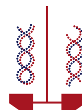

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Solid-phase analytical derivatization for gas-chromatography–mass-spectrometry-based metabolomics

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A novel derivatization method for gas chromatography/mass spectrometry (GC/MS)-based metabolomics was developed, based on solid-phase analytical derivatization (SPAD) with methoximation followed by trimethylsilylation. This SPAD method realized derivatization on solid phases combining strong anion exchange with strong cation exchange. To omit a sample condensation process, GC/MS injection was performed using a large-volume injection mode. This mode uses a stomach-shaped insert, and enables a large quantity of sample to be vaporized and introduced into the GC/MS system. In the present study, several parameters were investigated for each SPAD step. The optimal derivatization conditions were determined to be 3-min-methoximation with 5 μL of >5% methoxyamine solution, and 10-min-trimethylsilylation with 25 μL of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA). Derivatized analytes were effectively eluted with 25 μL of *n*-hexane. The influences of coexisting substances were also investigated. Coexisting saccharides did not significantly affect the derivatization of analytes. Moreover, saccharides were efficiently washed out using 80% (v/v) acetonitrile in water. The influences of coexisting sodium chloride were negated by dilution of the sample solution with water. The developed method enables the derivatization of both anionic and cationic metabolites, and high-throughput sample preparation. The coverage of detectable metabolites for the developed method was similar to that of the conventional method. This is the first report of a SPAD-based human plasma metabolome analysis protocol.

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[**Key words:** Derivatization; Sample preparation; Solid phase; Gas chromatography-mass spectrometry; Metabolomics; Amino acids; Organic acids; Organic bases]

Gas chromatography-mass spectrometry (GC/MS) has been frequently used for analyzing volatiles and hydrophilic low-molecular-weight metabolites. GC/MS provides efficient analyses and reproducible results (1). However, improvements may still be made in GC/MS methods, including sample preparation steps in metabolomics.

Many metabolites have polar functional groups, such as carboxyl, amino, and hydroxyl groups. Hence, these metabolites are thermally labile at the temperatures required for GC. Additionally, these metabolites typically exhibit low volatility, or may be irreversibly adsorbed onto the column stationary phase. Hence, it is necessary to derivatize these metabolites prior to GC analysis. Derivatization using methoximation followed by trimethylsilylation (TMS) has been widely used in GC/MS-based metabolomics (2–4). Generally, derivatization is performed after extraction (for example, liquid–liquid extraction (LLE)), and dehydration using a vacuum freeze dryer to prevent hydrolysis of the TMS reagents. This dehydration process makes the derivatization step time-consuming and laborious.

Various approaches have been developed which combine the extraction and derivatization steps, to reduce the complexity and number of experimental steps (5–8). Derivatization on a solid phase, termed solid-phase analytical derivatization (SPAD), has been widely used (9,10). Because SPAD realizes solid-phase extraction (SPE) and derivatization simultaneously, it exhibits high selectivity, low organic solvent consumption, automation with any chromatographic system, compatibility with a wide range of complicated matrices, and an absence of emulsions. Moreover, the solid phases used in SPAD are same as those used in SPE, and are commercially available.

SPAD has been used for the derivatization of the following compounds. For phenols, methods using either a strong anion-exchange solid phase with pentafluoropyridine (11) or a C_{18} solid phase with acetic anhydride (12) have been reported. For organic acids, methods using a strong anion-exchange solid phase with either methyl iodide (13) or *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) (14) have been reported.

Although these approaches have been reported to be effective in improving throughput, they have not yet been applied to metabolomics because of their selectivity. Abundant organic ions (anionic and cationic) are important analytical targets in metabolomics. Hence, the selectivity of SPAD hinders the realization of broad and high-throughput analyses. However, simultaneous derivatization of a comprehensive range of ionic metabolites may overcome this problem. Solid-phase derivatization may be achieved by combining strong anion-exchange solid phases with strong cation-exchange

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solid phases. Additionally, large-volume injection (LVI) to a GC/MS system would omit the need for concentration of the eluates from the solid phase, thus shortening the sample preparation time.

In the present study, a novel approach for the SPAD of a large variety of ionic metabolites was developed. A proof-of-concept was demonstrated and evaluated using selected standard compounds to realize simple and high-throughput methods for GC/MS-based metabolomics. Subsequently, the developed method was applied to human serum samples and compared with a conventional method. Concept and scheme examined in this study are shown in Fig. 1.

MATERIALS AND METHODS

Chemicals L-valine, L-(−)-threonine, and L-methionine standards were purchased from Tokyo Chemical Industry (Tokyo, Japan). L-(−)-proline, glycine, β-alanine, L-aspartic acid, 4-aminobutyric acid (GABA), anthranilic acid, L-(−)-phenylalanine, L-(+)-lysine monohydrochloride, fumaric acid, α-ketoglutaric acid, phosphoenolpyruvic acid monopotassium salt (PEP), citric acid monohydrate, 50% gluconic acid solution, and cadaverine dihydrochloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). DL-asparagine monohydrate, maleic acid, and D-glucosamine hydrochloride were purchased from Nacalai Tesque (Kyoto, Japan). 3-Hydroxytyramine (dopamine) was purchased from Sigma−Aldrich (MD, USA). Liquid chromatography−mass spectrometry (LC-MS) grade ultrapure water, chromatography grade *n*-hexane, infinity pure grade pyridine, 0.1 M hydrochloric acid (HCl) and 0.1 M sodium hydroxide (NaOH) solutions were purchased from Wako Pure Chemical Industries. LC-MS grade acetonitrile was purchased from Kanto Chemical (Tokyo, Japan), and 35% HCl was purchased from Kishida Chemical (Osaka, Japan). Methoxyamine hydrochloride (Sigma−Aldrich) and MSTFA (GL Sciences, Tokyo, Japan) were prepared for use in methoximation and trimethylsilylation, respectively. Phenanthrene was purchased from Wako Pure Chemical Industries, for use as an internal standard. D-(−)-Fructose, D-(+)-galactose, D-mannose, and sodium chloride (Wako Pure Chemical Industries) and anhydrous D-(+)-glucose (Nacalai Tesque) were used for the evaluation of coexisting substances. For experiments with human plasma samples, SRM 1950 (Metabolites in Human Plasma) was obtained from NIST (MD, USA) and stored at −80 °C until analysis.

Selection of analytes for method evaluation To examine the analytical conditions of our proposed methods, twelve amino acids, seven organic acids, and three organic bases were selected as analytes, based on their functional groups. The corresponding values of pK_a, retention index (RI), *log P*, and the *m/z* values of their ions are listed in Table S1.

Preparation of standard solutions Standard solutions were prepared by dissolving analytes in either 0.2 M HCl (for aspartic acid) or water (for the remaining analytes). A stock mixed standard solution (final concentrations: 0.5 mM for asparagine; 0.1 mM for cadaverine, glucosamine, and dopamine; 0.2 mM for the remaining analytes) was prepared and stored in dark conditions at −30 °C. The solvent for the stock mixed standard solution was 2.0 mM HCl. An internal standard solution of 0.1 mM phenanthrene in *n*-hexane was prepared and stored under dark conditions at 4 °C.

Standard solutions were prepared by mixing 100 μL of a stock standard solution, 70 μL of water, and 30 μL of 10 mM NaOH. Subsequently, the standard solution was adjusted to pH 7, which is representative of physiological pH. Standard solutions were mixed with acetonitrile at a ratio of 1:4 before loading onto the solid phase. This process was consistent with conditions after deprotonation. The phenanthrene internal standard solution was diluted with *n*-hexane to a concentration of 0.01 mM.

Solid phases Solid-phase cartridges, AiSTI-SPE Deriv-AOS (each containing a total of 3 mg of resins), were supplied by AiSTI SCIENCE (Wakayama, Japan). AiSTI-SPE Deriv-AOS cartridges contain a polymer-based strong cation-exchange resin in the upper layer, and a polymer-based strong anion-exchange resin in the lower layer. All of the SPAD steps, including conditioning, loading, washing, and elution were performed using 1.0-mL syringes (HSW NORM-JECT; Henke Sass Wolf, Tuttlingen, Germany).

Derivatization on solid phases SPE cartridges were conditioned with 100 μL of water, 100 μL of acetonitrile, and 100 μL of 80% (v/v) acetonitrile in water. Fifty microliters of 20% (v/v) working composite standard solution in acetonitrile was slowly loaded onto the SPE cartridge. Then, the cartridge was washed and dehydrated by passing 100 μL of acetonitrile through the SPE cartridge. The solid phase was then loaded with 5 μL of 20% (w/v) methoxyamine reagent in 10% (v/v) acetonitrile in pyridine. After 3 min, 25 μL of MSTFA was applied to the cartridge and allowed to percolate through the solid phase, to prevent elution of the methoxyamine reagent. After 10 min, the derivatization reagent was extruded, and the analytes were eluted from cartridge using 100 μL of *n*-hexane. Lastly, 100 μL of internal standard solution and 270 μL of *n*-hexane were added to the eluate.

Preparation of human plasma samples Firstly, 50 μL of plasma was mixed with 150 μL of distilled water and incubated incubated at 37 °C for 30 min. During incubation, samples are mixed at the mixing frequency of 1200 rpm. Subsequently, 800 μL of acetonitrile was added and the resultant solution was incubated in the same manner. After centrifugation at 16,000 ×*g* for 3 min at 4 °C, a 50 μL aliquot of the supernatant was loaded onto the SPE cartridge. Derivatization on the solid phase was conducted as described above.

For conventional sample preparation, extraction and derivatization of samples were performed according to the previous report (15). Plasma (50 μL) was mixed with 1000 μL of a solvent mixture ($MeOH/H_2O/CHCl_3$ = 2.5/1/1, v/v/v). The mixture was incubated at 1200 rpm for 30 min at 37 °C, and then centrifuged at 16,000 ×*g* for 3 min at 4 °C. A 600 μL aliquot of the supernatant was transferred to a clean 1.5-mL tube, and 300 μL of water was added to the tube. After mixing, the solution was centrifuged at 16,000 ×*g* for 3 min at 4 °C. The supernatant (400 μL) was transferred into a clear tube, and then dried overnight in a vacuum-centrifuge dryer and a freeze dryer. After drying, 100 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) was added, and the resulting mixture was incubated at 30 °C for 90 min. Lastly, 40 μL of MSTFA was added, and the mixture was incubated at 37 °C for 30 min.

GC/MS conditions and data analysis Analyses were performed using a GCMS-TQ8040 gas chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan). The GC capillary column was an InertCap 5MS/NP (I.D. = 0.25 mm, L = 30 m, df = 0.25 μm) (GL Sciences). Helium was used as the carrier gas, at a flow rate of 1.2 mL/min. Transfer-line and ion source temperatures were maintained at 250 and 230 °C, respectively. For mass spectrometry, electron ionization (EI) was performed using an electron energy of 70 eV and an emission current of 60 μA. Positive ions were detected from *m/z* 85 to 500 at a scan speed of 5000 unit/s. Data were analyzed using GCMS solution software, version 4.20 (Shimadzu).

Injection modes and GC oven conditions For the samples prepared using the SPAD method, injections were performed in programmed temperature vaporizer

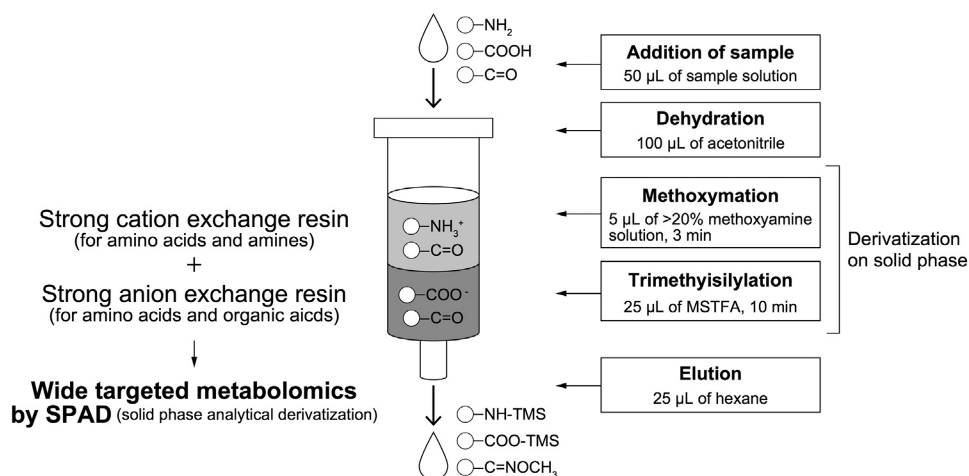

FIG. 1. Concept and scheme optimized in this study. Two kinds of solid phase resins were combined in a solid phase for metabolomics study.

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