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Identification of very long chain fatty acids from sugar cane wax by atmospheric pressure chemical ionization liquid chromatography–mass spectroscopy

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Abstract

A method is described for the enrichment of very long chain fatty acids (VLCFAs) from total fatty acids of sugar cane wax and their identification as picolinyl esters by means of liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization (LC–MS/APCI). The method is based on the use of preparative reversed phase HPLC of 100 mg amounts and their subsequent identification by microbore APCI LC–MS. The combination of these two techniques was used to identify unusual saturated VLCFAs up to C_{50}.

Keywords: Sugar cane wax; Very long chain fatty acids; Liquid chromatography–mass spectrometry – atmospheric pressure chemical ionization

1. Introduction

Fatty acids are important constituents of waxes, in which they occur in free and esterified forms. These compounds are found in animal, plant, and microbial tissues and have a variety of functions, such as energy stores and waterproofing (Kolattukudy, 1976; Hamilton, 1995). These waxes contain mostly very long chain fatty acids (VLCFAs), i.e., fatty acids with more than 20 carbon atoms. The main problem in the identification of VLCFAs in natural materials, i.e., in plants, animals and also in microorganisms is their low concentration (Rezanka, 1989).

In previous papers (Rezanka and Sokolov, 1993; Rezanka, 2002), we published methods for enriching mixtures of natural fatty acids. These methods are based predominantly on chromatographic techniques, i.e., on thin-layer chromatography or HPLC, both in reversed phase mode. The method of choice turned out to be RP-HPLC (Rezanka, 1990), which could be used not only for enrichment of total fatty acids with VLCFAs but also for separation of individual compounds.

The detection of eluted components is complicated as fatty acids hardly absorb in the UV region. It is therefore necessary to either use the absorption of the terminal carboxylic group, of the double bond(s), or derivatize this carboxylic group.

Picolinyl esters are the only derivatives that can be detected via UV absorption and, at the same time, by gas chromatography–mass spectrometry (GC–MS) (Christie and Stefanov, 1987; Christie, 1998).

The individual components were resolved by mobile phases based on methanol with a small amount of pyridine in water (Christie and Stefanov, 1987). In our previous paper (Rezanka, 1990), undesirable interactions between the picolinyl moiety and the stationary phase were eliminated by triethylamine. A column with phases based on C8/C18 multi-alkyl bonding and exhaustive endcapping minimizes the use of organic modifiers. It was also recently successfully used by Christie (1998), pure acetonitrile being used as mobile phase. Gas chromatography has recently

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been used for the separation of VLCFA (Rezanka, 1989; Van Pelt et al., 1999). This method is suitable for the analysis of compounds which are volatile as such or after derivatization. It is known from the literature (Rezanka and Mares, 1991), that the discrimination of higher homologues increases with increasing number of carbons and double bonds. The technique used in this paper, i.e., APCI, avoids this shortcoming (Rezanka, 2000a,b; Rezanka and Votruba, 2005).

Based on our previous experience with semi-preparative RP-HPLC (Rezanka, 2002) we have now used its preparative mode for the rapid enrichment of the VLCFA fraction with minimal losses. FA mixture containing VLCFAs prepared from the sugar cane wax was utilized to demonstrate the suitability of the method. The presence of VLCFAs in this sample was demonstrated by a newly developed method, i.e., identification of picolinyl esters of the VLCFAs by means of LC–MS/APCI.

A natural mixture of higher primary aliphatic acids (24:0 to 36:0) was obtained from sugar cane (*Saccharum officinarum* L.) wax (Gonzales et al., 2002). This product was found to be responsible for some biological properties related to health, such as antioxidant (Menendez et al., 2002) and cholesterol-lowering effects, determined in both in vivo and in vitro models (Gamez et al., 2000; Rodriguez et al., 2004), beneficial effects on platelet aggregation in healthy volunteers (Arruzazabala et al., 2005), as well as antiplatelet and antithrombotic activities (Molina et al., 2000; Mas, 2004). The nutritional significance and metabolism of very long chain fatty alcohols and acids from sugar cane has recently been reviewed (Hargrove et al., 2004). It was also found that their mixture does not show any evidence of cytotoxic or genotoxic activity on either somatic or germ cells in rodents (Gamez et al., 2004).

2. Results and discussion

Fatty acids isolated from the sugar cane wax have already been identified and quantified using a GC method (Delange et al., 2002; Gonzales et al., 2002; Antolin et al., 2004). As mentioned above, long chain fatty acids are extremely sensitive to thermal degradation, i.e., to conditions under which they are eluted from the GC column. To minimize the possibility of thermal degradation, we used two HPLC steps; the FA derivatives were first concentrated by preparative RP-HPLC (Fig. 1) and individual molecular types of VLCFA derivatives were separated, identified, and quantified by LC–MS.

The difficulty in identifying the VLCFA in the whole complex of fatty acids was eliminated by separating shorter chain fatty acids (up to C_{36}) by RP-HPLC, i.e., enriching longer chain FA. Although the formation of higher homologues of VLCFA drops dramatically with proceeding chain elongation, the C_{50} is still detectable even though it constitutes only 0.028% of total fatty acids.

The enrichment of the individual fractions with VLCFA enabled us to identify as yet unknown homologues (Fig. 2). The total ion current was used for quantification, as shown also in Fig. 2.

Table 1 shows the percent representation of VLCFA in the enriched complex of fatty acids of sugar cane wax. The fatty acids were saturated from C_{35} up to C_{50}, odd and even numbered with a straight chain.

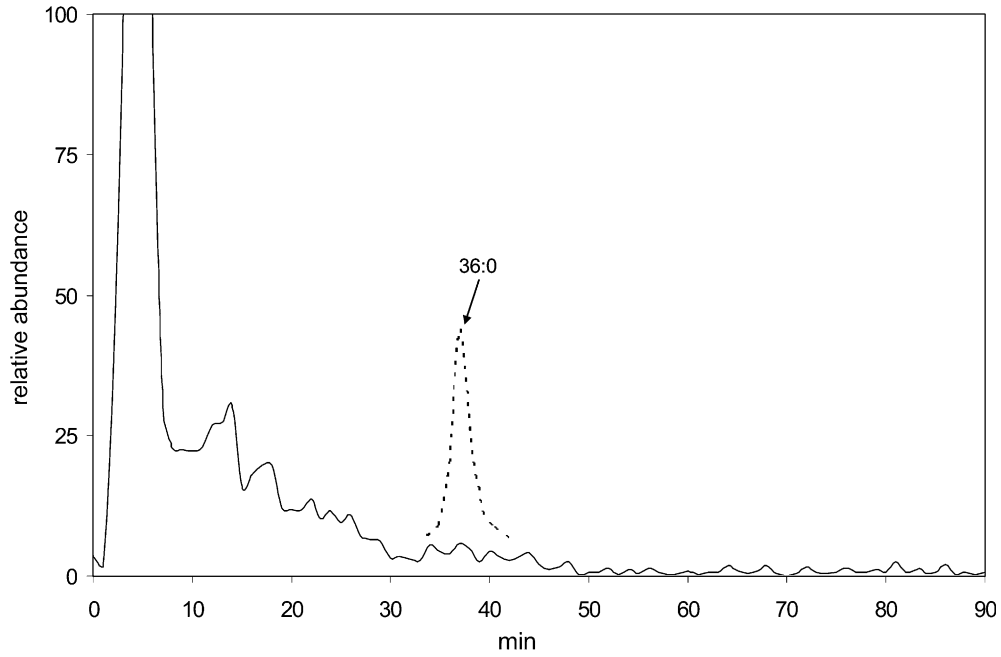

Fig. 1. Preparative RP-HPLC chromatogram of FA derivatives from the sugar cane wax. Peak identification: 36:0 – derivative of the FA with chain length 36 carbon atoms. Broken line, chromatogram after the addition of a standard-picolinyl ester of 36:0 synthetic acid. The fraction within the interval 37.5–90 min was used for further analysis.

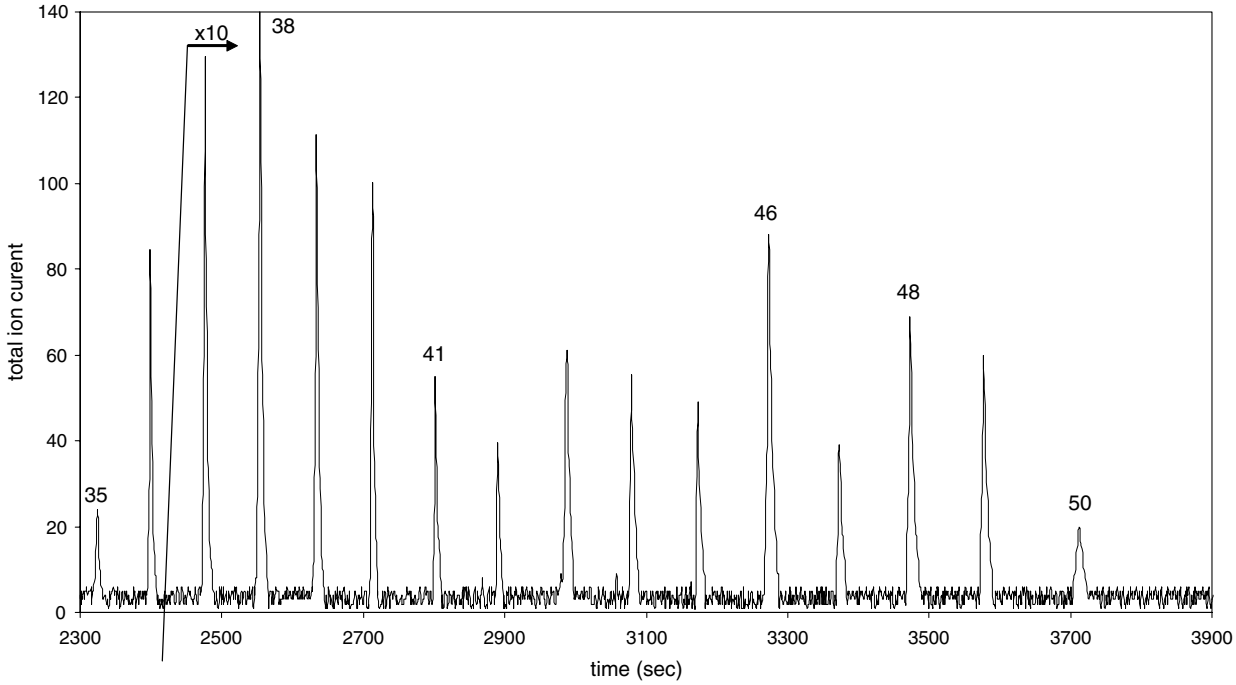

Fig. 2. LC–MS/APCI chromatogram of enriched fraction (after preparative RP-HPLC, see Fig. 1). Peak identification: 35 – picolinyl ester of pentatriacontanoic acid, etc.

In contrast to electron impact ionization (Christie, 1997) where the mass spectra of the picolinyl esters of straight chain saturated fatty acids contain ions from the lower part of mass spectra, i.e., ions *m/z* 92, 108, 151 and 164, these ions are in minority in APCI as shown in Fig. 3A (e.g., 46:0). Even-numbered ions differing by 14 amu and representing cleavage between methylene groups also have low intensity. Quite a distinct situation can be found in the M^+ region. Here, we can observe the formation of two abundant ions $[M-1]^+$ and $[M+1]^+$ including the generation of adducts with the mobile phase such as $[M+40]^+$ $(M+C_2H_2N)$ and $[M+54]^+$ $(M+C_3H_4N)$. In all investigated VLCFA spectra, the $[M+H]^+$ ion is the most abundant one. Fig. 3B shows the APCI spectrum of synthetically prepared 46:0 picolinyl ester; there is obviously hardly any difference between this compound and the natural one.

Table 1
Total fatty acids from sugar cane wax longer than C34

Fatty acid	Molar% ± SD ($n = 5$)
24:0–34:0	98.723
35:0	0.130 ± 0.099
36:0	0.398 ± 0.054
37:0	0.087 ± 0.023
38:0	0.096 ± 0.012
39:0	0.073 ± 0.007
40:0	0.070 ± 0.031
41:0	0.043 ± 0.015
42:0	0.025 ± 0.018
43:0	0.044 ± 0.017
44:0	0.041 ± 0.010
45:0	0.038 ± 0.013
46:0	0.063 ± 0.021
47:0	0.032 ± 0.018
48:0	0.050 ± 0.016
49:0	0.047 ± 0.009
50:0	0.040 ± 0.015

The industrial process of sugar refining results in the accumulation of by-products. One of them, mill mud, comprises crude wax and fats, fiber, sugar, crude protein, and ash. Typical components of wax are described in US Patent (Valix, 2004). The members of individual groups exhibit mostly straight saturated chains. No chains with more than 36 carbon atoms were found in any of the cases mentioned below. For instance, fatty acids with up to 34 carbon atoms and hydrocarbons with up to 35 carbon atoms were identified in different fractions obtained by extraction of sugar cane wax with different solvents (Nuissier et al., 2002). Interestingly, in the hydrocarbon fraction pentatriacontane accounted for 12% of the total alkane content. Alcohols up to tetratriacontanol have been described in a US patent (Laguna et al., 1999) and fatty acids up to hexatriacontanoic in another US patent (Gonzales et al., 2002). Aldehydes with chains of the same length were identified in two studies (Purcell et al., 2005; Lamberton and Redcliffe, 1960).

In contrast to these data, we demonstrated two basic features of sugar cane wax:

(1) Chain length of 36 carbon atoms is not final because, as seen in Figs. 2 and 3, homologues containing up to 50 carbon atoms have been identified. The reasons for their not being discovered earlier can include, e.g., insufficient sensitivity of the analytical techniques

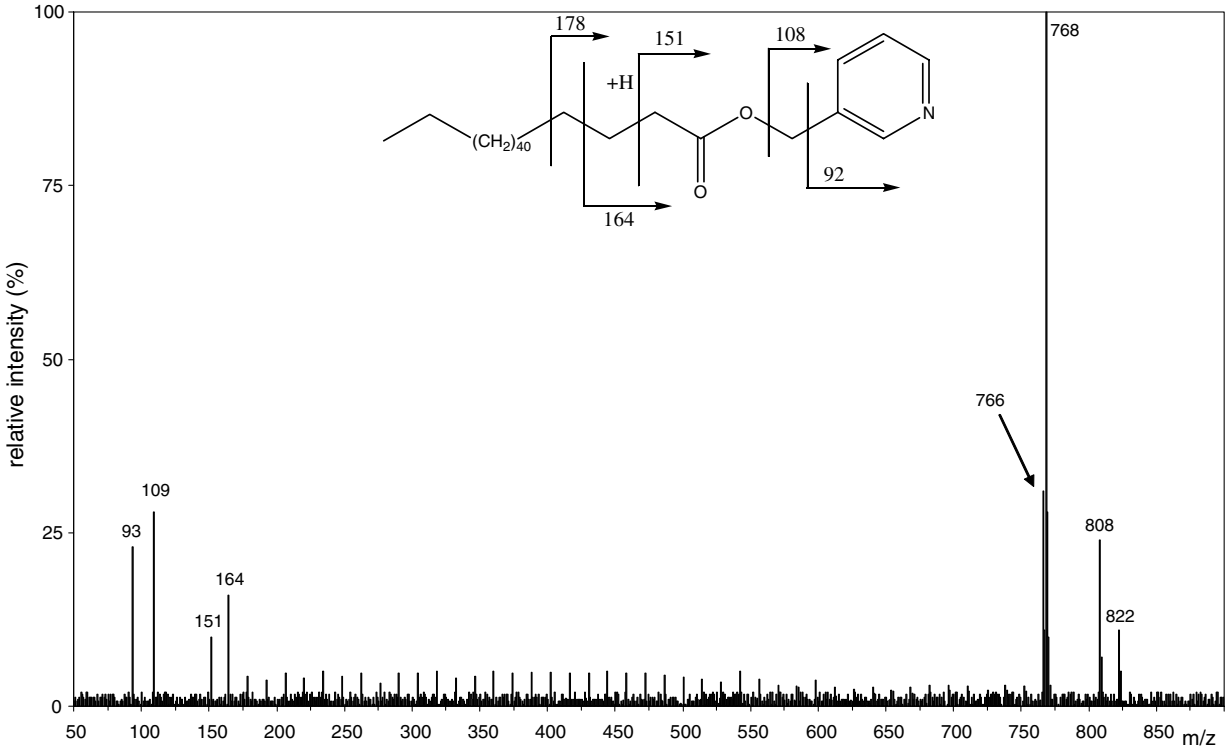

Fig. 3A. Mass spectrum of picolinyl ester of 46:0 natural acid; the structures of major ions are shown. For explanation of values see the text.

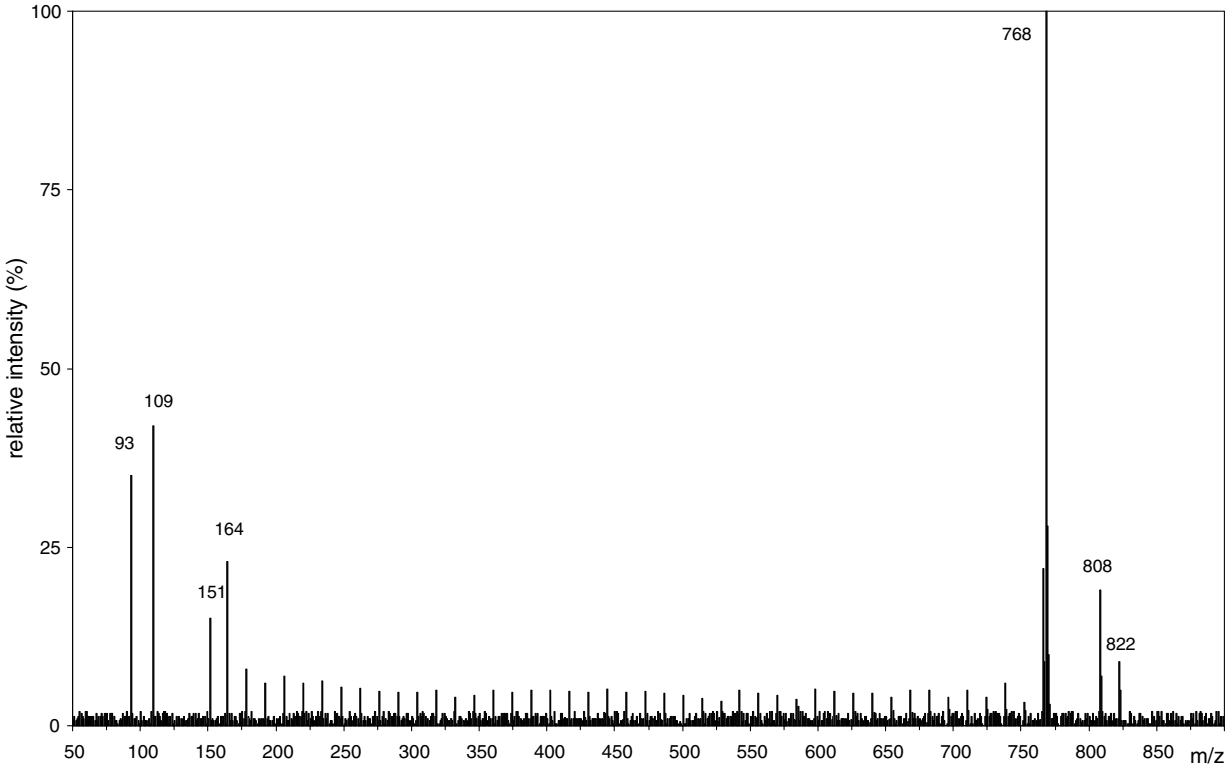

Fig. 3B. Mass spectrum of picolinyl ester of 46:0 synthetic acid.

used for the purpose (all relevant studies used gas chromatography). The LC–MS method with APCI used in our study is much more efficient in identifying compounds with very long chains. The identification was also greatly facilitated by the enrichment of the samples by VLCFAs that we used.

(2) Table 1 clearly implies that differences in proportion (in molar%) between chains with odd and even numbers of carbon atoms are obliterated. Although this phenomenon has been observed repeatedly (Rezanka, 2002), we have no satisfactory explanation.

Although conventional theory suggests successive chain elongation C16 and C18 acids as a possible mechanism for the biosynthesis of this VLCFA, further research into the pathways of long chain fatty acid biosynthesis in marine organisms is needed.

GC–MS can be recommended as a method of a first choice for fatty acid analysis. It is well suited for the analysis of a sample that contains VLCFA with chain lengths exceeding to common length to a certain degree – to about C34. On the other hand, the more expensive LC–MS technique is more convenient when it is necessary to analyze longer-chain VLCFA. This method may spur further development of VCLFA analysis and extend the options in the exploration of metabolism of recently examined organisms.

When analyzing VLCFA with chain length identified here in sugar cane wax one should keep in mind that these compounds are much more like triacylglycerols than fatty acids. An example is, e.g., the C50 acid that we identified, when compared with the common plant triacylglycerol 1,3-dioleoyl-2-palmitoylglycerol (POO). The acid derivative has 56 carbon atoms, POO has 55 and the degree of unsaturation of both compounds is five. The analysis of triacylglycerols and VLCFA therefore poses similar problems (see below).

High-temperature GC has until recently been used largely to separate molecular species of simple lipids according to the combined chain-lengths of the fatty acid moieties. It is an analytical technique that is capable of a high degree of precision, and can be married well with mass spectrometry. However, the technique may be nearing the technical limits before pyrolytic breakdown both of samples and of stationary phases occurs and a greater attention must be paid to instrumental parameters than with other types of gas chromatography.

The first problem concerns the injection system – the injected compound can be very easily degraded. The same holds for the passage of the compound through the column, during which both the eluted compound and the stationary phase may be degraded. Still another vulnerable point is the entry into the MS and the mass spectrometer itself. Probably for these reasons the GC–MS has seldom been used for the analysis of triacylglycerols – see, e.g., Buchgraber et al., 2004; Laakso, 2002. As mentioned by Christie (2003), "although useful separations of intact triacylglycerols by high-temperature GC can be achieved routinely, the technique can still be fraught with difficulties. The conditions necessary to elute lipids of such high molecular weight from the columns approach the limits of thermal stability both of the stationary phases and of the compounds themselves". On the other hand, HPLC does not suffer from these problems with sample thermal lability and, on top of it, permits the use of any soft ionization method in the mass spectrometer. This method has as yet been developed for the analysis of relatively saturated compounds but it can be readily extended to compounds with more than 10 double bonds and a chain of corresponding length, for which GC is not applicable.

Even though the presence of VLCFA in cane wax has not yet been described, the probability of their detection is likely to increase due to the advances in physico-chemical separation and identification methods as well as improvement of methods of sample collection, and also due to the currently rising interest in higher plants. Like other lipids, waxes and hydrocarbons, very long chain fatty acids act predominantly as components of the hydrophobic layer on the surface of leaves, which decreases their wettability. It can be speculated that they could participate in the formation of surface structures responsible for the so-called lotus effect (Furstner et al., 2005; Neinhuis and Barthlott, 1997).

The presence of a homologous series of very long chain fatty acids in the material used in this study, i.e., cane wax from Egypt, contrasts with the absence of similar compounds documented by previous studies (Delange et al., 2002; Gamez et al., 2000). It is difficult to say whether this striking difference is caused by differences in the geographical origin of the analyzed cane (Egyptian cane in our case, Cuban cane in the case of Gonzales et al., 2002), or if it can be ascribed to different instrumentation and methodology. The geographical factors might include different types of climate (dry weather with very low amounts of precipitations in Egypt versus a much more rainy weather in Cuba). The more abundant precipitations in Cuba could, e.g., wash the VLCFA off the leaf surface. Also, due to these different environmental conditions, the cane cultivars grown in the two regions might differ in metabolic patters resulting in different proportions of VLCFA being produced – plants growing in the arid regions might need these acids more than those from more rain-exposed localities.

In conclusion, we succeeded in identifying some of the longest fatty acids found in nature, and extending the knowledge of the sugar cane wax composition. The combination of preparative RP-HPLC and microbore LC–MS thus extends the analytical possibilities of both methods and contributes to the acquisition of new information on biological materials.

3. Experimental

3.1. Standards and isolation

Standards of fatty acids were prepared as described below. All solvents were double-distilled and degassed before use. 1-Bromotetracosane (**1**) and 12-bromododecanoic acid (**2**) were obtained from Sigma–Aldrich, Prague. The sugar cane wax from the 2003 sugar cane harvest (imported from Egypt) was obtained from a local sugar refinery.

3.1.1. Standards (Fig. 4)

To the stirred mixture containing a large excess of magnesium turnings (20 mg; 0.8 mmol) and 15 ml of dry diethyl ether was added dropwise a solution of 1-bromotetracosane (**1**) (91.6 mg; 0.22 mmol) in 25 ml of dry diethyl ether. At the end of the addition (2 h), the mixture was stirred for 1 h, whereupon the Grignard reagent solution was transferred under nitrogen into a dropping funnel (see Fig. 4).

To the solution of 12-bromododecanoic acid (**2**) (55.6 mg; 0.2 mmol) in 8 ml ethanol was added sodium ethanolate (from 5 mg Na; 0.22 mmol) in 8 ml ethanol and the mixture was stirred for 1 h. Ethanol was evaporated away and the sodium salt was dried at a pressure of 1 Torr (Zakharkin et al., 1988).

A suspension of 60 mg the sodium salt of **2** (0.2 mmol) in 8 ml absolute THF at −10 °C was supplied under continuous stirring and in argon atmosphere with Li_2CuCl_4 (4.38 mg, 0.02 mmol) and dropwise with the solution of 0.22 mmol of alkylmagnesium bromide of **1**, see above. The mixture was stirred at −10 °C for 4 h, acidified with 10% sulfuric acid and extracted with 3 × 5 ml diethyl ether. The extract was washed with water, dried, and evaporated to dryness. The yield of **4** referred to compound **2** was 87.4%, i.e., 93.8 mg, colorless, waxy compound, LC–MS/APCI: *m/z* 537 $[M+H]^+$; HREIMS *m/z* 536.5530 $C_{36}H_{72}O_2$ $[M]^+$, calculated for $[M]^+$ 536.5532; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.91 (3H, *t*, *J* = 6.8, H-36), 1.22-1.32 (62H, *m*, H-4–H-35), 1.59 (2H, *m*, H-3) and 2.26 (2H, *m*, H-2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.1 (C-36), 19.8 (C-35), 24.8 (C-3), 29.3-29.8 (C-4–C-33), 32.1 (C-34), 34.2 (C-2), 180.1 (C-1).

The acid (**4**) (40.1 mg; 0.07 mmol) was dissolved in 50 ml of dry ether. Fifteen milliliters of a 10% solution of $LiAlH_4$ (3.8 mg; 0.1 mmol) in 5 ml of dry diethyl ether was added and the mixture refluxed for 1 h. Water was added dropwise until the evolution of gas ceased. 10% Sulfuric acid was then added until the white precipitate had dissolved. The mixture was transferred to a separating funnel. The ether layer was washed twice with water (2 × 15 ml) and dried by means of sodium sulfate and the ether was removed by distillation. A solution of alcohol (31.9 mg, 0.061 mmol) and carbon tetrabromide (50 mg, 0.15 mmol) in dry acetonitrile (2 ml) was heated under reflux with stirring, and a solution of triphenylphosphine (58 mg, 0.22 mmol) in dry acetonitrile was added dropwise over 2 min. The mixture was heated under reflux for 2 h and cooled, the solvent was removed in vacuo, and the residue was extracted with benzene (3 × 10 ml). The combined extracts were filtered, and the solvent was removed in vacuo. The yield of product (**6**) was 85.5% (30.6 mg), MS *m/z* (relative intensity) 726 ($[M]^+$, 1), 646 ($[M-HBr]^+$, 19).

Bromide **6** (37.3 mg; 0.064 mmol) was used to prepare alkylmagnesium bromide by the procedure described with compound **1**. Sodium salt of acid **3** (14.5 mg; 0.058 mmol) was obtained by using a procedure analogous to that used with acid **2**. Reaction of the sodium salt of **3** with the alkylmagnesium bromide of **6** gave acid **7** as a colorless, waxy compound in a 86.1% yield (33.8 mg). LC–MS/APCI: *m/z* 677 $[M+H]^+$; HREIMS *m/z* 676.7100 $C_{46}H_{92}O_2$ $[M]^+$, calculated for $[M]^+$ 676.7097; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (3H, *t*, *J* = 6.8, H-46), 1.22–1.32 (84H, *m*, H-4–H-45), 1.59 (2H, *m*, H-3) and 2.26 (2H, *m*, H-2); ^{13}C NMR

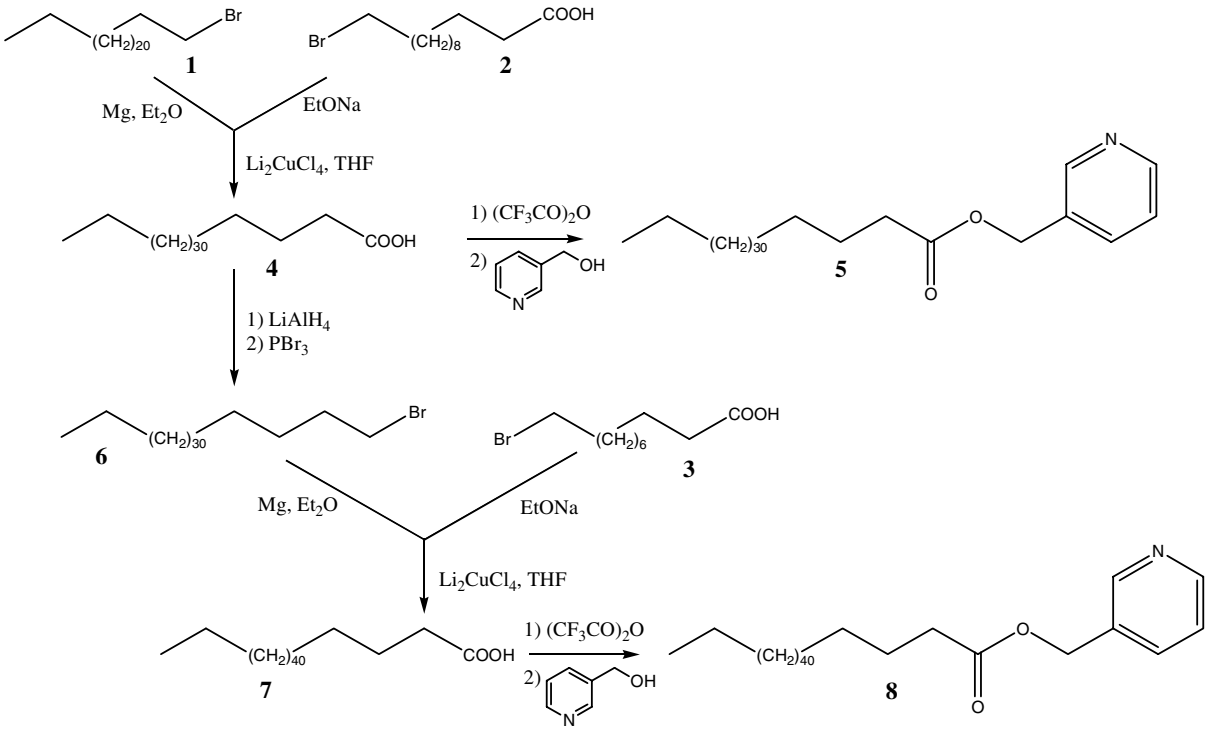

Fig. 4. Scheme of the preparation of hexatriacontanoic and hexatetracontanoic acids (**4**, **7**) and corresponding picolinyl esters (**5**, **8**).

(100 MHz, $CDCl_3$) δ 13.2 (C-46), 19.9 (C-45), 24.7 (C-3), 29.3–29.8 (C-4–C-33), 32.2 (C-44), 34.2 (C-2), 181.0 (C-1).

The synthesis of both picolinyl esters (**5** and **8**) is described below.

5 as colorless, waxy compound, LC–MS/APCI: *m/z* 628 $[M+H]^+$; HREIMS *m/z* 627.5958 $C_{42}H_{77}NO_2$ $[M]^+$, calculated for $[M]^+$ 627.5954; ^1H NMR (400 MHz, $CDCl_3$) δ 0.91 (3H, *t*, $J = 6.8$, H-36), 1.22–1.32 (62H, *m*, H-4–H-35), 1.48 (2H, *m*, H-3), 2.23 (2H, *m*, H-2), 5.10 (2H, *m*, H-7′), 8.40 (1H, *m*, H-2′), 7.15 (1H, *m*, H-5′), 8.37 (1H, *m*, H-6′), 7.50 (1H, *m*, H-4′), ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.2 (C-36), 19.8 (C-35), 24.8 (C-3), 28.9–29.7 (C-4–C-33), 32.0 (C-44), 34.4 (C-2), 174.1 (C-1), 124.4 (C-5′), 135.7 (C-4′), 136.5 (C-3′), 147.4 (C-2′), 147.9 (C-6′), 65.4 (C-7′).

8 as colorless, waxy compound, LC–MS/APCI: *m/z* 768 $[M+H]^+$; HREIMS *m/z* 767.7522 $C_{52}H_{97}NO_2$ $[M]^+$, calculated for $[M]^+$ 767.7519; ^1H NMR (400 MHz, $CDCl_3$) δ 0.91 (3H, *t*, $J = 6.8$, H-46), 1.22–1.32 (84H, *m*, H-4–H-45), 1.48 (2H, *m*, H-3), 2.23 (2H, *m*, H-2), 5.10 (2H, *m*, H-7′), 8.40 (1H, *m*, H-2′), 7.15 (1H, *m*, H-5′), 8.37 (1H, *m*, H-6′), 7.50 (1H, *m*, H-4′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.2 (C-46), 19.8 (C-45), 24.8 (C-3), 28.9–29.7 (C-4–C-43), 32.0 (C-44), 34.4 (C-2), 172.5 (C-1), 124.4 (C-5′), 135.7 (C-4′), 136.5 (C-3′), 147.3 (C-2′), 147.9 (C-6′), 65.4 (C-7′).

3.1.2. Fatty acids from natural source and picolinyl esters

An amount of 10 g of refined sugar cane wax was mixed with 2 g of KOH dissolved in 15 ml of water. The saponification process was maintained for 30 min with periodical stirring. The fatty acid was extracted from the solid obtained in the process using acetone in a solid–liquid extraction system. The ensuing residue was cooled to room temperature, extracted with heptane, the pH was adjusted to approximately 3 by dilute sulfuric acid and another extraction with heptane was performed. The yield of VLCFAs was 2 g with a purity amounting to 94%.

These free fatty acids (2 g) were dissolved in diethyl ether (100 ml) and converted to the mixed anhydride derivatives by reaction with trifluoroacetic anhydride (15 ml) at 50 °C for 2 h. The excess reagent was evaporated, a 10% solution of nicotinyl alcohol in tetrahydrofurane (15 ml) was added and the mixture was left at 50 °C for 2 h. Diethyl ether (100 ml) and hexane (20 ml) were added and the mixture was washed with water (50 ml), 1 M HCl (20 ml, three times) and water (20 ml, three times) and dried by anhydrous sodium sulfate. The solvents were evaporated under reduced pressure and the picolinyl esters were purified by silica gel chromatography with the hexane–diethyl ether (1:1 v/v) mixture as the eluting agent; the yield was 754 mg (Christie et al., 1986).

The sample of 754 mg of derivatized fatty acids (as picolinyl esters) was submitted to preparative chromatography to obtain 11.8 mg of picolinyl derivatives of C_{35}–C_{50} fatty acids. This fraction was then analyzed by LC–MS.

Picolinyl esters (**5** and **8**) were prepared from acids **4** and **7** (see Fig. 4) by the procedure described above.

3.2. Preparative chromatography

A Chromatospac Prep 100 preparative chromatograph (Jobin-Yvon, Longjumeau, France) with axial compression of the chromatographic bed (61.2 × 8 cm I.D.) was used for the isolation of the fraction enriched with C_{35}–C_{50} picolinyl esters. The column efficiency was 6510 theoretical plates, V_0 was 1625 ml and t_R of the picolinyl ester of hexatriacontanoic acid (36:0) was 37.5 min. The mobile phase flow rate was 64 ml/min. The mean particle diameter of the Separon C18 reversed-phase packing was 15 μm (formerly Laboratorní přístroje, Prague, Czechoslovakia). A Holochrome H/MD variable-wavelength detector (Gilson, France) with a 40-μl flow-through cell was set at 235 nm. All experiments were carried out at room temperature.

The amount of 754 mg picolinyl esters (see above) was dissolved in 19 ml of the mobile phase (injection volume) (methanol–isopropanol–triethylamine, 87:13:0.05). In the time interval 0–37 min the column was eluted with mobile phase that was later replaced with diethyl ether (76 min). The column was again conditioned with mobile phase for 14 min. The fraction up to 37.5 min was discarded and that obtained within the interval 37.5–90 min was used for further analysis. After evaporation of the mobile phase, the total yield of a mixture of fatty acids with the chain longer than 34 carbon atoms was 11.8 mg. These were separated and identified by LC–MS.

3.3. LC–MS/APCI

HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett–Packard, USA) and two Hichrom columns HIRPB-250AM 250 × 2.1 mm ID, 5 μm phase particle, in series. This setup provided us with a high-efficiency column – approximately ~53,000-plates/50 cm. A quadruple mass spectrometer system Navigator (Finnigan MAT, San Jose, CA, USA) was used for analysis. The instrument was fitted with an atmospheric pressure chemical ionization source (vaporizer temperature 400 °C), capillary heater temperature 220 °C, corona current 5 μA, sheath gas – high-purity nitrogen, pressure 0.38 MPa, and auxiliary gas (also nitrogen) flow rate 15 ml/min. Positively charged ions with *m/z* 200–900 were scanned with a scan time of 0.5 s. The whole HPLC flow (0.37 ml/min) was introduced into the APCI source without any splitting. Fatty acid picolinyl esters were separated using a gradient solvent program with acetonitrile (ACN), dichloromethane (DCM) and propionitrile (EtCN) as follows: initial ACN/EtCN/DCM (60:30:10, vol/vol/vol); linear from 10 to 40 min ACN/EtCN/DCM (30:40:30, vol/vol/vol); held until 60.5 min; the composition was returned to the initial conditions over 8 min. A peak threshold of 0.3% intensity was applied to the mass spectra. Data acquisition and analyses were performed

using PC with MassLab 2.0 for Windows XP applications/ operating software.

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