	−0.43	20.6	7.9×10^4	1.87	40.5	9.5×10^4	32.3	1.2×10^5	0.12	2.4	1.2×10^5	−0.04	2.8	1.2×10^5	2.5
Alanine_3TMS	8.322	1.1×10^4	44.25	47.0	N.D.			N.D.		3.8×10^3	8.37	44.7	N.D.			N.D.	
Threonine_3TMS	8.561	4.4×10^4	−0.07	14.9	5.0×10^4	1.90	27.2	5.7×10^4	22.8	6.1×10^4	0.20	2.2	6.1×10^4	−0.01	3.7	6.2×10^4	3.0
Thymine_2TMS	8.667	1.9×10^5	0.46	6.5	1.8×10^5	0.80	12.5	1.9×10^5	10.0	1.7×10^5	0.05	2.8	1.7×10^5	−0.11	4.9	1.7×10^5	3.8
Methionine_1TMS	8.718	5.5×10^4	−0.60	12.3	5.2×10^4	−1.91	27.3	4.4×10^4	25.1	1.1×10^4	−0.49	11.5	1.1×10^4	1.34	14.2	8.0×10^3	12.4
Aspartic acid_2TMS	8.828	4.4×10^4	−0.71	15.8	3.9×10^4	−2.19	30.1	3.5×10^4	23.1	7.4×10^3	0.39	8.9	5.5×10^3	2.05	17.3	N.D.	
Malic acid_3TMS	9.429	4.8×10^4	0.34	4.2	4.3×10^4	0.74	7.1	4.5×10^4	5.6	3.6×10^4	0.13	2.8	3.6×10^4	0.03	3.6	3.4×10^4	3.2
Methionine_2TMS	9.703	5.5×10^4	1.38	24.2	6.3×10^4	5.41	50.4	7.8×10^4	28.4	9.9×10^4	−0.05	1.8	9.9×10^4	−0.35	4.4	1.0×10^5	3.1
Aspartic acid_3TMS	9.705	9.9×10^4	1.48	26.4	1.2×10^5	5.08	45.1	1.4×10^5	23.8	1.7×10^5	0.09	2.2	1.7×10^5	−0.20	3.8	1.8×10^5	3.5
Pyroglutamic acid_2TMS	9.730	1.8×10^5	3.80	19.3	1.5×10^5	5.04	23.9	1.0×10^5	4.6	1.2×10^5	2.98	16.0	1.2×10^5	3.34	17.3	7.5×10^4	2.9
Cytosine_2TMS	9.733	6.5×10^4	0.66	16.2	1.3×10^4	−2.98	107.7	6.7×10^4	23.6	6.5×10^4	−0.35	5.5	6.5×10^4	−3.31	83.4	6.4×10^4	3.5
4-Aminobutyric acid_3TMS	9.799	1.7×10^5	0.71	8.3	1.8×10^5	1.13	11.1	1.9×10^5	7.2	1.9×10^5	−0.04	1.2	1.9×10^5	−0.09	3.6	1.9×10^5	2.3
Cytosine_1methoxim_2TMS	9.944	1.9×10^3	−0.40	51.7	4.2×10^3	32.36	77.4	2.8×10^3	31.4	5.7×10^3	0.24	9.4	5.7×10^3	5.81	21.9	4.7×10^3	8.4
Phenylalanine_1TMS	9.928	1.0×10^5	−1.23	27.2	8.4×10^4	−1.78	35.2	6.9×10^4	29.7	9.8×10^3	0.58	13.8	9.8×10^3	1.39	17.8	8.1×10^3	18.7
Cysteine_3TMS	10.016	2.3×10^4	1.61	23.6	3.9×10^4	6.02	37.9	3.1×10^4	18.4	3.6×10^4	0.10	5.5	3.6×10^4	−0.80	12.2	3.5×10^4	6.0
α-Ketoglutaric acid_1_methoxim_2TMS	10.130	1.0×10^4	0.54	5.3	1.3×10^4	2.37	14.8	9.9×10^3	7.5	7.9×10^3	−0.10	4.2	7.9×10^3	0.84	10.3	7.8×10^3	5.7
Glutamic acid_3TMS	10.501	6.8×10^4	−1.66	22.2	1.1×10^5	1.64	19.1	1.2×10^5	11.4	8.6×10^4	−1.67	15.7	8.6×10^4	−0.10	3.8	1.1×10^5	2.8
Phenylalanine_2TMS	10.604	1.1×10^5	1.49	24.1	1.3×10^5	2.66	27.7	1.4×10^5	15.7	1.5×10^5	0.00	2.1	1.5×10^5	−0.25	3.6	1.5×10^5	2.6
Asparagine_3TMS	10.919	2.5×10^4	−3.20	57.9	6.6×10^4	1.09	21.5	8.6×10^4	15.7	2.3×10^4	−3.40	51.4	2.3×10^4	−0.49	5.2	5.1×10^4	2.5
Glutamine 4TMS	11.426	N.D.			N.D.			5.3×10^3	35.2	1.6×10^4	−0.34	6.3	1.6×10^4	−2.83	30.2	2.3×10^4	4.3
Aconitic acid_3TMS	11.454	2.6×10^4	−0.21	4.0	2.6×10^4	0.59	10.0	2.6×10^4	7.1	1.8×10^4	−0.16	6.7	1.8×10^4	−0.28	5.9	2.0×10^4	6.1
Putrescine_4TMS	11.480	4.6×10^5	0.36	4.0	4.8×10^5	0.67	6.8	4.9×10^5	5.1	4.2×10^5	0.05	2.2	4.2×10^5	−0.02	3.0	4.0×10^5	2.1
Glutamine_3TMS	11.676	2.6×10^4	−3.00	42.1	3.4×10^4	−2.38	31.2	6.6×10^4	10.2	3.6×10^4	−1.33	11.8	3.6×10^4	−2.57	33.4	3.5×10^4	3.2
Ornithine_4TMS	12.045	2.6×10^5	0.20	4.0	2.7×10^5	0.56	6.2	2.8×10^5	5.5	2.3×10^5	0.06	1.5	2.3×10^5	−0.06	2.9	2.2×10^5	1.8
Isocitric acid_4TMS & Citiric acid_4TMS	12.056	3.7×10^5	0.45	4.2	3.3×10^5	0.60	7.1	3.3×10^5	5.1	3.0×10^5	0.12	2.7	3.0×10^5	−0.01	4.2	2.6×10^5	4.1
Caffeine	12.255	7.7×10^4	0.17	3.5	7.5×10^4	0.34	5.8	7.6×10^4	5.1	6.3×10^4	0.37	3.4	6.3×10^4	−0.18	2.8	6.2×10^4	3.4
Adenine_2TMS	12.375	2.3×10^5	0.11	4.1	2.3×10^5	0.86	8.1	2.4×10^5	6.2	1.9×10^5	−0.14	3.2	1.9×10^5	−0.14	4.8	1.9×10^5	2.8
Fructose_1	12.511	2.5×10^5	0.30	3.7	2.3×10^5	0.64	6.5	2.2×10^5	4.7	2.0×10^5	0.06	1.7	2.0×10^5	0.11	3.1	1.8×10^5	2.0
Fructose_2	12.579	1.5×10^5	0.26	3.4	1.4×10^5	0.63	6.3	1.4×10^5	4.7	1.2×10^5	0.05	2.5	1.2×10^5	0.06	3.2	1.1×10^5	1.5

Glucose_1	12.701	1.3×10^5	0.25	3.9	1.2×10^5	0.59	7.4	1.1×10^5	6.4	1.2×10^5	−0.07	3.5	1.2×10^5	0.13	6.1	9.6×10^4	4.6
Lysine_4TMS	12.749	1.4×10^5	0.08	4.0	1.4×10^5	0.54	5.8	1.5×10^5	4.4	1.2×10^5	0.06	2.4	1.2×10^5	−0.08	3.7	1.2×10^5	2.5
Histidine_3TMS	12.750	2.1×10^4	−2.25	42.8	9.1×10^4	3.69	26.6	9.8×10^4	22.6	6.6×10^4	−1.91	21.5	6.6×10^4	0.03	4.8	1.0×10^5	4.1
Glucose_2	12.840	2.4×10^4	0.25	3.7	2.1×10^4	0.53	7.8	2.1×10^4	6.0	2.2×10^4	−0.05	5.0	2.2×10^4	−0.13	6.8	1.8×10^4	5.8
Tyrosine_3TMS	12.870	4.1×10^5	0.14	3.3	4.3×10^5	0.63	6.5	4.4×10^5	5.2	3.6×10^5	0.02	2.9	3.6×10^5	−0.05	3.2	3.5×10^5	2.2
Xanthine_3TMS	13.394	1.3×10^5	0.34	4.3	1.3×10^5	0.90	7.8	1.3×10^5	7.0	1.1×10^5	0.20	4.3	1.1×10^5	−0.23	5.9	1.1×10^5	5.3
Palmitic acid_1TMS	13.449	3.2×10^5	0.62	5.4	2.0×10^5	0.54	5.4	2.0×10^5	5.3	2.4×10^5	−0.03	3.2	2.4×10^5	0.03	2.9	1.7×10^5	2.3
Inositol	13.987	3.2×10^5	0.36	3.9	3.1×10^5	0.60	5.9	3.2×10^5	5.6	2.6×10^5	0.08	3.7	2.6×10^5	−0.14	3.6	2.5×10^5	2.4
Guanine_3TMS	14.061	2.4×10^5	0.43	4.2	2.3×10^5	0.52	6.8	2.3×10^5	6.0	1.9×10^5	0.29	4.1	1.9×10^5	−0.28	5.1	1.8×10^5	4.4
Histidine_4TMS	14.283	2.7×10^4	27.41	38.5	3.7×10^3	0.98	16.5	3.4×10^3	12.2	2.0×10^4	18.41	42.5	N.D.			N.D.	
Tryptophan_1TMS	14.315	2.8×10^4	0.21	21.7	2.0×10^4	−1.17	14.7	1.9×10^4	11.9	7.1×10^3	−1.27	18.2	7.5×10^3	−0.96	16.7	7.0×10^3	15.3
Tryptophan_2TMS	14.499	1.1×10^5	−0.59	6.2	1.3×10^5	0.41	5.0	1.3×10^5	4.2	9.4×10^4	−0.72	6.2	1.0×10^5	−0.28	5.3	1.0×10^5	3.5
Stearic acid_1TMS	14.653	2.5×10^5	0.64	5.2	1.9×10^5	0.41	5.0	1.9×10^5	4.3	2.0×10^5	0.07	3.0	1.7×10^5	0.02	3.0	1.6×10^5	3.1
Tryptophan_3TMS	14.684	3.8×10^4	12.51	35.8	1.6×10^4	0.10	5.3	1.6×10^4	6.0	7.4×10^4	7.37	28.9	3.0×10^4	0.53	8.6	2.9×10^4	15.4
Cystine_4TMS	15.104	1.6×10^4	−0.34	6.2	1.7×10^4	−0.07	5.6	1.7×10^4	5.0	1.6×10^4	0.02	5.4	1.5×10^4	−0.13	3.9	1.5×10^4	4.4
Inosine_4TMS	16.614	6.0×10^4	0.42	4.8	6.0×10^4	0.24	4.3	6.0×10^4	5.0	5.2×10^4	0.19	4.5	5.0×10^4	−0.07	5.0	5.0×10^4	3.3
Sucrose	17.114	7.3×10^5	0.22	3.1	7.1×10^5	0.21	3.6	7.0×10^5	3.9	6.1×10^5	0.07	4.8	6.0×10^5	−0.32	5.7	5.9×10^5	3.5
β-Lactose_1	17.403	2.1×10^5	−0.25	2.8	2.0×10^5	−0.10	3.4	1.9×10^5	2.3	1.8×10^5	−0.10	2.8	1.8×10^5	−0.05	4.7	1.7×10^5	2.9
β-Lactose_2	17.492	6.9×10^4	−0.21	5.2	6.5×10^4	0.06	3.9	6.3×10^4	3.6	5.0×10^4	0.09	3.4	5.1×10^4	−0.19	4.9	5.2×10^4	5.9
Trehalose & Maltose_1	17.649	1.1×10^6	0.20	2.2	1.0×10^6	0.26	3.9	1.0×10^6	3.2	9.1×10^5	0.12	3.6	9.1×10^5	−0.25	6.2	8.7×10^5	2.3
Maltose_2	17.801	7.6×10^4	0.07	2.1	7.1×10^4	0.09	5.0	7.0×10^4	2.6	6.4×10^4	0.02	4.3	6.5×10^4	−0.50	6.7	6.3×10^4	4.2
Ergosterol_1TMS	19.969	2.3×10^4	−0.55	9.0	2.4×10^4	−0.44	6.4	2.3×10^4	3.8	2.2×10^4	−0.82	8.0	2.4×10^4	−0.30	5.9	2.2×10^4	4.8
Raffinose	21.150	8.5×10^5	−0.39	4.2	8.1×10^5	−0.32	5.9	7.9×10^5	1.7	6.8×10^5	−0.10	3.7	6.6×10^5	−0.37	6.7	6.4×10^5	4.2

Δ_A(%/h), time course changing rate of peak areas when 12 samples were measured at every 2 h after silylation. Δ_B(%/h), time course changing rate of peak areas when 12 samples were measured at every 2 h after oximation. The interval from silylation to introduction into GC-MS was the same. N.D., not detected.

RESULTS AND DISCUSSION

Investigation of the oximation and silylation derivatization conditions We initially attempted to optimize the two-step derivatization conditions using the mixed standard containing the 52 metabolites mentioned previously. For oximation, a solution of methoxyamine HCl in pyridine was employed. To date, a range of reagent concentrations, reagent quantities, derivatization temperatures, and derivatization times have been reported, including 30–100 μL of a 20–40 mg/mL solution of methoxyamine HCl in pyridine, 25–50°C, and 1.5–24 h (2–5,7,11–13,15–22). For the silylation reaction, the most commonly employed reagents are MSTFA, MSTFA+1% trimethylchlorosilane (TMCS), *N*,*O*-bistrifluoroacetamide (BSTFA), and BSTFA+1% TMCS. Both MSTFA and MSTFA+1% TMCS have been used in a number of metabolomics methods (7,13,17). However, Fiehn et al. (7,23) suggested the use of *N*-methyl-*N*-*tert*-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) instead of MSTFA for the determination of amino acids and polyamines. However, they also reported that sugars and sugar alcohols would be incompletely derivatized due to the steric hindrance arising from the *tert*-butyldimethylsilyl group. As for the silylation procedure, a range of reagent quantities, derivatization temperatures, and derivatization times have been examined, i.e., 30–200 μL, 25–60°C, and 30–240 min (2–5,7,11–13,15–22).

Therefore, for the methoximation and silylation procedures reported in this research we employed 80 μL of a 20 mg/mL solution of methoxyamine HCl in pyridine and 40 μL of MSTFA, which was commonly used in batch derivatization. Then, we examined the effects of derivatization temperature and time.

More specifically, for the methoximation process, the derivatization temperature was set to 37°C or 70°C, and the derivatization time was set to 30, 60, or 90 min. Since drug molecules and sterols are often derivatized at 60–100°C (24,25), we also examined the transformation at 70°C. The results obtained for these methoximation investigations are shown in Table S2. Shepherd et al. (20) reported that glucose and fructose are only partially methoximated at 30°C after 45 min, and sucrose is hydrolyzed to glucose and fructose at 100°C over 45 min (20). Also, the reducing disaccharide β-lactose was incompletely methoximated at 37°C after 30 min, which resulted in significant peak broadening caused by multi peaks. Therefore, it was necessary to employ the following methoximation conditions: 70°C for 30 min or 37°C for a minimum of 60 min (optimal, 90 min; Fig. S1). Methoximation conditions of 37°C for a minimum of 60 min were also preferred for glucose and maltose. The peak area of the sucrose derivative decreased slightly at 70°C, while those of the glucose and fructose derivatives increased upon increasing the reaction time at the same temperature. However, such changes were not observed at 37°C. Moreover, it was found that the peak areas of pyruvic acid_1methoxim_1TMS and α-ketoglutaric acid_1methoxim_2TMS, both of which are α-keto acid derivatives, increased significantly at 70°C, as did that of pyroglutamic acid_2TMS. At the same time, the signals corresponding to glutamine (i.e., Gln_3TMS, Gln_4TMS) essentially disappeared at this temperature. Similarly, the peak corresponding to cytosine_2TMS decreased in intensity at 70°C (Fig. S2). The data in Figs. S2 and S3 were obtained from different analytical batches at the derivatization temperature, which led to a difference in sensitivity. Therefore, the peak areas of the metabolites were divided by that of caffeine unaffected by derivatization.

For silylation, derivatization temperatures of 37°C and 70°C were investigated in addition to derivatization times of 15, 30, 60, and 90 min. The results obtained for these silylation investigations are shown in Table S3. Interestingly, we found that no differences in

the peak area were observed between the transformations carried out at 37°C and 70°C for 48 of the metabolites (i.e., excluding tryptophan, asparagine, alanine, and glutamine, Fig. S3). However, in the case of tryptophan derivatization, three chromatographic peaks were observed, namely Trp_1TMS, Trp_2TMS, and Trp_3TMS. The peak area of Trp_3TMS was found to increase with the longer reaction times at both 37 and 70°C, although the reaction at 70°C over 90 min was not complete. In contrast, at 37°C, the area of the most intense signal, which corresponded to Trp_2TMS, remained relatively constant. Similarly, the silylation of asparagine was confirmed by the presence of two chromatographic peaks corresponding to Asn_3TMS and Asn_4TMS. In this case, the peak area of the Asn_4TMS signal was enhanced upon increasing the reaction time for both 37°C and 70°C reactions, although the reaction at 70°C over 90 min was not complete. However, at 37°C, the area of the most intense signal, i.e., corresponding to Asn_3TMS, remained relatively constant. Furthermore, the silylation of alanine was also confirmed by the presence of two chromatographic peaks (Ala_2TMS and Ala_3TMS), where the area of the Ala_3TMS signal increased with longer reaction times at both the examined temperatures. The fact that the peak corresponding to glutamine (i.e., Gln_3TMS, Gln_4TMS), which essentially disappeared at 70°C, indicates that it might be necessary to consider the effects of the methoximation conditions before continuing with the silylation process.

Considering the aforementioned results, we determined that the optimal conditions for the methoximation and silylation transformations were 90 min at 37°C and 30 min at 37°C, respectively. The fact that these results corresponded to those reported by Abbiss et al. (17) and Zarate et al. (18) indicated that the same temperature could be employed for both derivatization processes.

Comparison of the batch and sequential derivatization processes We then compared the three different derivatization processes, namely batch derivatization, sequential derivatization, and batch oximation and sequential silylation. Using the mixed metabolite sample (i.e., containing the aforementioned 52 metabolites), measurements were taken at 2-h intervals for each method to obtain data over a 24 h period ($n = 12$, Fig. 1). In the batch derivatization process, the derivatization reagents were added manually to each tube using a pipette, and all samples were derivatized simultaneously using a shaker. Since the methoximation and silylation times of the tubes were maintained constant, the time between completing the derivatization reaction and commencing the GC analysis differed among samples. In terms of the sequential derivatization process, the derivatized samples were analyzed by GC following the addition of the derivatization reagent, heating, and shaking for a predetermined time using the GC autosampler. In this case, the methoximation and silylation derivatization times and the time prior to GC analysis were maintained constant. Moreover, in the batch oximation and sequential silylation process, the methoximation reaction was carried out using the previously described batch technique, while silylation and introduction of the samples into the GC apparatus were carried out using the GC autosampler. In this case, the methoximation and silylation times were maintained constant, but the time between completing the methoximation reaction and beginning the silylation process differed among samples.

The results obtained for these three transformation methods are shown in Table 1 and Fig. 2. It was apparent that the addition of fatty acids and fatty acid methyl ester (i.e., heptanoic acid, decanoic acid, eicosanoic acid, and methyl eicosanoate) to the derivatization reagents present in the mixed metabolite sample resulted in good repeatability (i.e., 1.7–6.7%, $n = 12$).

Interestingly, we found that the peak areas of palmitic and stearic acids were larger following the batch derivatization compared to the sequential derivatization. It was expected that this phenomenon was caused by the elution of detaching agent from the polypropylene tube employed as the reaction vessel for batch derivatization in the additional control experiments.

A comparison of the results obtained from the treatment with various volumes of methoxyamine HCl solution in pyridine (20 mg/mL) and MSTFA (i.e., 80 and 40 μL, 40 and 80 μL, total volume 120 μL) revealed that the peak areas of a number of amino acid derivatives increased when the volume of MSTFA employed for the derivatization increased. In addition, in the case of proline_2TMS and methionine_2TMS, the peak area intensities and repeatability were enhanced (Table 1, Fig. S4). Furthermore, the trimethylsilylation of histidine was confirmed by the observation of chromatographic peaks corresponding to His_3TMS and His_4TMS. Upon increasing the volume of MSTFA employed, the His_3TMS peak area decreased in intensity, while that of His_4TMS increased upon lengthening the time between derivatization and GC analysis during the batch process. It was therefore apparent that the peak area intensity and repeatability were again enhanced upon increasing the quantity of MSTFA employed.

The peak areas of the pyruvic acid, α-ketoglutaric acid, cytosine, and glutamine derivatives were found to vary depending on the total methoximation time employed (i.e., from the beginning of the methoximation process to commencing the silylation reaction). More specifically, slight increases in the peak area were observed for pyruvic acid_1methoxim_1TMS and α-ketoglutaric acid_1-methoxim_2TMS upon increasing the methoximation time. In contrast, the peak areas of cytosine_2TMS and glutamine (i.e., Gln_3TMS, Gln_4TMS) demonstrated the opposite trend. Although the mass spectrum of cytosine_1methoxim_2TMS is not registered in any of the databases examined, we confirmed the structure of this product as indicated in Fig. S5. An increase in its peak area was observed upon increasing the methoximation time. Furthermore, the derivatization of glutamine was confirmed by the presence of chromatographic peaks corresponding to Gln_3TMS and Gln_4TMS, the peak areas of which decreased upon increasing the methoximation time. In terms of repeatability, at a constant methoximation time, the glutamine peak areas for the batch and sequential derivatization methods were 12% (mean area: 3.6×10^4) and 3.2% (mean area: 3.5×10^4) for Gln_3TMS, and 6.3% (mean area: 1.6×10^4) and 4.3% (mean area: 2.3×10^4) for Gln_4TMS. These results suggest that an increased contact time between glutamine and the methoximation reagent resulted in increased degeneration of glutamine, likely due to the known decomposition of glutamine under basic conditions (26,27). As such, either batch or sequential derivatization was deemed suitable for the derivatization of glutamine. Other metabolites showed no apparent variation in the peak area upon increasing the methoximation time.

Upon extending the time between commencing the silylation derivatization reaction and GC analysis, the peak areas of the alanine, asparagine, tryptophan, histidine, glutamine, and glutamic acid derivatives were found to vary. It is possible that such variation took place because the formation of the alanine (Ala_2TMS, Ala_3TMS), asparagine (Asn_3TMS, Asn_4TMS), tryptophan (Trp_1TMS, Trp_2TMS, Trp_3TMS), histidine (His_3TMS, His_4TMS), and glutamine (Gln_3TMS, Gln_4TMS) derivatives was affected by a combination of the steric hindrance and the reactivity of the TMS group towards secondary amines. However, the formation of Glu_3TMS was confirmed, while that of Glu_4TMS was not. Furthermore, glutamine has been reported to undergo spontaneous cyclization to form pyroglutamic acid with a loss of ammonia under extreme acidic, alkaline, or high-temperature conditions (26,27). Upon heating, glutamine is converted to glutamic acid, which is subsequently converted to pyroglutamic acid (14,25,26), thereby

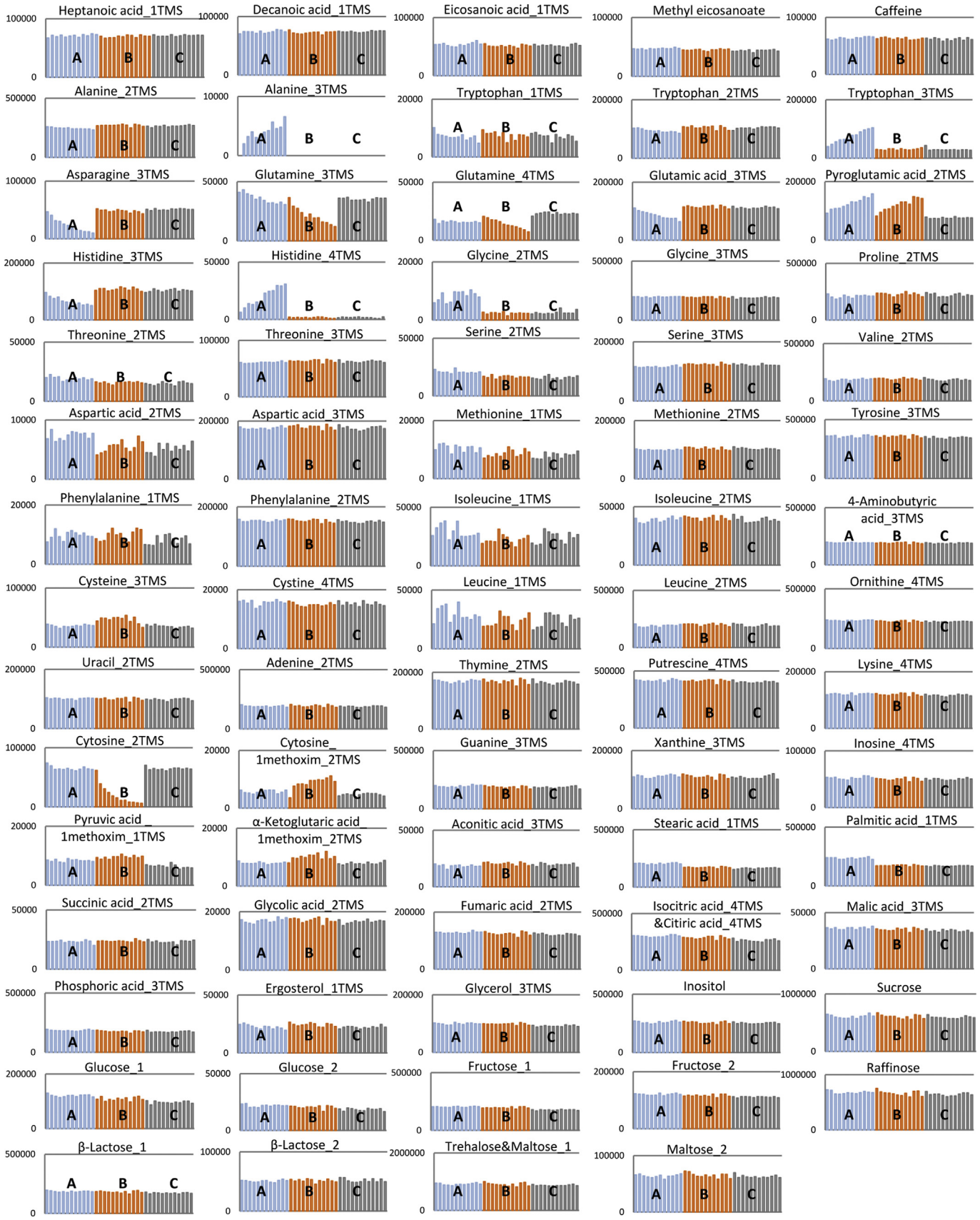

FIG. 2. Comparison of the three methods in measuring the peak area of the 52 metabolites contained in the mixture. A, batch derivatization; B, batch oximation and sequential silylation; C, sequential derivatization. The graphs are presented in the order measured, from left to right, for each method. Measurements used a 20 mg/mL solution of methoxyamine HCl in pyridine:MSTFA = 40 μL:80 μL.

TABLE 2. Time course changing of metabolites in sake sample comparing with the three methods A, B, C and MSTFA quantity (n =12).

Compound	RT (min)	Methoxyamine HCl in pyridine 80 μL, MSTFA 40 μL							Methoxyamine HCl in pyridine 40 μL, MSTFA 80 μL						
		A: Batch deriv.			B: Batch methoximation and sequential silylation			C: Sequential deriv.	A: Batch deriv.			B: Batch methoximation and sequential silylation			C: Sequential deriv.
		Peak area av.	Δ_A (%/h)	RSD (%)	peak area Av.	Δ_B (%/h)	RSD (%)	Peak area av.	RSD (%)	Peak area av.	Δ_A (%/h)	RSD (%)	Peak area av.	Δ_B (%/h)	RSD (%)	peak area Av.	RSD (%)
Heptanoic acid_1TMS	6.315	1.2×10^5	0.13	3.7	1.2×10^5	0.18	2.3	1.2×10^5	2.1	6.3×10^4	0.32	4.0	6.2×10^4	0.53	5.5	6.0×10^4	5.0
Decanoic acid_1TMS	9.111	1.3×10^5	0.05	3.0	1.2×10^5	−0.08	1.7	1.2×10^5	2.1	6.2×10^4	0.33	3.8	6.0×10^4	0.48	5.9	5.8×10^4	3.8
Eicosanoic acid_1TMS	15.780	9.9×10^4	0.10	2.0	9.5×10^4	−0.09	2.8	9.4×10^4	2.6	4.3×10^4	0.42	5.7	4.2×10^4	0.18	3.8	4.1×10^4	4.0
Methyl eicosanoate	15.160	1.7×10^4	0.29	3.4	1.8×10^4	−0.08	4.1	1.7×10^4	3.3	3.4×10^4	0.14	2.4	3.3×10^4	−0.04	2.6	3.3×10^4	2.6
Pyruvic acid _1methoxim_1TMS	5.008	3.2×10^3	0.28	9.3	3.4×10^3	0.32	8.3	2.9×10^3	7.9	3.0×10^3	−0.40	17.8	3.3×10^3	−0.13	14.2	3.0×10^3	16.4
Alanine_2TMS	5.643	1.2×10^6	−0.16	9.6	1.4×10^6	0.39	15.5	1.6×10^6	13.2	2.6×10^6	0.20	4.7	2.7×10^6	0.57	7.0	2.7×10^6	7.2
Glycine_2TMS	5.646	2.7×10^4	6.49	32.3	5.4×10^3	−0.62	10.0	4.8×10^3	7.0	1.9×10^4	1.45	28.8	8.2×10^3	−0.13	10.6	8.0×10^3	11.5
Leucine_1TMS	6.197	2.2×10^5	0.79	11.8	2.2×10^5	0.74	8.5	1.9×10^5	14.0	2.3×10^4	1.07	11.8	2.3×10^4	0.05	15.1	1.8×10^4	13.9
Isoleucine_1TMS	6.435	1.3×10^5	0.65	12.2	1.3×10^5	0.77	9.1	1.1×10^5	15.3	1.2×10^4	0.93	13.5	1.2×10^4	−0.06	14.5	9.6×10^3	33.8
Valine_2TMS	6.891	1.7×10^5	−0.11	7.8	1.7×10^5	−0.12	11.1	1.9×10^5	12.7	3.4×10^5	0.32	5.1	3.5×10^5	0.53	5.8	3.4×10^5	7.5
Serine_2TMS	7.287	9.3×10^4	0.75	11.3	9.6×10^4	0.82	8.6	8.4×10^4	12.4	1.1×10^4	−0.22	7.9	1.1×10^4	−0.29	5.6	9.1×10^3	10.6
Leucine_2TMS	7.457	1.6×10^5	−0.40	9.7	1.6×10^5	−0.40	12.9	1.9×10^5	15.7	4.2×10^5	0.32	5.9	4.4×10^5	0.39	5.4	4.2×10^5	7.5
Phosphoric acid_3TMS	7.475	8.8×10^5	0.06	5.0	8.8×10^5	−0.08	5.1	8.5×10^5	4.3	8.3×10^5	0.49	6.2	8.5×10^5	0.40	4.8	8.3×10^5	6.8
Glycerol_3TMS	7.492	6.7×10^6	−0.17	3.4	6.7×10^6	−0.15	3.0	6.6×10^6	3.0	6.4×10^6	0.25	3.0	6.5×10^6	0.25	3.8	6.4×10^6	4.2
Threonine_2TMS	7.675	8.7×10^4	0.71	10.5	8.7×10^4	0.83	8.3	7.7×10^4	9.9	3.0×10^4	0.40	6.3	3.2×10^4	0.27	5.3	2.8×10^4	7.5
Isoleucine_2TMS	7.680	2.3×10^4	−0.51	9.4	2.3×10^4	−0.51	12.7	2.6×10^4	15.4	5.0×10^4	0.35	6.5	5.4×10^4	0.46	6.6	5.1×10^4	8.5
Proline_2TMS	7.727	2.2×10^5	−1.23	20.7	2.7×10^5	−0.33	21.1	3.7×10^5	27.6	1.1×10^6	0.03	4.2	1.2×10^6	0.43	5.4	1.2×10^6	6.9
Succinic acid_2TMS	7.812	1.9×10^5	0.21	5.7	1.8×10^5	0.15	3.6	1.8×10^5	4.1	1.8×10^5	0.63	6.2	1.8×10^5	0.53	5.5	1.8×10^5	6.6
Glycine_3TMS	7.823	9.5×10^5	0.03	5.2	9.6×10^5	0.11	4.7	9.2×10^5	5.0	9.5×10^5	0.31	4.6	6.2×10^5	0.53	7.8	5.0×10^5	10.4
Uracil_2TMS	8.095	2.1×10^4	0.20	5.8	2.0×10^4	0.18	4.6	2.0×10^4	4.4	2.0×10^4	0.51	7.4	2.2×10^4	0.90	10.0	2.1×10^4	7.3
Fumaric acid_2TMS	8.114	4.4×10^3	0.55	12.8	4.9×10^3	−0.08	6.6	4.4×10^3	9.1	5.9×10^3	0.91	12.4	6.4×10^3	0.13	14.0	6.1×10^3	6.7
Serine_3TMS	8.318	9.5×10^4	−0.65	13.4	9.6×10^4	−0.74	16.9	1.1×10^5	19.0	3.1×10^5	0.41	5.9	3.3×10^5	0.55	6.2	3.2×10^5	7.2
Threonine_3TMS	8.581	2.9×10^4	−0.72	13.3	2.8×10^4	−0.69	12.7	3.2×10^4	16.1	7.5×10^4	0.53	5.9	7.9×10^4	0.46	5.5	7.5×10^4	8.0
Thymine_2TMS	8.690	3.4×10^3	−0.16	11.9	3.0×10^3	−0.41	6.7	3.0×10^3	10.1	3.2×10^3	0.08	16.2	3.3×10^3	0.44	17.2	3.3×10^3	15.0
Methionine_1TMS	8.742	5.0×10^3	2.71	22.6	5.2×10^3	1.66	14.4	4.2×10^3	16.6	N.D.			N.D.			N.D.	
Aspartic acid_2TMS	8.848	9.5×10^4	2.39	21.2	9.4×10^4	2.01	14.9	8.0×10^4	13.4	4.8×10^3	0.59	10.2	4.6×10^3	0.16	15.8	3.5×10^3	12.4
Malic acid_3TMS	9.448	5.0×10^3	−0.21	8.9	4.7×10^3	−0.15	5.9	4.6×10^3	8.2	4.8×10^3	−0.48	9.9	4.9×10^3	0.64	9.0	4.8×10^3	9.5
Methionine_2TMS	9.721	9.7×10^3	−0.61	14.8	9.7×10^3	−0.69	13.1	1.0×10^4	12.9	1.8×10^4	−0.27	5.8	1.9×10^4	0.33	7.9	1.8×10^4	9.1
Aspartic acid_3TMS	9.726	3.2×10^5	−0.72	15.3	3.2×10^5	−0.76	13.2	3.4×10^5	9.5	6.1×10^5	0.50	5.8	6.3×10^5	0.60	5.9	6.1×10^5	7.0
Pyroglutamic acid_2TMS	9.750	4.1×10^5	1.23	9.4	4.2×10^5	1.08	8.0	3.5×10^5	4.6	3.8×10^5	0.65	6.7	4.2×10^5	1.16	8.5	3.7×10^5	7.4
Cytosine_2TMS	9.758	3.6×10^3	−0.20	8.6	1.9×10^3	−3.09	49.0	3.7×10^3	16.4	3.4×10^3	0.93	12.5	2.8×10^3	−2.63	30.0	3.8×10^3	6.9
4-Aminobutyric acid_3TMS	9.818	3.7×10^4	0.04	5.3	3.6×10^4	0.08	4.0	3.5×10^4	4.6	3.6×10^4	0.30	5.1	2.4×10^4	0.18	7.2	1.9×10^4	10.7
Phenylalanine_1TMS	9.948	5.5×10^4	4.79	33.8	5.4×10^4	4.07	23.0	4.4×10^4	18.3	2.1×10^3	−1.49	26.5	2.3×10^3	−0.74	19.0	2.3×10^3	25.2
Cysteine_3TMS	10.040	1.8×10^4	−2.30	26.2	1.9×10^4	−2.34	26.3	1.8×10^4	22.0	2.8×10^4	−0.78	15.0	3.2×10^4	0.03	14.2	3.0×10^4	21.2
α-Ketoglutaric acid_1_methoxim_2TMS	10.150	7.4×10^3	0.36	8.4	7.1×10^3	−0.32	8.2	7.0×10^3	5.8	7.1×10^3	0.25	10.0	7.5×10^3	0.21	7.9	6.9×10^3	8.7
Glutamic acid_3TMS	10.520	5.0×10^5	−0.58	9.5	5.3×10^5	−0.44	6.8	5.2×10^5	5.3	6.3×10^5	0.35	6.2	6.8×10^5	0.52	6.0	6.3×10^5	8.1
Phenylalanine_2TMS	10.620	1.2×10^5	−0.76	13.6	1.2×10^5	−0.70	10.5	1.2×10^5	5.8	1.6×10^5	0.47	5.9	1.7×10^5	0.48	4.8	1.6×10^5	7.8
Asparagine_3TMS	10.940	1.8×10^4	−0.60	8.0	1.9×10^4	−0.43	5.9	2.0×10^4	4.1	1.3×10^4	0.19	7.4	1.4×10^4	0.13	6.5	1.3×10^4	10.7
Glutamine 4TMS	11.430	3.4×10^3	−1.42	21.0	3.1×10^3	−3.39	35.8	6.8×10^3	10.4	3.0×10^4	0.32	4.3	2.1×10^4	−1.91	19.6	3.3×10^4	5.1
Putrescine_4TMS	11.500	1.2×10^4	0.54	7.5	1.2×10^4	0.36	4.0	1.1×10^4	4.5	1.2×10^4	−0.28	7.8	9.8×10^3	0.48	12.6	7.3×10^3	16.3
Glutamine_3TMS	11.700	6.1×10^4	−1.43	13.7	4.2×10^4	−2.41	28.5	7.4×10^4	5.9	5.7×10^4	0.27	6.8	3.9×10^4	−1.85	21.2	5.1×10^4	11.3
Ornithine_4TMS	12.060	6.1×10^4	0.21	5.3	5.9×10^4	0.34	4.9	5.5×10^4	4.6	6.2×10^4	0.23	6.9	5.1×10^4	0.52	8.3	4.0×10^4	12.2
Isocitric acid_4TMS & Citiric acid_4TMS	12.080	6.7×10^4	0.46	6.7	6.4×10^4	0.38	4.7	6.2×10^4	3.6	6.2×10^4	0.43	5.2	6.3×10^4	0.33	3.8	6.0×10^4	7.1
Adenine_2TMS	12.400	3.2×10^3	0.29	13.2	3.0×10^3	−0.67	11.4	2.9×10^3	15.2	3.3×10^3	−0.75	14.4	3.2×10^3	−0.56	14.7	3.2×10^3	12.5
Glucose_1	12.770	1.6×10^7	−0.15	4.7	1.6×10^7	−0.10	4.0	1.6×10^7	2.5	1.5×10^7	0.31	3.8	1.5×10^7	0.28	3.2	1.5×10^7	4.7
Lysine_4TMS	12.790	7.5×10^4	0.12	8.3	8.7×10^4	0.01	5.4	8.4×10^4	2.9	8.6×10^4	0.10	3.8	9.0×10^4	0.38	5.5	8.5×10^4	2.7
Histidine_3TMS	12.780	1.6×10^5	−0.36	3.2	1.6×10^5	0.55	3.5	1.5×10^5	3.7	1.5×10^5	−0.20	3.3	1.3×10^5	−0.04	3.3	1.1×10^5	8.6
Glucose_2	12.880	6.6×10^6	−0.26	3.6	6.4×10^6	−0.27	3.7	6.4×10^6	2.8	6.0×10^6	0.22	2.5	6.0×10^6	0.30	4.4	6.0×10^6	3.8
Tyrosine_3TMS	12.910	5.0×10^5	0.08	5.5	5.0×10^5	0.03	4.9	4.8×10^5	5.3	4.7×10^5	0.34	7.3	5.1×10^5	0.32	5.5	4.7×10^5	6.9
Xanthine_3TMS	13.410	2.2×10^4	0.42	8.0	2.1×10^4	0.22	6.0	2.2×10^4	6.3	2.2×10^4	0.52	7.9	2.3×10^4	0.20	6.1	2.1×10^4	8.6
Palmitic acid_1TMS	13.470	9.8×10^4	0.38	11.8	7.1×10^3	1.28	22.6	4.5×10^3	23.9	4.4×10^4	2.28	16.7	1.1×10^4	1.01	11.6	9.3×10^3	14.6
Inositol	14.010	5.6×10^3	0.01	9.9	5.2×10^3	−0.37	8.2	5.1×10^3	7.5	5.2×10^3	0.49	14.2	5.5×10^3	0.28	6.6	5.4×10^3	12.8

Guanine_3TMS	14.080	7.4×10^3	−0.28	10.8	7.4×10^3	0.02	6.2	6.6×10^3	7.1	6.7×10^3	−0.02	9.8	7.4×10^3	−0.04	7.5	6.6×10^3	8.4
Histidine_4TMS	14.300	3.3×10^3	21.40		N.D.			N.D.		N.D.			N.D.			N.D.	
Stearic acid_1TMS	14.670	3.7×10^4	1.67	21.3	8.1×10^3	0.02	10.2	7.4×10^3	9.6	2.9×10^4	0.91	11.6	1.7×10^4	−0.30	7.0	1.7×10^4	7.1
Tryptophan_3TMS	14.700	7.5×10^4	0.22	6.6	7.4×10^4	0.23	5.1	7.1×10^4	5.3	7.7×10^4	0.10	6.2	8.0×10^4	0.23	4.8	7.7×10^4	7.6
Cystine_4TMS	15.120	2.4×10^4	1.09	8.5	2.5×10^4	0.84	9.6	2.3×10^4	4.8	2.7×10^4	0.27	6.3	2.9×10^4	−0.18	4.5	2.5×10^4	8.5

$\triangle_A$(%/h), time course changing rate of peak areas when 12 samples were measured at every 2 h after silylation. $\triangle_B$(%/h), time course changing rate of peak areas when 12 samples were measured at every 2 h after oximation. The interval from silylation to introduction into GC-MS was the same. N.D., not detected.

resulting in an increase in the pyroglutamic acid peak area with longer derivatization time. However, no difference was observed for the sequential derivatization process. Moreover, no differences were observed in the peak areas corresponding to the organic acids, sugars, nucleobases, and other amino acids (i.e., valine, leucine, isoleucine, serine, threonine, proline, glycine, methionine, aspartic acid, 4-aminobutyric acid, putrescine, ornithine, phenylalanine, tyrosine, lysine, and cysteine) with longer silylation times.

Verification of the developed procedure using a sake sample Finally, we employed a sample of sake in order to compare the three derivatization methods described herein, targeting only the compounds present in the mixed metabolite standard (Table 2). As observed for the mixed metabolites, the peak areas of the cytosine and glutamine derivatives were found to vary depending on the total methoxymation time employed (i.e., from the beginning of the methoxymation process to commencing the silylation reaction). Upon extending the time between commencing the silylation derivatization reaction and GC analysis, the peak areas of the alanine and glutamine derivatives were found to vary. The derivatization of glutamine was confirmed by the presence of chromatographic peaks corresponding to Gln_3TMS and Gln_4TMS, which decreased upon increasing the methoxymation time. In terms of repeatability, at a constant methoxymation time, the glutamine peak areas for the batch and sequential derivatization methods were 6.8% (mean area: 5.7×10^4) and 11% (mean area: 5.1×10^4), respectively, for Gln_3TMS, and 4.3% (mean area: 3.0×10^4) and 5.1% (mean area: 3.3×10^4), respectively, for Gln_4TMS. As observed for the mixed metabolites, an increase in the quantity of MSTFA employed resulted in the increased peak areas for a number of the amino acids in the sake sample. However, no increases were observed for glycine, 4-aminobutyric acid, putrescine, ornithine, or lysine, and the peak areas after the sequential derivatization were 50−65% of those observed after the batch derivatization when increased volumes of MSTFA were employed. This may be due to incomplete derivatization caused by insufficient reaction times and temperatures. Finally, no differences in peak areas were observed for the organic acids, nucleobases, and sugars upon increasing the quantity of MSTFA.

In this study, a GC/MS-based metabolomics consisting of oximation and silylation was investigated in an attempt to improve the peak area repeatability during the batch derivatization. This was a challenging task, as the time between completing the derivatization process and GC analysis differs from sample to sample, thereby leading to an extended derivatization and/or degeneration of the metabolites. Interestingly, we found that the time between the derivatization and GC analysis could be maintained relatively constant using an autosampler capable of performing both these processes. More specifically, differences in the metabolite peak areas of the various derivatives were observed upon changing the silylation time, the methoxymation time, and the quantity of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide employed during the treatment. In addition, in the batch derivatization process, variations in the peak area were observed for alanine, asparagine, tryptophan, histidine, glutamine, and glutamic acid. In the process, the silylation time exhibited the largest influence on the derivatives of histidine, alanine, asparagine, and tryptophan, while both the methoxymation and silylation times influenced the peak areas of the glutamine and glutamic acid derivatives. As metabolomics involves the simultaneous analysis of multiple components, it is practically impossible to perform complete derivatization for all metabolites and employ derivatization conditions that inhibit the denaturation of all species. However, we confirmed that it is possible to acquire data of high quality and high reproducibility by developing an improved understanding of the sensitive

metabolites and carefully managing the derivatization temperature and time using the systems described herein.

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