A Fully Automated Procedure for the Preparation and Analysis of Fatty Acid Methyl Esters

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The determination of the fatty acid composition of lipids is an analysis that is widely performed. Typically a manual procedure is used to prepare the lipid sample for analysis, by forming fatty acid methyl esters from the triglycerides, prior to analysis by gas chromatography of the resulting mixture. An automated analogue of a manual procedure using boron triflouride and methanol as derivatisation reagents was developed and a comparison made between the two procedures. The study showed that automation of the sample preparation was viable and gave results, at least as good as the manual version of the method.

Introduction

When an analytical laboratory is faced with a large increase in the number of samples to be run; the economics of the analysis changes significantly. From a cost accounting perspective, the costs associated with any analysis can be seen as composed of two elements: variable costs (those costs that rise in proportion to the increase in samples analysed) and fixed costs (those costs that remain the same as the number of samples analysed increases). If the aim is to minimise the total cost of the activity, when sample numbers are low, it is relatively important to minimise fixed costs, since these costs will tend to represent the major component of the total cost. However, as sample numbers rise, the proportion of the total cost represented by the fixed costs will fall sharply, the reverse argument will become increasingly true and it will become relatively more important to minimise variable costs (even at the expense of increasing fixed costs). Labour costs associated with sample preparation are usually a large part of the variable cost component of any analysis. As a consequence , when faced with an increasing sample workload, automation of the process will be favourable when the savings in the labour component of the variable cost, out-weighs the increase in fixed cost due to the capital spend required.

Once it becomes economically favourable, increased automation also offers other significant advantages such as opportunities to: improve the precision of the analysis by reducing the effect of human variability, reduce exposure of laboratory staff to hazardous materials and to minimise the use of environmentally undesirable substances.

Triglycerides are the main components of fats and oils from animals and plants. The commercial and dietary significance of these substances makes the analysis of these compounds a common one. Triglycerides are made by the combination of three fatty acid molecules with a single glycerol molecule, by the formation of ester linkages between the three OH groups of the glycerol molecule and individual fatty acids. The most common methods used in their analysis involve breaking the ester linkages, forming the methyl esters of the fatty acids and analysing the mixture of fatty acid methyl esters (FAMEs) to determine the fatty acid composition of the fat or oil.

This article describes work done to automate the preparation of fatty acid methyl esters from lipid samples and deuterated fatty acid surrogates, prior to analysis by gas chromatography and includes a comparison of results obtained automatically with results obtained using the existing manual procedure. The lipids samples in this example were derived from polymer specimens via accelerated solvent extraction (ASE), followed by evaporation of the extract to dryness.

Experimental

The use of boron triflouride and methanol for the preparation of fatty acid methyl esters (FAMEs) from lipids is a commonly used and well documented procedure [1]. FAMEs are non-polar and more volatile than their corresponding fatty acids; therefore they are much more amenable

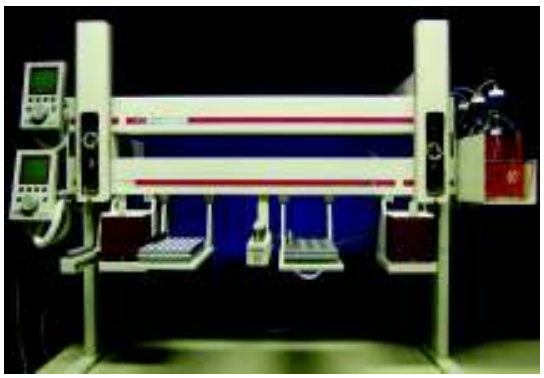

Figure 1. GERSTEL MPS PrepStation configured to perform off line preparation of FAMEs

to analysis by gas chromatography than free fatty acids. In this study, to maintain comparability, an automated version of the procedure was devised to mimic the manual version as closely as possible. Samples were prepared on a "just-in-time" basis to ensure that all samples would be treated in the same fashion. The lipid samples were presented in the form of a dried extracts in a 10ml vials.

A GERSTEL MPS PrepStation (Figure 1), set to accommodate 10ml sample vials, was configured with 10 µl and 1 ml syringes, dual heated agitators, and a four place solvent delivery station. Two of the solvent delivery stations were filled, one with HPLC grade water, and one with acetone (used as a co-solvent for rinsing of the syringes). Two separate 100ml vials were used to contain the hexane/internal standard mixture (bromotetradecane in hexane) and the derivatisation reagent (14% BF3 in methanol) Programming and control was via GERSTEL Maestro software. The processed samples were analysed using an Agilent 6890 GC with 5973 Mass Selective Detector and 7673A auto sampler. The configuration of instrumentation arrived at, allowed for the entire sample preparation, injection and analysis to run as a seamless process or for the sample preparation and analysis to run independently, whichever mode of operation best suited the workload of the laboratory.

The GCMS conditions used for the FAME analysis are summarised below:

Inlet:	Automated cool on-column in oven track mode (oven t +3 °C)
Injection volume:	1 µl from a 10µl syringe
Analytical column:	Phenomenex Zebron ZB1 30 m x 250 µm (0.1 µm film)
Pre-column:	Approx 1 meter 0.53 µm i.d. deactivated
Carrier gas:	Helium at 1ml/min (constant flow), vacuum compensated
Temperature program:	Initial 40 °C, hold for 1 min. Ramp to 300 °C at 10 °C min-1 Final hold, 300 °C for 5 min
Detection mode:	SIM (Selection Ion Monitoring)
Interface temperature:	280 °C
MSD tune:	Standard Auto tune (STUNE)
MSD solvent delay:	5 Minutes

All test analytes and reagents were sourced commerciall.

The automated process was as follows:

1. The 10 ml sample vials were placed in the sample tray of the MPS PrepStation by hand and the PrepStation was started
2. 1ml of the BF_3/methanol mixture was taken from the solvent reservoir and added to a sample vial using the 1ml syringe.
3. The sample vial was moved to the first heated agitator and shaken for 5 minutes at a temperature of 70 °C.
4. The vial was removed from the agitator and placed back into the sample tray 1ml of the hexane/internal standard solution added using the 1ml syringe.
5. 3 ml of water was then added to stop the reaction from proceeding further.
6. The vial was moved to the second agitator and mixed at room temperature for 35 minutes to extract the fatty acid methyl esters into the hexane.
7. The sample vial was then removed from the agitator and placed back into the sample tray and left to stand for 1 minute for the phases to separate.
8. 1ul of the organic layer was aspirated into a 10 µl syringe and injected into the GC-MS.

For the purposes of this comparative exercise, the process was halted after step 7 and a portion of the organic layer removed with a Pasteur pipette and sealed in a 2 ml auto sampler vial, for subsequent analysis with a parallel set of samples that had been prepared manually by a skilled and experienced technician.

Since the time taken to process a single sample was significantly greater than the cycle time of the gas chromatograph, the fact that the robot processed the samples were in a serial fashion meant that in order to gain maximum productivity from the GC-MS, it was important to ensure that the preparation of two samples was in progress at any one time. To this end, the PrepStation was used in "prep ahead" mode, which triggered the Maestro software to interleave the processing of each pair or samples to ensure that samples were processed at a rate that matched the rate that the GC could run samples. This approach is made possible in this example, by the fact that the process involves two lengthy mixing stages during which time the robot is free to begin processing the next sample in the sequence.

Results

The chromatogram shown in Figure 2 is typical of those obtained from samples prepared automatically by this method. In this example, the largest peak is the internal standard, 1-bromotetradecane.

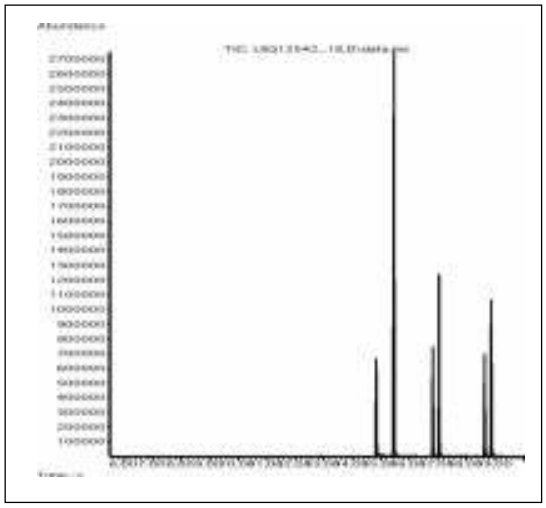

Figure 2. Peaks in order of elution from left: to right: deuterated methyl myristate, methyl myristate, 1-bromotetradecane (internal standard) deuterated methyl palmitate, methyl palmitate, deuterated methyl stearate, methyl stearate.

Tables 1 and 2 contain the peak areas for the methyl esters of both the endogenous fatty acids and the spiked deuterated fatty acids used to check recoveries.

Table 1 contains data from the manually processed control samples; Table 2 contains data from the extracts produced by the PrepStation. Table 3 contains summarised results from Tables 1 and 2.

The variability in the data for the target endogenous methyl esters include a contribution from the accelerated solvent extractions (ASE) which was performed individually on each sample, prior to submission for derivatisation. Known concentrations of the deuterated acids were spiked into the extracts post ASE and prior to drying ready for derivatisation. The derivatised extracts prepared

Manual Sample Derivatisation	Target Compound Esters			Deuterated Recovery Compounds		
	Methyl Myristate	Methyl Palmitate	Methyl Stearate	Methyl Myristate	Methyl Palmitate	Methyl Stearate
Extract 1	5911552	560429	6651084	22453164	5917192	20516698
Extract 3	5214954	464150	5963759	19187919	5314788	17406052
Extract 5	6299444	526698	6967218	24595728	6292183	22172220
Extract 7	5947941	428824	6595360	22733879	5813124	20426225
Extract 9	5592389	509097	6143239	21871223	5455742	19616556
Extract 11	5377264	409680	5737022	19631692	5376005	17588047
Mean	5723924	483146	6342947	21745601	5694839	19620966
SD	403271	58740	468868	2031093	380398	1843817
% RSD	7.0	12.2	7.4	9.3	6.7	9.4

Table 1. Peak Areas for Methyl Esters from Manually Derivatised Extracts [2]

Automated Sample Derivatisation	Target Compound Esters			Deuterated Recovery Compounds		
	Methyl Myristate	Methyl Palmitate	Methyl Stearate	Methyl Myristate	Methyl Palmitate	Methyl Stearate
Extract 4	6344831	532349	6898268	22216496	6108466	21780161
Extract 6	6116601	502786	6809711	21822825	6002122	19655581
Extract 8	5738027	533162	6325660	23609555	5619961	21100648
Extract 10	5788570	499413	6257615	21053158	5814404	18986733
Extract 12	5711370	529985	5955155	20983184	5299360	18702875
Mean	5939880	519539	6449282	21937044	5768863	20045200
SD	278869	16915	396131	1069993	321877	1341459
% RSD	4.7	3.3	6.1	4.9	5.6	6.7

Table 2. Peak Areas for Methyl Esters from Automatically Derivatised Extracts [2]

	Target Methyl Esters			Deuterated Methyl Esters		
	Myristate	Palmitate	Stearate	Myristate	Palmitate	Stearate
Manual Mean	5723924	483146	6342947	21745601	5694839	19620966
PrepStation Mean	5939880	519539	6449282	21937044	5768863	20045200
% Difference in Means	3.8 %	7.5 %	1.7 %	0.9 %	1.3 %	2.2 %

Table 3. Comparison of Mean Peak Areas of manually prepared and automatically prepared extracts [2]

by each technique were alternated within the GC-MS sequence to compensate for potential drift in the performance of the GC-MS throughout the sequence of samples.

Conclusions

It has proved possible to use a sample preparation robot to successfully mimic a common manual process used for the preparation of fatty acid methyl esters. The results obtained using automated sample preparation compare well with results obtained when a skilled and experienced technician prepared identical samples by hand. As expected, the automatically derivatised samples demonstrate better precision than the manually prepared samples and, in this case, recoveries were also found to be slightly better. Using the robot, it is easy to arrange the sample processing sequence such that several samples can be worked on at any one time while maintaining a regime where each sample is processed in an identical fashion and is also completed just as the GC comes ready to run each sample, something that even the most experienced technician would find difficult to achieve.

Automation was shown to be a practical alternative to the manual preparation of FAMEs and offered the prospect of freeing valuable staff from a time consuming routine task to spend time on more cerebral activities.

References

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2. *Summerhill K. and Angove J., Anatune Technical 0Note No AS54*