Volume 8 | Number 8 | 28 February 2016 | Pages 1731–1954

Analytical Methods

www.rsc.org/methods

ISSN 1759-9660

COMMUNICATION
Jessica Pandohee and Oliver A. H. Jones
Evaluation of new micro solid-phase extraction cartridges for on-column derivatisation reactions

175 YEARS

Analytical Methods

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2016, **8**, 1765

Evaluation of new micro solid-phase extraction cartridges for on-column derivatisation reactions

Jessica Pandohee* and Oliver A. H. Jones

Received 1st October 2015
Accepted 13th January 2016

DOI: 10.1039/c5ay02618k

www.rsc.org/methods

Published on 14 January 2016. Downloaded on 19/02/2016 04:17:43.

A novel on-column derivatisation technique using micro solid-phase extraction (μ-SPE) cartridges has been evaluated and applied to the derivatisation of short-chain fatty acids in olive oil. The results show that μ-SPE is a good candidate for on-column derivatisation, giving good results with only a few microlitres of sample.

1. Introduction

Developed in the late 1970s, solid-phase extraction (SPE) is a well-used and exceedingly valuable sample preparation technique that has been in widespread use in analytical chemistry since the mid 1980s.[1] SPE is most commonly utilised for quantitative extraction and pre-concentration of analytes and/or sample cleanup. It is simple to perform and uses minimal amounts of time and reagents. Recent developments in SPE include the use of nanoparticles,[2] antibodies[3] and molecular imprinted polymers[4] to provide highly selective extraction mechanisms.

SPE is not just limited to separation science however. Once analytes are loaded onto a cartridge it is theoretically possible to perform additional chemistry on them rather than just simple elution. For example antibody-based sorbent material could be used as the basis of a quantitative and/or quantitative colorimetric test for the presence of a particular compound in a sample, and refraction index matching of sorbents and solvents in see-through columns could be used to facilitate on-column visualisation of sample migration.[5] This latter method could theoretically also allow on-column spectroscopic analysis. A simpler methodology is the chemical modification of the compounds of interest to facilitate their later analysis. Such derivatisation reactions are often sensitive to atmospheric conditions such as moisture so they are generally not suitable for use in traditional SPE cartridges, which are often open to the air. Newly developed, μ-SPE cartridges however, bring the possibility of on-column derivatisation reactions closer.

The development of μ-SPE cartridges is very recent; the system tested in this study was only brought to market in 2014 and have never been investigated for on-column derivatisation methods before. The cartridges are packed with a very small particle size sorbent of 3 μm, compared to 50–60 μm used in traditional SPE, and thus offer increased efficiency and resolution. This not only provides a more effective separation of the analytes from the sample matrix but the design of the system means the sorbent bed is sealed from the external environment so environmentally sensitive reactions are possible in the cartridge itself (see Fig. 1).

One field that commonly uses derivatisation to modify compounds of interest is metabolomics. In such studies trimethylsilyl (TMS) and acid-catalysed esterification is often used to modify fats and lipids into forms suitable for analysis using gas chromatography coupled with either a flame ionisation detector (GC-FID) or mass spectrometer (GC-MS). Derivatisation also reduces analyte adsorption and improves detector response, peak separation and peak symmetry, which are all desirable for accurate analysis.[6,7]

High Performance Liquid Chromatography (HPLC) has also been used to analyse fatty acids as it avoids the degradation of heat-sensitive functional groups and can be also used on a preparative scale. However, fatty acids also need to be

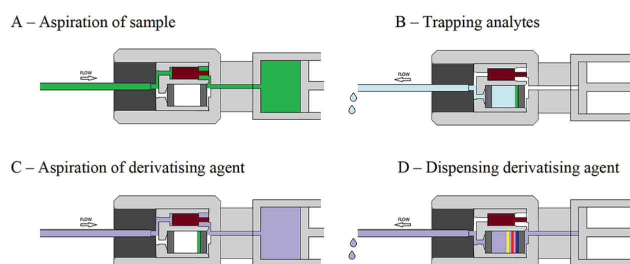

Fig. 1 Derivatisation steps using micro SPE cartridges. Image courtesy of Eprep Pty Ltd.

Australian Centre for Research on Separation Science (ACROSS), School of Science, RMIT University, GPO Box 2476, VIC 3001, Australia. E-mail: jessica.pandohee@rmit.edu.au; Tel: +61 (0)3 9925 2850

This journal is © The Royal Society of Chemistry 2016

Published on 14 January 2016. Downloaded on 19/02/2016 04:17:43.

derivatised for HPLC analysis, not to make them volatile but to add a chromophore to make them detectable by ultraviolet light (UV).[8]

Fatty acids themselves are small biomolecules consisting of a carboxylic acid with a saturated or unsaturated aliphatic chain, usually 12 to 28 carbon atoms long. These acids play an important role in metabolism; linoleic and linolenic acids for example are precursors of arachidonic, eicosapentaenoic and docosahexaenoic acids, which are vital components of all membrane lipids and are also used in the synthesis of other species that are involved in regulating blood pressure and inflammatory responses.[9] Fatty acids also act as secondary messengers for the translation of external cellular signals and are involved in regulating gene expression, especially genes that encode proteins with roles in fatty acid transport or metabolism.[10] Moreover, fatty acids are the smallest units of complex lipids such as triacylglycerols or phospholipids that are responsible for many structural components of cells and provide the distinctive properties of cell membranes.[9,11]

Fatty acids are of great interest in biology and are the focus of many biochemical studies.[12] The present study was undertaken as a proof of principle study and designed to assess the possibility of performing on-column derivatisation of fatty acids. Such a procedure could save a significant amount of glassware and chemicals and also potentially allow for subsequent direct injection into a mass spectrometer, which would allow faster sample analysis in a range of studies.

2. Experimental

2.1 Materials

All chemicals used were analytical grade reagents and solvents were HPLC grade unless stated otherwise. Milli-Q water (18.2 MΩ) was obtained in-house. Acetonitrile was purchased from Ajax Fine Chem (Taren Point, New South Wales, Australia). Individual fatty acids standards (enanthic, caprylic; pelargonic; capric; undecanoic; palmitic and stearic acids) were purchased from AccuStandard, Inc (New Haven, Connecticut, U.S.A) and the 37-component FAME mix (10 mg mL^{-1} in methylene chloride) and boron trifluoride–methanol (10% w/w) were bought from Supelco (Bellefonte, Pennsylvania, USA). Olive oil was purchased from a local market (Melbourne, Victoria, Australia). The ePrep micro SPE cartridges packed with the C18 stationary phase tested were a kind gift from ePrep Pty Ltd (Mulgrave, Victoria, Australia).

2.2 Standard and sample preparation

A solution of 100 μg mL^{-1} of the 37 fatty acid methyl esters standard was prepared to assess the GC-MS method. Once the GC-MS method was established an acid stock solution (10 μL mL^{-1}) of enanthic, caprylic; pelargonic; capric; undecanoic; palmitic and stearic acids was made in acetonitrile for use in optimising the on-column derivatisation method. The stock solution was then further diluted to make solutions of 1, 2.5, 10, 50 and 100 ng mL^{-1} to create the calibration curve. The olive oil (10 μL) was dissolved in acetonitrile to a final concentration of 10 μL mL^{-1}.

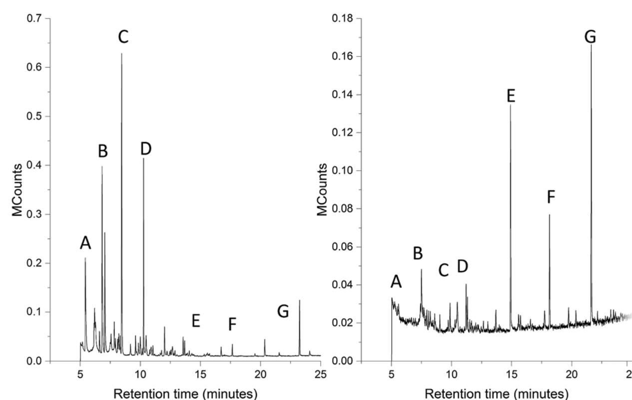

Fig. 2 Chromatograms comparing the performance of derivatisation of the 7 fatty acids standards on micro SPE cartridges (left) and in control vials (right); (A): enanthic; (B): caprylic; (C): pelargonic; (D): capric; (E): undecanoic; (F): palmitic and (G): stearic acid.

2.3 Optimisation of derivatisation procedure

2.3.1 Comparison of derivatisation time between previous procedures. Conventionally, during an esterification, boron trifluoride in methanol (BF_3–MeOH) is added to the sample and incubated at 80 °C for 90 minutes using the method outlined in Gullberg *et al.*[13] The organic layer is transferred in a different vial, evaporated and reconstituted in hexane. The same procedure was used for the derivatisation in the SPE cartridges except there was no need to transfer the sample or reconstitute in hexane. Ribeiro *et al.*[14] later showed that heating the samples at 90 °C for 10 minutes was more efficient in some cases. Therefore these two esterification methods were compared.

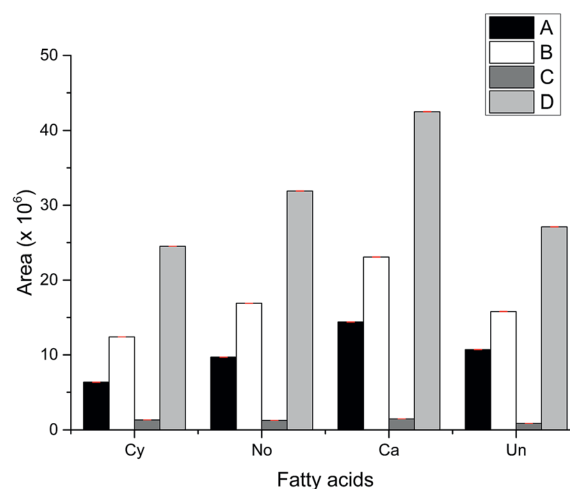

Fig. 3 Comparison of derivatisation method for caprylic (Cy), pelargonic (No), capric (Ca) and undecanoic (Un) acids using micro SPE cartridges; $n = 3$. (A): 10 μL fatty acid aliquot with the cartridge left for 1 minute after which 10 μL BF_3–MeOH was added and the sample, heated at 80 °C for 90 minutes. (B): 10 μL fatty acid and 10 μL BF_3–MeOH (in that order), heated at 80 °C for 90 minutes. (C): as with B but with the order of reagents reversed. (D): 10 μL fatty acid then addition of, 10 μL BF_3–MeOH (as with (B)), heated at 90 °C for 10 minutes. All derivatives were eluted with 150 μL of acetonitrile. NB: error bars are very small but are highlighted in red.

 This journal is © The Royal Society of Chemistry 2016

In short, 10 μL of sample and BF_3–MeOH (10% v/v) was passed through the SPE cartridges and the latter were incubated for 80 °C for 90 minutes or 90 °C for 10 minutes. The resulting FAMEs were then eluted with 150 μL of acetonitrile.

2.3.2 Investigating mixing of reagents. To the best of the authors' knowledge the μ-SPE cartridges have not been tested as a medium for on-column derivatisation. The first step in developing the method is to ensure that the derivatisation efficiency is optimal. Therefore mixing procedures in the micro SPE cartridges were investigated. Fig. 3 shows the different steps involved to trap and derivatise the analytes on the cartridge. The following studies were carried out:

• Study A: 10 μL fatty acids, the cartridge was left for 1 minute and 10 μL BF_3–MeOH was added, then heated at 80 °C for 90 minutes;

• Study B: 10 μL fatty acids, 10 μL BF_3–MeOH, heated at 80 °C for 90 minutes;

• Study C: withdrew 10 μL BF_3–MeOH and 10 μL fatty acids, inject all through cartridge and heated at 80 °C for 90 minutes;

• Study D: 10 μL fatty acids, 10 μL BF_3–MeOH (as experiment B), heated at 80 °C for 10 minutes.

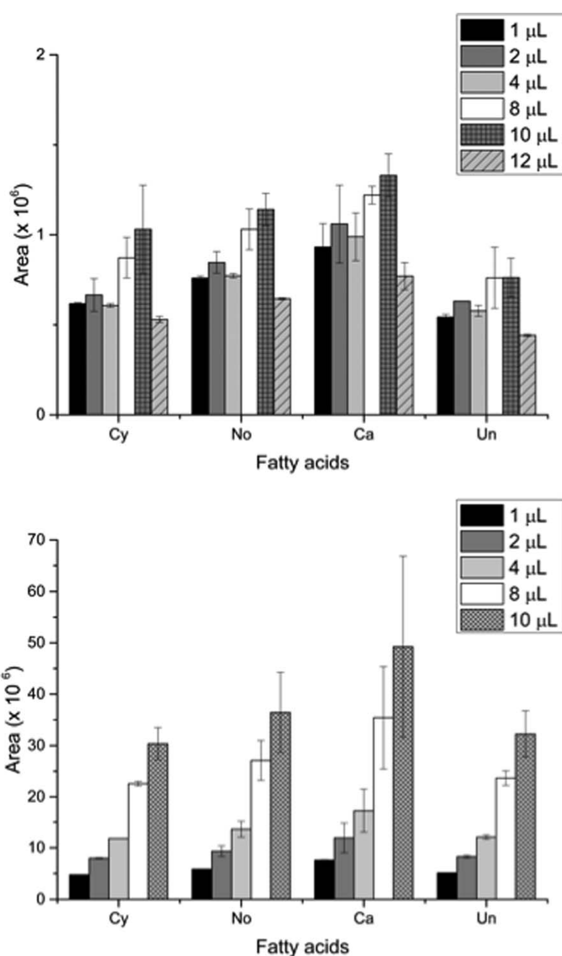

Fig. 4 Effect of volume of derivatising agent on derivatisation of caprylic (Cy), pelargonic (No), capric (Ca) and undecyl (Un) acids; $n = 3$. Derivatisation undertaken on micro SPE cartridges (top) and in vials (bottom).

• All derivatives were eluted with 150 μL of acetonitrile.

2.3.3 Comparison of derivatisation time between repeated derivatisation procedures. The cartridge has a volume of 8 μL. The effect of sample volume and solvents were also tested from a range of 2–12 μL to determine the optimal saturation volume needed to obtain maximum conversion of fatty acids to fatty acids methyl esters (FAMEs).

2.4 Instrumentation

All analyses were carried out on a Varian Saturn 2200 ion trap system with CP-3800 GC consisting of a split/splitless injector with a Varian Saturn 3000 ion trap mass spectrometer (Agilent Technologies, Mulgrave, Australia). Experimental parameters were as follows: column BPX5 (30 m × 0.25 mm × 0.25 mm film) (SGE Analytical Science, Australia); temperature program, 70 °C heated to 130 °C at a rate of 10 °C min^{-1}, then to 230 °C at a rate of 5 °C min^{-1} and finally to 310 °C at a rate of 20 °C min^{-1} and then held constant for 5 min. Nitrogen was employed as the carrier gas with a flow of 1 mL min^{-1}. The injector was programmed to return to split mode after 2 minutes from the beginning of the run. The split flow was set at 50 mL min^{-1} and the injector temperature was held constant at 260 °C. The mass spectrometer was used in the positive electron impact mode at 70 eV. A mass range of 45 to 450 m/z was scanned. Saturn GC-MS workstation software, version 6.8, was used to monitor the instrument and to perform data analysis (measurement of area, signal to noise ratio) and % relative standard deviation (RSD).

3. Results and discussion

3.1 Chromatographic conditions

Prior to the optimisation of the derivatisation technique, a method was developed to separate the 37-component FAME mix using GC-MS. This method was used throughout the experiment to study the performance of the SPE cartridges.

The choice of solvent for SPE is dictated by factors including solubility of the analytes and compatibility to the stationary phase of the sorbent bed. In the case of fatty acids, which are hydrophobic, non-polar solvents are preferable for elution. The compatibility of the cartridges with methyl *tert*-butyl ether, ethyl acetate, chloroform, hexane and acetonitrile was therefore tested. The non-polar solvents such as chloroform and hexane were found to dissolve the SPE cartridge valve and thus were not used further. Acetonitrile was found to give the best results and also provided less backpressure in the SPE system than any other solvent and was therefore used for the remainder of the study.

3.2 Optimisation of the derivatisation method

In the initial investigations, the performance of the SPE on-column reaction was compared to the conventional method of derivatisation; that is, the reactions were performed in glass vials as controls (for comparison) using the method developed by Gullberg *et al.*[13] This method is known to work well for 100 microliter volumes of fatty acids in many metabolomics studies but is not well tested at low (∼10) microliter sample volumes.

Published on 14 January 2016. Downloaded on 19/02/2016 04:17:43.

Published on 14 January 2016. Downloaded on 19/02/2016 04:17:43.

Derivatisation of the fatty acids (10 μL) was carried with 10 μL boron trifluoride in methanol (10% v/v) in an oven at 80 °C for 90 minutes in SPE cartridges and in control vials. After cooling, the FAMEs in the control vials were reconstituted with 150 μL of acetonitrile and the FAMEs eluted from the cartridges with the same amount of acetonitrile. Fig. 2 shows a comparison of the chromatograms obtained when using the SPE cartridges (left) and vials (right). The most prominent feature is the selectivity of the SPE sorbent bed for short-chain fatty acids.

The reaction is shown to be very efficient yielding a FAME content five times higher than was observed from the reaction in the vials for short-chain fatty acids. It is important to note however, that the fatty acid mix tested also contained fatty acids with long aliphatic chains (undecanoic, hexadecanoic and octadecanoic acids). While the latter were derivatised in both situations they only appear in the samples from the glass vial based reactions indicating that the long chain fatty acids do not elute from the cartridge. It is likely that the interactions between the long chain fatty acids and the C18 in the sorbent are stronger than the interactions between the fatty acids and the acetonitrile, making this technique only useful for short chain fatty acids analyses if acetonitrile is used as the elution solvent. Conversely, as can be seen in Fig. 2 the glass vials did not allow good recovery of short chain fatty acids at the ten-microliter level.

3.3 Investigating mixing of reagents

The next step in developing the method was to ensure that efficiency was at its maximum. Therefore mixing procedures in the SPE cartridges were investigated. Fig. 3 shows the different steps involved to trap and derivatise the analytes on the cartridge. The following studies were carried out: study A: 10 μL fatty acids, the cartridge was left for 1 minute and 10 μL BF_3–MeOH was added, heated at 80 °C for 90 minutes; study B: 10 μL fatty acids, 10 μL BF_3–MeOH, heated at 80 °C for 90 minutes; study C: withdrew 10 μL BF_3–MeOH and 10 μL fatty acids, inject all through cartridge and heated at 80 °C for 90 minutes; and study D: 10 μL fatty acids, 10 μL BF_3–MeOH (as experiment B), heated at 80 °C for 10 minutes.

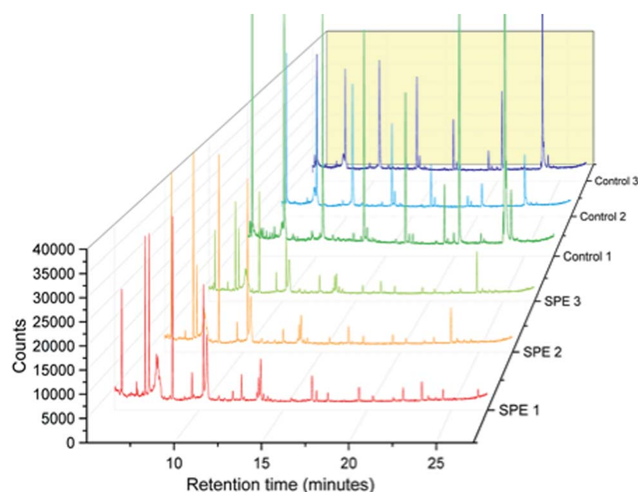

Fig. 5 Stacked chromatograms of the FAME present in olive oil using both the micro SPE and the standard vial based analysis.

Table 1 Calibration curve results

Fatty acids	Cy	No	Ca	Un
R^2	0.987	0.994	0.892	0.959
Slope	7099	911	641	3529
Intercept	−47 295	−329	7268	83 335

It was important to ensure that the reaction was time-effective and the main factor that can decrease the time taken for the reaction is the temperature of said reaction. Two derivatising procedures specifically those of Gullberg *et al.*[13] and Ribeiro *et al.*[14] and two solvent mixing methods were compared, with the results being displayed in Fig. 3 (study B and D). It can be seen from Fig. 3 that method D is not only the best performing reaction but was also the least consuming reaction. It produced the most efficient conversion of fatty acids to FAME and was faster four times shorter in length than the standard vial based method. This has clear advantages for biological studies.

The derivatisation procedures were optimised by assessing the effects of variables such as minimum volume of sample required, temperature and duration of heating. Two main esterification methods were compared; the first one involved heating the organic phase with BF_3–MeOH at 80 °C for 90 minutes and the second method was adapted from the method of Ribeiro *et al.*,[14] which involved heating the samples at 90 °C for 10 minutes.

The effect of volume of sample and solvents used was also investigated in order to determine the minimum volume required to achieve the maximum conversion of fatty acids to FAME. Since the volume of the sorbent bed is about 8 μL, the range tested was from 1 to 12 μL. The results are shown in Fig. 4.

As expected for the control reaction the amount of FAME obtained increased with the volume of fatty acids used (Fig. 4). This is because as the reactions were carried out in vials, the reaction volume was not a limiting factor. A similar observation can be made with the SPE cartridges from 1 to 10 μL; reagent volumes greater than 12 μL however yield less FAME indicating that 10 μL (of sample and derivatising agent) is the optimum volume for this reaction. This means the on-column derivatisation technique is well suited for studies using small sample volumes.

Calibration curves for each FAME were made from solutions of 1, 2.5, 10, 50 and 100 ng mL^{-1} prepared from the 10 μL mL^{-1} stock solution. It was found that detection limits of caprylic, capric and undecylclic were 2 ng mL^{-1} and that of pelargolic was 2.5 ng mL^{-1}, which was lower than the detection limit using a vial. This result further indicated the potential of the on column derivatisation reaction (Table 1).

3.4 Application

Fig. 5 shows the chromatograms obtained from the derivatisation of olive oil on three different individual micro SPE cartridges with the C18 stationary phase and three control vials. This was undertaken to test the method on a real sample. Olive oil is a very valuable substance that is tested on a daily basis

 This journal is © The Royal Society of Chemistry 2016

Published on 14 January 2016. Downloaded on 19/02/2016 04:17:43.

worldwide. Adulteration of olive oil with cheaper plant oils such as hazelnut or canola can be detected via the alterations in short chain fatty acid profiles that such mixing causes and thus our method has potential applications in the food industry. It was possible to use the on-column derivatisation method to quantify FAME in olive oil where the concentrations of pelargonic, capric and undecyclic were found to be 54.4, 83.3 and 43.9 ng mL^{-1}, respectively.

4. Conclusions

Our results show that on-column derivatisation suitable for metabolomics and lipidomics studies was possible with the micro SPE system and that the method allowed fast and accurate analysis with minimal sample separation. We have previously also shown it to be suitable for sample clean-up of complex matrices and to provide a short, fast separation prior to mass spectrometry based analysis.[15,16] Although the present study focused on the analysis of short chain fatty acids the principle demonstrates great potential for a variety of other novel chemical modifications to be made quickly and simply to samples prior to analysis. This method therefore has the potential to be used by scientists in many fields where such reactions are commonly used including (but not limited to) the pharmaceutical, biological/biomedical, environmental, food and forensic sciences.

Acknowledgements

J. P. is grateful for the award of an RMIT PhD Scholarship and a Commonwealth Scientific and Industrial Research Organisation (CSIRO) top-up scholarship (grant number 13/03634). Both authors thank Andrew Minett from ePrep Pty Ltd for providing micro SPE cartridges.

Notes and references

1 C. W. Huck and G. K. Bonn, *J. Chromatogr. A*, 2000, **885**, 51–72.
2 Y. Song, S. Zhao, P. Tchounwou and Y.-M. Liu, *J. Chromatogr. A*, 2007, **1166**, 79–84.
3 D. Stevenson, *J. Chromatogr. B: Biomed. Sci. Appl.*, 2000, **745**, 39–48.
4 D. Stevenson, *TrAC, Trends Anal. Chem.*, 1999, **18**, 154–158.
5 R. A. Shalliker, B. S. Broyles and G. Guiochon, *Anal. Chem.*, 2000, **72**, 323–332.
6 F. Orata, *Derivatization reactions and reagents for gas chromatography analysis*, INTECH Open Access Publisher, 2012.
7 O. A. H. Jones and H. M. Hügel, in *Metabolomics Tools for Natural Product Discovery*, ed. U. Roessner and D. A. Dias, Humana Press, New York, USA, 2013, vol. 1055, ch. 18, pp. 245–266.
8 J. Pandohee, P. G. Stevenson, X.-R. Zhou, M. J. S. Spencer and O. A. H. Jones, *Curr. Metabolomics*, 2015, **3**, 10.
9 C. Scrimgeour, in *Bailey's Industrial Oil and Fat Products*, John Wiley & Sons, Inc., 2005, DOI: 10.1002/047167849x.bio005.
10 W. A. Khan, G. C. Blobe and Y. A. Hannun, *Cell. Signalling*, 1995, **7**, 171–184.
11 G. van Meer and A. I. P. M. de Kroon, *J. Cell Sci.*, 2011, **124**, 5–8.
12 O. A. H. Jones, L. D. Roberts and M. L. Maguire, *Sample Preparation in Biological Mass Spectrometry*, Springer, Netherlands, 2011.
13 J. Gullberg, P. Jonsson, A. Nordström, M. Sjöström and T. Moritz, *Anal. Biochem.*, 2004, **331**, 283–295.
14 B. Ribeiro, P. Guedes de Pinho, P. B. Andrade, P. Baptista and P. Valentão, *Microchem. J.*, 2009, **93**, 29–35.
15 L. D. Alexandrou, M. J. S. Spencer, P. D. Morrison, B. J. Meehan and O. A. H. Jones, *Sci. Total Environ.*, 2015, **512–513**, 210–214.
16 J. Pandohee, A. Minett and O. A. H. Jones, Separating the separation; evaluation of μ-solid-phase extraction for on-column derivatisation and direct injection mass spectrometry sample preparation, *The 63rd American Society of Mass Spectrometry Conference*, St. Louis, Missouri, USA, 2015.