Determining FAMEs with just-in-time sample preparation

Every laboratory is under pressure to quickly and consistently deliver accurate results and to provide clear answers faster than ever before while reducing the cost per analysis. Analysis systems that meet these criteria are always welcome. Application scientists from Bespak Europe Ltd. and from Anatune Ltd., both based in the U.K., have provided such a solution for automated derivatization and GC/MS determination of fatty acids. The approach taken to the challenge: Automated sample preparation combined with discrimination free introduction of the derivatized analytes to the GC/MS system. Automated sample preparation in this case includes adding an internal standard and derivatizing the fatty acids.
The more complex the method, the higher the demands on the laboratory robotics used. A system that is able to perform two independent robotic functions is by default the more flexible and can frequently provide better productivity and throughput. The GERSTEL PrepStation happens to be such a system; it was successfully used to automate the determination of fatty acid profiles and concentrations in dried extracts of polymer materials used in the pharmaceutical industry. Extraction was first performed using accelerated solvent extraction (ASE). The work was performed in collaboration between John Colwell from Bespak Europe Ltd. and Ray Perkins, Keith Summerhill and Jonathan Angove from Anatune Ltd. in Cambridge, U.K., and it was reported in Chromatography Today (Vol. 1, Issue 4, Sept./Oct. 2008, p. 17-19) as well as in Anatune Application note AS54 (www.anatune.co.uk).

Derivatizing fatty acids for GC/MS determination

Animal and vegetable fats are key components in our nutrition, but lipids are also used in various industrial applications such as polymers used for packaging. This means that there is a significant analytical market for determination of fat content in foods and fatty acid profiles in both foods and polymers used for packaging. Fats and fat oils are mainly triglycerides, glycerol esters of monocarboxylic fatty acids (glycerol is also known as propane-1,2,3-triol). Most triglycerides are made up of three different, linear, saturated fatty acids, each with an even number of carbon atoms. Triglycerides of fatty acids cannot be analyzed directly by gas chromatography (GC), they must first be hydrolyzed and derivatized. The ester bonds are hydrolyzed and the free fatty acids that are formed in the process are converted to the corresponding fatty acid methyl esters (FAMEs). FAMEs are moderately apolar and sufficiently volatile to be determined by GC or GC/MS. The derivatization step is typically quite labor intensive, which makes the work by Colwell, Perkins, Summerhill and Angove even more interesting. Using the PrepStation, the scientists implemented and automated a widely used manual derivatization method that is based on boron trifluoride and methanol (Journal of Liquid Research, 1965. 5: p. 600-608). Using an established method as a base enabled the authors to compare their results with those from existing methods. First the dried polymer extracts containing fatty acids were placed in 10mL vials, and deuterated fatty acids were added as recovery standards. All further steps were performed fully automated by the PrepStation. The quantification was performed using 1-bromotetradecane as internal standard and calibration curves were prepared from FAME standards.

Technical Details

All steps of the derivatization process for the fatty acids were performed using the GERSTEL PrepStation, which has two independent parallel rails, each fitted with independent robotic towers capable of performing liquid handling steps. The upper robot of the GERSTEL PrepStation covers the entire spectrum of liquid handling. This includes liquid sample introduction, the addition of an internal standard, dilution and/or derivatization. The lower robot complements the upper robot, enabling other types of analyte enrichment and sample introduction such as Headspace (HS), Solid Phase Micro-Extraction (SPME) or automated Solid Phase Extraction (SPE). “The MPS PrepStation enables efficient automation of complex tasks”, says Ray Perkins, owner and General Manager of Anatune. “Sample preparation is performed during GC or LC analysis of the preceding sample, there is no loss of productivity, samples are prepared just-in-time for introduction to the GC or LC exactly when it is ready for the next run. This means that the analysis system is never waiting idly for the next sample. Equally,
Acetone was used as a syringe rinsing solvent in the work reported here. Acetone is miscible with water and with most organic solvents in any ratio. This means that acetone is especially well suited for conditioning and rinsing solvent syringes to avoid sample carry over and surface adhesion problems when changing from aqueous to oily phases and vice versa.

Prepared samples are never kept waiting in the autosampler. The risk of decomposition of labile analytes or labile derivatized analytes is thereby greatly reduced, all samples are treated exactly the same, which reduces the risk of variations in results.

Intelligent scheduling of sample preparation and analysis

The GERSTEL MAESTRO software was used to control all sample preparation steps. The MAESTRO Scheduler provides a complete at-a-glance overview of sample preparation and sample introduction timing including total sample preparation and analysis time for all samples. This functionality facilitates planning and scheduling of the laboratory work load. The analysis was performed on a 6890 GC/5973 MSD GC/MS system from Agilent Technologies. The PrepStation can be used as a benchtop WorkStation, independent of the GC/MS system or it can be mounted on top of the GC/MS system enabling it to perform synchronized, overlapping sample preparation and sample introduction in one automated analysis system.

Using isotopically labelled standards

In the work reported here, deuterated fatty acids were used as recovery standards to demonstrate that the derivatization process had been completed satisfactorily.

As an aside, the widely used technique of adding deuterated internal standards to your sample brings a number of benefits: 1) The analyst can be almost certain that he or she is using internal standards that do not occur naturally; there can be little doubt that the concentration of the standard compound is equal to what was added to the sample. 2) The properties of the internal standard compounds closely resemble the target analytes, which means that deviations in response factors, retention times and recoveries will be minimal. 3) The isotopes effectively provide a quality check on the analysis: Less check standards will be needed to run; productivity and sample throughput per instrument can be increased. 4) In general, using isotopically labelled standards can serve as a calibration or a calibration check: Less calibration standards will need to be run; productivity and sample throughput per instrument can be increased.

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The PrepStation used in the work reported here was equipped with a 1 mL syringe and a 10 µL syringe respectively in the lower and upper robotic towers. Two heated agitators and a Solvent Filling Station (SFS) configured with four solvent reservoirs were mounted on the system as well. One solvent reservoir was filled with HPLC-grade water, one with acetone and one with internal standard in hexane. The derivatization reagent (BF3 in methanol) was kept in a separate 100 mL vial.

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Automated derivatization and addition of an internal standard

10 mL sample vials were manually placed on the MPS PrepStation. The vials contained ASE extracts that had been concentrated by solvent evaporation. All further steps were fully automated: 1 mL of the BF3/methanol mixture was aspirated from the 100 mL storage vial and added to the sample. The sample was then transferred to the agitator, where it was kept at 70 °C for 5 minutes under agitation before being returned to the sample tray. The 1 mL syringe was subsequently used to add 1 mL internal standard.
Fatty acid methyl esters (FAMEs) are formed by esterification of fatty acids with methanol. The fatty acids are initially formed when oils (triglycerides) are hydrolyzed. A triglyceride normally contains different fatty acids and a mixture of different FAMEs is therefore formed in the reaction.

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\text{R}O\text{H} + \text{HO-CH}_3 \xrightarrow{\text{Kat.}} \text{H}_2\text{O} + \text{R'-O-CH}_3
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标准 (1-Bromotetradecane in hexane) to the sample. The derivatization reaction was stopped by adding 3 mL HPLC-grade water to the sample.

### Partitioning of FAMEs followed by sample introduction

The FAMEs were partitioned into the organic hexane phase, the process was accelerated by agitating the vial in the second agitator at room temperature for 35 minutes. Following an equilibration time of 1 minute in the sample tray, the organic phase that contained the FAMEs had separated out and settled. Using the 10 µL syringe, the PrepStation aspirated 1 µL of the organic phase and introduced it to the GC/MS system.

### High sample throughput and accurate results

"Since the sample preparation steps for this analysis require much more time than the GC run, the PrepStation gives us significant time savings and it greatly improves productivity," says Ray Perkins. The reason for this can be found in the intelligent MAESTRO PrepAhead function that enables the user to perform sample preparation of one or more samples in parallel with ongoing GC/MS analysis. Using the PrepAhead function, samples can be prepared well ahead of the time when they must be ready for injection. This is a win-win situation: The chromatography system wins in terms of productivity; it never has to wait idly for the next sample. The samples win in terms of uniform treatment: Every sample is introduced immediately after it has been prepared; this means that there is less risk of sample to sample variations in terms of, for example, analyte degradation. Of course, the laboratory gains in terms of productivity and quality of results.

The MAESTRO Scheduler even provides a complete, at-a-glance overview of sample preparation and sample introduction timing including total sample preparation and analysis time for all samples. This functionality facilitates planning and scheduling of the laboratory work load.

### Higher recovery and improved accuracy through automation

"This work has shown", the scientists said, "that our proven manual derivatization method for methylation of free fatty acids can easily and successfully be automated." Furthermore, a comparison between the results obtained from manual and automated derivatization procedures clearly showed the advantages of automation: “The results we got from the automated system with the MPS PrepStation showed better recovery and much lower RSDs for all compounds”, Ray Perkins stated, while noting that a part of the already low RSDs could even be attributed to the Accelerated Solvent Extraction (ASE) procedure performed prior to the derivatization process.

### Bio diesel

Did you know? Bio diesel is a vegetable oil based fuel that is comparable to diesel fuel even though it is not produced from crude oil, but rather from vegetable oils, most often rapeseed oil, or from animal fats. Bio diesel is considered a renewable source of energy; chemically it is based on FAMEs.

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