White Paper 017
March 2018

Reliable speciation of fatty acid methyl esters (FAMEs) by flow-modulated GC×GC–TOF MS/FID with Tandem Ionisation

This study demonstrates the use of flow-modulated GC×GC to analyse a FAME standard and FAMEs from a butter extract, with simultaneous detection by TOF MS and FID for improved speciation. We show that inverse-phase GC×GC provides the chromatographic resolution necessary for the separation of closely-related isomers in a single analytical run, while Tandem Ionisation adds an additional level of confidence for compounds exhibiting weak molecular ions and/or similar spectra at 70 eV.

Introduction

Triglycerides of fatty acids are major components of the fats and oils present in foods and living organisms, and along with fatty acids themselves, play a central role in human health. In addition, fatty acid triglycerides from vegetable oils are increasingly used in the production of biodiesel, and consequently they are also valuable in environmental forensics, for example in oil-spill attribution.

Research into both these areas relies on knowing the chemical structure of the fatty acid side-chains (specifically the chain length, degree of unsaturation and double-bond stereochemistry), and the proportions of particular classes of triglycerides present.

Analysis of fatty acids is therefore an area of considerable interest. To avoid reproducibility problems in the analysis of free fatty acids, it is common to extract the lipid fraction from the substrate and convert it to the corresponding mixture of fatty acid methyl esters (FAMEs). These are volatile enough to be analysed by gas chromatography (GC), typically with flame ionisation detection (FID).

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The separation of FAMEs is typically performed using highly polar capillary GC columns that are able to separate saturated and unsaturated FAMEs, as well as positional and *cis*/*trans* isomers of the unsaturated FAMEs.[1] However, such columns have a higher degree of retention for the polyunsaturated FAMEs, which can result in co-elutions with the higher-chain-length saturated FAMEs. The usual solution to this problem is to use very long columns (up to 200 m in some cases) or multiple analyses employing different flows or temperature ramps. However, none of these solutions are ideal – longer columns are not only expensive but also lead to longer run times, while multiple runs and extra extraction steps decrease productivity.

Addressing such issues, two-dimensional gas chromatography (GC×GC) using two columns of differing selectivity can provide the chromatographic resolution necessary to separate larger numbers of unsaturated and saturated FAME homologues and isomers, in a single run. In this study, we use the INSIGHT™ flow modulator for robust, repeatable and affordable GC×GC, and in addition, describe the benefits of parallel detection with time-of-flight mass spectrometry (TOF MS) and FID. This includes confident identification of targets and unknowns through the use of Tandem Ionisation for simultaneous acquisition of 70 eV and soft ionisation mass spectra.

Experimental

Samples: A 37-component FAME standard was prepared in hexane at a concentration of 200–400 µg/mL. The butter extract was prepared by saponification, derivatisation and solid-phase extraction clean-up.

GC×GC: Injector: OPTIC-4™ multi-mode inlet; Injection volume: 1.0 µL; Split 25:1; Flow modulator: INSIGHT™ (SepSolve Analytical).

FID: H_2 flow: 30 mL/min; Air flow: 400 mL/min; Temperature: 300°C.

TOF MS: Instrument: BenchTOF-Select™. A bespoke three-port splitter (available from SepSolve Analytical) was used to direct the flow to the TOF MS and FID detectors in the ratio 1:4.

Software: Instrument control and GC×GC data processing by ChromSpace®.

Please contact SepSolve for full analytical parameters.

Results

1. Analysis of FAME standard

GC×GC separation was optimised using the commercial FAME standard (Figure 1). The full separation of all 37 components was achieved with an analytical run time of under 30 minutes, which would not be possible with conventional one-dimensional GC.

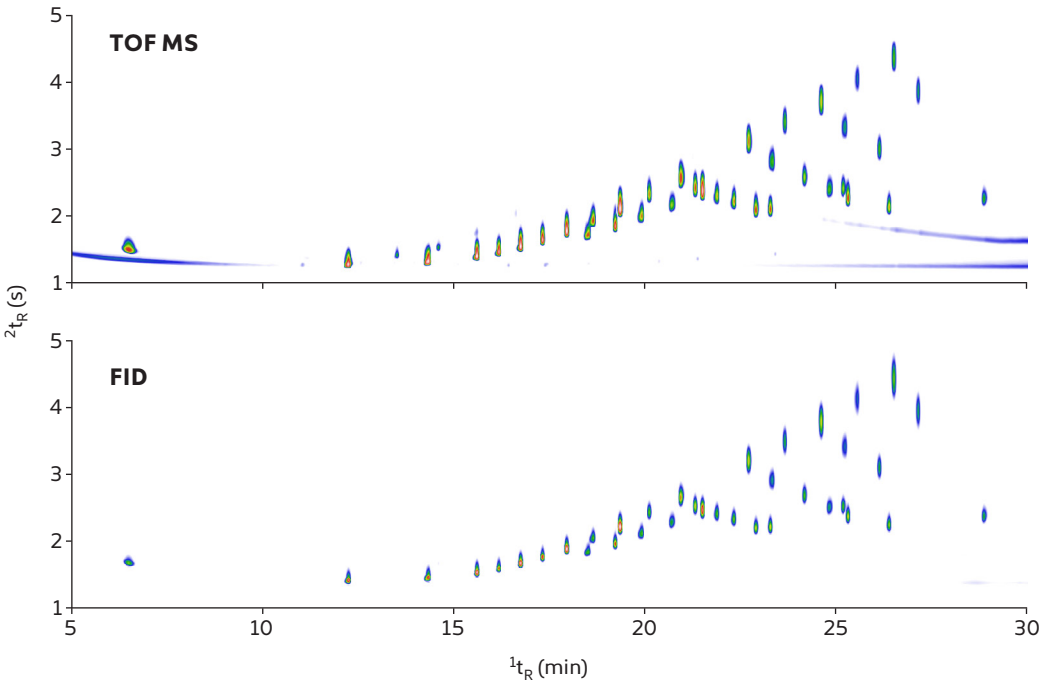

Figure 1

GC×GC colour plots produced by parallel detection using TOF MS (top) and FID (bottom).

The stacked colour plots shown in Figure 1 illustrate the excellent retention time alignment between the two datasets, facilitating the simple transfer of processing methods from TOF MS to FID. In this study, this is aided by the use of the ChromSpace® software platform, which as well as streamlined instrument control, is able to process data (in various file formats) generated by parallel detection methods, so avoiding the need to use multiple software packages.

Acquiring both FID and TOF MS datasets simultaneously has great benefit when analysing real samples – FID enables robust quantitation over a wide dynamic range, while TOF MS provides trace-level identification of both targets and unknowns.

2. Preliminary speciation using the roof-tiling effect

The separation shown in Figure 1 uses a polar column in the first dimension and a non-polar column in the second dimension (commonly referred to as an inverse separation). An additional benefit of GC×GC is the elution of structurally similar components in bands – a phenomenon known as 'roof tiling'.[2] This feature – apparent in Figure 2 – enables the easy application of stencil regions for group-type classification of FAMEs, which is especially important if an MS detector is not available and only FID is in use.

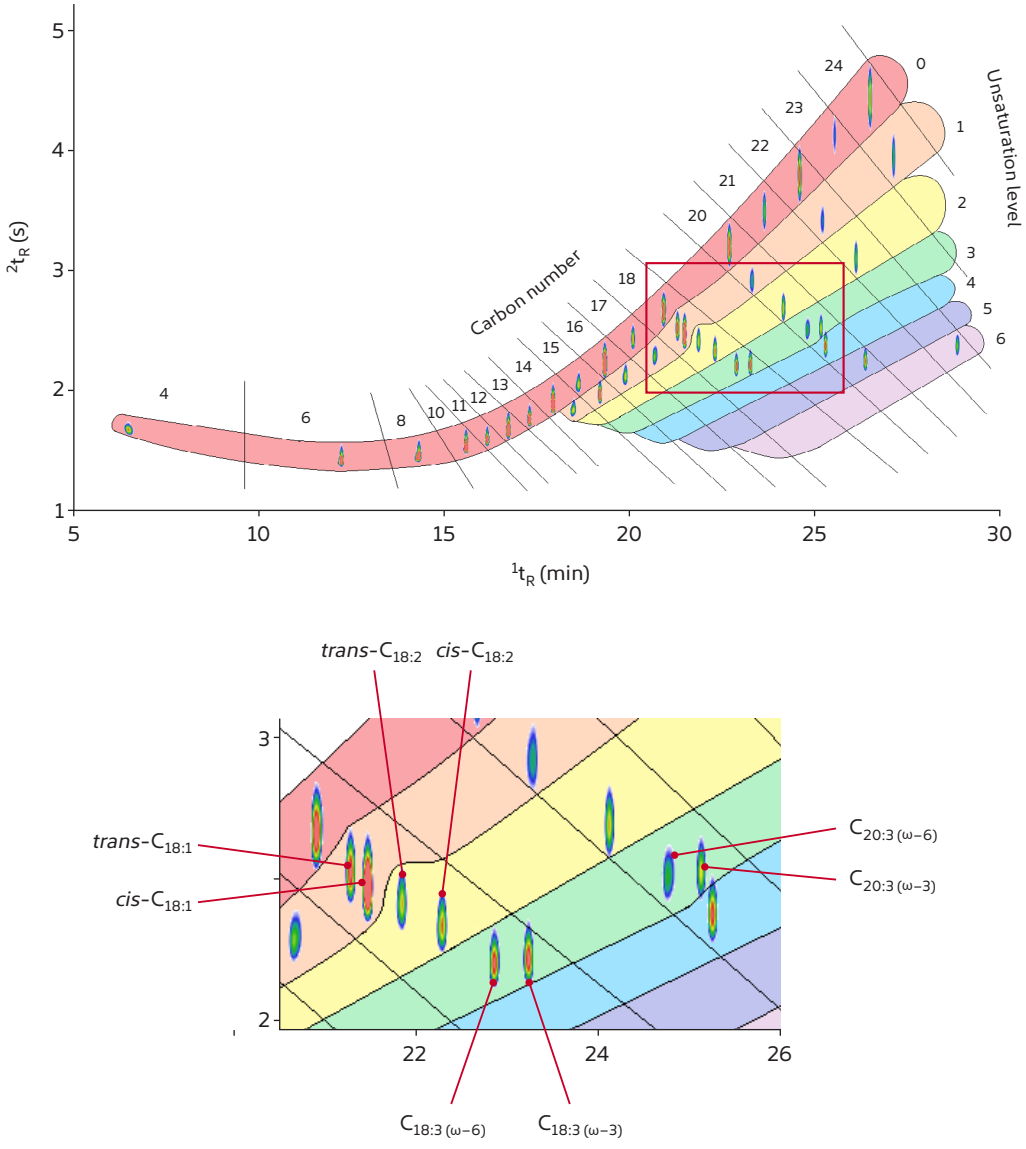

Figure 2

GC×GC–FID colour plot showing the 37-component FAME standard, and highlighting the roof-tiling effect. The coloured bands indicate successively higher levels of unsaturation, from 0 (red, with no C=C bonds) to 6 (violet, with six C=C bonds). The expansion shows separation of *cis* and *trans* isomers and ω–3 and ω–6 isomers.

3. Isomer speciation in a butter extract

Even within a relatively simple standard such as the one described above, there are compounds that would co-elute in a one-dimensional separation – and when using FID, quantitation can be problematic even when peak overlap is slight. Co-elutions are particularly prevalent when analysing complex food samples, such as the butter extract shown in Figure 3. This illustrates the improved separation achievable with GC×GC for the case of the C_{14}–C_{18} FAMEs, where some of the monounsaturates would closely elute with the branched saturated FAMEs. Moreover, simply from using the 'roof tiling' effect it is possible to tentatively identify carbon number and degree of unsaturation by using GC×GC–FID.

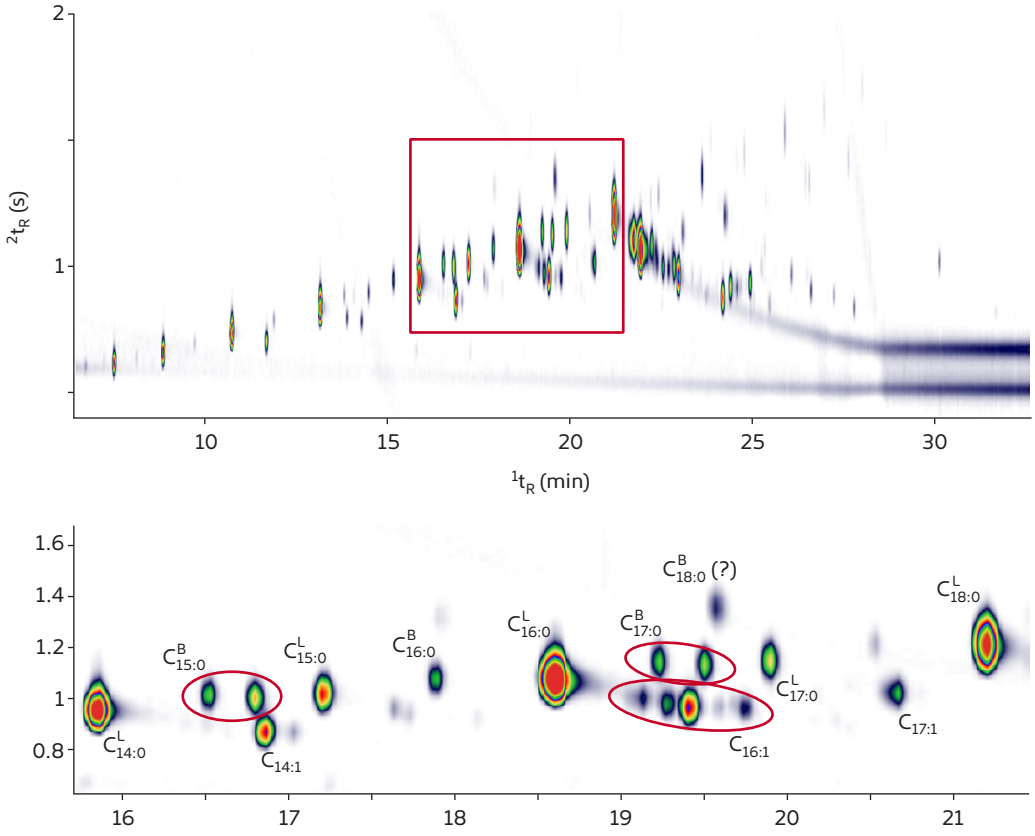

Figure 3

GC×GC–FID colour plot showing a FAME-derivatised butter extract, with the inset highlighting the improved separation achieved using GC×GC for linear (L) and branched (B) C_{14}–C_{18} FAMEs.

4. Reference-quality mass spectra

Although detection by FID allows target identities to be inferred from their retention times, independent confirmation of identity by mass spectra is often desirable. The narrow peak widths generated by GC×GC require a detector with fast acquisition rates (typically at least 50 Hz), making time-of-flight (TOF) mass spectrometers an ideal choice.

An advantage of the BenchTOF instrument used in this study is that (unlike other TOF MS instruments) it does not exhibit mass discrimination. This means that spectra can be compared directly to those acquired by quadrupole instruments

(such as those in commercial or in-house libraries), simplifying identification of unknowns. Figure 4 shows a good match between the TOF MS and library spectra for one FAME in the banding standard.

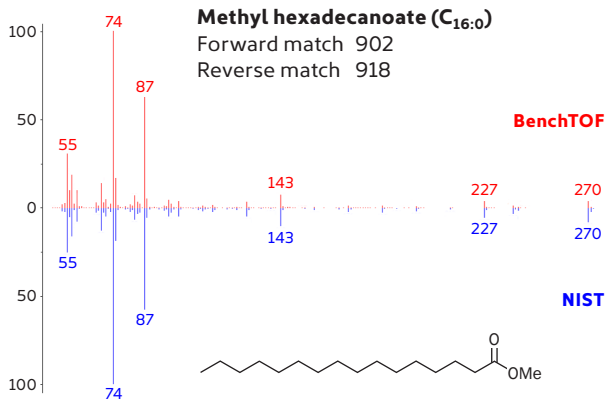

Figure 4

BenchTOF spectrum (top, red) and NIST 14 spectrum (bottom, blue) for $C_{16:0}$ from the banding standard.

5. Tandem Ionisation for confident identification

Despite the benefits of GC×GC with dual TOF MS and FID detection, certain FAMEs can be challenging to identify using standard 70 eV ionisation alone, due to the high degree of fragmentation and subsequent low response for the molecular ion.

The BenchTOF-Select model used in this study can address this issue through its Tandem Ionisation® capability, which enables fast switching between conventional 70 eV EI and low-energy 'soft' EI for improved isomer speciation (without an inherent loss in sensitivity). Used in conjunction with FID, the result is that three information-rich datasets are acquired per run.

Figure 5 shows how Tandem Ionisation at 70 eV and 12 eV can assist discrimination between the ω–3 and ω–6 isomers of methyl octadeca-trienoate ($C_{18:3}$). The 70 eV spectra are very similar, with a high degree of fragmentation and weak molecular ions making it difficult to confirm the carbon chain length. However, the 12 eV spectra show a much stronger molecular ion, as well as structurally significant fragment ions.

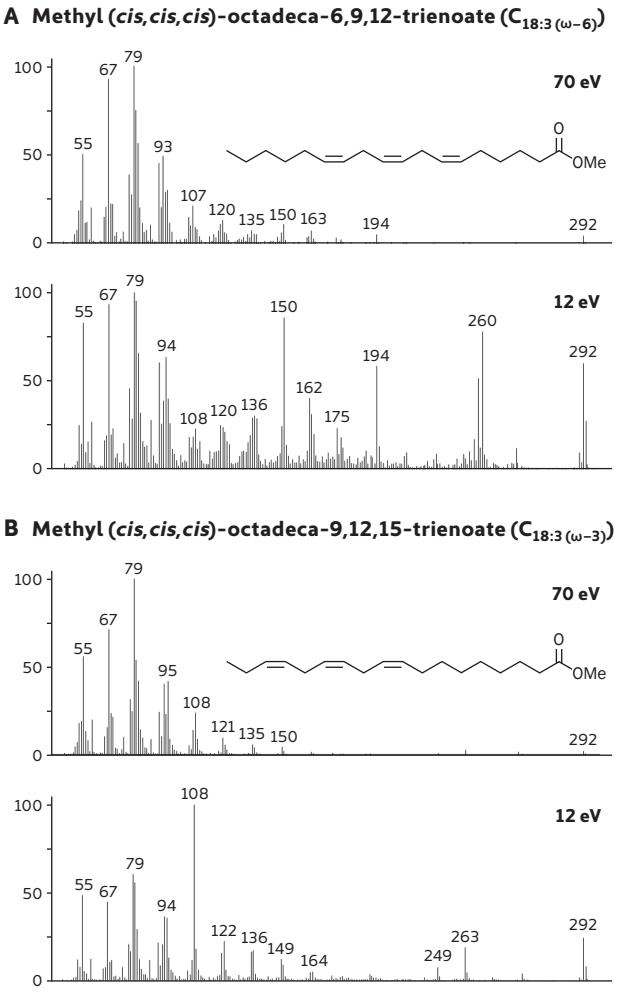

Figure 5

Comparison of Tandem Ionisation mass spectra at 70 eV and 12 eV for:
(**A**) $C_{18:3\ (\omega-6)}$ and
(**B**) $C_{18:3\ (\omega-3)}$.

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Such impressions are confirmed by a more rigorous examination of match factors for four FAMEs (the ω–6 and ω–3 isomers of $C_{18:3}$ and $C_{20:3}$) that all returned a match of methyl octadecatrienoate when the 70 eV spectra were searched against the NIST 14 library. Unlike many soft ionisation techniques, soft EI is repeatable and the spectra retain a degree of fragmentation, meaning that spectral libraries can be created for comparison.

Using the FAME standard, mass spectra at 70 eV and 12 eV were generated for all four compounds, and match factors then calculated for each pairwise comparison (Table 1). The greater range of match factors for the 12 eV data confirms the improved capability to discriminate between these compounds, leading to a reduced risk of incorrect identification and greater data confidence.

70 eV		Library spectrum			
		$C_{18:3\ (\omega-6)}$	$C_{18:3\ (\omega-3)}$	$C_{20:3\ (\omega-6)}$	$C_{20:3\ (\omega-3)}$
Acquired spectrum	$C_{18:3\ (\omega-6)}$	999	845	881	811
	$C_{18:3\ (\omega-3)}$	841	999	867	896
	$C_{20:3\ (\omega-6)}$	876	866	999	868
	$C_{20:3\ (\omega-3)}$	810	899	870	999

12 eV		Library spectrum			
		$C_{18:3\ (\omega-6)}$	$C_{18:3\ (\omega-3)}$	$C_{20:3\ (\omega-6)}$	$C_{20:3\ (\omega-3)}$
Acquired spectrum	$C_{18:3\ (\omega-6)}$	999	769	751	697
	$C_{18:3\ (\omega-3)}$	772	999	743	771
	$C_{20:3\ (\omega-6)}$	751	745	999	778
	$C_{20:3\ (\omega-3)}$	690	773	778	999

Table 1

Match factor matrices showing comparison of sample spectra against bespoke libraries for four compounds in the FAME standard at (top) 70 eV and (bottom) 12 eV.

Conclusions

This study has illustrated the power of flow-modulated GC×GC to provide simple, robust and affordable separation of FAMEs. The increased peak capacity enabled full separation of a 37-component FAME mix, including saturated and unsaturated homologues, *cis* and *trans* isomers and ω–3 and ω–6 isomers – all in a single 30-minute analytical run.

In addition, the simultaneous acquisition of FID and TOF MS data improves the ability to identify and quantify targets and unknowns over a large dynamic range, while Tandem Ionisation adds an additional level of confidence in situations where 70 eV data alone cannot speciate similar compounds, without impacting laboratory workflow.

For more information on this application, or any of the techniques or products used, please contact SepSolve.

Acknowledgements

SepSolve Analytical would like to thank Dr Albert Koulman (NIHR BRC Nutritional Biomarker Laboratory, University of Cambridge, UK) and Keith Summerhill (MRC Elsie Widdowson Laboratory, Cambridge, UK), for supplying the FAME standard and the butter extract, and for their assistance in compiling this report.

Notes

[1] Positional double-bond isomers in fatty acids are designated by their position relative to the methyl terminal carbon. For example, the isomer with a double bond between the third and fourth carbons from the chain end is termed the ω–3 (or n–3) isomer.

[2] All FAMEs with a given degree of unsaturation are structurally fairly similar, meaning that there are not major differences in their affinity for the polar (first-dimension) and non-polar (second-dimension) phases. This means that within each group, increasing the carbon number causes later elution in both dimensions, largely in accordance with the rising GC oven temperature.

In contrast, increasing the number of C=C bonds results in a less flexible carbon chain that is not so compact and is less effective at shielding the ester group, resulting in an increase in polarity. This causes the molecules to elute later on the polar (first-dimension) column and earlier on the non-polar (second-dimension) column.

The separation of *cis* and *trans* isomers rests on a more subtle point, whereby the greater ability of the sterically less encumbered *cis*-C=C bonds to form π–π interactions with the cyanopropylphenyl groups on the polar column leads to a later elution in the first dimension. This effect does not arise in the second dimension.

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