Review Article

Beyond diazomethane: Alternative approaches to analyzing non-esterified fatty acids

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In many branches of lipid science, researchers are interested in non-esterified fatty acid (NEFA) content and composition. For many years, diazomethane was the reagent of choice to selectively derivatize and then detect NEFA due to its highly specific methylation of the carboxylic acid functional group. While the activity of this derivatizing reagent is very defined, it is dangerous and can be difficult to obtain. In this brief review, we have compiled a collection of methods which allow for the detection of NEFA and hydroxy NEFA without the use of diazomethane. We have concentrated on methods that employ three distinct approaches of selective quantification/extraction, purification from total lipids and derivatization techniques.

Keywords: Derivatization / Extraction / GC-MS / Hydroxy fatty acids / Quaternary ammonium salts

Received: August 21, 2014 / Revised: December 23, 2014 / Accepted: January 15, 2015

DOI: 10.1002/ejlt.201400404

1 Introduction

Diazomethane has been utilized by lipid analysts for many years as a highly effective and selective means of derivatizing non-esterified fatty acids (NEFA) to their corresponding methyl esters for subsequent analysis by gas chromatography [1]. However, the use of this reagent poses several serious risks to the analyst, primarily due to its toxic and explosive nature. Researchers have been aware of these risks for some time and the first report of diazomethane poisoning dates back to 1938 [2]. During recent research efforts we had hoped to identify short-chained hydroxylated NEFA in *Saccharomyces pastorianus* which earlier studies had achieved with diazomethane [3, 4]. In light of the danger and scarcity of diazomethane, we sought out and evaluated alternative means to analyze these hydroxylated NEFA. It quickly became apparent that while other methods existed within the literature, there was not a body of work which aggregated these methods into one succinct review. Thus, the goal of this short review is to explore and report on methods that provide an alternative to diazomethane and allow for selective quantification/extraction, purification, and derivatization of NEFA, with a particular focus on short-chained hydroxylated NEFA. It is our hope that this report and the methods, and approaches we highlight herein will aid future researchers and analysts in their detection of NEFA and hydroxy-NEFA.

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Abbreviations: 3-OH10:0-3, hydroxy decanoic; **DMAP,** 4-dimethylaminopyridine; **DMP-2,2,** dimethoxypropane; **FFA,** free fatty acid; **NCI-MS,** negative chemical ionization-mass spectrometry; **NEFA,** non-esterified fatty acid; **PCI-MS,** positive chemical ionization-mass spectrometry; **PUFA,** polyunsaturated fatty acids; **SIM,** selected ion monitoring; **SPE,** solid-phase extraction; **TBDMS,** tert-butyldimethylsilyl; **TLC,** thin-layer chromatography; **TMA,** tetramethylammonium; **TMAH,** tetramethylammonium hydroxide; **TMPH,** trimethylphenylammonium hydroxide; **TMS,** trimethylsilyl; **TMSH,** trimethylsulfonium hydroxide; **TMTFTH,** trimethyl (α, α,α-trifluoro-m-tolyl) ammonium hydroxide

2 Selective quantification and extraction

Diazomethane is particularly useful for free fatty acid detection largely because this compound will only react with the free carboxyl end of NEFA, leaving esterified fatty acids intact. An alternative approach to diazomethane use is to employ a selective quantification or selective extraction scheme. In the literature, we found numerous such methods and have grouped them as general selective quantification/extraction methods involving a base [5, 6, 7], a reportedly true selective extraction [8] and selective extractions involving quaternary ammonium salts [9, 10, 11]. We then go on to discuss

extraction of short-chained hydroxylated NEFA, and describe the method we used in our work (paper to be published, Potter, G., Budge, S.M., and Speers, R.A., 2014).

The carboxyl functional group at the head of NEFA is a convenient target to selectively quantify and extract these molecules. Due to the acidic nature of the carboxyl group, NEFA can be quantified by titration with alkali. Similarly, NEFA may be selectively extracted by harnessing their altered solubility in organic or aqueous solvents upon exposure to acid or base. A number of methods have achieved selective quantification and extraction with serum samples [5, 6, 7] and each follows the same series of preliminary steps. NEFA are first acidified to (1) ensure protonation of the carboxyl functional group and they are then (2) extracted into a suitable organic solvent. Thereafter, the NEFA can be quantified in the organic layer by titration with alkali or can be further extracted and concentrated from the solvent using base deprotonation to yield salts of the NEFA in an aqueous layer. For instance, Dole et. al. [5] presented an early alkali titration option for selective quantification of NEFA in serum where the sulphuric acid acidified samples were first extracted using heptane, and were then titrated with NaOH and a thymol blue indicator. This quantification scheme was found to be quite selective for NEFA with less than 2% co-extraction of other organic acids commonly found in biological samples.

A similar selective extraction approach that used hexane as the solvent and KOH to deprotonate NEFA also added spikes of radioactively-labeled triolein [6]. By adding the triolein, Schotz et al. [6] demonstrated that there was less than 0.1% carryover of the labeled triacylglycerol into the NEFA concentrate, which implied quantitative extraction of long chain NEFA. Hušek et al. [7] described a second selective extraction method using oxalic acid, isooctane, and KOH and reported that some polar phospholipids were co-extracted in their system. However, they were able to confirm that none of the NEFA later detected resulted from hydrolysis of the co-extracted phospholipids. Any similar method where salts of NEFA are recovered in aqueous base with minimal interference from other lipid classes may employ a final acidification step to reform NEFA. This pure fraction of NEFA can then be analyzed by gas chromatography, in the derivatized or underivatized form, to determine their structures and concentrations.

There is a single report of a purportedly true NEFA-specific extraction described by Höckel et al. [8]. This method used a 1:1 mixture of n-heptane:chloroform with 2% methanol to extract NEFA from serum or plasma in a phosphate buffer at pH 6.4. The resulting extractant contained principally NEFA; cholesterol esters, triacylglycerols, and phospholipids were co-extracted but only in trace amounts. Quantitative recovery of NEFA ranging in lengths from lauric (12:0) to lignoceric (24:0) was demonstrated, indicating that the method is suitable for both medium and long-chain NEFA. While applicable to serum samples, this NEFA-specific extraction method has not been validated with other sample types that may contain more complex lipid mixtures. Basic solutions of quaternary ammonium salts can also be used to selectively extract NEFA. As with the aforementioned protocols involving basic aqueous solutions, basic quaternary ammonium salts contain hydroxide ions and are able to deprotonate the carboxyl group of NEFA (Fig. 1a), thus concentrating NEFA from the organic extraction solvent. These quaternary ammonium salts are particularly useful because they allow for direct pyrolytic derivatization of NEFA in a hot injector of a GC (Fig. 1b). A number of basic quaternary ammonium salt preparations have been employed including trimethyl (α,α,α-trifluoro-m-tolyl), ammonium hydroxide (TMTFTH), and trimethylphenylammonium hydroxide (TMPH). These salts have facilitated selective extraction of NEFA from a variety of starting materials including serum, tissue samples, and edible oils, demonstrating their versatility and utility in numerous different applications (Table 1).

MacGee and Allen [9] reported on such a selective extraction approach to isolate NEFA from serum samples with TMTFTH. These authors found that their method was rapid and suggested that food samples may also be analyzed for NEFA so long as appropriately small initial samples were used. Kishiro and Yasuda [10] also used TMTFTH to detect free

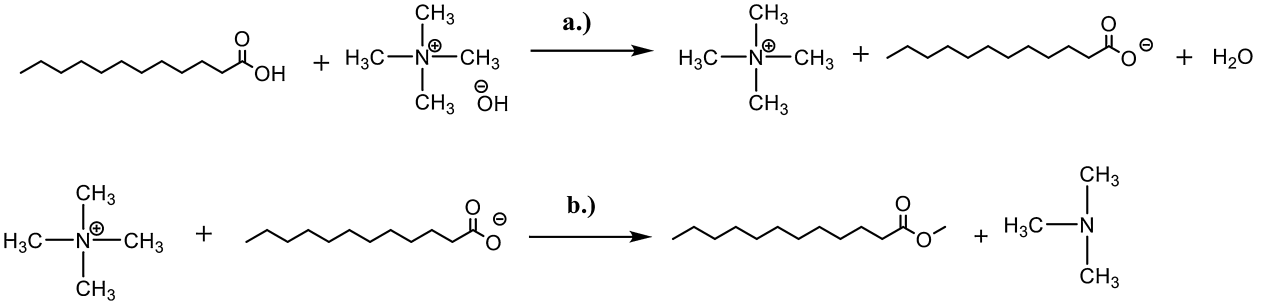

Figure 1. Chemical reactions that occur during selective extraction of NEFA using a quaternary ammonium salt (a) and the later pyrolytic derivatization when the mixture is placed in the hot injection port of a GC (b).

Table 1. A summary of the selective quantification and extraction methods described

Type	Applications	Advantages	Disadvantages	References
a	Serum containing all chain lengths of NEFA	Ease of removal of lipid-containing organic phase No evaporation step	Tedious titration required	[5]
b	Serum with long-chain NEFA	Quantitative for long-chain NEFA	Less ideal for short-chain NEFA	[6]
b	Serum with NEFA prone to breakdown	Rapid	Co-extraction of phospholipids	[7]
c	Serum with medium and long-chain NEFA	Supposedly, truly selective for NEFA	Timely concentration step required Other lipid species co-extracted	[8]
d	Serum and food with NEFA prone to breakdown	Rapid	TMTFTH solution laborious to prepare	[9]
d	Tissue samples with long-chain PUFA	Sensitive and quantitative	N/A	[10]
d	Edible oils with medium and long-chain PUFA	Rapid and quantitative	Only validated with ≤ 5 concentrations % NEFA	[11]
e	Yeast and other microbiological samples containing hydroxy lipids	Simple	Slow evaporation of ethyl acetate	Potter et al. [2014]

a, selective quantification involving a base; b, selective extractions involving a base; c, true NEFA selective extraction; d, selective extractions with quaternary ammonium salts; e, extraction for hydroxy-NEFA.

arachidonic acid (20:4) from Folch-extracted brain tissue with little co-extraction of triacylgylcerols, phospholipids, and cholesterol esters. Furthermore, these authors noted that the method was highly sensitive and was likely useful with other applications where NEFA must be removed from complex tissue matrices. Williams and MacGee [11] later developed a similar protocol to remove NEFA from vegetable oil containing no more than 5% FFA with TMPH. These authors reported that the method was quantitative and could extract saturated and unsaturated NEFA ranging in length from dodecanoic (12:0) to linoleic (18:2) acids.

While the previously described methods were undoubtedly suitable ones for NEFA, our recent study (paper to be published, Potter, G., Budge, S.M., and Speers, R.A., 2014) found that a hexane-based extraction was inappropriate for hydroxylated short-chained NEFA. When a hexane:isopropanol extraction solvent was applied to yeast cells, we noted poor recovery of 3-hydroxy decanoic acid (3-OH 10:0) because this molecule is more strongly solvated by isopropanol and the subsequent isopropanol/water mixture, than hexane. To achieve better recovery of 3-OH 10:0 from our material, we utilized an extraction solvent of intermediate polarity, ethyl acetate. This extraction approach does not necessarily exclusively remove short-chained hydroxylated NEFA from the material; however, it is an important preliminary step in their quantitative recovery.

In our ethyl acetate extraction procedure, the material was acidified to pH 3.5–4.5 to protonate the NEFA and ensure their recovery in the organic extraction solution [12]. A saturating amount of NaCl was also added to the mixture to reduce emulsion formation [12]. The 3-OH 10:0 and other lipids were then double extracted with successive aliquots of ethyl acetate. In another protocol designed to extract similarly polar compounds from an aqueous matrix [13], alkanocarboxylic herbicides were best extracted with ethyl acetate as the extractant and 4% sodium sulphate as a salting agent. Thus, the addition of NaCl to our extraction medium also likely improved recovery of 3-OH 10:0 by a "salting out" effect. A summary of our extraction procedure, and all those previously described, are presented in Table 1.

The work of Chappell et al. [14] also revealed pertinent information regarding extraction of short-chained hydroxy-fatty acids, particularly hydroxy-butyric acid (hydroxy-4:0). After testing the recovery of γ-hydroxy butyric acid (γ-hydroxy 4:0) in various organic solvents during a liquid-liquid extraction, they found that those with an oxygen-containing functional group, including methyl acetate and ethyl acetate, were most effective. They attributed this effectiveness to the hydrogen-bonding potential of the hydroxyl and the carbonyl groups. Interestingly, these authors also found that *n*-hexane achieved very little extraction of hydroxy-butyric acid (hydroxy-4:0) and that extraction efficiency was improved considerably when a saturating amount of salt was added to the extraction medium [14].

3 Purification of NEFA from total lipid extracts

NEFA can also be isolated by purifying total lipid extracts. Here, we present two approaches to purify NEFA by each of thin-layer chromatography [20, 21] and solid-phase extraction [23, 24]. In the solid-phase extractions, we describe one conventional and one static method.

One common and extensively used means to separate NEFA from total lipid extracts is thin-layer chromatography (TLC), and many early and recent authors have described protocols to achieve this separation [15, 16, 17, 18, 19]. Herein, we describe two representative methods where NEFA separation was accomplished with slightly different proportions of petroleum ether, diethyl ether, and acetic acid (Table 2). These techniques should allow analysts to purify NEFA from a variety of sample types. In the first report, Dudzinski [20] noted, unexpectedly, that FFA on a TLC plate could be selectively detected as a "rose-violet spot" on a "pale yellow background" when the spray reagents 2′,7′-dichlorofluoroscein, alcoholic aluminium chloride, and acid ferric chloride were used together. Additionally, Dudzinski [20] reported this method was widely applicable as it could purify short, medium, and long-chain as well as saturated, monounsaturated, and polyunsaturated NEFA. However, one drawback of this method is that alkaline reagents in the developing solvent interfered with color development of the spray reagents.

Rehncrona et al. [21] describe a TLC method to detect NEFA from Folch-extracted brain tissues. These authors noted that their method was able to sufficiently separate saturated (palmitic [16:0], stearic [18:0]), monoenoic (oleic [18:1]), and polyenoic (arachidonic [20:4], docohexaenoic [22:6]) NEFA, making this protocol better suited to the analysis of long-chain NEFA than that of Dudzinski [20]. However, the visualization reagents used in this method lack the specificity of the Dudzinski [20] approach, and genuine NEFA standards must be employed when identifying the NEFA band on the TLC plate.

Although frequently used, TLC does have drawbacks in that sizeable samples may be required, prep times can be long, and recoveries are often low [22]. As an alternative, other chromatography approaches have been developed, with solid-phase extraction (SPE) among the most simple and convenient. In many SPE applications for purification of lipid extracts, the samples are run through re-usable pre-packed cartridges that can be eluted under gravity or on a vacuum manifold. Notter et al. [23] describe such an approach to isolate NEFA from lipids recovered from samples of wet porcine adipose tissue. These authors described their approach as rapid, simple, and reproducible; however, they reported that in some instances there was co-elution of non-NEFA molecules in the NEFA fraction. Therefore, this purification technique must be used with caution if a sample free of contaminants is required. Summaries of each purification approach are presented in Table 2.

Table 2. A summary of the purification methods described

Type	Development/elution solvent	Applications	Advantages	Disadvantages	References
TLC	Petroleum ether:diethyl ether:acetic acid (80:20:1 v/v/v)	Short to long-chain saturated and unsaturated NEFA purification	Visualization reagent completely selective for NEFA	Alkaline development reagents interfere with specificity	[20]
TLC	Light petroleum:diethyl ether:acetic acid (55:45:2 v/v/v)	Purification of NEFA from tissue matrices	Better suited to long-chain PUFA then [20]	Standards needed for NEFA identification	[21]
SPE	Diethyl ether containing 2% acetic acid	Applicable to wet tissue samples	Rapid and simple	Co-elution of non-NEFA during elution	[23]
SPE	Dichloromethane:n-hexane (1:4 v/v) following selective adsorption to alkalized sodium carbonate	Purification of NEFA from triacylglycerols and phospholipids	Rapid and simple	Purification not validated for lipid species other than triacylglycerols and phospholipids	[24]

An alternative approach to the use of pre-packed cartridges is to carry out static SPE; this has the distinct the advantage of being more affordable when a large number of samples must be processed [24]. For instance, Paik et al. [24] have developed such a simple and rapid static SPE method for purification of NEFA from triacylglycerol in lyophilized algal samples. In their approach, lipids recovered with a modified Bligh and Dyer [25] extraction were added to sodium carbonate that had been pre-wetted with 0.1 M KOH to promote deprotonation of the NEFA and strong adsorption to the sodium carbonate. Following elution of neutral lipids, NEFA that were still adsorbed to the sodium carbonate were recovered by (1) acidification to protonate NEFA; and then (2) extraction with solvent. During method validation, Paik et al. [24] found that they could obtain at least 85% recovery, and usually more, of fatty acids ranging in length from caprylic (8:0) to behenic (22:0) acids. Furthermore, phospholipids in their initial sample did not interfere with NEFA recovery. However, these authors did not confirm the selectivity of their method for NEFA in the presence of lipid species other than triacylglycerols and phospholipids.

4 Derivatization

Here, we first describe several methods in the literature where derivatization[1] was achieved by placing quaternary ammonium salts of the fatty acids in a hot GC injector. The salts we describe include TMA [27, 9], TMTFTH [28, 9], and TMPH [29, 30]. Then, we mention pyrolytic derivatization techniques that did not use quaternary ammonium salts, but instead used dry salts of the fatty acids and ethyl potassium sulfate [32]. We then highlight several other techniques which used methyl iodide [33, 37, 38, 22, 8]. Later, we describe organic compounds, alkyl chloroformates [36, 7], and dimethoxypropane [40], used to derivatize NEFA and how similar compounds have been utilized with hydroxy-NEFA [41, 42]. Then, we comment on several protocols that identified NEFA amongst other lipid species by trimethylsilylating the lipid extract [43, 44, 46] and describe how the diazomethane derivative, trimethylsilyldiazomethane, can be used to esterify NEFA [47]. Finally, we explain how we used trimethylsilylation for identification of 3-OH fatty acids (paper to be published, Potter, G., Budge, S.M., and Speers, R.A., 2014) and then suggest alternative approaches to derivatize these analytes with TBDMS [50] and heptafluorobutyrates [51].

Diazomethane remains a powerful reagent for selective derivatization of NEFA, but there are other derivatization tools at the disposal of lipid researchers and analysts. When salts of NEFA are heated with an appropriate reagent, the aforementioned pyrolytic derivatization can occur. This was first reported by Prelog and Picentanida [26] who observed that when tetramethylammonium (TMA) salts of acids were heated, trimethylamine was driven off, and nearly pure methyl esters remained. Robb and Westbrook [27] later described the methylation of NEFA in a hot injection port of a gas chromatograph with TMA salts and noted that this happened in a near quantitative manner. Overall, this work [27] revealed that TMA can be used to derivatize each of short, medium, and long-chain NEFA and short-chain hydroxy NEFA without conversion of other organic acids. However, MacGee and Allen [9] detailed an important disadvantage of the hydroxide salt of TMA: tetramethylammonium hydroxide (TMAH) caused complete destruction of polyunsaturated fatty acids (PUFA). Recovery of PUFA was improved by adding methyl propionate to the quaternary ammonium hydroxide extracts and the authors postulated that the hydrolysis of the propionate ester in the hot injector neutralized additional alkalinity.

While simple quaternary ammonium salts such as TMA may be used, bulkier salts which pyrolize more readily seem to be favored. Accordingly, early researchers noted TMTFTH and TMPH were more suitable than TMA because dimethyltrifluorotoluidine and dimethylaniline were more stable leaving groups than trimethylamine [28, 29]. However, like TMAH, these other basic salt preparations require addition of a suitable neutralizing agent to prevent degradation of PUFA. For example, MacGee and Allen [9] did not detect any degradation of the PUFA linoleic (18:2), linolenic (18:3), and arachidonic acid (20:4) with a small addition of methyl propionate to their TMTFTH mixture. Similarly, methyl acetate was found to be an effective neutralizing reagent to prevent PUFA breakdown during TMPH use [30]. Despite TMFTH's utility, several authors have opted in favor of TMPH due to its low cost, easy preparation, and more uniform pyrolytic derivatization of fatty acids [30, 31].

We initially considered a selective extraction and pyrolytic derivatization technique similar to that described by Williams and MacGee [11] to detect 3-OH oxylipins. We assumed that the gas-liquid chromatography conditions of the Williams and MacGee [11] protocol could be adapted for GC-MS analysis as quaternary ammonium salts have been reported in GC-MS applications [13]. However, upon further consultation with experts in the field, we were urged not to inject these non-volatile salts into our system as they would likely be damaging. In fact, we confirmed that Sigma–Aldrich, who manufactures a TMPH solution in methanol for GC derivatization, had not validated the use of this reagent with GC-MS. Therefore, strong consideration should be taken before choosing certain quaternary ammonium salts for analysis by GC-MS.

Pyrolytic derivatization has been widely achieved using quaternary ammonium salts of NEFA but pyrolytic derivatization can also been produced with a potassium salt/NEFA approach [32]. In Hunter's [32] protocol, dry potassium salts of fatty acids mixed with ethyl potassium sulfate placed in a hot injection port of a GC produced ethyl esters that eluted as sharp, distinct peaks. However, this procedure required a complicated

[1] Here derivatization is concerned with alteration of the carboxylic acid functionality of non-hydroxy NEFA. When the derivatizaion of 3-OH fatty acids is discussed, this refers to the alteration of both the carboxylic acid and the hydroxyl functionality.

setup where an argon gas source was connected to the injection needle. Summaries of each of the pyrolytic derivatization approaches are presented in Table 3.

Iodides have also been reported to efficiently derivatize NEFA as these compounds undergo a nucleophilic substitution reaction in the presence of fatty acids, but methyl iodide in particular has been widely used [33, 34, 35]. Furthermore, methyl iodide represents a true alternative to the specificity of diazomethane when the reaction occurs in a polar aprotic solvent [36] and derivatization of NEFA with this reagent can be quite rapid when conditions are optimized. Some reports have suggested methyl iodide methylation could take upwards of 8 h [37, 38], but Allen et al. [22] obtained complete methylation of fatty acids in 10 min in aqueous KOH at 65°C with N,N-dimethylacetamide as a catalyst. Hockel et al. [8] also describe a quick methyl iodide approach where methylation was complete 10 min after reflux of a mixture of fatty acids with crown ether (dibenzo-18-crown-6), dried potassium carbonate, and methyl iodide. However, considerable care should be taken with the use of this chemical because it is toxic and can be damaging to human health [39].

Chloroformates, a class of compounds used in organic chemistry to derivatize amino and hydroxy groups, can also be used to derivatize NEFA. Hušek et al. [36] developed an approach to prepare methyl esters of NEFA using methyl chloroformate. This method was quantitative and almost instantaneous at room temperature when methyl chloroformate was added to a reaction medium that contained the fatty acids, a pyridine catalyst and the solvents acetonitrile (for non-aqueous systems), or acetonitrile-water-methanol (for aqueous systems). Several of the same authors [7] developed a three-step procedure for selective extraction and subsequent methylation of NEFA in serum, using 4-dimethylaminopyridine (DMAP) as a catalyst in place of pyridine. With this method, >95% of NEFA in the sample were methylated, and carryover of the DMAP into the sample did not have an impact on quantitation.

Provided the proper reaction conditions are used, dimethoxypropane (DMP), like methyl iodide, offers an alternative to the selectivity of diazomethane. Research has verified that this compound will derivatize NEFA while intact, esterified acyl lipids are unaffected. For instance, Tserng et al. [40] developed a NEFA-specific derivitzation technique by adding 2,2-DMP and hydrogen chloride to serum samples. In this system the DMP was multi-functional and acted as a water-scavenger, a protein precipitant and a source of methyl groups for NEFA methylation.

Other researchers have attempted to methylate hydroxy NEFA at the carboxyl and hydroxyl functional groups with mixed success. Vosmann et al. [41] pyrolytically derivatized these compounds with trimethylsulfonium hydroxide (TMSH) in a hot injector. The group noted complete conversion to the corresponding methyl esters but only around 3–5% conversion to *O*-methyl ethers. Hušek [42] also assessed whether methyl chloroformate reagents could be applied to

Table 3. A summary of each of the pyrolytic derivatization approaches described

Reagents	Applications and advantages	Disadvantages	References
TMA	Applicable to NEFA of different chain lengths Can be used for hydroxy-NEFA	Alkalinity of TMAH completely destroys PUFA	[27]
TMTFTH	Applicable for long-chain PUFA when methyl propionate co-injected	Ghost peaks can occur if insufficient TMTFTH used	[9]
TMPH	Easily prepared Low cost More uniform derivatization than other salts	Degradation of PUFA without co-injection of methyl acetate	[30, 31]
TMSH	High conversion efficiency to methyl esters Useful in screening for hydroxy-NEFA in GC-MS	Low conversion to *O*-methyl ether for hydroxy-NEFA	[41][a]
Ethyl potassium sulphate	Sharp, distinct peaks produced by GC	Specialized injection technique required	[32]

[a]Used in hydroxy-NEFA derivatization.

Table 4. A summary of the non-pyrolytic derivatization reagents described

Reagents	Applications and advantages	Disadvantages	References
Methyl iodide	Fast with appropriate catalyst True alternative to diazomethane Simpler than other NEFA derivatization approaches	Toxic, must be used with care Potential degradation of modern capillary columns	[33, 37, 38, 22, 8]
Methyl chloroformate	Methylation is instantaneous at room temperature Useful in screening for hydroxy-NEFA in GC-MS	NEFA unreacted with certain catalysts Carryover of catalyst into sample Side chain reactions when used with hydroxy-NEFA	[36, 7, 42][a]
Dimethoxypropane	Helps to precipitate proteins in sample True alternative to diazomethane	N/A	[40]

[a]Used in hydroxy-NEFA derivatization.

hydroxy fatty acids. Similarly, Hušek [42] noted *O*-methoxycarbonyl methyl esters were the main products with certain hydroxy fatty acid standards, but also reported side-chain reaction products that included lactones and inter-ester oligomers. Overall, derivatization with TMSH and methyl chloroformate are likely suitable derivatizing reagents to screen for hydroxy-NEFA in GC-MS applications, but they are not ideal for quantitative analysis. The key features of methyl chlorformates and other non-pyrolytic derivatization compounds are described in Table 4.

Methyl esters are undoubtedly the most common derivatives of fatty acids formed prior to chromatographic analysis but other approaches can be equally effective. One such popular derivatization technique which allows for NEFA detection is silylation, which adds a trimethylsilyl (TMS) moiety to the acid carboxyl head of fatty acids to produce TMS esters. Preparation of these derivatives has facilitated resolution of NEFA without a selective extraction by silylation of total lipid extracts from plasma and serum samples [43, 44]. Additionally, silyl esters offer lipid analysts several advantages in that their elution order is similar to methyl esters and they are easily and quickly made at room temperature [43, 44]. Unfortunately, TMS esters and ethers are hydrolytically unstable [45] and may only be stored for 24–72 h before noticeable degradation occurs [44, 46]. Nonetheless, silylation is a simple and versatile approach with broad applications which D'Alonzo et al. [46] demonstrated by separation of NEFA from intact glycerides following silylation of soy bean oil lipid extracts.

Trimethylsilylation is an attractive derivatizing reagent, but trimethylsilyldiazomethane, a safer and more stable derivative

Table 5. Comparison of techniques utilizing trimethylsilylating reagents, trimethylsilyldiazomethane, TBDMS, and heptafluorobutyrates

Reagents	Applications and advantages	Disadvantages	References
Trimethylsilylating reagents	Derivatives can be produced at room temperature Silyl esters elute like methyl esters but give better response in FID Good for hydroxy-NEFA detection	Very prone to hydrolysis Limited storage time	[43, 44, 46], Potter et al. [2014][a]
Trimethylsilyl-diazomethane	Methyl esters formed instantly Near 100% yield Can be used for hydroxy-NEFA	Produces artifacts and byproducts that obscure chromatograms	[47a, 48, 49]
TBDMS	Intense $[M^+ - 57]$ peak produced; can achieve low limits of detection	Require longer elution times May not be good for long-chain hydroxy-NEFA	[50][a]
Heptafluorobutyrates	Produce diagnostic spectra in PCI-MS and NCI-MS Very low limits of detection (femtogram) in NCI-MS	Specialized instrument calibration required	[51][a]

[a]Used in hydroxy-NEFA derivatization.

of diazomethane, can also be used to esterify NEFA [47]. For instance, Presser and Hüfner [47] demonstrated that methyl esters of long-chain NEFA and hydroxy long-chain NEFA were prepared almost instantaneously by dropwise addition of trimethylsilyldiazomethane. Furthermore, the yields of the methyl esters were nearly 100%. However, the same authors found partial *O*-methylation of tertiary alcohols, implying trimethylsilyldiazomethane lacks the specificity of diazomethane. Not surprisingly, other research has found use of trimethylsilyldiazomethane can lead to artifact and byproduct formation [48, 49], so some consideration should be taken when using this reagent to methylate NEFA.

Our recent study (paper to be published, Potter, G., Budge, S.M., and Speers, R.A., 2014) identified trimethylsilylation of hydroxy-fatty acids, in our case 3-OH oxylipins, as an effective derivatization approach. In this procedure, TMS moieties were added to both the carboxyl and hydroxyl functional groups on the molecule. In earlier works which analyzed 3-OH fatty acid methyl esters by GC-MS [3, 4], a base peak at 103 m/z was indicative of a hydroxyl group on the β-carbon. When we double silylated the 3-OH 10:0 molecule at the carboxyl and β-hydoxyl functionalities, we observed that the same fragmentation at the β-carbon produced a 232 m/z fragment, although the base peak was now at 147 m/z. The advantage then of methylating 3-OH fatty acids is that the limits of detection in GC-MS selected ion monitoring (SIM) mode can be very low as the diagnostic fragment is also the base beak. However, trimethylsilylation still represents a safer and easy to use substitute to diazomethane.

Another alternative approach to still achieve low limits of detection with 3-OH and other short-chained hydroxy fatty acids is to prepare *tert*-butyldimethylsilyl (TBDMS) derivatives. For example, Mamer et al. [50] have developed a protocol to quantify all the intermediates of the Kreb's cycle, including α-hydroxyglutaric acid, by preparing TBDMS derivatives. These authors noted that the TBDMS derivatives of the acids were characterized by an intense $[M^+ - 57]$ fragment, which should allow for lower limits of detection then the identical TMS derivatives. Of course, preparation of these heavier derivatives would require higher oven temperatures and result in longer elution times. Therefore, TBDMS derivatives may not be appropriate for long-chained hydroxylated NEFA.

Heptafluorobutyrate derivatives of hydroxy methyl esters offer a third option to achieve low detection limits, on the order of 1 fg (10^{-15} g). The key to this technique is the use of negative chemical ionization (NCI)-MS [51], which showed 20× greater response than positive chemical ionization (PCI)-MS of the same analytes. Furthermore, Stan and Schuitwinkel-Reich [51] noted hydroxy methyl ester standards with varying numbers of heptafluorobutyrate derivatized OH groups had conserved fragmentation patterns in both PCI-MS and NCI-MS. A pseudomolecular ion (M + H) as the base peak, M + 29, and M + 41 were reported in PCI-MS from heptafluorbutyrate derivatized mono-hydroxy, di-hydroxy, and tri-hydroxy fatty acid methyl esters. Meanwhile, in NCI-MS, large spectral peaks of 213, 194, and 178 m/z were noted as diagnostic. Comparison of trimethylsilylating reagents, trimethylsilyldiazomethane, TBDMS, and heptafluorobutyrates are summarized in Table 5.

5 Conclusion

Despite diazomethane's danger and toxicity, current research efforts still focus on more efficiently producing and more effectively utilizing this reagent [52, 53]. We elected not to use this derivatizing chemical largely because we were concerned for the general safety of our laboratory personnel. Once we delved into the literature, there were clearly numerous replacement methods for the specificity of diazomethane derivatization which still allowed for detection of both NEFA and hydroxylated NEFA. We found that by selectively quantifying/extracting, purifying from total lipids, and applying alternative derivatization approaches, NEFA and hydroxylated NEFA could be resolved from other lipids species. For our own purposes, we chose to trimethylsilyate our lipid extract to detect 3-OH 10:0 in yeast cells (submitted for publication, Potter, G., Budge, S.M., and Speers, R.A., 2014), but this detection could have been achieved with several of the other protocols we described.

The authors wish to thank the Natural Sciences and Engineering Research Council of Canada for providing funding to SMB and RAS.

The authors have declared no conflicts of interest.

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