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# A simple modification of a silicic acid lipid fractionation protocol to eliminate free fatty acids from glycolipid and phospholipid fractions

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ABSTRACT

When fractionating natural and standard lipid mixtures according to the most widely used method for the analysis of soil and other environmental materials, free fatty acids (FFAs) were not recovered quantitatively in the fraction expected to contain all simple lipids. Rather than being eluted from activated silicic acid adsorption chromatographic columns with chloroform, FFAs in standard lipid mixtures appeared only in the two subsequent fractions eluted with acetone and methanol, respectively. Substantial quantities of FFAs from cow dung lipid samples were eluted using chloroform as expected but appreciable amounts also eluted in the acetone (glycolipid) fraction. The fatty acid distribution of the methanol (phospholipid) fraction from dung displayed slightly more FFA compositional character than when FFAs were excluded but this influence was not significant ($p = 0.08$). A simple modification to the silicic acid column technique ensures that FFAs are reproducibly eluted in the chloroform/acetic acid (100:1*v*/*v*; simple lipid) fraction. The modification had no deleterious effect upon the elution characteristics of any phospholipid from a variety of headgroup classes tested. The carry-over of FFAs into glycolipid fractions was confirmed by nanospray ionisation mass spectrometry of the intact polar lipid fractions. As far as could be inferred from the distinctive molecular weight distributions of the glycolipid and phospholipid fractions, none of the complex lipids in dung eluted in both fractions.



## 1. Introduction

The use of silicic acid adsorption column chromatography to separate environmentally derived lipid mixtures into fractions according to their broad chemical class is commonplace. When using fatty acids in their various chemical forms as biomarkers or isotopically labelled tracers, it is especially important that they are separated without loss or cross-contamination. In environmental studies phospholipid fatty acids (PLFAs) have been used widely to derive information about the living microbial community. The matrix has usually been defaunated soil (e.g. Frostegård and Bååth, 1996) although examples also exist for dung (Frostegård et al., 1997), charcoal (Pietikainen et al., 2000), compost (Steger et al., 2007) and gut contents (Oravecz et al., 2004). The analytical protocol most frequently cited (>350 citations) is that of Frostegård et al. (1991, 1993) in which a single phase extraction procedure, based upon that of Bligh and Dyer (1959), yields a total lipid extract (TLE) which is separated on a silicic acid column. Fractions obtained are identified as 'simple lipid' (SL), 'glycolipid' (GL) and 'phospholipid' (PL) and all three are eluted using unmixed solvents. The PL fraction, often the only fraction analysed further, may be investigated directly (e.g. Sturt et al., 2004) but is more usually transesterified for analysis of its fatty acids as methyl esters (FAMEs). These FAMEs, known to originate from PLs that were intact at the time of sampling, are termed phospholipid fatty acid methyl esters (PLFAMEs). Similarly, FAMEs originating from intact glycolipids may be termed GLFAMEs.

Frostegård et al. (1991) presented a thorough and comprehensive validation of their extraction and storage protocols but validation of their fractionation was not central to their paper. Natural lipid mixtures contain fatty acids in a number of forms besides GLs and PLs. In dung research free fatty acids (FFAs) are a pertinent example, being more abundant than any complex lipid. FFAs must therefore be accounted-for when relative abundances of fatty acids and/or their stable isotopic signatures are used to draw conclusions about transfers of organic matter within the ecosystem, for example, *via* trophic links. As was a reasonable expectation for a simple lipid, Frostegård et al. (1991) implied that they expected FFA to be recoverable from the chloroform fraction. Indeed, this is stated explicitly in the paper from which Frostegård et al. appear to have taken the fundamental column method (Kates, 1972) and in several other works (Ginger and Fairbairn, 1966; Rouser et al., 1967; Perkins, 1993; Gunstone et al., 1995). However, FFAs are also amongst the more polar SLs and therefore amongst the most likely to be retained by the silicic acid stationary phase while others are eluted. Furthermore, the resulting contamination of subsequent GL and PL fractions will remain unnoticed if base catalysed transesterification is used for derivatisation; these reactions being selective for ester-bound fatty acids. However, if an unselective

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derivatisation technique is used (e.g. an acid catalysed esterification) the efficacy of the preceding adsorption chromatography stage is vital in preventing FFAME contamination of GLFAME and possibly PLFAME fractions. The first aim of this work was therefore to assess the degree to which FFAs can contaminate GL and PL fractions, the second aim being to modify the adsorption chromatography method of Frostegård et al. (1991) to enable FFAs to be recovered quantitatively in the SL fraction without affecting the other components. Described herein is a validation of such a modification with an analysis of the elution characteristics of simple and complex molecular forms of fatty acids using the unmodified and modified methods. Conclusions derive from tests using both standard and dung-derived lipid mixtures.

## 2. Materials and methods

### 2.1. Reagents and solvents

All solvents were of HPLC or glass distilled grade. Aqueous solutions were formulated using double distilled water and then extracted three times with DCM. Anhydrous sodium sulphate columns were pre-washed with the solvent of the solution to be dried.

### 2.2. Lipid fractionation

Two fractionation protocols were compared:

(i) "Frostegård" is the standard literature method (Frostegård et al., 1991): A column of oven-activated (125 °C) silica gel (0.5 g, 60 Å particle size) was conditioned with chloroform (4 ml) and the lipid mixture was applied to the column in the same solvent (2×400 μl). A SL fraction was then eluted with chloroform (5 ml) followed by a GL fraction eluted with acetone (20 ml) and a PL fraction eluted with methanol (5 ml).
(ii) "Modified" was the same as "Frostegård" except chloroform/acetic acid (100:1 *v*/*v*) was used in place of pure chloroform.

Solvent was evaporated from all eluted fractions under a gentle flow of nitrogen before further fractionation or preparation of FAMEs for analysis.

### 2.3. Isolation of FFAs from SL fractions

FFAs in dung SL fractions were separated using the column chromatographic method of Ansari and Shoeman (1988). Briefly, activated silica gel columns (~0.3 g, 60 Å particle size) were conditioned with chloroform that had recently been saturated with ammonium hydroxide (4 ml). SL fractions, dissolved in the same solvent (2×400 μl) were applied to these columns and all SLs other than FFAs were then eluted using a further 9 ml of this solvent. FFAs that remained on the column were then protonated and eluted using chloroform/acetic acid (100:1 *v*/*v*).

### 2.4. Preparation of FAMEs

Two methods were used to prepare FAMEs for gas chromatography (GC) (Christie, 1993):

(i) Acid catalysed methylation: Samples were dissolved in 5% $HCl_{(dry\ methanol)}$ and heated at 50 °C in closed vials. After 2 h 5% $NaCl_{(water)}$ (5 ml) was added followed by hexane (1 ml). The hexane layer was decanted and the remaining aqueous phase extracted twice more with hexane (1 ml). The combined hexane layers were washed with 2% $KHCO_{3(aq.)}$ and then dried over anhydrous sodium sulphate. The solvent was evaporated under a gentle flow of nitrogen and the residue dissolved in hexane for GC analysis.
(ii) Base catalysed transesterification: Samples were dissolved in a mixture of sodium-dried diethyl ether (0.5 ml) and methyl acetate (20 μl). Sodium $methoxide_{(MeOH)}$ (1 M; 20 μl) was added and, after brief mixing, the reaction was left to proceed at room temperature for 5 min. The reaction was stopped by the addition of acetic acid (2 μl) and the solvent evaporated under a gentle flow of nitrogen. Hexane (1 ml) was added and the sample centrifuged (2500 rpm) after which the solution of FAMEs was decanted into a clean vial from which the solvent was evaporated, leaving a residue to be dissolved in hexane for analysis by GC.

### 2.5. Artificial TLE formulation

Artificial TLEs contained FFA (5 μg) and PLFAs (5 μg). *n*-Nonadecanoic acid ($C_{19:0}$) was always used as the FFA but the limited choice in the FA composition of the available PL headgroup classes dictated that the identity of the standard PLFAs varied. Distearyl-phosphatidyl choline (PC) was used for all quantitative comparisons with FFA. In addition, qualitative elution characteristics were assessed for phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG) and the acidic phospholipids, phosphatidyl serine (PS), phosphatidyl inositol (PI), phosphatidic acid (PA) and cardiolipin.

### 2.6. Experimental design — artificial TLEs

Four replicate artificial TLEs were fractionated according to the "Frostegård" and "Modified" protocols. In order to ensure methylation of free and esterified fatty acids, all twelve fractions were methylated using the acid catalysed protocol. FAME peaks were quantified against an *n*-nonadecane internal standard by GC as detailed below.

### 2.7. Experimental design — dung TLEs

Three replicate samples of fresh cow dung were freeze-dried and extracted according to a modified monophasic extraction procedure (Bligh and Dyer, 1959). The monophasic solvent was made up from potassium dihydrogen phosphate solution buffered at pH 7.2, methanol and chloroform in a ratio of 4:10:5 (*v*/*v*/*v*). Monophasic solvent (4 ml) was added to 250 mg of dung and ultrasonicated for 15 min prior to centrifugation for 5 min at 3000 rpm. The supernatant was removed and retained and the extraction procedure repeated a further 3 times with 3 ml of monophasic solvent each time. The supernatants were combined and buffered water (2 ml) added followed by chloroform (2 ml) thereby breaking the solution into aqueous and organic phases. The organic phase was removed and retained and the aqueous phase extracted twice with 2 ml volumes of chloroform. All organic layers were combined and solvent evaporated under $N_2$ yielding the TLE.

Aliquots of all replicate dung TLEs were separated according to the "Frostegård" and "Modified" protocols. The resulting GL and PL fractions were transesterified using the base catalysed method. This entire set of treatments was then repeated with the exception that the GL and PL fractions were methylated by the acid catalysed (unselective) derivatisation reaction. FFAs eluting in the SL fraction were isolated from neutral compounds using the FFA separation protocol and methylated (acid catalysed).

### 2.8. GC and GC/mass spectrometry (GC/MS)

Fatty acids in artificial TLEs were quantified against an *n*-heptadecanoic acid internal standard using a GC equipped with a flame ionisation detector. Fatty acids originating in dung TLEs were quantified against an *n*-nonadecane internal standard, making a systematic adjustment for the effect of compound class difference on detector response. Identification was by means of GC/MS using a Factor Four VF23-MS fused silica capillary column (high cyanopropyl modified methyl polysiloxane; Varian Chrompack, 60 m×0.32 mm, 0.15 μm film thickness). For GC analyses, the carrier gas was hydrogen with the GC

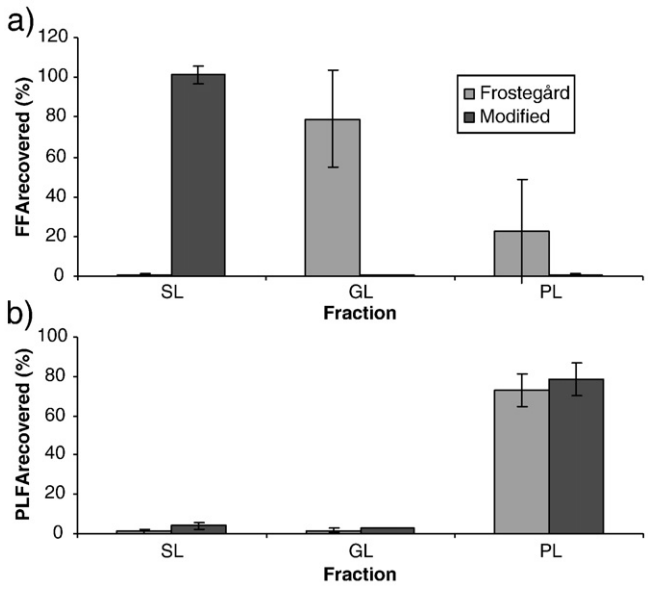


**Fig. 1.** Separation of a) FFA and b) PLFA (applied as distearyl PC) into SL, GL and PL fractions as produced by the "Frostegård" and "modified" protocols. PLFA recovery is expressed as a percentage of that applied to the column.

(Hewlett Packard 5890 Series II) oven temperature programmed to rest at 50 °C for 1 min then from 50 °C to 100 °C at 15 °C $min^{-1}$, then from 100 °C to 240 °C at 4 °C $min^{-1}$ then from 240 °C to 260 °C at 15 °C $min^{-1}$ and held at 260 °C for 10 min. For GC/MS (TraceMS, ThermoQuest, Hemel Hempstead, UK) analyses, the MS was set to scan the range $m/z$ 50–650 in a total cycle time of 0.6 s. Additional operating conditions were as follows: ion source temperature 200 °C; emission current, 300 μA; electron ionisation potential, 70 eV; the GC-MS interface was maintained at a temperature of 300 °C; helium was used as the carrier gas. Data were acquired and processed using the XCalibur (ThermoFisher Scientific, Hemel Hempstead, UK) data acquisition and processing software.

### 2.9. Nanospray ionisation MS

Nanospray mass spectrometry was performed on a QStar XL (Quadrupole time-of-flight) Instrument (Applied Biosystems, Warrington, UK) using a Nanomate HD 'chip based' nanospray system (Advion Biosciences, Norwich, UK). The Nanomate was set for 5 μl of an approximately 200 ng $\mu l^{-1}$ solution of complex lipid dissolved in chloroform/methanol (2:1 $v/v$) to be aspirated and sprayed through a Nanomate 400 chip at 1.45 kV, with a nitrogen back pressure of 0.4 psi.

## 3. Results

### 3.1. Artificial TLEs

The hypothesis that FFAs are too polar to be eluted quantitatively from activated silicic acid by chloroform was confirmed by the observation that standard FFA ($C_{19:0}$) never eluted in the SL fraction when using the "Frostegård" protocol. Rather, the applied $C_{19:0}$ was recovered in the GL fraction (79 ± 24%) and the PL fraction (23 ± 26%). The large standard deviations quoted are a consequence of the applied FFA eluting entirely in the GL fraction for one batch of two replicates and partly in each of the GL and PL fractions for the other batch of two replicates. Using the "modified" protocol all FFA eluted in the SL fraction (see Fig. 1a). The fatty acid applied as PL (distearyl PC), was detected above background levels only in the PL fraction regardless of the fractionation protocol used (see Fig. 1b). The same was observed in experiments with the other phospholipid headgroups (PS, PI, PE, PG, PA and cardiolipin).

### 3.2. Dung TLEs

In contrast with the results from standard mixtures, substantial proportions of FFAs in dung were recovered in the SL fraction even when using the unmodified Frostegård fractionation. Nevertheless, the quantity of FFA recovered in the SL fraction (1090 ± 189 μg $g^{-1}$, SD $n = 3$) was significantly lower than that obtained using the modified scheme (1830 ± 382 μg $g^{-1}$, SD $n = 3$) and the significance of this comparison extended to testing the three most abundant FAs individually

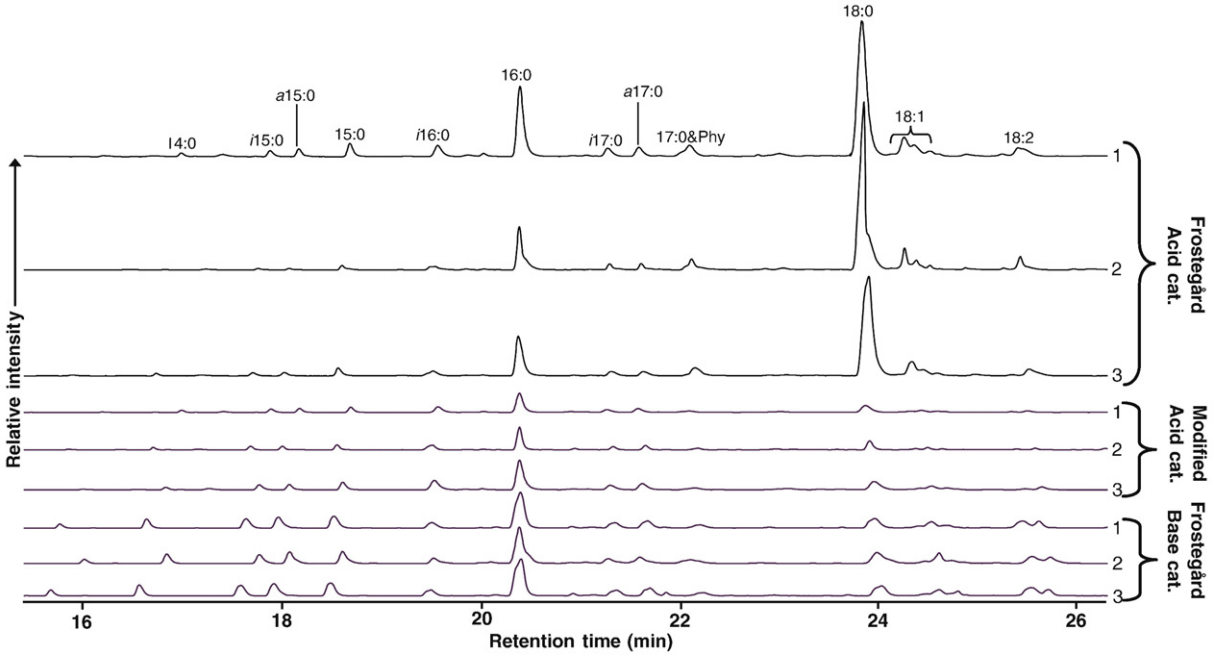


**Fig. 2.** Partial gas chromatograms of the GLFAME fractions of three numbered replicate dung extractions prepared for GC using three combinations of separation and derivatisation methods. Top — "Frostegård" separation, acid catalysed esterification; Middle — "Modified" separation, acid catalysed esterification; Bottom — "Frostegård" separation, base catalysed transesterification (Phy — phytanic acid).

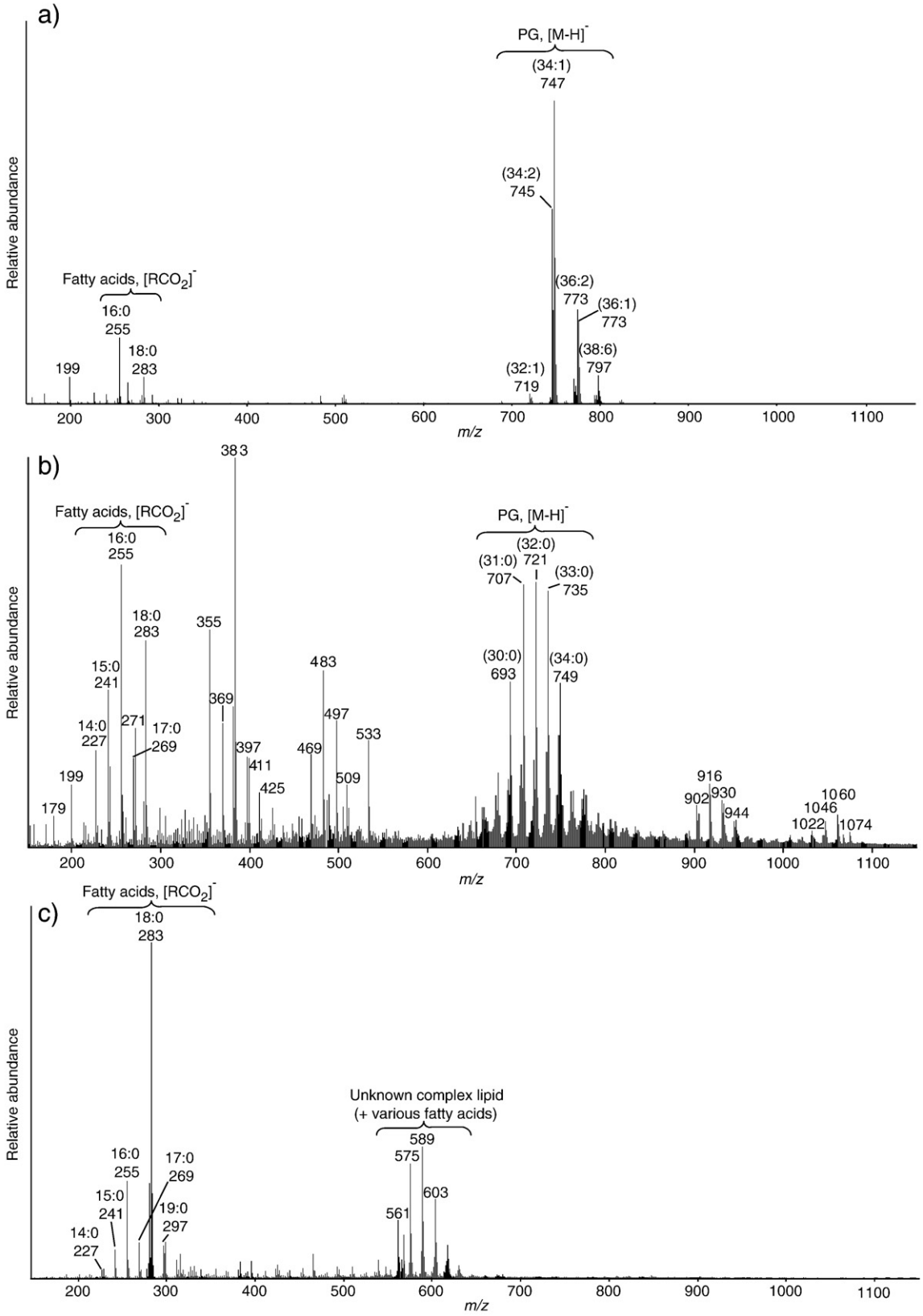


**Fig. 3.** Negative ion nanospray ionisation mass spectra: a) A PG standard (derived from egg yolk). Note that the only $RCO_2^-$ species correspond to saturated fatty acids despite GC analysis of the PLFAMEs identifying major $C_{18}$ and $C_{20}$ unsaturates; b) The PL fraction of a cow dung TLE, obtained using the modified silicic acid column method; c) The glycolipid fraction of a cow dung TLE obtained using the unmodified method. The spectrum exhibits a relative abundance of $CH_3(CH_2)_{16}CO_2^-$ ions over forty times that measured when the modified method was used (data not shown). Fatty acid compositions for the each ion are indicated in the form: (total C number:total double bonds).

($p<0.05$). Non quantitative elution of dung FFAs in the SL fraction using the "Frostegård" protocol was confirmed by evidence of FFA carry-over in the GLFAME chromatograms (see Fig. 2). These showed serious contamination with FFA carried-over from the SL fraction (seen most obviously as the many times greater abundance of the, now dominant, $C_{18:0}$ species). This finding was confirmed by comparison of the negative ion mode nanospray mass spectra of dung GL fractions obtained by the unmodified and modified protocols. Using the unmodified protocol, the ion at $m/z$ 283 (corresponding to $CH_3(CH_2)_{16}CO_2^-$) was the base peak, being more than twice as intense as the major high mass ions. Relative to the same high mass ions in negative ion mode nanospray mass spectra, adoption of the modified protocol reduced the intensity of $m/z$ 283 in the GL fraction (see Fig. 3c) by a factor of over 40 whilst showing no effects on the distribution of high mass ions in either the GL or PL fractions of dung.

In contrast to the unequivocal evidence of FFA carry-over in the PL fraction, as was found for the artificial TLEs, only a small indication of FFA carry-over was visible in PLFAME chromatograms of dung samples. It would appear that a favourable matrix effect resulted in $C_{18:0}$ in these fractions not being significantly more abundant having used the unmodified protocol than having used the modification ($p=0.08$). This being said, it is worth noting that the major dung FFA species ($C_{18:0}$ and, to a lesser extent, $C_{16:0}$) were more abundant in the "Frostegård" than in the "modified" PLFAME fractions whereas all other fatty acids were less abundant.

## 4. Discussion

The body of evidence presented above demonstrates that a modification of the commonly used silicic acid adsorption chromatographic procedure of Frostegård et al. (1991) is necessary to elute FFAs quantitatively in the SL fraction. The proportion not eluted by chloroform is liable to be eluted by either or both of the subsequent, more polar solvents, acetone and methanol (the eluotropic strengths of the three solvents on silica being: chloroform, 0.26; acetone, 0.50; methanol, 0.72). The selective derivatisation (i.e. base catalysed transesterification) stage of the original method has probably been responsible for the presence of FFAs in GL and PL fractions going unnoticed until now. However, researchers restricted to unselective derivatisation reactions (e.g. acid catalysed esterifications) go without this second selective step and should therefore be aware that TLEs with high concentrations of FFAs will yield GL and possibly PL fractions with important FFA input.

If FFAs are of interest alongside GLFAs and PLFAs, and an attempt is made to recover them from a SL fraction obtained using the unmodified method, their concentrations would be underestimated. This effect could even potentially extend to selective underestimation of the more polar FFA species. Analysts interested in quantifying FFAs or in obtaining GL and PL fractions with no FFA cross-contamination must therefore use a modified method.

The technique of Ansari and Shoeman (1988) has been validated for isolation of FFAs from SL fractions obtained using the modified silicic acid column protocol detailed above. The need to recover FFAs from a SL fraction using another column makes a silicic acid TLE fractionation less appealing than a method that would require only one column. For this reason an attempt was made to combine the Frostegård et al. (1991) TLE fractionation and Ansari and Shoeman (1988) FFA separation using the same silicic acid column (i.e. the first fraction was neutral lipids, eluted with chloroform saturated with ammonium hydroxide and then the second, third and fourth fractions were FFA, GL and PL eluted using the three solvents of the modified protocol). However, recovery of PLFA was very poor (data not shown), even when the methanol was amended with various cations (including acids) and anions in attempts to reverse the effect of the ammonium hydroxide upon the stationary phase or PL analytes. A second adsorption chromatographic step would therefore seem necessary for obtaining FFAs separately to other SLs.

Although the insufficient polarity of chloroform for the elution of FFAs from anhydrous ("activated") silica meant that standard FFA ($C_{19:0}$) was never observed in the SL fraction, the division of standard FFA between the two complex lipid fractions differed greatly between batches of replicates. Notwithstanding measures taken to ensure that the silica remained fully activated during the preparation of columns, it seem most likely that the variation was due to partial hydration of one batch of silicic acid columns before use; $C_{19:0}$ eluting more in the GL and less in the PL fractions from this batch. Hydration not only reduces the polarity of silicic acid but may also potentiate partition chromatography effects whereby regions containing trapped water act as an extra phase through which analytes may move (Christie, 1973). Even if hydration is eliminated, the properties of silicic acid can differ between suppliers and even between individual batches from the same supplier (Christie, 1973) so the potential for inter-batch chromatographic variability of FFAs must be borne in mind at all times.

Partial deactivation of silica gel might have been an option for ensuring FFAs eluted in the SL fraction. However, controlled hydration would add to preparation time and make it more difficult to ensure that the stationary phase had reproducibly uniform properties. Altering the acidity, rather than the polarity, of the SL eluent was also considered to be more favourable because such an alteration is more likely to result in an effect on the elution of FFAs specifically. This seems to be the case because the addition of acetic acid in a volume ratio of 1:100 for the SL eluent, in order to disrupt the interaction of FFAs with the stationary phase, was not enough to prematurely elute any of the standard PLs tested. Notably, this list includes PS, PI, PA and cardiolipin, acidic PLs expected, from the work of Rouser et al. (1967) and Gunstone et al. (1995), to elute partly in the GL fraction even when the preceding SL elution had used a neutral solvent. Nanospray mass spectra of intact complex lipid fractions support the interpretation that the modification did not cause legitimate components of the GL or PL fraction to elute in the wrong complex lipid fraction. The most intense high mass ions in the nanospray mass spectra of dung PL fractions were part of a bell-shaped series of five ions separated by $m/z$ 14 (methylene units) and centred on $m/z$ 721 (see Fig. 3a,b). These correspond to PG possessing two saturated fatty acids with a total of 30–34 carbon atoms, consistent with the distribution of fatty acids found in dung PL fractions. However, under nanospray ionisation conditions a caveat of potential differential ionisation of the various PL components applies, especially relating to their differing acidities (i.e. ability to yield negative ions). Other PL species could therefore be quantitatively more important than PG and yet account for fewer ions in the nanospray mass spectrum of a PL fraction. Indeed, a dung PL fraction spiked with PC in a molar ratio of 1:5 with the natural PLs yielded a mass spectrum with the PC pseudomolecular deprotonated chlorine adduct ion ($m/z$ 824; $[M\text{-}H+Cl]^-$) accounting for only 5% of the combined peak intensities of the PG pseudomolecular ions ($[M\text{-}H]^-$).

Although this paper shows that it has been wrongly assumed that complex lipid fractions are devoid of FFAs, the deleterious interference of FFAs with measured complex lipid fatty acid profiles seems to have been largely avoided in the past by virtue of two factors. Firstly, their elution, in the most part, in the GL rather than the more widely used PL, fraction has shielded them from attention. Secondly, the resistance of FFAs to methylation with a base catalyst, as confirmed in this work, has led to FFA contaminants reaching the GC underivatised and therefore remaining in the injector and/or GC column. When using an unmodified "Frostegård" separation, analysts using unselective methylation reactions should therefore be alert to the possibility of FFA carry-over into their GL and PL fractions, especially if FFAs are abundant in their extracts.

To conclude:

(i) This work has shown that the destination fraction of FFAs in mixtures of standard compounds applied to the silicic acid columns is not the SL fraction as expected but rather the GL ($79\pm24\%$, SD $n=3$) and PL ($23\pm26\%$, SD $n=3$) fractions.

(ii) For dung lipid samples, carry-over of FFAs from the SL to the GL fraction is less pronounced but not to be neglected if (a) FFAs are to be analysed or (b) GLs are to be analysed by their FAMEs *via* a derivatisation reaction that does not discriminate against FFA.

(iii) FFA contamination of dung PLFAME fractions was only bordering on statistical significance ($p = 0.08$) when fractionating dung lipids. However, using the unmodified method, the major variability in standard FFA elution characteristics between batches of column and between lipid matrices was noted. This should be taken as a warning that what has been observed for one batch of dung TLEs may not always apply to all PL fractions obtained and for all types of natural TLE.

On the basis of the above, it is recommended that FFA analysts and/or those analysing complex lipids *via* unselective derivatisation reactions adopt the chromatographic modification validated here in order to fully quantify FFAs and eliminate their interference with GLFA and PLFA analyses.

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