Development of a New Metabolomics Method using GC-TOFMS with Automated Derivatization System

Fumihiko Tsuchiya[1]; Tomoko Hiraishi[2]; Ryoichi Sasano[3]; Kenichi Suzumura[2]

[1]LECO Japan Corporation, Tokyo, Japan ; [2]Astellas Pharma Inc, Tsukuba, Japan; [3]AiSTI Science, Wakayama, Japan

Overview

Purpose: Development of a metabolomics workflow by using an automated derivatization system in order to make metabolomics workflow simpler. We call the system "Online Derivatization System".

Methods: In order to compare the offline derivatization sample (Manual derivatization) and the online derivatization sample (Automated derivatization), we applied two kinds of sample mixtures (e.g. amino acids and fatty acids) to them. Both of the samples were dissolved in 20mg/ml methoxyamine/pyridine solution to check chemical noise when the online derivatization method is used for clinical samples. To make online derivatization samples, the sample solutions were diluted with Acetonitrile. After that, the CombiPAL first picked up 20 uL of 10% MSTFA solution, and then picked up 4 uL of the diluted sample solution and injected into a Large Volume Injection port. The sample solution with MSTFA was simultaneously evaporated and derivatized in the inlet. The data was acquired by using a LECO TruTOF GC-TOFMS.

Results: Almost all compounds in the amino acids and fatty acids mixture sample were detected at the same retention times of those in the offline derivatization samples with even peak intensities. The other amino acids, Glycine and Lysine, also were detected with enough amplitude as the form that is modified with trimethylsilyl group. They were detected at retention times earlier than our anticipation, because the numbers of the TMS groups on the amino acids were one less than the amino acids in the offline sample. The results indicate that the derivatization of the online sample was completed without a long incubation time.

Introduction

GC-TOFMS is a powerful tool for investigation of metabolic profiling and is widely used as a standard workflow world-wide. The protocol was completed by some pioneer scientists. In the workflow, derivatization such as trimethylsilylation is the key to get reasonable results with system biological metabolites. The standard operating procedures is widely used and requires a long incubation for the trimethylsilylation. These steps are usually operated as a batch process manually. Since it is known that the trimethylsilyl group is not stable during long storage, we think that development of an online derivatization system that is coupled to each GC-MS acquisition which derivatizes all components quickly would allow the stabilization and the simplification of experiments.

Large Volume Injection (LVI) systems are commonly used to enhance analytical sensitivity. One type of the LVIs, LVI-S200, has a spiral shaped insert and can receive about 100 uL of sample solution. In this study, we applied the system to build an online derivatization method because the shape of the insert befits to replicate the trimethylsilylation in sample vials.

The aim of this study is confirmation of performance of the LVI as an automated derivatization system. We have optimized experimental performance of the online derivatization system and will discuss the application for metabolomics study.

Sample preparation and materials

The flow chart of the protocol to make the samples for the online and the offline derivatization experiments is shown at figure 1. An Amino Acid Standard mixture and a Fatty Acid Mixture(FA61) were purchased from Fluka Analytical and Larodan Fine Chemical, respectively. A 10 uL aliquot of the Amino Acid Standard solution was dispensed to each vial insert and dried using a vacuum evaporator. The dried amino acid standard samples were used for online and offline derivatization testing. Fatty acid solutions were diluted to 1mg/mL of total fatty acid by using heptane and 10 uL of the solutions were used for each experiment. The amino acid samples were treated the same. Methoxyamine hydrochloride was purchased from WAKO. Twenty milligrams of the powder were dissolved into 1 mL of pyridine. A 10 uL aliquot of each solution was added to the sample vial. The MSTFA+1% TMCS was purchased from PIERCE. A 20 uL aliquot of the MSTFA solution was directly added to the sample for the offline derivatization measurement to derivatize before sample injection. The 10% MSTFA solution diluted with heptane was also used for the online derivatization experiment.

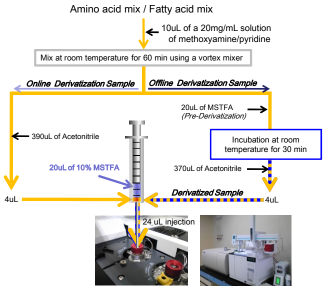

Figure 1. Flow chart of the sample preparations for making the online and the offline derivatization samples.

Large Volume Injection System

The LVI system consists of four consecutive periods. The first period is for sample injection (See Figure 2). During that time, the insert is held at 80ºC which is less than the boiling point of the solvent. The second period is for sample derivatization. Almost all of the solvent is evaporated and ejected from the solvent vent by using a 150 mL/min purge flow. The derivatization is finished during this time. The derivatized sample components are then transferred to the column by heating the insert to 300ºC. In the final period, the insert is cleaned and cooled in preparation for the next experiment.

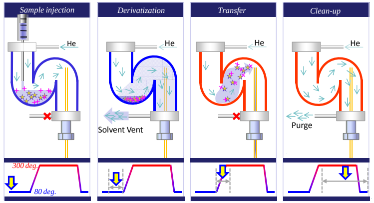

Figure 2. Derivatization steps using the LVI system

Experiments

Auto Sampler	CombiPAL	
	Syringe size	100 uL
GC	Agilent 7890	
	column	DB-5MS 30m, 250um, 0.25um
	Ramping	4 min at 50 deg. -> 20 deg./min -> 320 deg. hold for 5min
	LVI	3min at 80 deg. -> 150deg./min -> 300 deg. hold for 13min
Mass Spec.	LECO TruTOF® HT GC-TOFMS	
	Acquisition Rate	20 Hz
	Mass range	*m/z* 35 - 500

Table 1. Analytical Conditions

Results and Discussion

Result of the Amino Acid Sample

In the GC-TOFMS data for both the online and offline derivatization samples, 16 amino acids were detected in a region from 519.5 to 940.4 seconds (see Table 2 and Figure 3). The sample acquisition was repeated 5 times for the each sample. The Relative Standard Deviation (%) of the offline and the online derivatizations were 3.0 to 17.9%, and 2.1 to 9.3%, respectively.

Peak	TMS	Mass	R.T. (s)	Offline Derivatization		Online Derivatization	
				Average Area (N=5)	RSD (%)	Average Area (N=5)	RSD (%)
Alanine	di	116	519.5	28872303	17.9	27613356	5.1
Glycine	di	102	529.4			21528850	9.3
Valine	di	144	571.1	39419571	6.7	31872783	4.2
Leucine	di	158	596.8	45322924	7.2	35052751	4.8
Isoleucine	di	158	606.7	39893755	7.0	30721006	4.9
Proline	di	142	610.3	55185140	7.8	51486906	3.1
Glycine	tri	174	613.4	33411208	6.8		
Serine	tri	204	634.5	21634107	6.4	17839381	4.0
Threonine	tri	219	645.1	10437267	6.2	7483628	3.7
Aspartic acid	tri	232	698.2	24014574	6.1	19288389	3.7
Methionine	di	176	700.0	24878852	5.8	17537991	5.3
Glutamine	tri	246	734.9	15984493	6.9	12528735	5.4
Phenylalanine	di	218	740.6	22394508	5.8	18705882	4.0
Lysine	tri	156	765.8			11378722	4.0
Histidine	tri	254	835.3	5986316	4.6	18648108	7.4
Lysine	tetra	317	835.7	7451832	4.3		
Tyrosine	tri	218	841.5	42830826	3.3	35564342	2.1
Cystine	tetra	218	940.4	21252323	3.0	10398828	5.6

Table 2. Comparison data for the peak lists of the amino acid standards

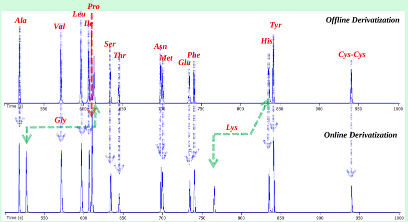

Figure 3. Analytical Ion Chromatograms of the amino acid mixture samples for both the online and the offline derivatizations.

Almost all retention times of amino acids in the online derivatization sample correspond exactly to those of the offline derivatization sample. In contrast, two amino acids, Glycine and Lysine, were eluted in regions earlier than each amino acid of the offline sample. In this case, the two amino acids were detected as the **Glycine N-(trimethylsilyl)-, trimethylsilyl ester** and the **Lysine, N2, N6-Bis(trimethylsilyl)-, trimethylsilyl ester**, respectively. At the same time, in the results of the off-line derivatization sample, these two amino acids were identified as the **Glycine N,N-Bis(trimethylsilyl)-, trimethylsilyl ester** and the **Lysine, N2, N6,N6-Tris(trimethylsilyl)-, trimethylsilyl ester**, respectively. Thus, the online derivatization technique yielded one less TMS group than the offline technique for these two amino acids. Interestingly, the two amino acids have the form "N,N-BIS(TMS)-"in the offline sample data. Since there was no such structure in the online sample data, we think such trimethylsilylation needs a longer incubation time or a higher concentration of the MSTFA. However, we think that the online derivatization is a useful derivatization method because all of the Glycine and Lysine derivatives were chromatographically resolved.

Result of the Fatty Acid Sample

In our results for the different derivatization techniques, 9 fatty acids were detected in a region from 748.6 to 978.5 seconds (see Table 3 and Figure 4). The sample acquisition was repeated 5 times for each sample. The Relative Standard Deviation (%) for offline verses online derivatization was 1.1 to 4.5% and 3.0 to 13.7%, respectively. All peak areas were normalized using Linoleic acid as the internal standard.

Benefit of the Peak shifting of the Lysine

Since retention time of the lysine-4TMS (R.T=835.7 s) approximates with the peak of the Histidine-3TMS (R.T=835.3 s), there is an overlapped peak in the offline derivatization data (see figure 5). Since the GC-TOFMS can collect full mass spectrum with higher time resolution (more than 20 Hz), that can provide enough data points to each peak to separate compound's spectrum mathematically by using the Signal deconvolution algorism.

Peak	TMS	Mass	R.T. (s)	Offline Derivatization		Online Derivatization	
				Average Area (N=5)	RSD (%)	Average Area (N=5)	RSD (%)
Lauric acid	mono	117	748.6	469410	4.5	499945	3.8
Myristoleic acid	mono	117	810.6	393872	4.3	442482	3.0
Myristic acid	mono	285	814.0	4077022	2.2	5281647	8.5
Palmitelaidic acid	mono	117	868.0	2848357	1.2	3524085	6.7
Palmitic acid	mono	313	873.7	6580820	3.2	9148502	12.6
Linoleic acid (IS)	mono	337	920.4	143244	-	167776	-
Oleic acid	mono	339	921.9	3502081	3.2	4788541	13.7
Stearic acid	mono	117	928.1	11792896	3.8	15685436	13.6
Arachidic acid	mono	117	978.5	1483255	1.1	1821136	5.6

Table 3. Comparison data for the peak areas of the fatty acids

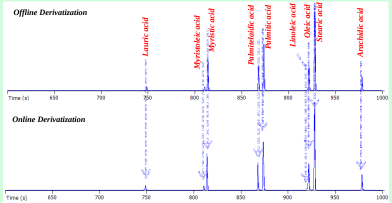

Figure 4. Analytical Ion Chromatogram of the Fatty acid mixture samples. Comparison data between the Online and the offline Derivatization

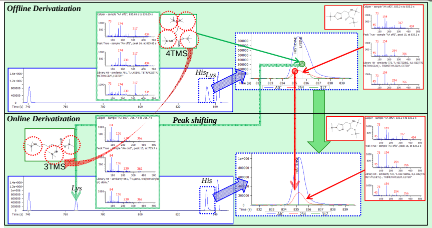

Figure 5. Peak shifting of the Lysine.

However, we think that the risk for misidentification may increase when there is a big concentration difference between the lysine and the histidine, as concentration may affect retention times of the target ion. In the online derivatization data they were completely separated and the data provided good shaped peaks.

Discussions about the Online derivatization mechanism

This study confirmed that the online derivatization is complete within a few minutes. The incubation time is significantly shorter than in the offline method. We think this is because of a concentration process in the stomach shaped insert that is heated at 80ºC. A large majority of the solvent was heptane (boiling point 98ºC). The insert temperature was below the boiling point of heptane. The rate of the trimethylsilylation reaction was increased due to the temperature. The subsequent 150 mL/min flow of purge gas accelerated the evaporation of the solution, concentrating the reactants.

The new method in the Metabolomics studies

GC-TOFMS is an advantageous tool for non-target analysis of complex samples and befits metabolic profiling because metabolomics samples, such as tissue extracts, contain many metabolites. Some are listed in metabolic pathway maps, however it is possible that new pathways would be discovered from diseased samples. Therefore, non-target analysis is a key approach for metabolomics discovery. In such studies, many samples are required for multivariate analysis. Our new method using the online derivatization system is simpler than the common method. Therefore, we expect the method to streamline such work and improve the work efficiency.

Conclusions

1 Online derivatization of Fatty Acids and Amino Acids was confirmed. This technique can replace manual trimethylsilylation.
2 The online derivatization method did not produce the "N,N-BIS(TMS)-"type trimethylsilyl group. Therefore, peak position of some amino acids shifted.
3 The shift of the lysine peak due to online derivatization, improved chromatographic separation.

Acknowledgments

The authors would like to thank Hiroshi Hike of AMR Incorporated for their support about making injection program of CombiPAL. The authors also would like to thank Scott Pugh and Peter Gorst-Allman from LECO Corporation for their support.

References

1 Oliver Fiehn; *Plant Mol. Biol.,* 48 (2002)
2 Carsted Denkert et. al, *Cancer Res.,* 66, 22 (2006)