Chapter 2

A rapid microwave-assisted derivatization of bacterial metabolome samples for gas chromatography/mass spectrometry analysis

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This report was conceived and planned by MaLi, AW and MiLa. AW made a series of measurements and statistical analysis under the supervision of MaLi and MiLa. All the authors interpreted the data and MaLi and MiLa wrote the paper.

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Notes & Tips

A rapid microwave-assisted derivatization of bacterial metabolome samples for gas chromatography/mass spectrometry analysis

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ABSTRACT

Analysis of metabolome samples by gas chromatography/mass spectrometry requires a comprehensive derivatization method to afford quantitative and qualitative information of a complex biological sample. Here we describe an extremely time-effective microwave-assisted protocol for the commonly used methoxyamine and *N*-methyl-*N*-trimethylsilylfluoracetamide silylation method of primary metabolites. Our studies show that microwave irradiation can decrease the sample preparation time from approximately 120 min to 6 min without loss of either qualitative or quantitative information for the tested synthetic metabolite mixtures and microbial-derived metabolome samples collected from *Bacillus subtilis* and *Staphylococcus aureus*. Comparisons of metabolic fingerprints and selected metabolites show no noticeable differences compared with the commonly used heating block methods.

To study the link between genotype and phenotype, so-called metabolomics [1], requires a high-throughput analytical technique that can characterize all imaginable phenotypes of living organisms and plants as well as fluids or other matrices in regard to metabolites. The main aim of most laboratories working in this field is to develop a reliable and fast method for the generation of comprehensive metabolome data from the original sample [2]. Much research has been devoted to sample collection and metabolite extraction, for example, from bacterial cells [3] or plant tissues [4]. The extraction of metabolites is followed by separation and detection procedures. In most cases, liquid chromatography or gas chromatography coupled to mass spectrometry detection (LC/MS or GC/MS, respectively)[1] is used in metabolomics labs [5,6]. Whereas LC/MS methods require a pre- or postcolumn derivatization of analytes in rare cases, in GC/MS analysis sample derivatization is mandatory before the sample can be subjected to GC due to the fact that most of the metabolites of interest are nonvolatile in their original composition.

The entire metabolome sample is a very complex mixture of biochemicals. The main metabolites are organic acids, amino acids, sugars, sugar phosphates, fatty acids, and steroids, all of which are of interest for the organism's physiology. In most protocols, the polar groups and active hydrogen atoms, such as those in –OH, NH, –COOH, –SH, and other chemical groups, are trimethylsilylated by *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) or *N,O*-bis-trimethylsilyltrifluoroacetamide (BSTFA) to gain more volatile compounds [7]. By adding methoxyamine (MeOx) or ethoxyamine to the sample before silylation, the number of tautomeric forms of monosaccharides can be reduced while aldehyde or keto groups are converted to hydroxyamines or alkoxyamines [8,9]. Many different protocols for the derivatization procedure of primary metabolites exist in the literature [10–13]. In the majority of these, two steps are required: first an oximation with ethoxyamine or MeOx at 37 °C for 90 min and then a trimethylsilylation at 37 °C for 30 min in a heating block. Shortening this time-consuming pretreatment step in metabolome analysis was the goal of our studies.

We describe here the implementation of a power-adjustable microwave oven to accelerate the derivatization process for oximation and silylation without loss of quality in identification and quantification of the metabolite data. This approach was recently described for another derivatization procedure, namely methylation of herbicides and fatty acids via microwave assistance (MA) [14] and silylation by MA with delimited metabolite approaches [15,16]. We have compared our procedure with the existing one for synthetic metabolite mixtures as well as for microbial-derived cell extracts from *Bacillus subtilis* and *Staphylococcus aureus* to cover more complex sample compositions.

First, a synthetic metabolite mixture was prepared at different concentrations to validate the derivatization by MA against the commonly used procedure in a heating block (HB). For this purpose, a mixture of 30 compounds (see note at bottom of Table 1), including deuterated standards, was prepared to ensure coverage of a broad range of different metabolites. All standards and MeOx were

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[1] *Abbreviations used:* LC/MS, liquid chromatography/mass spectrometry; GC/MS, gas chromatography/mass spectrometry; MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; BSTFA, *N,O*-bis-trimethylsilyltrifluoroacetamide; MeOx, methoxyamine; MA, microwave assistance; HB, heating block; MST, mass spectral tag; AUC, area under the curve; RSD, relative standard deviation.

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Table 1
Results for all tested derivatization conditions.

	RSD_{HB} 37 °C (90/30 min)	RSD_{MA} 80 W (3/3 min)	Δ_{MA-HB}	R^2	RSD_{MA} 80 W (1/1 min)	Δ_{MA-HB}	RSD_{HB} 37 °C (45/15 min)	Δ_{MA-HB}
Synthetic mixture (20 nmol)	13.8	11.2	+12.4	0.9825	16.7	−9.3	–	–
Synthetic mixture (200 nmol)	6.1	2.5	+2.7		–	–	3.4	−7.8
Synthetic mixture (500 nmol)	4.6	4.7	−7.3		–	–	–	–
Mean	8.2	6.1	+7.5					
	37 °C (90/30 min)	240 W (3/3 min)						
Synthetic mixture (500 nmol)		8.5	−0.2					
Bacillus subtilis	20.0	22.5	−27.5	0.9998				
Staphylococcus aureus	19.0	29.1	−10.0	0.9993				

Note. Values are the mean relative standard deviations (RSDs) of integrated areas for all metabolites contained in the synthetic mixture and for a subset of metabolites in the microbial-derived metabolome samples ($n = 3$). Mean differences (delta, Δ) between microwave-assisted derivatization (MA) and the commonly used heating block method (HB) are given in percentages for direct response signals from the MS. –, not determined. The synthetic metabolite mixture consisted of glucose, xylose, *ribitol,* cellobiose, pyruvic acid, citric acid, succinic acid, 2-oxoglutaric acid, fumaric acid, malic acid, cis-aconic acid, aspartate, cysteine, glutamic acid, methionine, isoleucine, leucine, *norvaline,* valine, serine, threonine, tyrosine, phenylalanine, phosphoenolpyruvate, *phenylalanine-2,2-d2, urea-d4, stearic acid-2,2-d2, leucine-2,3,3-d3, glucose-6,6-d2,* and *alanine-2,3,3,3-d4* (with internal standards in italics). The mixture was measured at concentrations of 20, 200, and 500 nmol/vial.

purchased from Sigma–Aldrich, and MSTFA used for derivatization was purchased from CS Chromatography Service. Metabolite derivatization, as commonly used for plant, bacterial, and eukaryotic samples [11,12], was performed by adding 80 µl of MeOx (20 mg ml^{-1} pyridine) to the freeze-dried sample and heating for 90 min at 37 °C. After this first heating phase, 80 µl of MSTFA was added and heated for another 30 min at 37 °C. The so-called microwave assistance method was accomplished with an adjustable microwave appliance by adding MeOx and MSTFA to the samples as described above, with each step requiring 3 min at 80 W (240 W for microbial samples). Samples were then subjected to GC/MS analysis under recently described conditions [17,18]. Defined mass spectral tags (MSTs, strongest mass fragment plus retention index) [4] were extracted from the total ion chromatogram for each metabolite and standard, and the areas under the curve (AUCs) were integrated by MetaQuant (version 1.3) [19]. In case of quantitative evaluations, data were normalized to the internal standards present in each sample, as suggested by Wu and coworkers [20].

To test the new MA protocol on complex biological matrices, microbial-derived intracellular metabolome samples were used. We selected *B. subtilis,* a gram-positive soil-derived microorganism known for its importance in biotechnology and basic bacterial physiology research. A second gram-positive but pathogen organism, *S. aureus,* was chosen. Bacterial growth conditions for *B. subtilis* strain 168 and *S. aureus* COL were as described elsewhere [21]. Bacterial metabolome samples were collected by fast filtration as published recently [3,17]. After washing the cells on the filter, the filter was dipped into an ice-cold solution containing internal standards, which quenched further cell metabolism. This solution was immediately frozen in liquid nitrogen. This was followed by further extraction with an organic solution and, in addition for *S. aureus,* by mechanical disruption with glass beads. After centrifugation (4 °C, 8000 rpm, 5 min), the cell debris was discarded and the supernatant was lyophilized and then derivatized for analysis as described above. To capture the constitutive metabolites, all peaks higher than 0.5% of the highest peak were analyzed.

Results for synthetic metabolite mixture and microbial samples are summarized in Table 1. The new fast derivatization protocol with MA gives results that agree well with the traditionally used HB method. The mean (of direct response signals) is more reproducible with the new protocol (mean $RSD_{MA} = 6.1\%$ vs. mean $RSD_{HB} = 8.2\%$). For all tested concentrations of the synthetic mixtures, there are no drastic negative differences in metabolite peak areas with microwave-assisted heating (3 min for both steps) compared with the classical HB method. The decrease of derivatization efficiency in the 500-nmol synthetic mixture can be improved by increasing the energy of the microwave to 240 W, which results in an increase of approximately 20% derivatization efficiency for a subset of critical metabolites such as pyruvate, glucose, and urea (see Table 1). On average, a 7.5% larger signal area was achieved with MA derivatization of synthetic samples. By chromatogram overlay of MA and HB samples, no missing or extra peaks were detected, so the selectivity of the derivatization reaction was tested under the new conditions. A reduction of time in both methods (45/15 min for HB, 1/1 min for MA) resulted in lower derivatization effectiveness (see last columns of Table 1) for some metabolites, especially carbohydrates. An evaluation of the samples from microorganisms also revealed no dramatic differences between the GC/MS fingerprints from both methods; in detail, the main compounds displayed small RSD values, whereas small peaks gave larger differences but for both methods were comparable (Fig. 1B and C; see also Supplementary Fig. 2S in supplementary material).

Fig. 1 illustrates the good correlation between results obtained by the traditional method and the new time-effective protocol. The measured relative responses of each MST in the chromatograms from both methods were plotted against each other to detect trends or outliers in the derivatization effectiveness. The correlation coefficients (MA vs. HB) are high for all samples; R^2 values higher than 0.98 were obtained for the synthetic mixture, *B. subtilis,* and *S. aureus.* The chromatographic fingerprints differ markedly between the two microorganisms, so that a broad analysis of unknown metabolites (in addition to the identified ones) was possible. The mean difference comparing MA with HB response areas for all selected MSTs is negative (Table 1). This fact must be considered for absolute quantification of trimethylsilylated metabolites via GC/MS. In addition, if a derivatized sample was subjected to repeated GC/MS analysis, a decrease in response (AUC, corrected to internal standards) was observed over time (1.5 and 3.0 h after derivatization with storage in autosampler at room temperature) and was independent of the derivatization method (see supplementary material). Therefore, we suggest that laboratories without an automatic sample-handling device should not prepare more than one sample at a time to avoid such metabolite loss in the last step of analysis. In principle, a test for microwave-assisted derivatization against traditionally used methods for each divergent metabolome sample is mandatory considering the complexity of the huge number of metabolites in the living cell.

In summary, microwave-assisted methoxylation/trimethylsilylation of metabolome samples significantly reduces the sample preparation time prior to GC/MS analysis without loss of experimental quality compared with the commonly used method. The new method has the potential to become an efficient and time-effective standard procedure in the preparation of metabolome samples of all kinds.

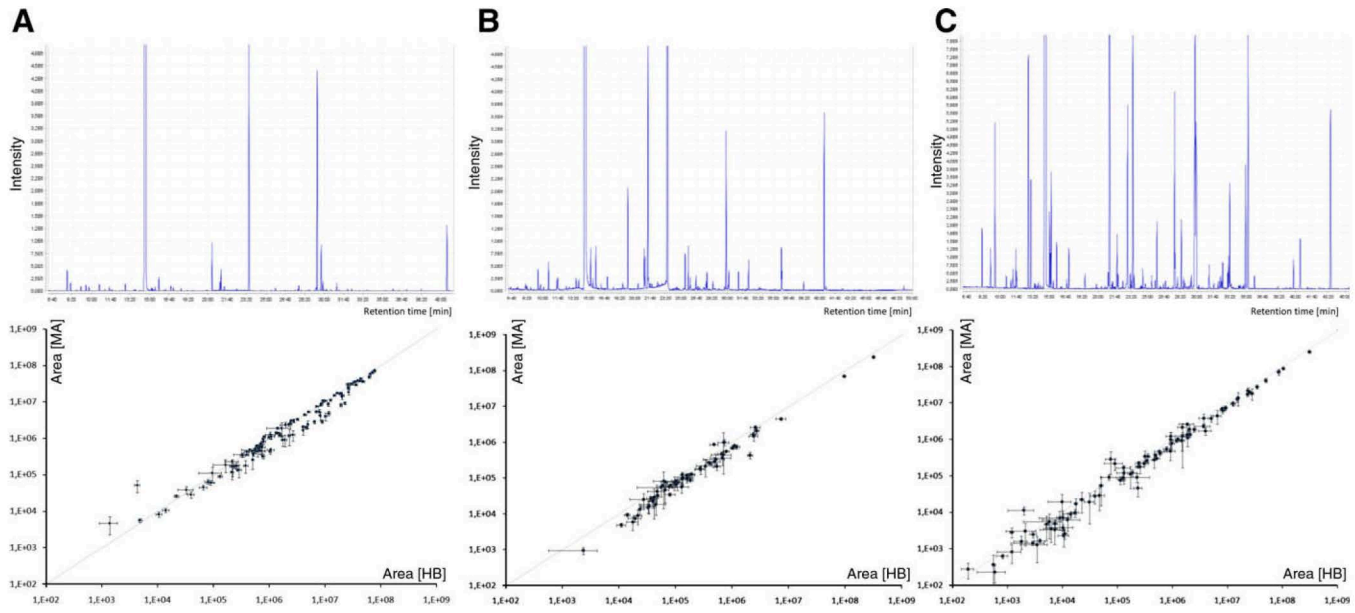

Fig. 1. Total ion chromatogram and plot of correlated metabolite signal areas from extracted mass spectral tags. The microwave-assisted fast method (MA, *y* axis) is plotted against the commonly used, time-consuming heating block procedure (HB, *x* axis) for synthetic metabolite mixture at different concentrations (A) and *Bacillus subtilis* (B) and *Staphylococcus aureus* (C) samples. The diagonal line in each correlation plot is the 1:1 graph of MA versus HB. For panels (B and C), metabolites presenting 95% of the total ion chromatogram were chosen for analysis.

Note added in proof

After receiving the proofs of our contribution we became aware of a similar method described by Villas-Boas and coworkers [Silas G. Villas-Bôas, Samantha Noel, Geoffrey A. Lane, Graeme Attwood, and Adrian Cookson: Extracellular metabolomics: A metabolic footprinting approach to assess fiber degradation in complex media. Analytical Biochemistry, Volume 349, Issue 2, Pages 297-305, doi:10.1016/j.ab.2005.11.019]. In that reference the usage of microwave irradiation for the derivatization of extracellular metabolome samples was described.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.04.030.

References

[1] O. Fiehn, Metabolomics: the link between genotypes and phenotypes, Plant Mol. Biol. 48 (2002) 155–171.
[2] J.C. Lindon, J.K. Nicholson, E. Holmes, H.C. Keun, A. Craig, J.T. Pearce, S.J. Bruce, N. Hardy, S.A. Sansone, H. Antti, et al., Summary recommendations for standardization and reporting of metabolic analyses, Nat. Biotechnol. 23 (2005) 833–838.
[3] C.J. Bolten, P. Kiefer, F. Letisse, J.C. Portais, C. Wittmann, Sampling for metabolome analysis of microorganisms, Anal. Chem. 79 (2007) 3843–3849.
[4] J. Kopka, Current challenges and developments in GC–MS based metabolite profiling technology, J. Biotechnol. 124 (2006) 312–322.
[5] M. Oldiges, S. Lutz, S. Pflug, K. Schroer, N. Stein, C. Wiendahl, Metabolomics: current state and evolving methodologies and tools, Appl. Microbiol. Biotechnol. 76 (2007) 495–511.
[6] S. Moco, R.J. Bino, R.C.H. De Vos, J. Vervoort, Metabolomics technologies and metabolite identification, Trends Anal. Chem. 26 (2007) 855–866.
[7] J. Drozd, Chemical derivatization in gas chromatography, J. Chromatogr. 113 (1975) 303–356.
[8] O. Fiehn, J. Kopka, R.N. Trethewey, L. Willmitzer, Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry, Anal. Chem. 72 (2000) 3573–3580.
[9] J.L. Little, Artifacts in trimethylsilyl derivatization reactions and ways to avoid them, J. Chromatogr. A 844 (1999) 1–22.
[10] M.M. Koek, B. Muilwijk, M.J. van der Werf, T. Hankemeier, Microbial metabolomics with gas chromatography/mass spectrometry, Anal. Chem. 78 (2006) 1272–1281.
[11] U. Roessner, A. Luedemann, D. Brust, O. Fiehn, T. Linke, L. Willmitzer, A. Fernie, Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems, Plant Cell 13 (2001) 11–29.
[12] S. Strelkov, M. von Elstermann, D. Schomburg, Comprehensive analysis of metabolites in *Corynebacterium glutamicum* by gas chromatography/mass spectrometry, Biol. Chem. 385 (2004) 853–861.
[13] J. Gullberg, P. Jonsson, A. Nordstrom, M. Sjostrom, T. Moritz, Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of *Arabidopsis thaliana* samples in metabolomic studies with gas chromatography/mass spectrometry, Anal. Biochem. 331 (2004) 283–295.
[14] A. Ranz, E. Maier, H. Motter, E. Lankmayr, Extraction and derivatization of polar herbicides for GC–MS analyses, J. Sep. Sci. 31 (2008) 3021–3029.
[15] R.Y. Xue, S. Zhang, C.H. Deng, L. Dong, T.T. Liu, J.Y. Wang, H.Y. Wu, J.X. Gu, X.Z. Shen, Simultaneous determination of blood glucose and isoleucine levels in rats after chronic alcohol exposure by microwave-assisted derivatization and isotope dilution gas chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 22 (2008) 245–252.
[16] Y.F. Sha, C.H. Deng, H.B. Zhang, W.Y. Xie, B.Z. Liu, Microwave-assisted silylation followed by gas chromatography/mass spectrometry for rapid determination of ergosterol in cigarettes, J. Sep. Sci. 31 (2008) 2451–2456.
[17] M. Liebeke, D.C. Pother, N. van Duy, D. Albrecht, D. Becher, F. Hochgrafe, M. Lalk, M. Hecker, H. Antelmann, Depletion of thiol-containing proteins in response to quinones in *Bacillus subtilis*, Mol. Microbiol. 69 (2008) 1513–1529.
[18] M. Liebeke, V.S. Brozel, M. Hecker, M. Lalk, Chemical characterization of soil extract as growth media for the ecophysiological study of bacteria, Appl. Microbiol. Biotechnol. 83 (2009) 161–173.
[19] B. Bunk, M. Kucklick, R. Jonas, R. Munch, M. Schobert, D. Jahn, K. Hiller, MetaQuant: a tool for the automatic quantification of GC/MS-based metabolome data, Bioinformatics 22 (2006) 2962–2965.
[20] L. Wu, M.R. Mashego, J.C. van Dam, A.M. Proell, J.L. Vinke, C. Ras, W.A. van Winden, W.M. van Gulik, J.J. Heijnen, Quantitative analysis of the microbial metabolome by isotope dilution mass spectrometry using uniformly ^{13}C-labeled cell extracts as internal standards, Anal. Biochem. 336 (2005) 164–171.
[21] F. Hochgrafe, C. Wolf, S. Fuchs, M. Liebeke, M. Lalk, S. Engelmann, M. Hecker, Nitric oxide stress induces different responses but mediates comparable protein thiol protection in *Bacillus subtilis* and *Staphylococcus aureus*, J. Bacteriol. 190 (2008) 4997–5008.