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Determination of fatty acid amides as trimethylsilyl derivatives by gas chromatography with mass spectrometric detection

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Abstract

Fatty acid amides are a newly emerging class of compounds with biological activity. The amides are formed enzymatically in vivo. Analysis of fatty acid amides has been accomplished by gas chromatography coupled with mass spectrometry. Fatty acid amides required derivatization prior to analysis at high temperatures due to thermal instability. Trimethylsilylation of fatty acid amides has been accomplished under optimum reaction conditions. The limit of detection for the silylated amides is approximately 1 pmol, with the lowest detected level being 700 fmol for the lauramide derivative. Quantitation of fatty acid amide derivatives can be accomplished by monitoring m/z 59 or m/z M-71, the only two major fragments formed in the ion trap mass spectrometer with electron impact ionization. The smaller fragment is the result of a newly reported, McLafferty-type rearrangement; M-71 resulted from loss of an *n*-pentyl fragment. Either peak gave four–five orders of magnitude linear dynamic range. Numerous trimethylsilylamides from C_7 to C_{20} were separated under standard conditions. Elution was linear with the number of carbons and was systematically affected by the number and position of the double bonds.

Keywords: Derivatization, GC; Fatty acid amides; Amides; Hormones; Biogenic amides; Lipids; Carboxamides

1. Introduction

Primary fatty acid amides (PFAMs) are a newly discovered class of mammalian hormone and neuromodulator. Oleamide (*cis*-9-octadecenamide, designated C18:1^{9}), erucamide (C22:1^{13}), and, at very trace levels, elaidamide (C18:1$^{trans\text{-}9}$) have been identified in the cerebrospinal fluid of sleep-deprived cats [1]. Oleamide initiates physiological sleep upon intravenous injection [1], potentiates serotonin receptor subtypes [2,3] and inhibits gap cell communication [4]. These functions have a moderate degree of structural specificity relative to the number of carbons and the degree and position of unsaturation [5,6]. The significance of these observations lies in the links between sleep disorders and other affective disorders, such as schizophrenia and bipolar disorder [2], as well as the links between these disorders and serotonin. It is conjectured that PFAMs play a heretofore unknown role in normal neurological activity, and abnormal levels of PFAMs may therefore be diagnostic for this class of disease. Oleamide has been the most widely studied molecule in this class, but several other PFAMs have been shown to have hormone-like activity [7–11].

Their potential importance as a class notwithstand-

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ing, little systematic work has been done to optimize the separation and quantitation of PFAMs. The majority of applications appear to be related to the plastics industry, where oleamide is used as a slip additive. Cooper and Tice [12,13] developed gas chromatography (GC) methods for long-chain FAMs using high-temperature, non-polar capillary columns and flame ionization detection. Detection limits were of the order of 50 ppb, but only a few compounds were separated. PFAMs extracted from plasma have been determined as their trimethylsilyl (TMS) derivatives by GC with mass spectrometric detection (GC–MS) [9]. Considerably more work has been done with the related *N*-acylethanolamines (e.g. [14]), particularly with regard to sample preparation.

High-performance liquid chromatography (HPLC) is also a viable separation technique. Merkler and Poore [15] have developed methods for a wide range of PFAMs, but sensitivity is quite poor when the amides are detected by absorbance at low UV wavelengths. Normal-phase LC–MS–MS was used in the discovery of the hormonal activity of oleamide [1], and LC with atmospheric pressure chemical ionization MS has been used for determination of *N*-acylethanolamines [16]. Because of its high efficiency and sensitivity, capillary electrophoresis with laser-induced fluorescence has begun to be explored as a means of analyzing PFAMs [17].

We report here optimal derivatization conditions for silylation of primary fatty acid amides from C_7 to C_{20}, including *cis* mono- and polyunsaturates. The silyl derivatives are analyzed by GC–MS and show characteristic elution and mass fragmentation patterns.

2. Experimental

2.1. Separations

All separations were performed on a Varian 3410 high-temperature gas chromatograph coupled to a Varian Saturn II ion trap mass spectrometer. Separations took place on a 30 m×0.25 mm I.D. Supelco Simplicity-5 (PDMS 5% phenyl, 0.25 μm film thickness) column, which was installed in between a 5 m×0.25 mm I.D. deactivated capillary guard column and a 5 m×0.25 mm I.D. deactivated capillary transfer line. The GC was programmed to an initial temperature of 55°C followed by a 40°C/min temperature ramp to 150°C. The oven was held at 150°C for 6 min followed by a temperature ramp of 10°C/min to a final temperature of 300°C. The transfer line was held at 280°C and the injection port was held at 250°C throughout the separation. The carrier gas was He. The injection volume was 1 μl unsplit. The ion trap was operated using electron impact ionization at an energy of 70 eV. The ion trap was run at one scan per second at a temperature of 220°C. The mass scan range was 50 to 350 u.

2.2. Synthetic methods

Fatty acids were converted to the acid chloride by either reaction with thionyl chloride or oxalyl chloride in hexane under nitrogen. The amides were formed by the addition of cold ammonium hydroxide to the acid chlorides. The amides were purified by recrystallization from methanol. The identity and purity of the amides was confirmed by NMR, IR and GC–MS. Silylation derivatizations were performed under dry nitrogen. Typical conditions for derivatization were an excess of silylating agent and 1 to 1000 nmol amide. Reaction volumes were kept below 100 μl in screw-topped vials and diluted to 1 ml for GC–MS analysis. Reactions were generally run at 95°C for 15 min. Molecular simulations were run on a Dell Dimension XPS 300 running Windows 95 using Gaussian 94 purchased from Gaussian (Pittsburgh, PA, USA).

2.3. Materials and reagents

Oxalyl chloride, enanthic chloride (C7:0) and capryloyl chloride (C8:0) were purchased from Aldrich (Milwaukee, WI, USA), thionyl chloride, bis-trimethylsilyltrifluoroacetamide (BSTFA), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), chlorotrimethylsilane (TMSCl), ricinoleic acid ($C18:1^{9,12\text{-OH}}$), eicosanoic acid (C20:0), myristic acid (C14:0), linoleic acid ($C18:2^{9,12}$), γ-linolenic acid ($C18:3^{9,12,15}$), palmitoleic acid ($C16:1^{9}$) and toluene were purchased from Acros Organics (Hampton, NH, USA), capramide (C10:0), myristoleic acid ($C14:1^{9}$), *cis*-vaccenic acid ($C18:1^{11}$) and pet-

roselinic acid (C18:1[6]) were purchased from Sigma (St. Louis, MO, USA), oleic acid (C18:1[9]), stearic acid (C18:0), ethyl acetate, methanol, acetonitrile, hexane and ammonium hydroxide were purchased from Fisher (Pittsburgh, PA, USA), palmitic acid (C16:0) was purchased from Eastman Kodak (New York, NY, USA), lauramide (C12:0) was purchased from TCI America (Portland, OR, USA), and erucamide (C22:1) was purchased from Janssen Chimica (Gardena, CA, USA). All unsaturated compounds were in the form of the *cis* isomer. All chemicals were used as received.

3. Results and discussion

3.1. Optimization of the silylation reaction

To show the influence of the choice of the silylating agent and solvent effects, myristamide and oleamide were chosen as model saturated and unsaturated aliphatic amides. Analysis of the reactions performed in the neat silylating agents is shown in Fig. 1. The intensity of the peak depended greatly on the silylating cocktail that was used. MSTFA and BSTFA alone gave the largest peak areas and had the most complete reactions. BSTFA was ultimately chosen over MSTFA, however, due to greater signal-to-noise and signal-to-background ratios. TMSCl alone did not react with primary amides and prevented the reaction of MSTFA with primary amides when present. The extent of the BSTFA reaction with primary amides was severely reduced in the presence of TMSCl. These ''catalytic'' amounts of TMSCl can potentially be used to selectively derivatize lipid components (for example, free fatty acids can be selectively derivatized in the presence of amides).

Myristamide and oleamide had a maximal peak areas when derivatized in the presence of ethyl

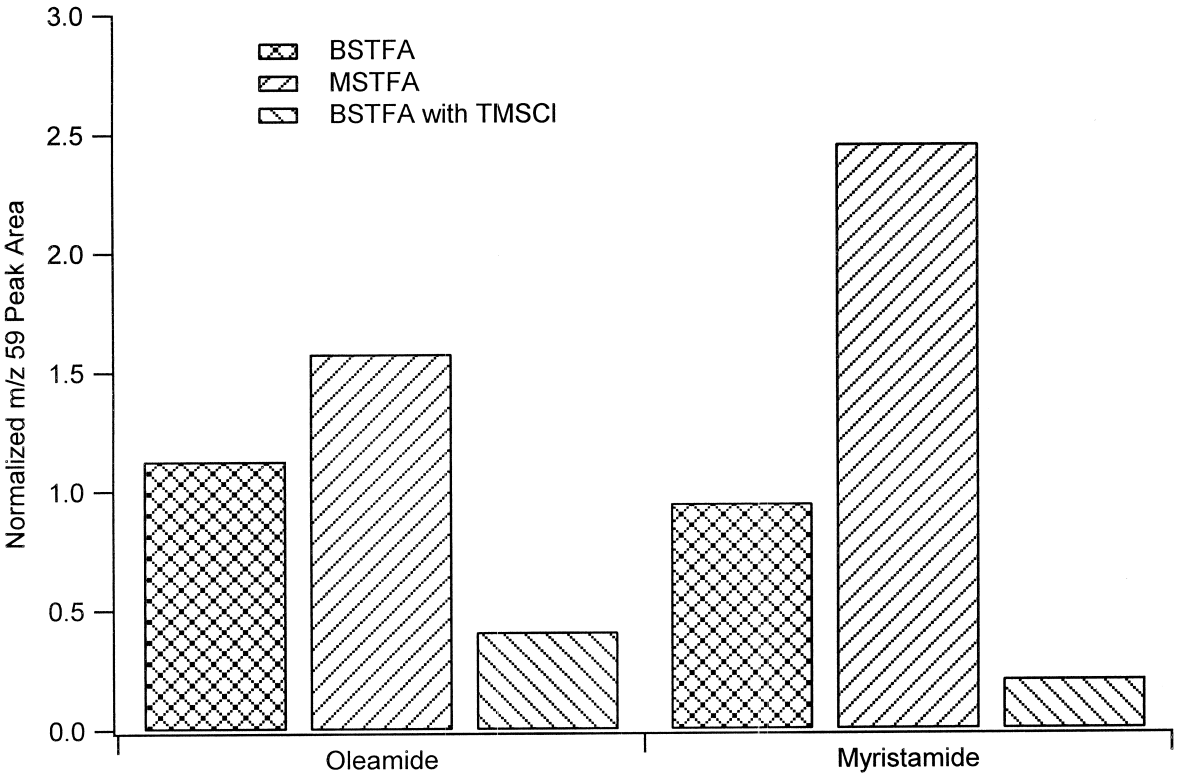

Fig. 1. Silylating cocktail implications on SIM (m/z 59) peak areas for 1 nmol oleamide and myristamide. Peak areas from the SIM were normalized to the SIM peak area for enanthamide.

acetate or toluene, with no significant differences with respect to the percentage of organic solvent present during derivatization, as shown in Fig. 2. Several other factors influenced the ultimate choice of reaction solvent, including amide solubility and chromatographic consequences. Reactions in toluene produced the highest signal-to-baseline and the highest signal-to-noise ratios due to a tenfold decrease in the baseline noise when compared to ethyl acetate. Toluene was beneficial in the reaction of BSTFA with amides, with dramatically increased peak areas relative to neat BSTFA (Fig. 2). All of the amides, as well as the silylated amides, were soluble in toluene, which made toluene the solvent of choice in amide silylation reactions. Acetonitrile was also examined as a possible solvent for use in the silylation reaction [9], but dramatically lower peak areas were found compared to those found using toluene and ethyl acetate.

The effects of reaction time and temperature were examined for oleamide in BSTFA–toluene (50:50, v/v). Reaction progress was monitored using GC–MS. The reactions were reasonably facile, which limited the amount of reliable kinetic information, but the reaction at 95°C had an estimated half-life of 10 min. Reactions appeared to be complete at approximately 40 min. Reaction completeness was assessed by examining the GC–MS chromatograms, which were typically very free of extraneous peaks, although it was possible that unreacted amide was pyrolized in the injection port and was subsequently adsorbed onto the guard column. The reaction rate appeared to be independent of temperature above 35°C. Pseudo-first-order kinetics would be expected

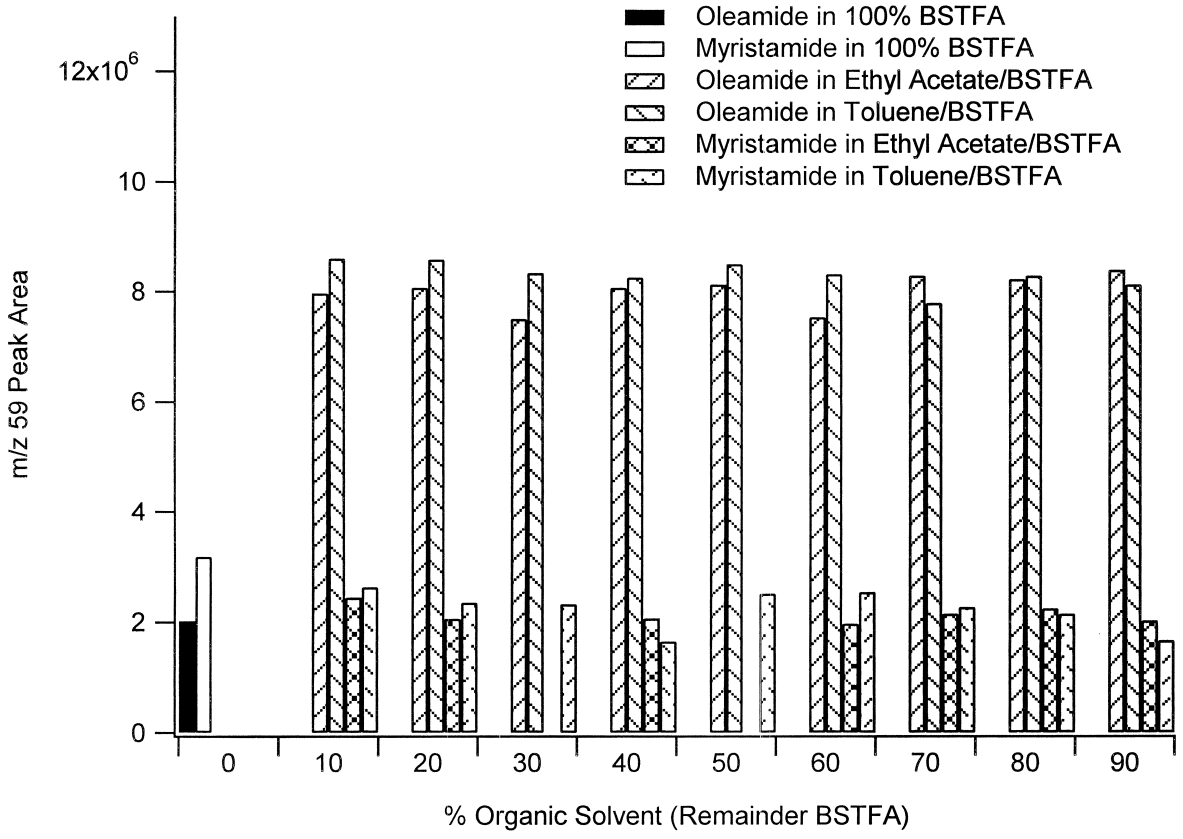

Fig. 2. Effect of organic modifier on SIM (m/z 59) peak areas of oleamide and myristamide derivatized with BSTFA in various percentages (v/v) of toluene and ethyl acetate. Results from the derivatization in neat BSTFA (see Fig. 1) are shown as a reference.

because of the large excess of silylating reagent, but the lack of a reaction rate increase with temperature suggested diffusion control of the reaction above 35°C. Thus, 40 min at 35°C was sufficient for complete reaction. Given these satisfactory results, no other reagents were investigated.

3.2. Separation of amides

The separation of a mixture of silylated amides from enanthamide to eicosanoamide, including unsaturated amides, is shown in Fig. 3 as the total ion chromatogram (TIC). The separation followed the typical trend for linear alkanes, with the retention time increasing with chain length. The elution time was linear with the number of carbons for saturated silylated amides (r^2=0.999, slope=76±1). The effect of unsaturation was also examined and the results are shown in Fig. 4. Unsaturated amides eluted before the saturated compounds, which is also shown with palmitamide and palmitoleamide in the insert in Fig. 3. Further analysis of a set of C_{18} compounds [stearamide (C18:0), petroselinamide (C18:1^{6}), oleamide (C18:1^{9}), *cis*-vaccenamide (C18:1^{11}), linoleamide (C18:$2^{9,12}$), linolenamide (18:$3^{9,12,15}$) and ricinoleamide (Cl8:$1^{9,12\text{-OH}}$)], Fig. 4, showed the details of the effect of unsaturation. Petroselinamide, oleamide and *cis*-vaccenamide all have one unsaturation in the alkyl chain, the only difference being the location of the double bond (6, 9 and 11, respectively). *cis*-Vaccenamide eluted first, followed by oleamide, then petroselinamide. The elution order was directly correlated to the movement of the carbon–carbon double bond towards the

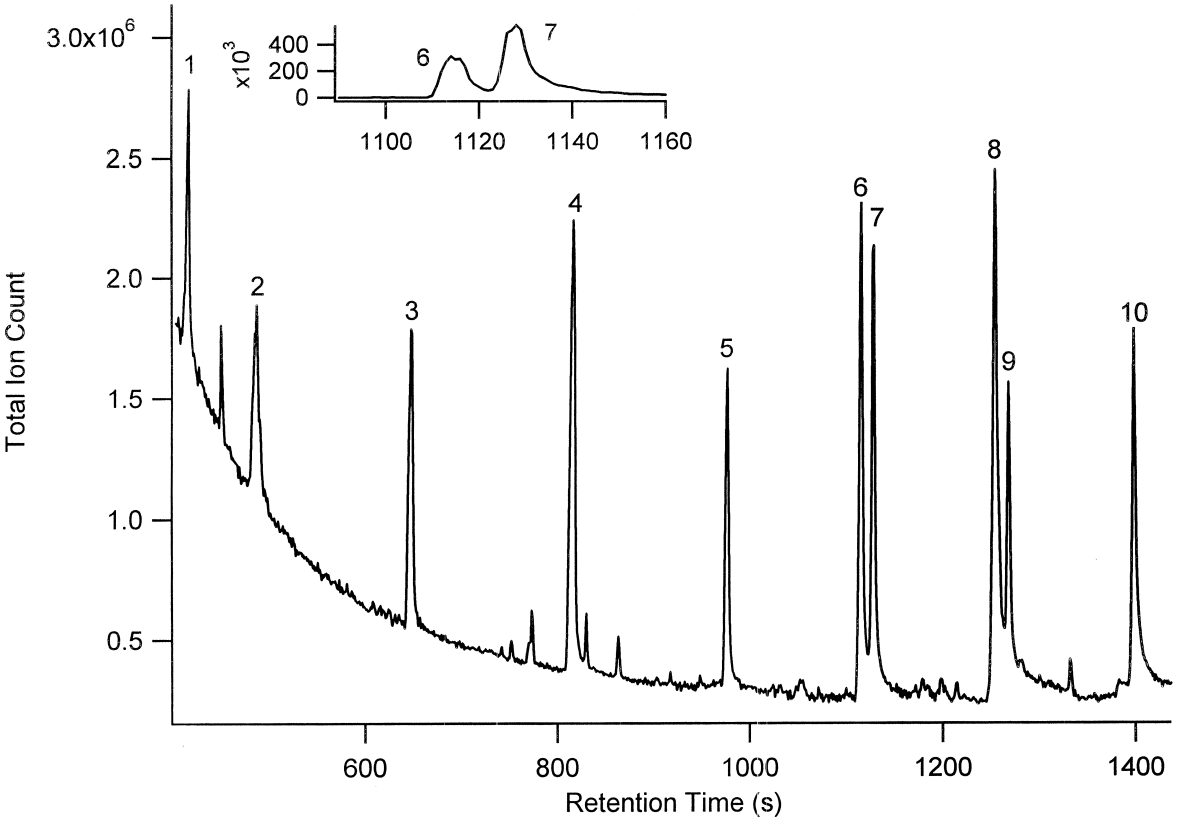

Fig. 3. Total ion chromatogram of ten amides derivatized with BSTFA–toluene (50:50, v/v). Enanthamide (1) 1.19 nmol, caprylamide (2) 1.09 nmol, capramide (3) 1.01 nmol, lauramide (4) 0.764 nmol, myristamide (5) 0.692 nmol, palmitoleamide (6) 0.596 nmol, palmitamide (7) 0.584 nmol, oleamide (8) 0.552 nmol, stearamide (9) 0.583 nmol, eicosanoamide (10) 0.486 nmol. See Experimental for separation conditions. The insert shows an expanded view of the *m*/*z* 59 SIM for palmitoleamide and palmitamide.

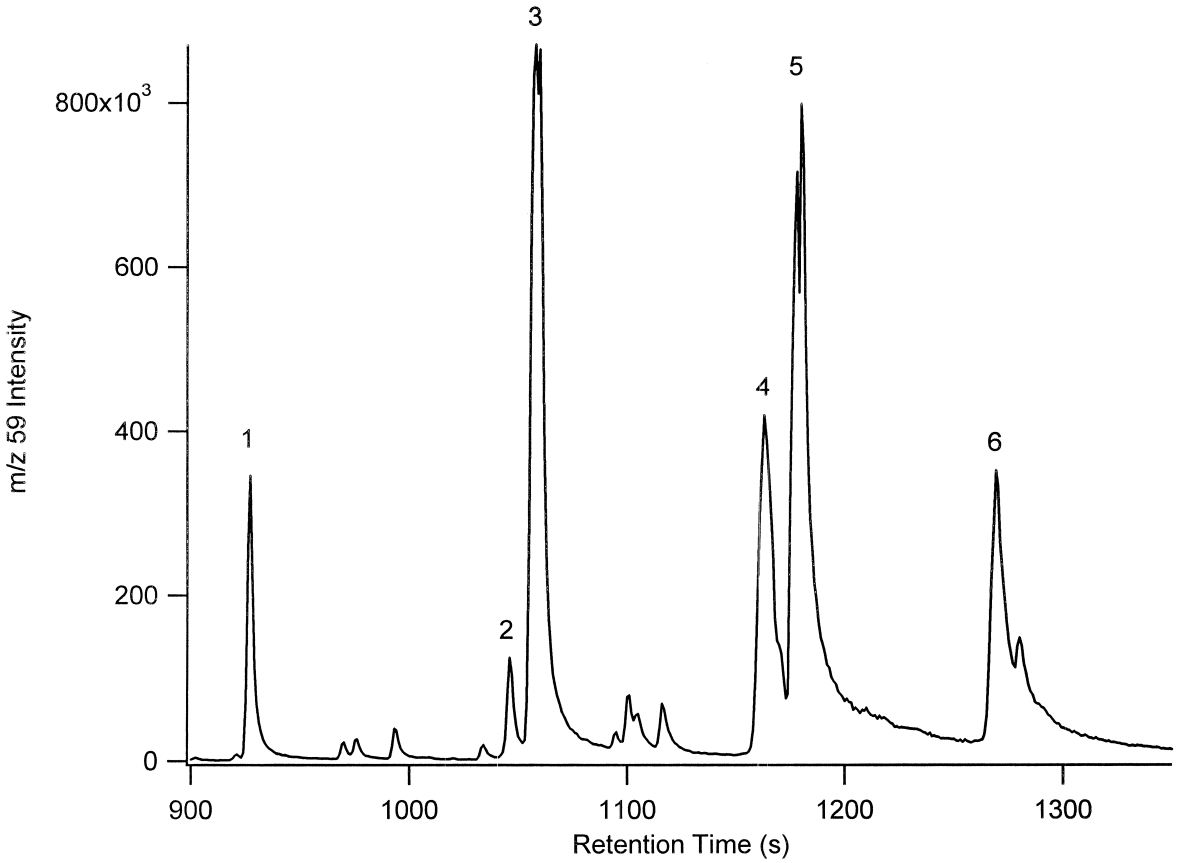

Fig. 4. Selected ion monitoring (SIM m/z 59) of C_{18} compounds derivatized with BSTFA–toluene (50:50, v/v). (1) *cis*-Vaccenamide, (2) linoleamide, (3) linolenamide, (4) oleamide, (5) stearamide and (6) petroselenamide.

carbonyl group. As the bond moved towards the carbonyl, the retention time increased, indicating a less polar molecule. Petroselinamide eluted after stearamide, the only amide examined with an unsaturation that eluted after the saturated compound. It was not clear why linoleamide (two double bonds) eluted before linolenamide (three double bonds). Ricinoleamide has two silylation sites: a free hydroxyl as well as the amide moiety. Ricinoleamide therefore eluted very early when compared to oleamide (retention time of ricinoleamide was approximately 400 s).

The elution order of the amides was similar to the order of fatty acid methyl esters (FAMEs) on nonpolar columns [18]. There was a difference noticed between the saturated and unsaturated compound elution order for fatty acid amides. With FAMEs, linoleic acid methyl ester and linolenic acid methyl ester elute before myristic acid methyl ester and palmitic acid methyl ester on a DB-1 column. This was in contrast to the amide series where the effect of the number of double bonds was not as strong, probably due to a slightly more polar column (DB-5 equivalent). Only *cis*-vaccenamide eluted before palmitamide. Myristamide eluted well before linoleamide and linolenamide. Closely following the elution order of the FAMEs, however, were oleamide and stearamide as well as the other long-chain-length amides [18].

3.3. Amide fragmentation patterns

Silylated fatty acid amides had a characteristic fragmentation pattern with only two major peaks in

most cases, as shown in Fig. 5. A prominent peak in most of the amides was found at m/z 59 with a typical base peak of either m/z 59 or m/z M-71. An explanation of the origin of the fragmentation patterns of oleamide is given in Fig. 6a. The m/z 59 peak was most likely the result of a McLafferty-like rearrangement. The proposed mechanism of the rearrangement is shown in Fig. 6b. The lack of isotopic peak distribution for silicon surrounding the m/z 59 fragment eliminated silicon from inclusion in the fragment. The mechanism was evaluated by molecular modeling of the gas-phase structure of the silylated amide (6–31 G* optimization with Gaussian). The critical distance was between the nitrogen and the closest hydrogen on the trimethylsilyl group, with a minimum computed distance of 2.942 Å. The minimum computed distance was well within the distance that migration of the proton can occur, making the proposed mechanism plausible. The other predicted fragment probably resulted in other daughter ions at low intensity (Fig. 5).

The other major fragment that was produced from the silylated amide originated from the loss of a pentyl group (M-71). The pentyl group loss was consistent throughout the saturated and monounsaturated amide series down to enanthamide, except for

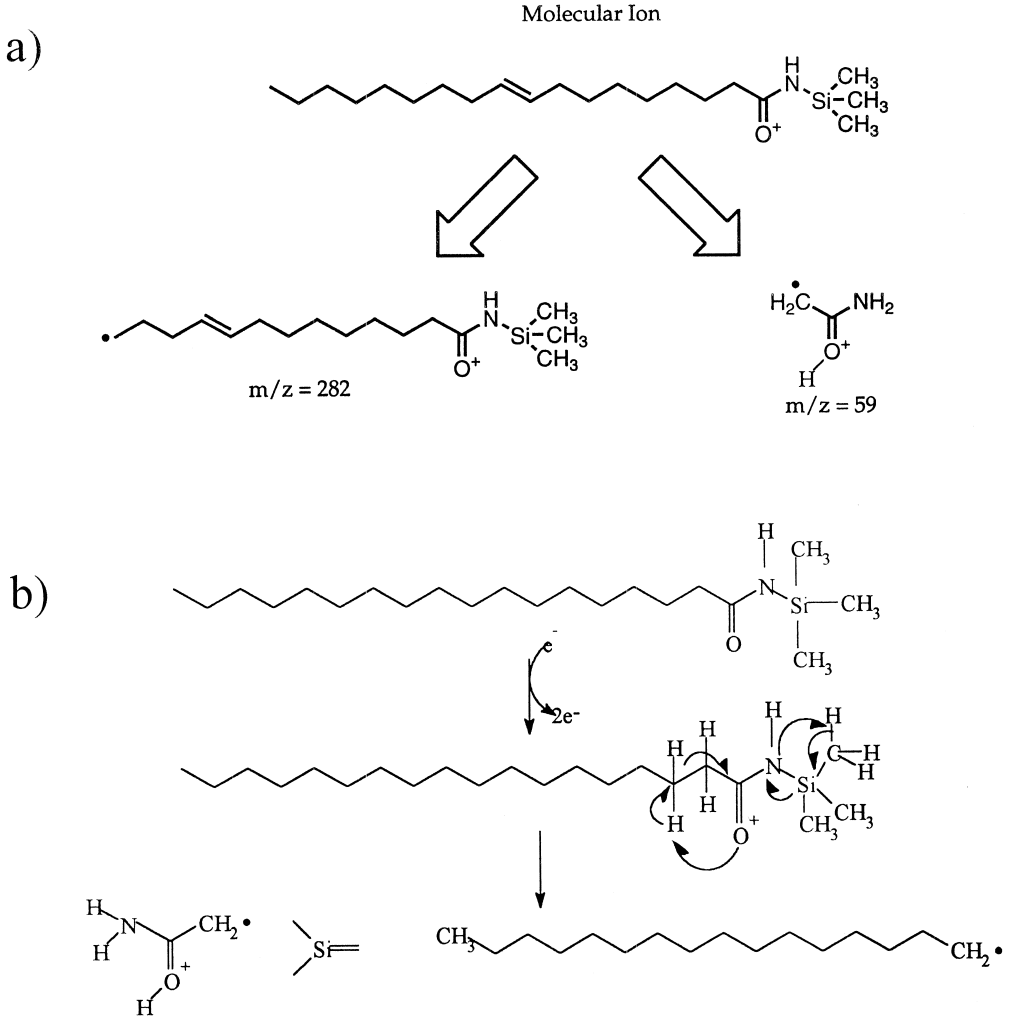

Fig. 5. Mass spectra obtained from GC–MS analysis of (a) stearamide and (b) oleamide. Scan range 50–350 u.

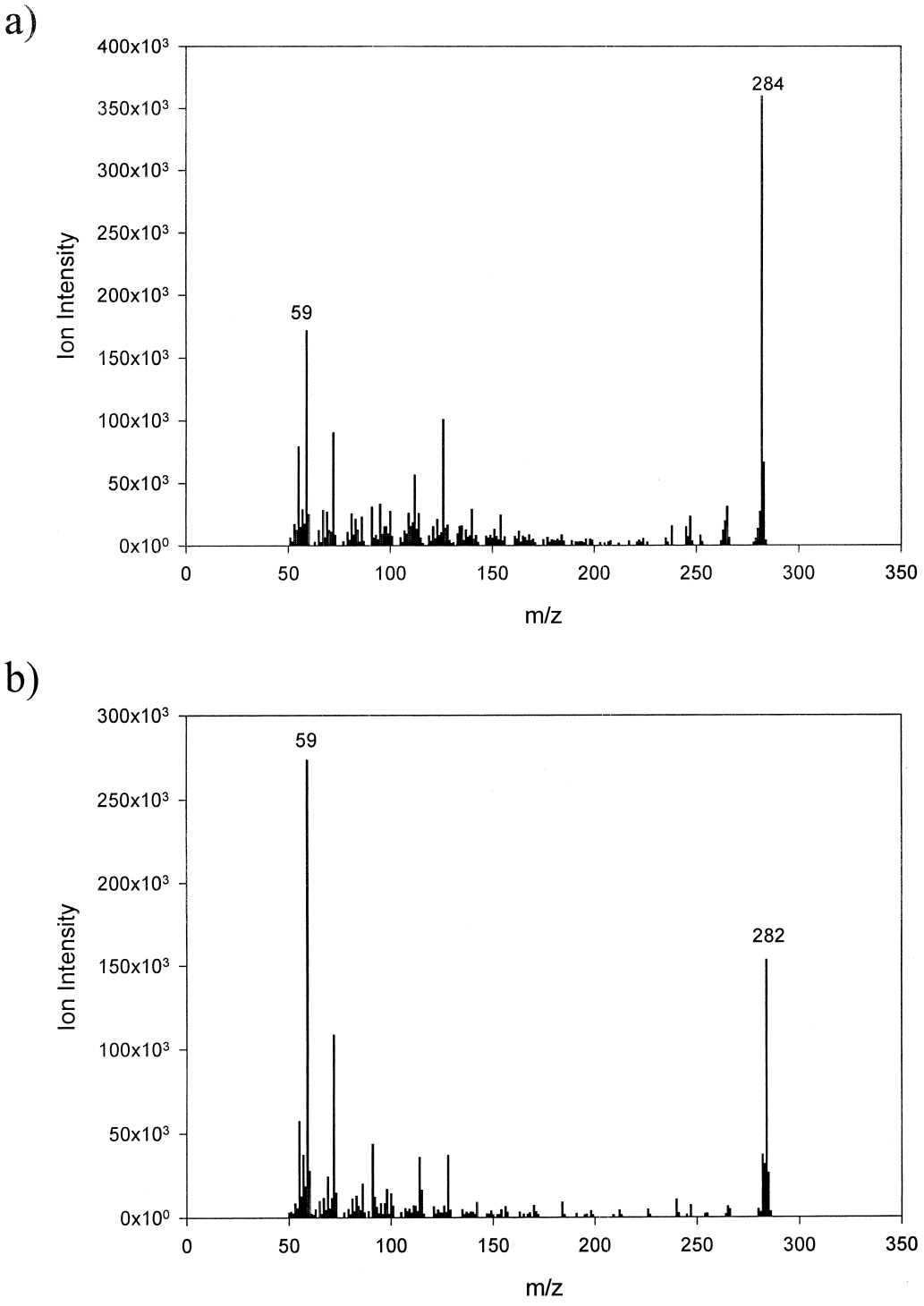

Fig. 6. (a) Origin of major oleamide fragments. (b) Mechanism of the McLafferty-like *N*-trimethylsilylamide rearrangement, with the resultant fragments.

those with double bonds that interfered with the pentyl fragment loss. For example, linoleamide, linolenamide, *cis*-vaccenamide all had double bonds at C-12/13, and the fragment cleavage site was at C-13/14. Fragmentation patterns for these compounds were considerably different. Caproamide (C6:0) eluted with the solvent front. Few other fragments occurred with any frequency, allowing the identity of an amide to be confirmed by the presence of the m/z 59 fragment and the fragment corresponding to the loss of a pentyl group. Stearamide and oleamide fragmented similarly (Fig. 5), but the unsaturation in oleamide resulted in the pentyl loss fragment having a mass of 282 instead of 284. Short chain amides had a much lower frequency of m/z 59, which contrasted with longer chain amides where the m/z 59 frequency increased. The intensity of the m/z 59 fragment increased linearly ($r^2=0.971$, slope= 37) with the number of carbons.

Aside from these two fragments, few other fragments of any intensity were observed. The peaks at m/z 72, 86, 100, 114, 128, 142 and 156 were common to all amides, and were probably the result of other, longer, McLafferty fragments with varying numbers of methylene groups. Bis-TMS adducts were not observed, and in no case was a molecular ion present. Details of the ion trap mass spectrometry of silylated amides are being investigated with isotopic labeling, and will be reported elsewhere.

This fragmentation differed from that seen by Arafat et al. [9], who also used TMS derivatization for PFAMs. They did observe fairly simple spectra, with a strong McLafferty rearrangement fragment at m/z 131 and a strong M-15 fragment. The former is a more typical McLafferty fragment in which the TMS group is retained; the latter is due to the loss of a methyl radical. They also observed a higher frequency of alkane chain loss fragments. The differences in fragmentation patterns are probably due to the fact that the current work was performed in an ion trap with a considerable amount of ionized solvent remaining in the trap, whereas Arafat et al. [9] used a quadrupole mass spectrometer. The large background observed in the total ion chromatogram was due entirely to toluene (m/z 91, which is visible but not labeled in Fig. 5). Oleamide has been chromatographed underivatized, with mass spectra similar to those found by Arafat et al. [9] and typical in fatty acids, but there was extreme broadening of the chromatographic peak, possibly due to pyrolysis in the injector.

3.4. Quantitation and trace detection

The total ion chromatogram suffered from a high baseline and a low signal-to-noise ratio. The relative simplicity of the fragmentation pattern of the amides has allowed a selected ion monitoring mode (SIM) to be used for lower detection limits. The fragment with a mass-to-charge ratio of 59 was common to all of the silylated amides and was simple to use to improve both the signal-to-noise ratio and the signal-to-baseline ratio. M-71 was also readily used for individual amides. Fig. 7 shows the improvement over the total ion count when the chromatogram is analyzed in the SIM mode, when compared to Fig. 3. The limit of detection for the amides was lowered a factor of 100 in SIM mode (m/z 59) compared to the total ion count. For lauramide, the limit of detection was 0.7 pmol injected. Injection of 1.4 pmol of lauramide yielded a signal-to-noise ratio of six and a signal-to-baseline ratio of 1.4 (m/z 59). The chromatogram is shown in Fig. 8 along with the total ion chromatogram.

Low level quantitation of fatty acid amides was easily accomplished in the SIM mode of operation, as shown in Table 1. Calibration was linear over four–five orders of magnitude. Limits of detection were of the order of 1 pmol, with a quantitation limit between 1 and 10 pmol. There was a slight, roughly linear, decrease in peak area with increasing chain length, which was attributed to a decrease in ionization efficiency. Precision was acceptable, with the relative standard deviations of peak areas shown in Table 1.

Some interesting effects were observed with the mass spectrometry in the ion trap. Fragmentation probability was concentration-dependent. The m/z 59 SIM peaks were split (Fig. 4 and Fig. 7); the M-71 was maximum at the peak split valley, indicating a change in ionization efficiency or mechanism favoring the M-71 fragment at higher concentrations. Sensitivity was generally equal for the two fragments (except for oleamide, for unknown reasons), partly because the concentration effect forced the M-71 peaks to be very narrow, while the m/z 59 peaks

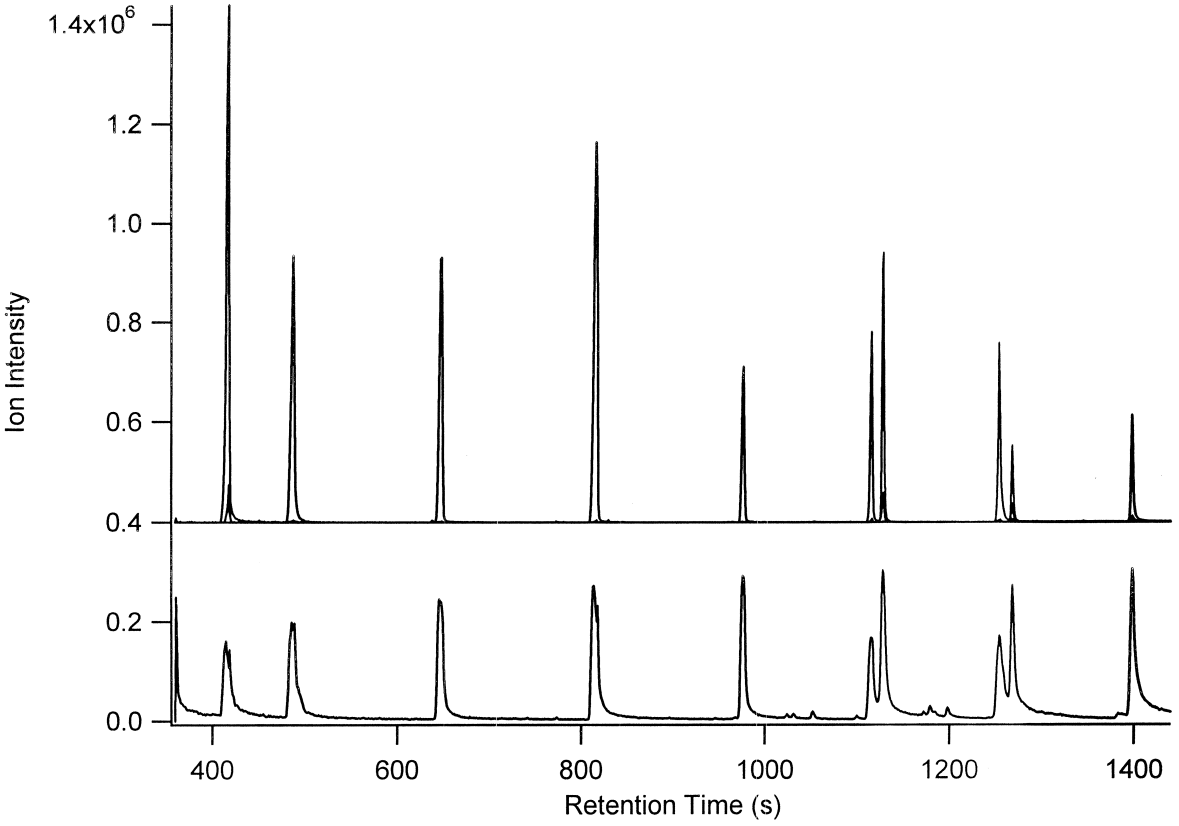

Fig. 7. Selected ion monitoring (m/z M-71 top trace, m/z 59 bottom trace) of ten amides derivatized with BSTFA–toluene (50:50, v/v). Peak identities and concentrations as in Fig. 3.

were badly tailed, and partly because of tradeoffs in ionization efficiency. Tailing was probably chromatographic in nature, which certainly affected calibration accuracy and precision. However, absolute detection limits were lower, by as much as a factor of ten, for m/z 59 peaks because, at the lowest injected concentration, significant amounts of M-71 fragment were not produced. Thus, the M-71 peak was found to be best for quantitative analysis, while the m/z 59 was best for trace analysis situations, such as screening, where quantitation was less important. The details of the ion trap ionization mechanisms are beyond the scope of this manuscript and will be published elsewhere.

While the sensitivity was quite adequate for the biological samples that will be analyzed and, in fact, was similar to that reported for *N*-acylethanolamines on a quadrupole MS [19], it was still several orders of magnitude worse than detection limits normally expected for GC–MS. The primary limitation appeared to be the fact that an ion trap rather than a quadrupole was used as the mass spectrometer. The ion trap did not allow a true SIM mode; furthermore, the ionization current duty cycle was quite low for this particular trap.

4. Conclusions

Trace level detection of trimethylsilylated fatty acid amides is possible using SIM methodology. TMS–amides undergo characteristic fragmentation, allowing rapid identification as well as quantitation. A new fragmentation mechanism has been proposed for silylated amides and has been investigated using molecular modeling. TMS–amides follow a pattern

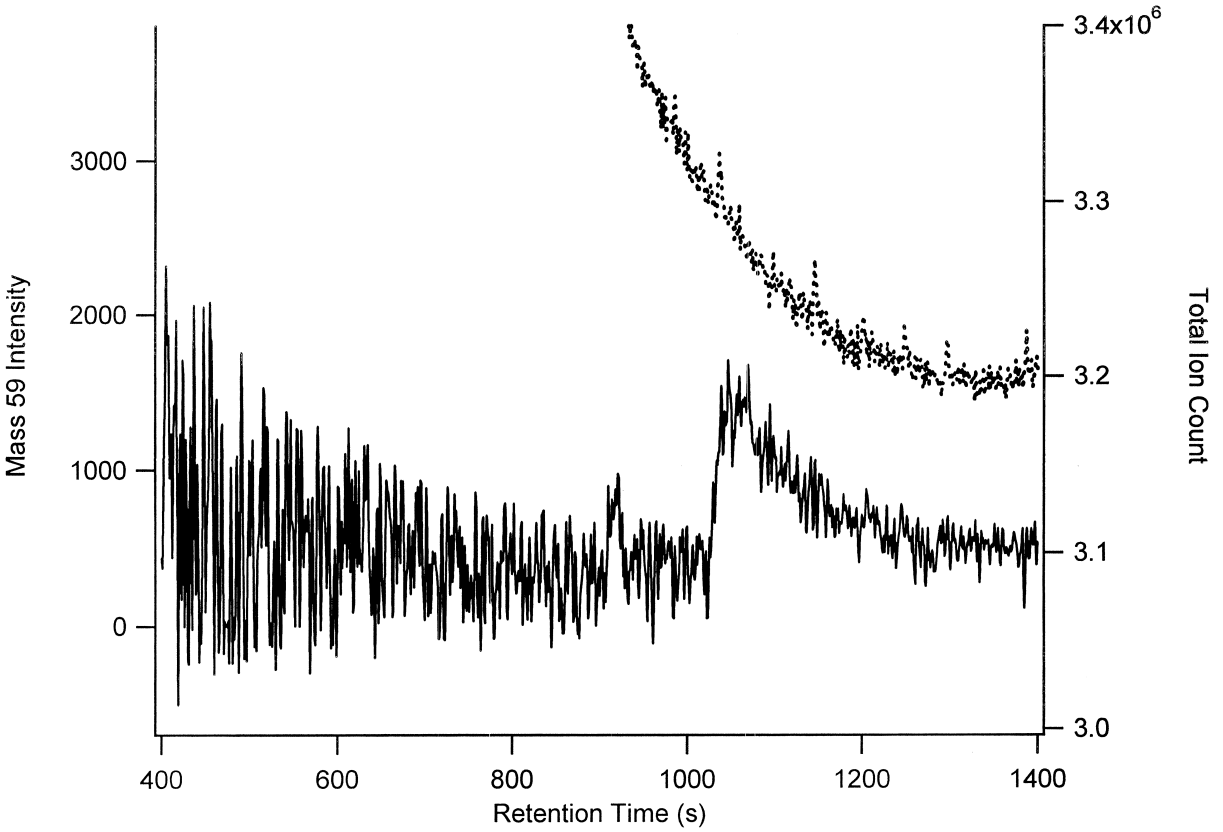

Fig. 8. Lauramide (1.4 pmol) derivatized with BSTFA in toluene. Solid trace: *m/z* 59 SIM (left axis); dashed trace, TIC (right axis).

similar to that of fatty acid methyl esters in their elution order but the number of carbons in fatty acid amides overrides the unsaturation effect seen in fatty acid methyl esters. There are many other considerations besides reaction efficiency when dealing with GC–MS following derivatization reactions. Baseline and signal-to-noise ratio improvements have led to the exclusive use of toluene and BSTFA as the reaction media and chromatographic solvent.

The use of *m/z* 59 is potentially problematic for analyzing complicated biological extracts, as envisioned here, which may prevent the detection of the lowest concentrations of amides. The mass is low enough that a large number of compounds are

Table 1
Calibration data for TMS derivatives of PFAMs in the selected ion mode[a]

Derivative	*m/z* 59				M-71			
	Log slope	r^2	LDR (nmol)	RSD[b] (%)	Log slope	r^2	LDR (nmol)	RSD (%)
Myristamide	0.95	0.96	0.01–1	5.0	1.03	0.95	0.01–100	3.4
Oleamide	0.22	0.94	0.001–10	5.7	1.04	0.96	0.001–100	5.0

[a] Injected mass at 1–3–5 intervals over 1 pmol to 100 nmol, three replicates each. Log slope is the slope of the plot of log (peak area) vs. log (pmol injected). LDR=linear dynamic range, determined by the elimination of data points until subsequent changes in r^2 were not significant.

[b] RSD=relative standard deviation of peak area; four replicate injections of 149 pmol (myristamide) and 332 pmol (oleamide).

expected to have fragments that interfere with the amides. However, sample clean-up and high-resolution capillary GC may obviate this problem. Given the general problem of self-chemical ionization in ion trap mass spectrometers, sample clean-up is required in any case. Furthermore, chloroform–methanol extracts of cellular material are relatively clean. We are exploring the use of normal-phase HPLC for preliminary isolation of the amides as a class before derivatization and injection.

Acknowledgements

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