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Biosynthetic Enzymes Regulating Ratios of Sex Pheromone Components in Female Redbanded Leafroller Moths

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Abstract—Changes in key enzymes in the biosynthetic pathways of sex pheromone components can produce differences in component ratios and structures. The sex pheromone communication system is critical to reproduction and the maintenance of a species and so changes in this system can play a major role in the speciation process. Artificial selection of female redbanded leafroller moths that produced either higher or lower ratios of 14-/12-carbon pheromone components was used to study how the biosynthetic pathways were affected in the high and low populations. The results showed that the chain shortening enzymes were selective for the (*E*) isomer and so left the 14-carbon acyl intermediates enriched in the (*Z*) isomer. Thus, the high population, which has a higher amount of 12-carbon components, also has a lower ratio of *E*11-/*Z*11–14:OAc pheromone components. The data also suggested that chain shortening occurred prior to reduction and acetylation of the 14-carbon components. These changes are not sufficient to isolate the redbanded leafroller populations, but we discuss some cases where significant changes in pheromone component ratios are affected by the chain-shortening enzymes. Copyright © 1996 Elsevier Science Ltd

Introduction

Sex pheromones in the Lepidoptera generally have been found to be blends of compounds that have evolved as species-specific mating signals.[1,2] Although many related species have been found to possess similar biosynthetic pathways, it has been found that the female-released pheromone blends exhibit a high degree of specificity by affecting the male's response behaviors with changes in the quality and quantity of blends in the odorous plume. Thus, it is important that both sender and receiver in this mating communication system be tuned to a specific blend and release rate of pheromone components.

In moths, the pheromone components generally have been found to be acetate esters (OAc), aldehydes and alcohols with carbon chains of 10–16.[3] In redbanded leafrollers (RBLR), *Argyrotaenia velutinana*, a seven-component blend was found to be produced and released by the female moths.[4] The blend consists of (*Z*)-11-tetradecenyl acetate (*Z*11–14:OAc; 79%), (*E*)-11-tetradecenyl acetate (*E*11–14:OAc; 7%), tetradecyl acetate (14:OAc; 4%), (*Z*)-9-dodecenyl acetate (*Z*9–12:OAc; 1%), (*E*)-9-dodecenyl acetate (*E*9–12:OAc; 2%), 11-dodecenyl acetate (11–12:OAc; 2%) and dodecyl acetate (12:OAc; 5%). Research has been conducted on this blend to determine how variable the component ratios might be among individuals, as well as to determine how enzymatic control is exerted in the biosynthetic pathways to produce the precise ratios.

Key words: Sex pheromone, chain shortening, redbanded leafroller moth, speciation, biosynthesis.

Variability of Pheromone Component Ratios in the Redbanded Leafroller Moth

*Z*11/*E*11–14:OAc ratio

The major pheromone components in RBLR consist of a mixture of *Z*11/*E*11–14:OAc. A study[5] on intrapopulation variation of these two acetates showed that the ratio of geometric isomers exhibited little variability. The individual female glands analyzed (ca. 600) had ratios between 4 and 15% of the (*E*) isomer, with means and standard deviations of 9.1 ± 1.8 and $7.0 \pm 1.4\%$ for a field and a laboratory population, respectively. Another study[6] was carried out to determine if there was a genetic basis to the small amount of observed individual variability of these components. Artificial selection protocols were carried out in attempts to increase the ratio of isomers. The protocols consisted of (a) a truncation selection of females, (b) truncation selection of females in concert with selection of males responding to abnormally high (*E*) blends of pheromone, and (c) a selection of females with inbreeding. Although slight differences in ratios were obtained as lines were selected for up to 25 generations, the selection gain always reverted back to normal several generations after selection removal and the ratio never moved beyond the range of the base population. It was concluded that this particular

component ratio is strongly canalized in this species and that it would be very difficult to shift this intractable blend with direct selection.

14:OAc's/12:OAc's ratio

Since the (*Z*/*E*) ratio was found to be intractable, it was thought that the higher variation found in the ratio of 14-carbon components/12-carbon components would provide a better basis for shifting the pheromone blend. A laboratory population of redbanded leafroller moths exhibited a blend with *E*9–12:OAc present at 20% of the *E*11–14:OAc component, whereas in a population from North Carolina the percentage was 31%. Hybrid populations from these two cultures were used in a two-way family truncation selection scheme in which families were selected for either the lowest (low line) or the highest (high line) ratio of *E*9–12:OAc/*E*11–14:OAc.[7] Artificial selection quickly produced a low line of 14% and a high line of 42% *E*9–12:OAc relative to *E*11–14:OAc. Although various selection protocols were used over several years, the ratios could not be moved much over 50% or below 10%. An interesting observation made in this study is that whereas the ratio of *E*9–12/*E*11–14 acetate is positively correlated to the total amount of 12-carbon components to 14-carbon components, it is negatively correlated to the amount of *E*11–14:OAc compared with *Z*11–14:OAc. Thus, a parent culture with 24% *E*9–12:OAc relative to *E*11–14:OAc had 5.8% *E*11–14:OAc compared with *Z*11–14:OAc, whereas the high line had percentages of 52.5 and 3.8%, respectively, and the low line had percentages of 10.1 and 8.2%, respectively. Although some plasticity was found in the 12-/14-carbon ratio, it reached a limit in how much it could be changed, apparently restricted by whatever factors control the canalized *Z*11-/*E*11–14:OAc ratio.

Enzymatic Control of Pheromone Ratios in the Redbanded Leafroller Moth

To determine how the component blend is produced and regulated in the redbanded leafroller moth, it was necessary to define the biosynthetic pathways, the composition of the precursor pools and the specificity of the various enzymes involved in these pathways. The pathways were defined by a series of studies with labeled precursors[8] and summarized in Figure 1. Since the major pheromone components, *Z*11- and *E*11–14:OAc, were produced in a specific ratio of ca. 92:8, it was anticipated that the corresponding acyl precursors, *Z*11- and *E*11–14:CoA, would also be in that approximate ratio. However, it was surprising to find that the (*E*) isomer was more abundant in the acyl intermediates, in proportions of 42:58 (*Z*/*E*). Analyses of lipid classes showed that the very abundant triacylglycerols and the trace quantities of diacylglycerols possessed a mix that was high in the (*E*) isomer [ca. 40:60 *Z*/*E*)]. The choline phosphatides and ethanolamine phosphatides possessed a small quantity of the unsaturated fatty acyl groups, but were found to be in a 70:30 (*Z*/*E*) ratio, which was intermediate between the 40:60 ratio of triacylglycerols and the 92:8 ratio of the pheromone components. Further analyses of the choline phosphatides with phospholipase A_2 showed that the *Z*11- and *E*11–14:Acyl groups were found only at the s_n2 position and, thus, the production of a 92:8

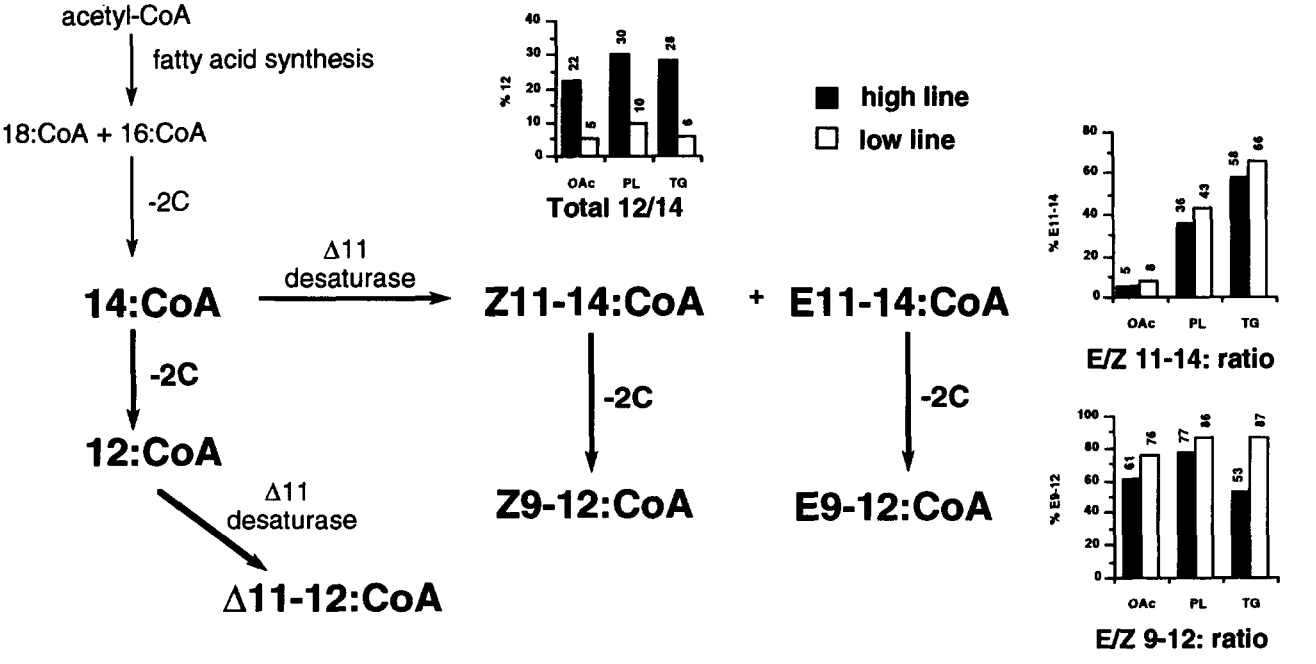

Figure 1. The pheromone biosynthetic pathway in the redbanded leafroller moth. The CoA derivatives shown in bold are further reduced and acetylated to make the pheromone blend of acetate esters. The graphs show the various ratios of interest found in the high and low lines and in three different lipid classes. OAc = pheromone acetate esters, PL = phospholipids, TG = triacylglycerols.

ratio could not be the result of a particular stereospecific position in the glycerolipids harboring this particular ratio.

Time-course studies[9] were conducted to define the sequence of fatty acyl intermediates in the biosynthesis of the *Z*11- and *E*11–14:OAc pheromone components. A large proportion of incorporated radiolabel from acetate was found quickly (8 min) in the pheromone components and the triacylglycerols. The free fatty acid, [1-^{14}C]tetradecanoic acid, also was incorporated readily into the pheromone components, although no label incorporation into pheromone was found when various labeled triacylglycerols prepared with this acid were used as possible biosynthetic precursors. The data suggested that there was a high preference of (*Z*) precursors in the conversion to pheromone components, possibly from the choline or ethanolamine phosphatides, and that the remaining (*E*)-enriched intermediates were converted to triacylglycerols as a chemical dump.

Specificity of acetyltransferase

In the redbanded leafroller moth it was found[10] that the Δ11 desaturase complex produces a 60:40 ratio of the *Z*11-/*E*11–14:acyl intermediates without the involvement of an isomerase. The Z-enriched blend of 92:8 *Z*11-/*E*11–14:OAc pheromone components must, therefore, be produced after the desaturation step in the pathway. This suggests some selectivity of the reductase and acetyltransferase that follow the desaturase. A study, therefore, was conducted to characterize the specificity of the acetyltransferase[11] in redbanded leafroller moths. This enzyme, which transfers an acetyl group from acetyl-CoA to a fatty alcohol to make acetate esters, was found only in the pheromone gland of female moths, and not in fat body or other epidermal tissue. Substrate competition assays between *Z*11- and *E*11–14:OH indicated that only one enzyme was involved and that it converts the (*Z*) isomer to product faster than the (*E*) isomer. The (*Z*) isomer always produced 1.5–2 times more product than the (*E*) isomer. The study showed that the specificity of the acetyltransferase in redbanded leafroller pheromone gland produced a 90:10 pheromone component ratio of acetates if the starting alcohol ratio was 75:25.

The specificity of this enzyme thus accounts for almost half of the enrichment of the (*Z*) isomer in the pheromone components from the 60:40 (*Z*/*E*) ratio of acyl intermediates. The remaining specificity would have to reside in the reductase step or in a change of the (*Z*/*E*) ratio in the precursors prior to the reductase step. Since the genetic studies discussed above showed a negative relationship between the 12-/14-carbon compounds and the *Z*11-/*E*11–14:OAcs, a study was carried out to determine if the chain-shortening step of 14-carbon acyl intermediates to 12-carbon acyl intermediates might be involved in changing the (*Z*/*E*) ratio in the precursor pool used by enzymes producing the 92:8 (*Z*/*E*) blend of pheromone components. The experimental details and results of this study will now be presented.

Results and Discussion

Populations of redbanded leafroller moth were selected that exhibited 'high' and 'low' ratios of 12-carbon pheromone/14-carbon pheromone components.[7] These populations provided the basis to study changes in the pool of acyl intermediates and to determine how these changes are reflected in the final *Z*11-/*E*11–14:OAc and *Z*9-/*E*9–12:OAc ratios. Although additional selection protocols were carried out, the high and low percentages of 12-carbon acetates could not be shifted to extremes any further than in the initial selection studies. Populations stabilized as high and low lines for several years were analyzed so that the composition of the precursor pools could be compared with the resulting pheromone-component ratios.

Ratios of the pheromone acetates were obtained from pheromone glands from the two lines of RBLR using a nonpolar column that could separate the various 12-carbon positional isomers (Fig. 2). Ratios of the

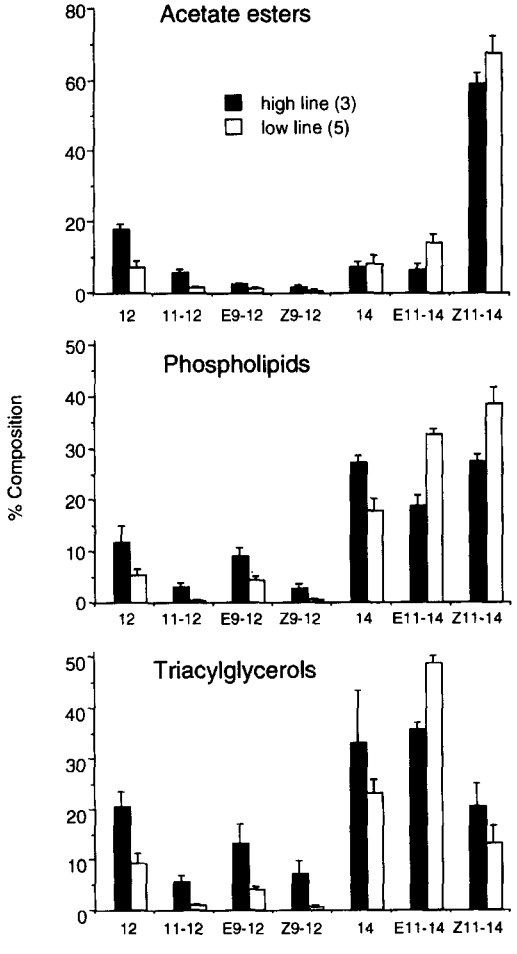

Figure 2. Percent compositions of the 12- and 14-carbon components found in three different lipid classes extracted from pheromone glands of female redbanded leafroller moths.

fatty acid intermediates analyzed as methyl esters also were determined (Figs 1 and 2). The lipids were first separated into triglycerides (TG) and phospholipids (PL) by TLC before conversion to methyl esters. The High line was characterized by having a higher percentage of the 12-carbon acetate esters, as well as intermediate fatty acids in both the TGs and PLs. The low line shows a lower percentage of 12-carbon compounds and unselected females have a percentage that is midway between the high and low lines (data not shown).

The ratios of interest based on the biosynthetic pathway are shown in Fig. 1. The main pheromone components *E*11–14:OAc and *Z*11–14:OAc are found in approximately an 8:92 ratio with the high line having a slightly lower amount of E11–14:OAc. This trend is true also for the *Z*11- and *E*11–14:Acyl components found in the PLs and TGs, with the low line showing a slightly higher amount of *E*11–14:Acyl. In contrast, the *E*9–12 to *Z*9–12 ratio is fairly constant amongst the lipid classes, with *E*9–12 being found in higher amounts than *Z*9–12. This indicates that more *E*11–14:CoA is being chain shortened to *E*9–12:CoA in all lines. However, in the high line, chain shortening has increased as indicated by the higher total 12/14 ratio (Fig. 1) and the higher amounts of 12-carbon components in all lipid classes (Fig. 2). These results indicate that chain shortening is increased in the High line and decreased in the low line, while the substrate specificity for the chain shortening enzymes was unaffected.

Chain shortening prior to reduction

There was a negative correlation between the percentage of *E*11–14:OAc relative to *Z*11–14:OAc and the amount of chain shortening (i.e. lower % *E*11–14:OAc with increased chain-shortening). A possible explanation for this is that the chain-shortening enzymes act before the reductase step due to differential positioning of the enzymes in the endoplasmic reticulum and peroxisomal membranes. Chain shortening of the Δ11–14:Acyl to Δ9–12:Acyl intermediates is selective for the (*E*) isomer over the (*Z*) isomer and, therefore, increased chain shortening leaves behind a blend of Δ11–14:Acyl intermediates that is enriched in the (*Z*) isomer. This (*Z*)-enriched blend of 14-carbon intermediates then is reduced and acetylated to produce a final pheromone blend that has a higher proportion of the (*Z*) isomer, while more of the (*E*) isomer ends up as E9–12:OAc due to chain shortening.

The acetyltransferase was found to act preferentially for the (*Z*) isomer with both 14- and 12-carbon compounds.[11] Therefore, the data from the high and low lines suggest that the (*E*/*Z*) ratios obtained for the Δ12-carbon acetates are generated from the PL blend of Δ12-carbon intermediates, and that the Δ14-carbon acetates are generated from the PL blend of 14-carbon intermediates after the chain-shortening sequence has enriched them in the (*Z*) isomer.

Changes in the chain-shortening step and speciation

Limited chain-shortening steps found in moth pheromone glands play an essential role in the generation of thousands of species-specific pheromone blends. Changes in the activation or inactivation of this step can produce pheromone components that differ by two carbons shorter or longer, with the double bond position ending up either two carbons closer or further from the carboxyl group, respectively. This type of structural change in the pheromone components could be sufficient to produce populations in which the males of each do not respond to the pheromone blend of the other and eventually the populations could become distinct species. In the present study, the increased chain-shortening exhibited in the high line of redbanded leafroller moths is not sufficient to isolate populations. The slight variations in (*E*/*Z*) ratios and in 14-/12-carbon ratios are well within the range of blends to which the male redbanded leafroller moths respond and so these artificially produced populations probably would be absorbed quickly back into the natural population if released in the field.

However, there are cases wherein the activation of a chain-shortening step appears to be playing a significant role in the speciation process. In 1971 the pheromone of the larch budmoth[12] was identified as *E*11–14:OAc. It was found to be attractive for males of this species in some forests, but was not active in others. Later it was found[13,14] that there was a population of larch budmoth that used *E*9–12:OAc as its pheromone. This compound would be produced by chain shortening the *E*11–14:Acyl intermediate to an *E*9–12:Acyl intermediate. The studies revealed that there are two morphologically indistinguishable populations of larch budmoth on different host plants and that they are distinguished by using different pheromone compounds. In one population the larvae are dark and feed on larch and the adults use *E*11–14:OAc, whereas with the other population the larvae are light and feed on cembran pine and spruce trees and the adults use *E*9–12:OAc. Mutations such as the appearance of this chain-shortening step probably can occur frequently in a population, but stabilization of a new pheromone system such as found with the larch budmoth would require unique circumstances in which some portion of the population producing the new pheromone is isolated (e.g. on another host tree) and males in that population gradually adapt to these new signals. With the larch budmoth, a study[15] of electroantennogram responses showed that the antennae of male moths of each population were maximally responsive to their own pheromone compound, indicating co-evolution of the antennal receptors in these two host races.

A less defined example of the effect of changes in the chain-shortening steps involves the turnip moth, *Agrotis segetum*. Generally, this species uses a combination of the chain-shortened cascade of compounds consisting of *Z*9–14:OAc, *Z*7–12:OAc and *Z*5–10:OAc. However, the blend ratio that is produced by female moths and

to which males respond behaviorally differs among various populations of this species in Europe and Western Asia.[16–23] Populations of this species in France, Denmark and Switzerland use a blend ratio of 13:40:47, respectively. Populations in Sweden and Britain have a reduced amount of the final chain-shortened product, Z5–10:OAc, and use a 44:52:4 blend ratio. Finally, Armenian/Bulgarian populations have very little of the last chain-shortening step in evidence as they use 47:52:1 and 57:42:1 blend ratios. The proportion of receptors sensitive to the Z5–10:OAc compound on male antennae also reflects the differences in the female blend. Male turnip moths from France had up to 87% of their receptors tuned to this 10-carbon compound, whereas only 6% of the antennal receptors on Bulgarian male moths were tuned to this compound.[22,23] Thus, there appears to be a stabilizing selection for the different pheromone systems, as produced by changes in the amount of chain-shortened product, to the point where now the Swedish and Armenian populations coexist in Hungary as parapatric populations.

One last example illustrates how a mutation affecting the chain-shortening sequence can produce large changes in the pheromone blend. The cabbage looper moth, *Trichoplusia ni*, was found to use a pheromone consisting of six components: Z7–12:OAc (100%), 12:OAc (6%), Δ11–12:OAc (2%), Z5–12:OAc (8%), Z7–14:OAc (0.8%) and Z9–14:OAc (0.6%).[24] During a study of interpopulational variation in this pheromone blend,[25] some female moths were found in a laboratory culture of cabbage looper that produced and released much reduced levels of the major component, Z7–12:OAc, 20 times as much of the Z9–14:OAc component and 30-fold less Z5–12:OAc. A laboratory population was generated from these mutants and an analysis of crosses between the two populations showed that one recessive autosomal gene was involved in producing the mutant blend.[25]

A study[26] involving a combination of in vivo labeling studies and in vitro enzyme assays showed that all three of the major changes listed above in the blend were produced by a mutation that affects chain-shortening through limited β-oxidation of fatty acyl-CoAs. The biosynthesis of the six pheromone components involves chain-shortening two different substrates.[27] One series produces a cascade of intermediates from Z11–16:Acyl, including Z9–14:Acyl and Z7–12:Acyl after two rounds of chain shortening. The other series produces a cascade of intermediates from Z11–18:Acyl, including Z9–16:Acyl, Z7–14:Acyl and Z5–12:Acyl after 3 rounds of chain shortening. The study on the mutants showed that the enzymes in the mutant females lacked the ability to chain shorten by more than one round of β-oxidation. Thus, in the first series, biosynthesis stalled after the first round, producing a large accumulation of Z9–14:Acyl intermediate and only a small proportion of Z7–12:OAc. In the other series, the Z9–16:Acyl intermediate accumulated and only traces of the subsequent intermediates, Z7–14:Acyl and Z5–12:Acyl, were produced.

The mutant pheromone blend did not attract cabbage looper males in the field, but did attract males of another species that used a blend similar to the mutant blend. Although males in the mutant colony were not very responsive to the mutant blend, indicating that there is no linkage between signal production and response, males in the mutant colony did broaden their response spectrum after 49 generations of laboratory rearing to respond as well to the mutant blend as they do to the normal blend.[28] This shows the possibility of tuning the signaling system in nature after a mutation in blend production has occurred.

Since limited chain shortening through β-oxidation plays a major role in the production of pheromone components in a large percentage of the moth species, it has become clear that any mutation affecting the activation or inactivation of even just one round of chain shortening can have a major effect on the resulting structures of the pheromone components produced by the mutant females. It can be speculated that the effect can be sufficiently great to produce mutant populations, which become reproductively isolated from the normal population via various combinations of different pheromone blends, geography and host plants, and through time become a new species.

Experimental

Insects

RBLR were reared as described previously.[29] The different lines of RBLR were obtained as described previously.[7] These lines have been maintained in the laboratory for at least 7 years with 3–4 generations per year. The ratios of pheromone components were relatively constant from generation to generation within these lines.

Pheromone extraction

Pheromone glands were removed during the photophase and extracted in hexane or chloroform: methanol (C:M; 2:1 v/v). Hexane extracts were analyzed for the ratios of acetate esters. C:M extracts were first separated by TLC into the lipid classes OAc, TG and PL. Plastic backed silica gel G TLC plates were used with hexane: ether (80:20 v/v) as the developing solvent. After developing, the plates were briefly exposed to iodine vapors and the areas corresponding to the different lipid classes were scraped from the plate and extracted in C:M. Methyl esters were then made from the TG and PL by base-catalyzed methanolysis as described.[9]

Ratio determinations

Ratios of pheromone components and fatty acid intermediates were determined by analysis by GC and GC–MS. A nonpolar column (SE-30, Alltech Assoc., 0.25 mm × 30 m) was temperature programmed in a GC (Hewlett Packard 5890) with a flame ionization

detector or coupled to a mass selective detector (Hewlett Packard 5970). Temperature programming was 80 °C for 1 m, 2 °C/min to 164°, 10°/m to 220° for analysis of ME and 80° for 1 m, 3°/m to 182°, 10°/m to 220° for analysis of OAc. The mass selective detector was run in the single ion mode and the following ions were used for identification: 12:OAc, 168, monounsaturated 12:OAc, 166, 14:OAc, 196, monounsaturated 14:OAc, 194, 12:ME, 183, monounsaturated 12:ME, 180, 14:ME, 211, monounsaturated 14:ME, 208.

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