Fully Automated Online Trimethylsilyl (TMS) Derivatization Protocol for Metabolite Profiling Using an Orbitrap GC-MS and High Resolution Accurate Mass Metabolomics Library

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ABSTRACT

A fully automated online derivatization protocol for metabolite profiling was evaluated on a Thermo Scientific™ TriPlus™ RSH™ autosampler coupled with a Thermo Scientific™ Q Exactive™ GC high resolution accurate mass (HRAM) mass spectrometer. This protocol was optimized under two-step derivatization: using methoxyamine (MOX) and N-methyl-N (trimethylsilyl) trifluoroamide (MSTFA) as derivatizing agents. The major advantage of this approach over offline (manual) derivatization is that it allows multiple samples to be overlapped in a sequence, and samples can be analyzed by gas chromatography mass spectrometry (GC-MS) right after derivatization to prevent breakdown and decrease variability among the samples in a batch injected at different times. In this study, a 24-hour non-stop sample preparation cycle was tested as an example of how this online derivatization protocol is able to deliver better reproducibility and precision for metabolite profiling analysis.

INTRODUCTION

GC-MS offers a relatively comprehensive range of metabolite coverage such as amino acids, sugars, sugar alcohols, fatty acids, and sugar phosphates. Silylation is one of the classic derivatization methods that has been widely used in metabolite profiling to generate stable, more volatile and less polar metabolite derivatives. However, most published derivatization protocols are manual approaches that are time consuming and labor intensive. Also, the majority of trimethylsilyl (TMS) derivatives are unstable due to moisture reactions. Thus, a fully automated online derivatization protocol is needed. The key benefits of this online derivatization protocol are listed below:

1. Fully automated for sample preparation – easy to set up and adjust parameters for each derivatization step.

2. Increased reproducibility and decreased breakdown – excellent average RSD% (<10%) was achieved; Injections were made right after derivatization to protect unstable derivatives.

3. Significantly maximized throughput – two incubators can be used simultaneously; multiple samples can overlap in one incubator.

MATERIALS AND METHODS

Sample Preparation

A 2.5 mm amino acid standard including L-alaine, L-cystine, Glycine, L-isoleucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-threonine, L-valine, L-aspartic acid, L-glutamic acid, L-leucine and L-serine was employed for both manual and online derivatization. An aliquot of 5 µL of the amino acid standard was transferred to a 350 µL insert in a GC vial. The samples were completely dried under nitrogen gas before derivatization.

Automated Online Derivatization

An automatic online derivatization protocol was used to analyze the dried amino acids using the TriPlus RSH autosampler with Thermo Scientific™ TraceFinder™ 4.1 software. Two-percent methoxyammonium chloride (MOX) in dried pyridine and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% 2,2,2-Trifluoro-N-methyl-N-(trimethylsilyl)-acetamide, chlorotrimethylsilane (TMCS) was placed in 2 mL amber GC vials and kept in the cooler drawer on the TriPlus RSH autosampler. The vials with dried amino acid standards were capped with metal caps and placed on Tray 1 of the cooler drawer. To begin the derivatization process, 10 µL of the MOX solution was added to a vial containing the amino acids and vortexed for 30 seconds at 2000 rpm to completely dissolve the dried sample. This mixture was incubated for 90 min at 600 rpm at 30 °C in incubator one. Then, 90 µL MSTFA with 1% TMCS was added to the vial and vortexed for 30 minutes at 600 rpm and 37 °C in the second incubator. The vial was then moved back to the cooler drawer to cool down for 5 minutes, and then the injection was made into the GC-MS. A total of 26 samples were derivatized and analyzed within a 24 h cycle. All samples were analyzed in electron ionization (EI) full-scan mode at 70 eV with 60,000 FWHM (measured at *m/z* 200) resolution. Details of the analytical conditions are given in Table 1.

Manual Derivatization

The manual derivatization was performed following the protocol published by Fiehn.[1] Samples were analyzed on the same column and GC-MS system using the same instrument method as in Table 1.

Data Analysis

Data was acquired and processed using TraceFinder 4.1 software, which allowed for both quantitative and qualitative sample analysis. This includes peak integration and calculation of compound concentration and recoveries, as well as data review and reporting. In addition, for qualitative analysis, TraceFinder automatically generates clean mass spectra through automated peak deconvolution and compound identification by library searching either a custom made or commercially available spectral library.

Table 1. Gas chromatograph and mass spectrometer analytical parameters.

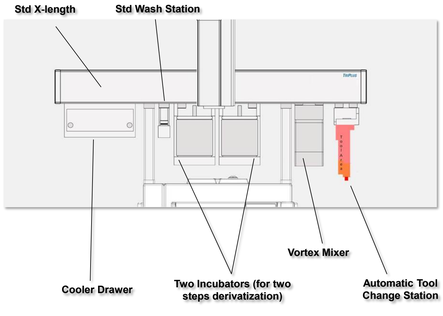
TRACE 1310 GC System Parameters	
Injection Volume:	1.0 µL
Liner:	Single taper without glass wool
Inlet:	250 °C
Inlet Module and Mode:	Split 5:1 (EI) Splitless 2 min .(CI)
Carrier Gas, mL/min:	He, 1.0 mL/min.
Oven Temperature Program:	
Temperature 1:	60 °C
Hold Time:	1 min.
Temperature:	325 °C
Rate:	10 °C/min.
Hold Time:	9.5 min.

Q Exactive GC Orbitrap GC-MS/MS System Parameters	
Transfer Line:	290 °C
Ionization Type:	EI / PCI
Ion Source:	230 °C
Electron Energy:	70 eV
Acquisition Mode:	Full-scan
Reagent Gas, mL/min	CH4, 1.5 mL/min.
Mass Range (*m/z*):	60-800 (EI) 100-1000 (CI)
Lock masses (*m/z*):	73.04680; 133.01356; 207.03235; 281.05114; 355.06990

Automated TriPlus RSH online derivatization system

Fully automated TriPlus RSH autosampler is shown in Figure 1 that sits on the top of a Thermo Scientific™ TRACE™ 1300 Gas Chromatograph. All the required tools for online derivatization are mounted on the standard TriPlus RSH autosampler rail. The cooler drawer keeps all the samples at a constant 4 °C environment with dried nitrogen gas continuously purged into it, which helps to prevent breakdown after derivatization. Two incubators can be used simultaneously on the standard TriPlus RSH rail for two-step derivatization at different temperatures. Multiple samples can be overlapped in one incubation chamber to maximize throughput. The automatic tool change station allows three syringes to be used in this preparation cycle.

Figure 1. TriPlus RSH autosampler with all the required tools for online derivatization protocol on the top of TRACE 1300 GC system.



RESULTS

Workflow of online derivatization protocol

A 24-hour non-stop sequence has been tested on this fully automated online derivatization system. A total of 26 samples was derivatized and analyzed within 24 hours (see Figure 2). Each injection was made right after derivatization to prevent breakdown and have no waiting time for the samples to be analyzed on GC-MS that might decrease variabilities on the tray holder. To the best of our knowledge, this automated online derivatization protocol is the only one that allows the use of two incubators simultaneously at different incubation temperatures on a standard autosampler rail (Figure 1).

Zoomed-in steps of one sample preparation are shown in Figure 3. Different colors represent the different steps. The blue and green bars show the tool change and solvent wash steps. Two-step derivatization using MOX and MSTFA were in red color. The yellow bar is the GC system acquisition time. In the TraceFinder software instrument editor, it is very easy to change parameters in each step according to user requirements. TraceFinder software automatically calculates the timing and then generates an overlapping sequence with the start and end times (Figure 2).

Figure 2. A total of 26 samples overlapped in a 24 hour non-stop sequence using automated online derivatization protocol.

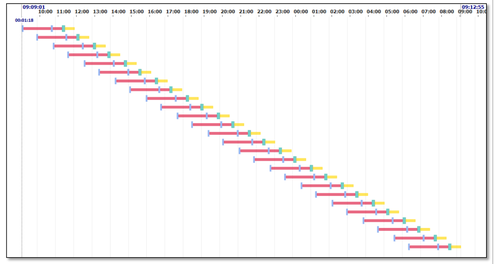


Figure 3. Steps for one sample preparation using two reagents for metaboloimcs derivatization approach.

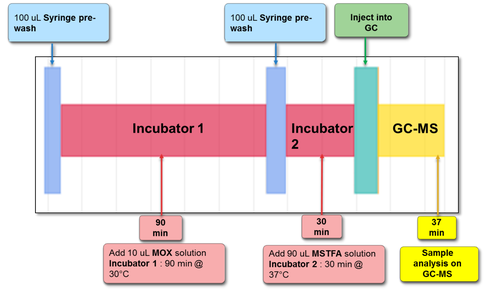
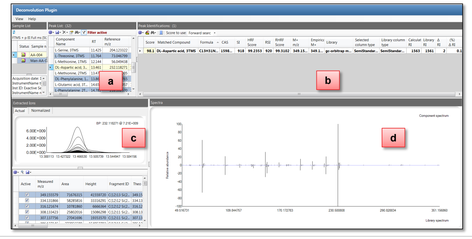


Figure 4. Example of metabolite identification in TraceFinder deconvolution software: (a) Peak list of all detected metabolites; (b) Library search results for current selected metabolite (aspartic acid, 3TMS); (c) Extracted ion overlaid; and (d) Mirror plot of spectral match between deconvoluted and reference spectra in an HRAM GC-Orbitrap metabolomics library.

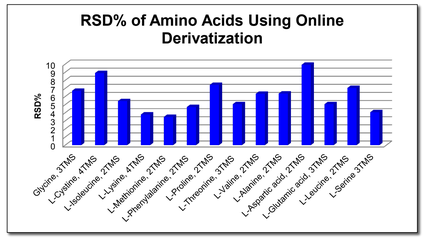


Compound identification using an HRAM GC-Orbitrap metabolomics library

Compound identification during GC-MS metabolomics studies is usually accomplished by comparing the measured spectra against in-house standard databases or unit mass spectral libraries such as NIST or Wiley. TraceFinder deconvolution software was used with NIST 2017 and the Thermo Scientific HRAM GC-Orbitrap metabolomics library. This HRAM metabolomics library was acquired using pure metabolite standards in EI mode at 70 eV with 60,000 FWHM (measured at *m/z* 200) resolution on a GC-Orbitrap MS. It contains ~1,000 unique metabolite spectra with retention indices, CAS numbers, PubChem CIDs and InChIKeys for both derivatized and native metabolites.

Aspartic acid, 3TMS is used as an example of spectral matching in the TraceFinder deconvolution software as shown in Figure 4. Under the peak identification tab shown in section b of Figure 4, excellent library search results are shown with a search index of 918 and reverse search index of 920. Furthermore, this deconvolution software can automatically calculate the retention index (RI) based on an *n*-alkane retention time list that was run on the system. In this case, the calculated RI of 1563 is almost identical to the library reference RI of 1561, which significantly increases the confidence of identification. As an additional convenience, the detected peak results can be easily filtered by the columns shown under the peak identification tab according to user preferences.

Figure 5. RSD% of amino acids using automated online derivatization protocol.

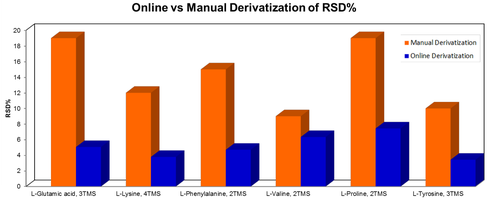


Repeatability

To evaluate the repeatability of this protocol, RSDs% (Relative Standard Deviation) were calculated for each amino acid being detected in the sample in Figure 5. Less than 10-15% RSD is generally accepted as a good repeatability for GC-MS metabolomics studies.[2,3] A total of 14 amino acids were analyzed using this online derivatization protocol. Less than 10% RSD for each analyte was achieved. The average RSD% is 5.85%, which demonstrated a very high reproducibility for this fully automated online metabolomics derivatization protocol.

In contrast, manual derivatization was also performed by preparing 26 samples manually using the same standard with the same derivatizing reagents. Human error is inevitable in measuring amount-of-substance, and a potential variation in waiting time for a whole batch being analyzed on GC-MS affects the quality and reproducibility of the results. Figure 6 compares the results between online and manual derivatization protocols. Obviously, the automated online derivatization protocol shows much lower RSD% 5.13% than the manual approach with 14% average RSD%.

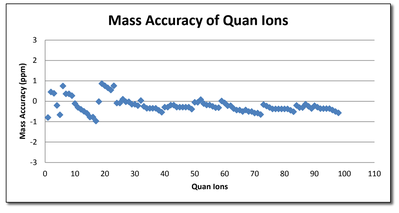
Figure 6. The comparison of RSD% between online derivatization (blue) and manual derivatization (orange).



Mass Accuracy

Acquiring reliable accurate mass measurements is critical for metabolomics studies using HRAM GC-MS. This is essential as any compromise in the accuracy of mass measurements can result in false identification, erroneous quantitation, and interferences from matrix ions in the extracted ion chromatogram. Low mass errors ensure that compound selectivity is high and detection is robust. Also, the low mass accuracy allows for tighter tolerances to be applied for extracted ion chromatograms, which results in fewer false positive detects, thus increasing efficiency by reducing the need for manual review. In Figure 7, outstanding mass accuracy (<1 ppm) is shown to be maintained across all quantitation ions during the 24-hour non-stop test for this automated online derivatization protocol.

Figure 7. Mass accuracy measurements across the whole sequence of online derivatization protocol.



CONCLUSIONS

This fully automated online TMS derivatization protocol provides a simplified sample preparation workflow for metabolomics studies by reducing manual, time-consuming lab work and increasing repeatability from batch to batch.

- This online derivatization protocol is easy to operate and all of the parameters are adjustable in the TraceFinder instrument editor according to the user's experiments
- Two incubators can be used simultaneously to increase sample overlapping in a sequence which can significantly help to maximize sample throughput
- Derivatized samples can be analyzed immediately after derivatization on the GC-MS which considerably improves the RSD% (< 10%) for each analyte
- Excellent mass accuracy of <1 ppm is consistently maintained during the whole process which increases the confidence of identification
- TraceFinder deconvolution software coupled with an HRAM GC-Orbitrap metabolomics library improves the confidence of library searching results

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