Chromatography Technical Note No AS157

Fully automated on-line Folch Extraction and trans-methylation of fatty acids in salmon tissue

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Introduction

Metabolomics focuses on the characterisation of small-molecule metabolite profiles (Molecular weight < 2000 Da) specific to investigated pathways.

Metabolomics studies usually require the preparation of extensive sample sets to allow significant differentiation between sample types in biological matrices. Hence, analytical data quality (i.e. low analytical variability) is essential to highlight true biological variability.

Sample preparation automation can provide the needed robustness and reproducibility to achieve good quality data.

Typical metabolomics workflow includes extraction, clean-up, derivatization, and pre-concentration, followed by GC-MS or LC-MS analysis, depending on the target category of metabolites. This application note describes the development of a fully automated on-line workflow including extraction, derivatisation and GC-MS analysis of fatty acids.

In fact, fatty acids are small molecules sharing similar physical chemical properties whose presence and abundance are key to many pathways in metabolic regulation. In this study, lipids were extracted from salmon tissue by Folch extraction and the extracts were subjected to direct transmethylation before injection on the GC-MS system.

Folch extraction[1] requires thorough tissue extraction. Standard Folch extraction was therefore compared to Folch extraction using bead beating. Bead beating is a mechanical cell disruption method for releasing biological molecules from inside a cell. Sample and beads are subjected to high level agitation by stirring or shaking; beads collision causes opening of the cell and releasing of the intercellular components.

Salmon tissue to be extracted via standard Folch extraction was blended to a paste whilst salmon tissue to be subjected to beads beating was left roughly chopped. Samples were analysed in triplicate on a Agilent GC 7890B GC coupled to the Agilent 7200B Q-TOF MS to provide accurate mass.

The fully automated workflow was developed on a GERSTEL Dual Head MPS system (Figure 1) equipped with the following objects:

-Solvent reservoirs (5 positions)
-Standard Wash station (2 washes and 2 wastes)
-Tray VT98
-GERSTEL Multiposition Vortexer (mVORX)
-GERSTEL Multiposition Evaporation station (mVAP)
-Anatune CF-200 Robotic Centrifuge

Figure 1: GERSTEL Dual Head MPS for fully automated Folch extraction and direct derivatisation

Instrumentation

GERSTEL MultiPurpose Sampler (MPS) 2 XL Dual head

Agilent 7890B Gas Chromatograph
Agilent 7200B Q-TOF High-Resolution Accurate-Mass Mass Spectrometer

Methods

Folch lipids extraction:

50 mg of salmon tissue was transferred to a 2 mL vial (glass vial for standard extraction, polypropylene vial for beads beating). 1 mL of Chloroform/MeOH 2:1 was added as effective extraction solvent of a broad range of lipid classes. The sample was then vortexed vigorously for 30 min at 2500 rpm using the mVORX. After centrifugation for 10 minutes at 4500rpm, 500 µL of supernatant were transferred to a clean vial and 200 µL LC-MS water added to extract the small organic molecules via liquid liquid extraction. The lower, organic layer containing the lipids was transferred to a clean 2 mL vial and evaporated to dryness in the mVAP. The dry extract was reconstituted in 100 µL Toluene before proceeding with the hydrolysis/transmethylation.

Direct transmethylation:

The salmon extract was added with 500 µL of freshly prepared 3N Methanolic HCl reacted at 70°C for 30 min.

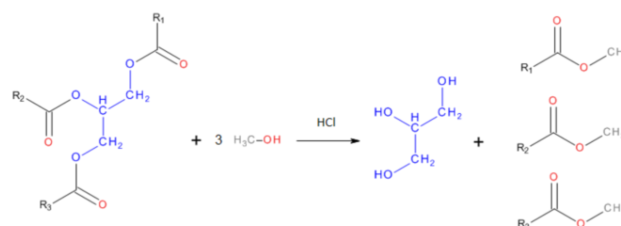

Figure 2: Direct transmethylation of lipids

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:

- High-Efficiency Removable EI source at 250 ºC
- Collision cell: Nitrogen as collision gas 1.5 mL/min
- Q-TOF in 2GHz mode, scan range 50-500 m/z

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Results and Discussion

Figure 3 shows the TIC chromatograms obtained for the standard Folch extraction (top) and for the Folch extraction using beads beating (bottom). The two extraction methods showed similar profiles in terms of peak numbers and peak responses.

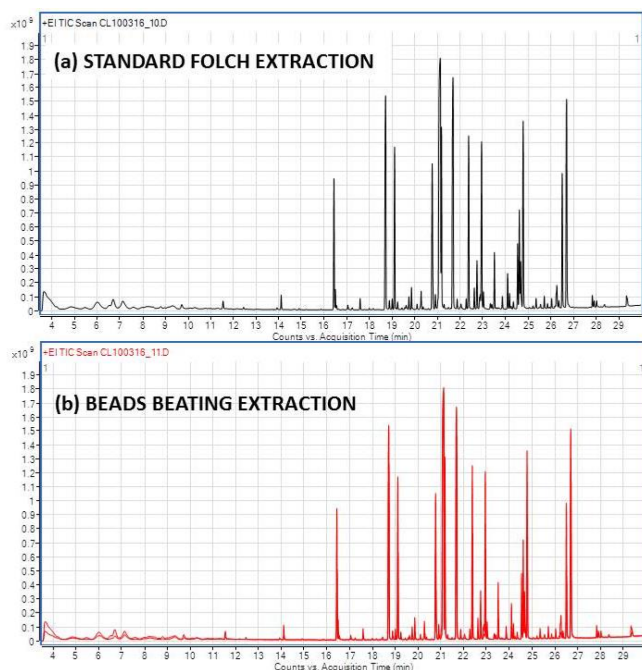


Figure 3: TIC QTOF MS chromatograms of salmon tissue extracts using (a) standard Folch extraction or (b) Folch extraction with beads beating

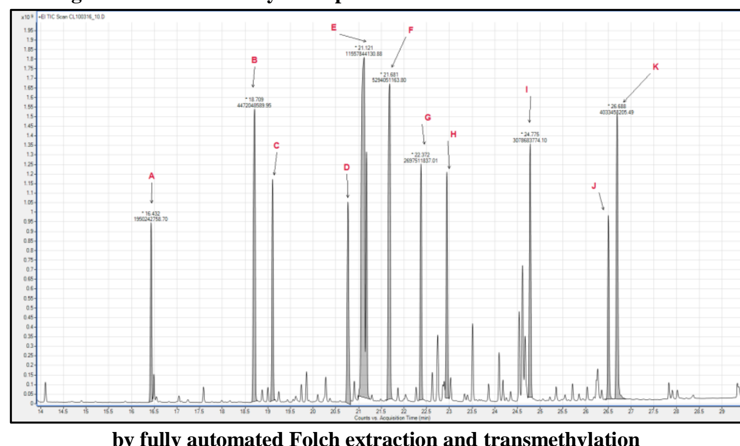
Mass spectra of the most intense peaks in the chromatograms were extracted and searched using NIST 14 MS and MS Interpreter for identification.
Table 1 and Figure 4 list the identity of the most intense peaks present in both methods extracts.

Compound ID	Compound name	Retention time [min]	Molecular ion m/z
A	methyl tetradecanoate	16.432	242.2246
B	methyl hexadecanoate	18.719	270.2559
C	methyl -9-Hexadecenoate	19.105	268.2402
D	methyl stearate	20.765	298.2872
E	methyl-9-octadecenoate	21.067	296.2715
F	methyl-9,12-octadecadienoate	21.661	294.2559
G	*methyl-9,12,15-octadecatrienoate	22.372	292.2402
H	*methyl-cis-13-eicosenoate	22.942	324.3028
I	*methyl-5,8,11,14,17-eicosaperntaenoate	24.775	N/A
J	*methyl-7,10,13,16,19 docosapentaenoate	26.497	N/A
K	*methyl-4,7,10,13,16,19-docosahexaenoate	26.688	N/A

*Omega 3 fatty acids

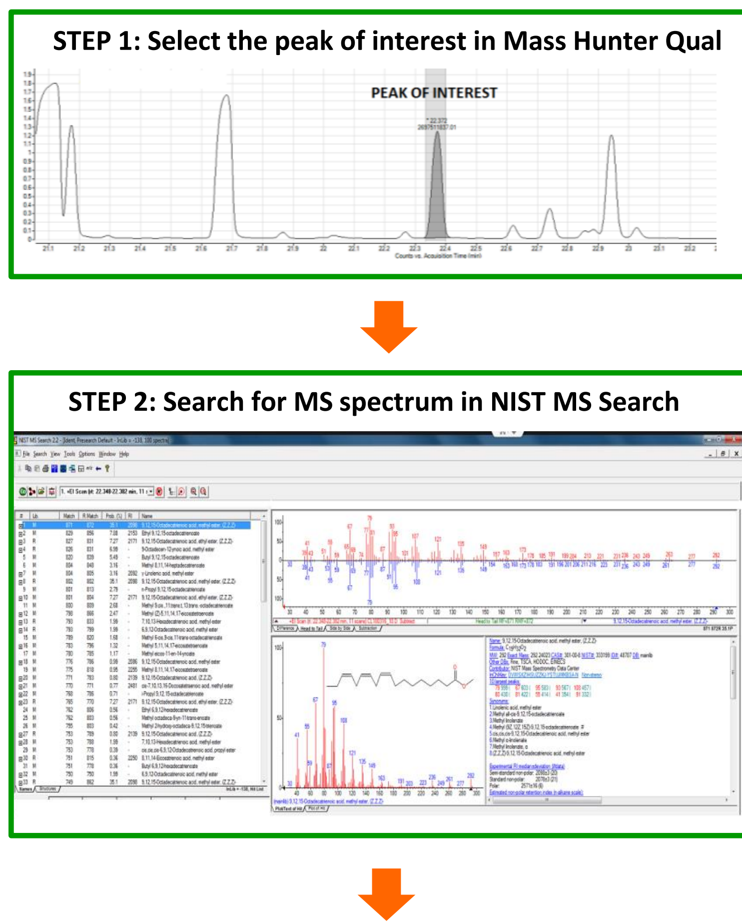
Table 1: Compound ID, retention time and molecular ion m/z for the most intense peaks present in the salmon tissue extracts using the fully automated Folch extraction plus direct trans-methylation.

Figure 4: Identified fatty acids peaks in salmon tissue extracts obtained



by fully automated Folch extraction and transmethylation

An example of the identification workflow for the peak of 9,12,15-Octadecatrienoic acid methyl ester, (Z,Z,Z) is summarised as an example in Figure 5.



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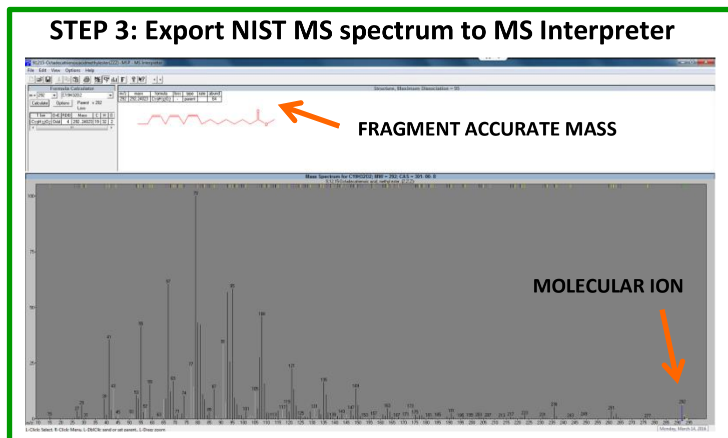

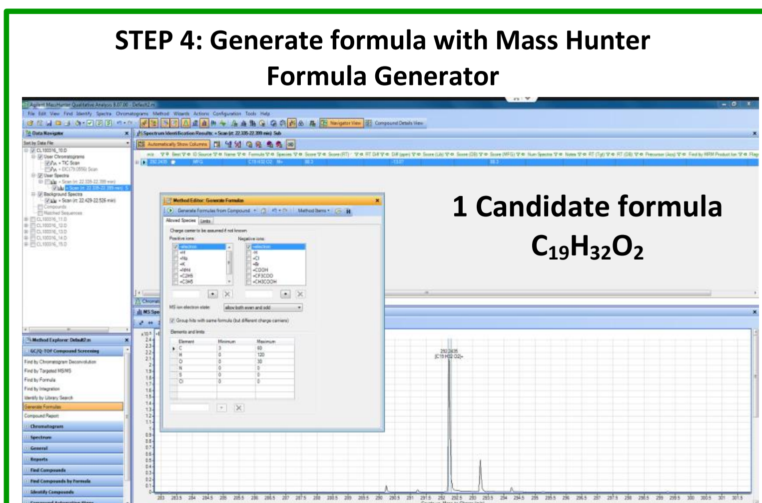

Figure 5: Compound identification workflow for 9,12,15-Octadecatrienoic acid methyl ester (Z,Z,Z) (Peak G from Table 1)

Very good agreement was achieved between the three replicates (RSD <10 %) and the two extraction approaches (standard Folch extraction and Folch extraction using beads beating). Figure 5 shows the average areas and relative standard deviations of three detected omega 3 fatty acids using the two extraction approaches (standard extraction in blue and beads beating in red).

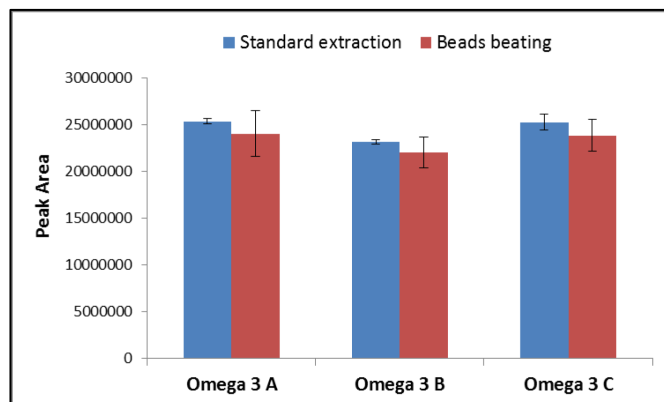

Figure 5: Average areas and standard deviation for three detected omega 3 fatty acids (Omega A: 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-, Omega B: ,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)- and Omega C: 4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-) using the fully automated Folch extraction (standard extraction versus extraction using beads beating).

Conclusions

A fully automated method for the online extraction and derivatisation of fatty acids in salmon was developed in our laboratory.

Folch extraction was fully automated using both homogenized salmon tissue and bead beating.

Fatty acids were successfully extracted and derivatised with very good reproducibility for both investigated methods.

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References

Folch J. et al, J. Biol. Chem.1957, 226: 497-509

Anatune Application Note AS 136 Sean O'Connor and Nathan Hawkins, The Automated Derivatisation and Extraction of Fatty Acids using the Gerstel MPS Autosampler and Agilent 7890 / 7200 GC/Q-TOF.

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