Chromatography Technical Note No AS158

The Power of Fully Automated Sample Preparation for Metabolomics Applications: Proof of Concept

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Introduction

Sample preparation is a most crucial part of the whole analytical workflow. No matter how sensitive, selective and highly performing our end analytical instrumentation might be, if the samples were not prepared correctly and reproducibly, our final data would never meet high quality standards.

This is particularly true in the field of metabolomics, where preparation of extensive sample sets is required to allow successful differentiation between sample types. Hence, analytical data quality is an essential requirement to highlight true biological variability.

Automation of sample preparation can indeed provide the robustness and reproducibility needed to achieve high quality, informative and reliable datasets.

This application note describes as "proof of concept" full automation of direct trans-methylation and liquid-liquid extraction of fatty acids followed by GC/Q-TOF analysis.

Dried herbs (Oregano and Marjoram) were used as matrix. Each herb type was purchased from three different brands (A, B, and C) and each sample was analysed in triplicate in random order.

The obtained dataset was then processed using the Agilent Mass Profile Professional software for Chemometrics analysis to extract all valuable information.

Direct trans-methylation combines triglycerides hydrolysis and fatty acids methylation in one step and very conveniently no prior extraction of the sample is required. A final liquid-liquid extraction step allows solvent exchange and concentration of the target analytes.

Instrumentation

The fully automated workflow 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 2 wastes)
- Tray VT98
- Agitator
- GERSTEL MultiPosition Vortexer (mVorx)
- 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

Data analysis was done using Agilent Mass Profiler Professional (MPP) software for Chemometrics Version 12.6.1

Figure 1: GERSTEL Dual Head MPS for fully automated direct fatty acids trans-methylation

Methods

Optimized Automated Sample Preparation

5 mg of dried herb was weighed in a glass 2 mL vial and it was added with 500 µL of freshly prepared 3N Methanolic HCl. The sample was transferred to the agitator and left reacting at 70°C for 30 min.

500 µL each LC-MS water and hexane was then added to, and the sample vortexed at 2500 rpm for 10 minutes. After centrifugation for 2 minutes at 4500 rpm, 1 µL of the top organic layer was directly injected on the GC-MS.

GC/MS conditions:

GC:

- Column: SGE BPX70 25m x 0.22 mm x 0.25 µm
- Injection mode: Split 10:1
- Flow: 2 mL/min
- GC ramp: 50 °C for 2 min, 7 °c/min to 260 °C, held for 5 min
- Auxiliary temperature: 260 °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 50-500 m/z

Results and Discussion

As an example, the obtained total ion chromatograms (TICs) for oregano and marjoram samples from brand A are shown in Figure 1.

The chromatogram complexity makes it challenging to identify by eye possible differences between species and/or brands

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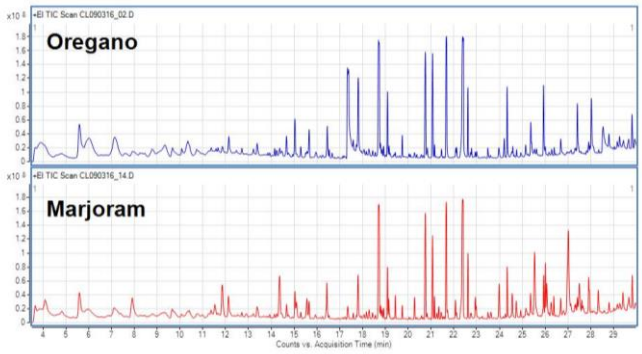

Figure 1: Examples of Total Ion Chromatograms (TICs) for oregano (top) and marjoram (bottom) samples (brand A).

On the other hand, statistical analysis of the data using Principal Component Analysis (PCA) can be particularly useful to emphasize variation and bring out strong patterns in a dataset.

Figure 2a and 2b show the 3D and 2D PCA plots, respectively, obtained for the oregano and marjoram samples from the three different brands (A, B and C). Marjoram data are shown in red while oregano data are in blue. The three different brands are represented by different shapes: circle, square and triangle, respectively.

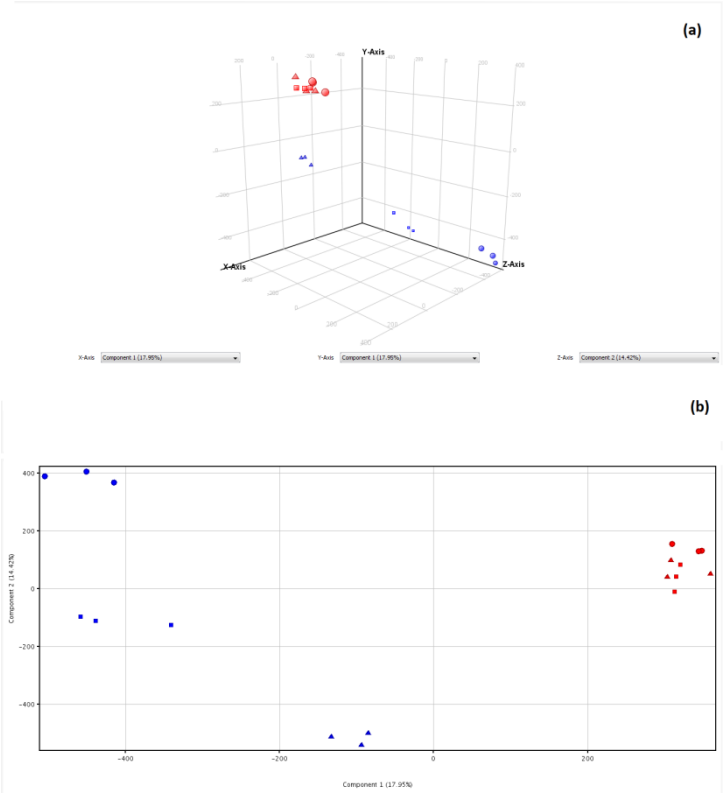

Figure 2: 3D- Principal Component Analysis (a) and 2D- Principal component Analysis (b) for the oregano (blue dataset) and marjoram (red dataset) samples of three different brands (A triangles, B circles and C squares).

The two herb types were clearly separated by the first component. The marjoram dataset was nicely clustered together whilst the oregano data revealed separation according to brand by the second component.

The data show very good repeatability across the three replicates allowing bringing up the real differences between the species and brands.

Indeed, good PCA data offers an advantageous starting point for further investigation of the dataset in order to identify features relevant to justify the biological variability.

In fact, analysis of the C-C plot (P-Correlation versus P-Covariance of the loadings) would highlight candidate entities responsible for the differentiation between the investigated species.

2-Butanone, 4-(4-methoxyphenyl)- (Retention time 21.53 min and *m/z* 121.0653) was identified by the C-C plot as entity responsible for the separation on the first principal component (i.e. differences between species).

This compound is also known as raspberry ketone methyl ether and is a natural occurring flavour and fragrance agent with a fruity and woody note.

Figure 3 shows that for all three brands this entity peak was present only in the marjoram samples (*n*=3, red trace) while it was absent in the oregano samples (*n*=3, blue trace).

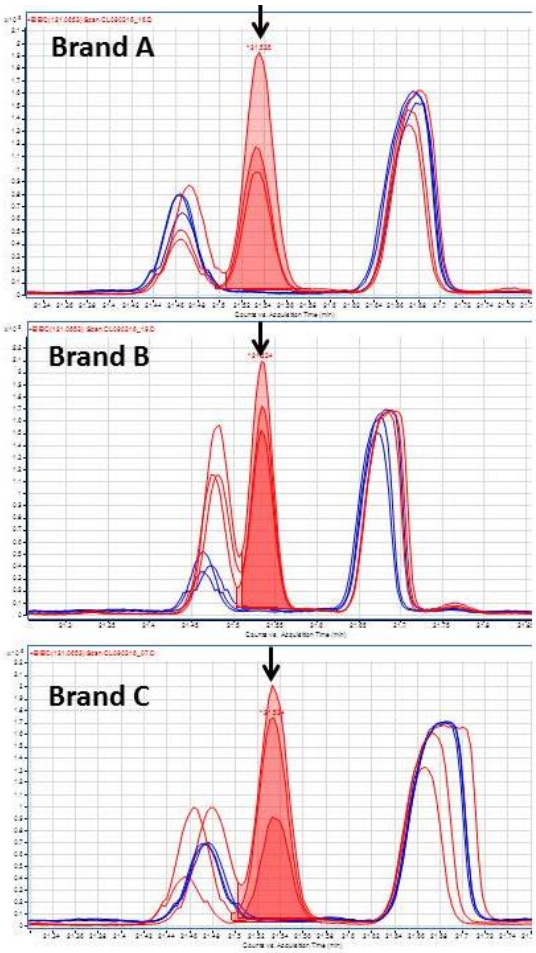

Figure 3: Overlaid extracted ion chromatograms (*n*=3) for 2-Butanone, 4-(4-methoxyphenyl)- responsible for differences between marjoram (red trace) and oregano (blue trace) in all investigated brands: brand A (top), brand B(middle) and brand C (bottom).

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Furthermore, 5,7-Octadien-4-one, 2,6-dimethyl-, (Z)- (Retention time 14.76 min *m/z* 95.0496) was suggested by the C-C plot as compound responsible for separation on the second component (i.e. differences between brands).

5,7-Octadien-4-one, 2,6-dimethyl-, (Z)- is also known as (Z)- Tagetone and is a natural occurring terpene.

Figure 4 shows 5,7-Octadien-4-one, 2,6-dimethyl-, (Z)- differences in response for the oregano samples (n=3) depending on the brand type. This compound was significantly more abundant in brand B (green) and brand C (purple) than brand A.

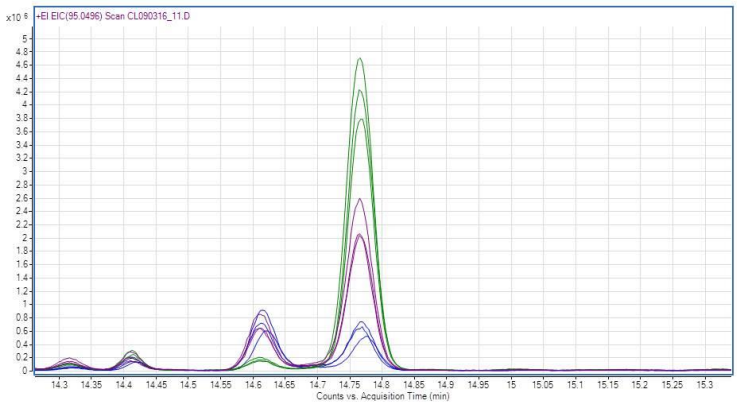

Figure 4: Overlaid extracted ion chromatograms (*n*=3) for 5,7-Octadien-4-one, 2,6-dimethyl-, (Z)- responsible for differences between brands (brand A in blue, brand B in green and brand C in purple).

As far as specifically concerns the fatty acids category, two entities, octanedioic acid methyl ester (left hand side, retention time 17.95 min *m/z* 129.0915) and hexadecanoic acid 3,7,11,15 tetramethyl methyl ester (right hand side, retention time 19.64 min *m/z* 101.0602) revealed different responses in the oregano and in the marjoram EICs as shown in Figure 5.

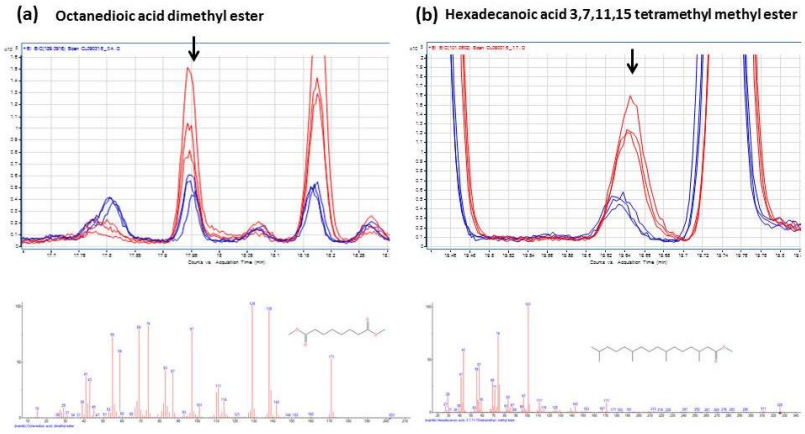

Figure 5: Overlaid extracted ion chromatograms (top) and relative mass spectra for the two fatty acids methyl esters responsible for the class separation between oregano (blue traces, *n*=3) and marjoram (red traces, *n*=3) for brand B.

Conclusions

A fully automated solution for on-line sample preparation for metabolomics applications was developed in our laboratory. As proof of concept, the solution was used to compare two dried herb species from different brands. Control of the analytical variability using automated sample preparation allowed to generate a high quality dataset which could effectively highlight the biological variability by means of powerful chemometrics tools.

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