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The fully on-line automation of MOX-TMS derivatisation for metabolomics applications

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Introduction

Metabolomics studies often rely on gas chromatography-mass spectrometry (GC-MS) as analytical platform because of its reproducibility and chromatographic resolution power. However, differently from LC-MS and NMR, GC-MS requires chemical derivatisation of the metabolites to increase their volatility and thermal stability and allow their successful detection.

Methoxymation (MOX) followed by silylation is the most commonly adopted derivatisation method. The preliminary methoxymation step allows reaction of carbonyl functional groups (>C=O) to form oxime derivatives. This step is crucial to prevent cyclization of reducing sugars/formation of keto-enol tautomers for aldehydes-ketones/decarboxylation, hence to reduce the complexity of obtained chromatograms due to presence of multiple peaks per metabolite. The successive silylation reaction replaces active hydrogens (e.g. -OH, -COOH, $-NH_2$, -SH) with an alkylsilyl group, most commonly trimethylsilyl group (TMS,$-Si(CH_3)_3$), as shown by the equation below:

Sample- X-H (active) + R_3Si-Y → Sample-X-SiR_3 + HY

X= N, O, S and **Y**= leaving group

Silylation is not only useful to encourage GC chromatographic properties but it also enhances mass spectrometric properties providing diagnostic fragmentation patterns for structure elucidation. On the downside, silylation reactions need to be carried out under strictly anhydrous conditions due to high sensitivity to moisture of the derivatising reagents and obtained derivatives.

Biological samples investigated in metabolomics are extremely complex matrices encompassing many interfering components. Hence, good sample preparation is crucial to achieve good quality chromatography and MS signals. The presence of proteins can cause difficulties in the sample preparation therefore a protein precipitation step is usually included to reduce the sample complexity. Commonly, proteins are precipitated by addition of a water-soluble organic solvent such as methanol, acetonitrile or acetone. After mixing and centrifugation, the supernatant is subjected to evaporation to provide the anhydrous conditions necessary to proceed with the MOX-TMS derivatisation.

This application note describes the automation of the MOX-TMS derivatisation for serum samples including a protein precipitation step. Blanks and serum samples were fully prepared by the MPS and then injected directly on a GC/Q-TOF MS system for instrumental analysis.

Instrumentation

The fully automated MOX-TMS workflow for metabolomics applications was developed on a GERSTEL Multipurpose Sampler (MPS) 2 XL Dual head (Figure 1) equipped with the following objects:

- Solvent reservoirs (5 positions)
- Standard Wash station (2 washes and 1 waste)
- Tray VT98
- Tray IS28
- Agitator
- GERSTEL Multiposition Vortexer (mVorx)
- GERSTEL MultiPosition Evaporation station (mVAP)
- Anatune CF-200 Robotic Centrifuge

GC-MS Analysis was performed using the Agilent 7890B Gas Chromatograph coupled to the Agilent 7200B Q-TOF High-Resolution Accurate-Mass Mass Spectrometer

Figure 1: GERSTEL Dual Head MPS for fully automated MOX-TMS derivatisation for metabolomics applications

Methods

Optimized Automated MOX-TMS

An aliquot of serum was transferred into a crimped high recovery vial. Methanol was then added to the vial to promote protein precipitation and the vial was vortexed at room temperature at 3000 rpm using the multiposition vortexer (mVorx).

Once mixing had been completed, the vial was moved to the CF-200 centrifuge whereby the sample was centrifuged at 4500 rpm to obtain a clear extract. Figure 2 shows the serum sample after addition of MeOH/mixing and after the centrifugation step.

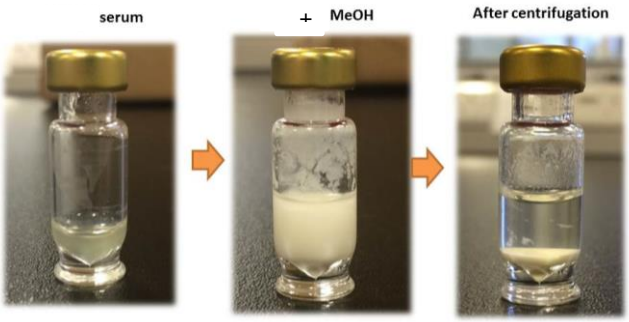

Figure 2: Serum sample protein precipitation before MOX-TMS derivatisation

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The supernatant was transferred to a clean vial and was then evaporated to dryness using the multiposition evaporation station (mVAP).

Once dry, the sample was firstly added with MOX solution (methoxyamine hydrochloride in pyridine containing adonitol as internal standard) and the mixture was incubated at 30 °C. The silylating reagent (either MSTFA + 1% TMCS or TMSCN) was subsequently added to the sample and reacted at 37°C (750 rpm agitation speed).

After derivatisation, the sample was cooled for 5 minutes at room temperature before injecting 1 µL for GC-MS analysis.

GC/MS conditions:

GC:

- Column: HP-5MS Ultra inert 30 m x 0.25 mm x 0.25 µm
- Injection mode: Split 10:1
- Flow: 1 mL/min
- GC ramp: 50 °C held for 2 min, 10 °C/min to 300 °C, held for 8 min
- Auxiliary temperature: 280 °C

MS:

- Removable Ion Source (RIS) in Electron impact (EI) mode at 250 ºC
- Collision cell: Nitrogen as collision gas 1.5 mL/min
- QTOF in 2GHz mode, scan range 35-500 m/z

Results and Discussion

Figure 3 shows the Total Ion Chromatograms (TICs) obtained for the procedural blank and the two serum sample replicates. The orange rectangular shape highlights the internal standard peak (IS, Adonitol 5TMS).

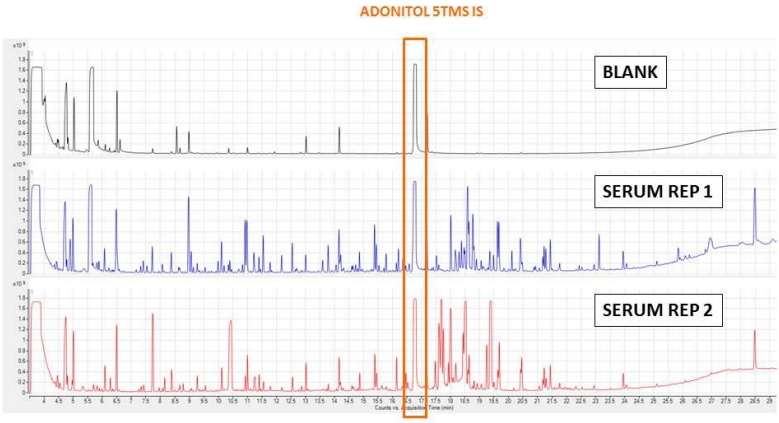

Figure 3: Total Ion Chromatograms (TIC) for MOX TMS Blank (top), Serum Replicate 1 (middle) and serum replicate 2 (bottom)

Table 1 summarises retention time (RT) and areas (TIC and EIC extracted ion m/z 307.1649) obtained for the IS for the shown samples. Observed RSD% were 1% for the TIC and 6% for the EIC traces, respectively.

ADONITOL 5TMS IS				
	TIC		m/z 307.1649	
	RT	Area	RT	Area
	[min]		[min]	
Blank	16.784	11534559938	16.727	50508376
Serum 1	16.759	11652152029	16.716	45323982
Serum 2	16.761	11856587701	16.721	50889819
Average		***11681099889***		***48907392***
SD		***162953840***		***3109179***
RSD		***1***		***6***

Table 1: Retention time and Areas (TIC and EIC) for the internal standard Adonitol 5TMS in the blank and two serum sample replicates

The data files were processed using Agilent Technologies MassHunter Workstation Software Unknowns Analysis. Unknowns Analysis is a very useful tool to perform deconvolution of the chromatograms and help in the detection and identification of relevant features present in the samples.

After analysis, the procedural blank revealed 1517 components whilst the two serum sample replicates showed 2623 and 2328 components respectively. The extracted components were searched against selected libraries (in this piece of work both NIST14 and Wiley 7 libraries were used).

Table 2 summarises the Unknowns Analysis results for both the procedural blank and the two replicate samples.

Sample	Total components	Library Search Hits	TMS derivatives
Procedural Blank	1517	642	123
Serum Replicate 1	2623	1060	304
Serum Replicate 2	2328	955	249

Table 2: Total components, Library Search Hits and found TMS derivatives using Unknowns Analysis for the investigated samples

Conclusions

A fully automated solution for on-line protein precipitation and MOX-TMS derivatisation of serum samples was developed and tested in our labs.

The MOX-TMS was successfully and reproducibly performed as shown by the internal standard Adonitol Areas and RSDs%.

The use of Unknowns Analysis software highlighted the presence of several components in the serum samples and approximately 30 % were identified as TMS derivatives.

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Nathan Hawkins, Account Manager, South East of England and Metabolomics Market Specialist

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