

Full Research Paper

## Analysis of Phenacyl Ester Derivatives of Fatty Acids from Human Skin Surface Sebum by Reversed-Phase HPLC: Chromatographic Mobility as a Function of Physico-Chemical Properties

Juliana Bodoprost and Helmut Rosemeyer \*

Organic Materials Chemistry - Bioorganic Chemistry, Institute of Chemistry, University of Osnabrück, Barbarastr. 7, D-49069 Osnabrück, Germany

\* Author to whom correspondence should be addressed; E-mail: [Helmut.Rosemeyer@uos.de](mailto:Helmut.Rosemeyer@uos.de)

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**Abstract:** A set of 13 fatty acids was transformed into their phenacyl esters by reaction with phenacyl bromide in acetonitrile using 18-crown-6 as phase-transfer catalyst. Conditions for the RP-18 HPL chromatographic separation of most of the esters has been worked out. Using this standard the fatty acid spectra from skin surface sebum lipids of 17 test persons was taken after microwave-assisted hydrolysis, neutralization and extraction with n-hexane. Quantitative evaluation of the chromatograms exhibits that oleic acid predominates in the sebum of all test persons. In the second part of the work the chromatographic mobility ( $R_E$  values) of fatty acid phenacyl esters is correlated with calculated physico-chemical parameters of the corresponding acids. The best linear correlation was found between the  $R_E$  and the  $\log P$  values. This is helpful for the structural elucidation of un-identified fatty acids in a chromatogram.

**Keywords:** fatty acid analysis, sebum lipids, RP-18 HPLC,  $\log P$

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### 1. Introduction

Human skin surface lipids consist of triglycerides (41%), wax mono esters (25%), free fatty acids (16%), squalene (12%) and other components (6%) such as cholesterol esters and cholesterol [1,2]. Besides antimicrobial peptides, especially cathelicidin and human  $\beta$ -defensin-2, antimicrobial lipids

from the stratum corneum are major contributors to cutaneous antimicrobial defence. The major classes of stratum corneum lipids with antimicrobial activity are free fatty acids as well as glycosylceramides and free sphingosides [3,4]. For a long time it was an orthodoxy that antimicrobially active fatty acids from the skin surface are only provided by lipase-catalysed hydrolysis of sebum triglycerides produced by the sebaceous glands [5]. Later, it was verified that also the keratinocytes of the epidermis form antimicrobially active lipids causing antimicrobial defence of the skin [6]. Obviously, atopic dermatitis can be traced back to disturbances of antimicrobial lipids. The superantigen staphylococcal enterotoxin B has been shown to reduce the suppressive effect of regulatory T cells on T-cell proliferation, thus augmenting T-cell activation in patients with atopic dermatitis [3]. The killing of superantigen-secreting bacteria with topically applied antimicrobial lipids such as long-chain fatty alcohols offers new antiseptic and immunomodulatory options for the treatment and secondary prevention of atopic dermatitis.

The fraction of free fatty acids of the stratum corneum represents a mixture of non-essential and essential fatty acids which are indispensable for the integrity of the epidermal barrier [7]. Not much is known about the mechanism of antimicrobial action of free fatty acids. In in-vivo experiments [6] on the antimicrobial activity of stratum corneum lipids against *Staphylococcus aureus*, free fatty acids proved to be most effective, particularly linoleic acid ((9Z,12Z)-octadecadienic acid) and the saturated fatty acids with the chain length C12:0 (lauric acid, dodecanoic acid) to C14:0 (myristic acid, tetradecanoic acid). A recent study on the antibacterial activity of long chain fatty alcohols against *Staphylococcus aureus* shows interestingly that dodecanol (C12:0) and tridecanol (C13:0) exhibit the highest antibacterial efficiency but not the highest membrane-damaging activity. Consequently, it appears that not only the antibacterial activity but also the mode of action of fatty alcohols (and fatty acids) might be determined by the length of the aliphatic carbon chain [8].

Skin lipids are unique with respect to their extraordinary complexity and structure. Functionally, this seems to allow each individual to have a distinct odor or chemical fingerprint. The particularity of skin surface sebum fatty acids becomes obvious when one compares the lipids synthesized by the skin with those synthesized by internal tissues: skin makes (i) odd instead of only even numbered carbon chains, (ii) branched (iso and ante-iso) instead of only straight chains, (iii) free instead of only esterified acids, (iv) places double bonds in unusual positions in the fatty acid chains, (v) extends chains to extreme length and (vi) accumulates intermediates in the synthesis of biologically valuable compounds such as cholesterol, farnesol, and squalene. Skin lipids may serve as olfactory messengers in the world of sense. Moreover, they are indicators of certain skin diseases.

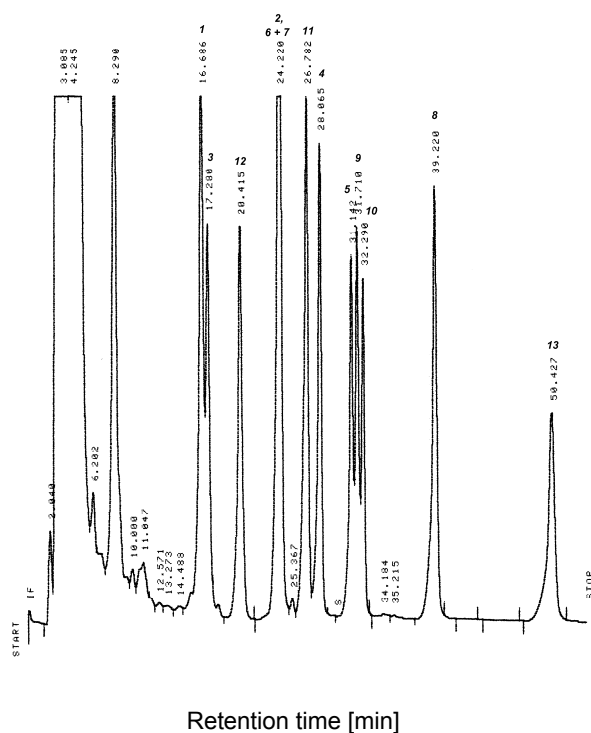
In this manuscript we report on the separation of human sebum fatty acids (Formula scheme, experimental section) of 17 test persons in form of their phenacyl esters by reversed phase high performance liquid chromatography (RP-18 HPLC) and their UV-spectrophotometric detection. Moreover, the HPL-chromatographic mobility of the various fatty acid phenacyl esters is correlated with various calculated physico-chemical properties of the parent fatty acids in order to predict the mobility of unknown congeners which offers the possibility for a limitation of their chemical structures.

## 2. Results and Discussion

### 2.1 The Fatty Acid Spectrum of Human Sebum Lipids

For a calibration an artificial mixture of phenacyl esters of the fatty acids **1** – **13** was prepared and injected onto the RP-18 HPLC column (Figure 1); furthermore, all individual phenacyl esters were chromatographed. A comparison of the results show that the  $R_E$  values of the single fatty acid esters do not deviate more than 0.1 – 1 min from those obtained from the chromatogram of the mixture. The esters of the acids **2**, **6**, and **7** could not be separated sufficiently by applying the gradient described in the experimental part. Generally, it was observed that (i) the retention time increases with increasing carbon chain length, (ii) a higher number of C=C double bonds reduces the  $R_E$  values of the phenacyl esters, and (iii) (E)-unsaturated compounds exhibit an intermediary mobility between the corresponding saturated derivatives and the corresponding (Z)-unsaturated esters.

**Figure 1.** HPLC-Profile of an artificial fatty acid phenacyl ester mixture.  
For the individual retention times see Table 3.



The chromatograms of all test persons can be found as complementary material from the correspondence author or from the editorial office. A critical inspection of the results with respect of the chromatographic separation shows that in all 17 chromatograms 9 of 13 fatty acid derivatives could be clearly identified. Table 1 shows the complete fatty acid spectra of the 17 test person [14-22]. The amounts of sebum fatty acids are given in arbitrary units by integrating the peaks. In row 3 the sum of the fatty acids **2**, **6**, and **7** is given. Table 2 presents for the test persons 1 and 9 – 17 the amounts of fatty acids **2** and **6 + 7** separately because in these cases a separation was at least partially possible.

**Table 1.** Fatty Acid Spectra of 17 Test Persons.

Sample	1	2+6+7	3	5	8	9	12	13
1	4.23	42.18*	3.81	17.76	9.01	20.43	2.59	-
2	2.08	48.55	6.38	5.21	7.91	26.52	3.34	-
3	-	51.76	8.40	7.27	5.48	23.23	3.86	-
4	-	48.93	9.41	11.42	3.88	21.39	4.97	-
5	2.47	39.97	4.19	15.01	7.78	26.43	4.15	-
6	-	51.37	9.13	10.25	3.88	19.97	5.41	-
7	-	54.90	9.87	9.13	3.61	17.10	5.39	-
8	-	51.28	6.77	5.99	4.39	25.57	6.00	-
9	-	42.09*	9.02	17.52	3.58	23.88	3.92	-
10	-	42.58*	10.95	19.28	3.65	19.28	4.25	-
11	1.72	40.97*	8.17	13.77	4.02	27.54	3.82	-
12	2.03	46.24*	3.89	11.12	10.41	15.53	1.79	8.98
13	-	44.40*	6.76	10.48	6.17	14.20	3.23	14.77
14	3.76	34.00*	3.48	11.85	11.41	34.11	1.41	-
15	7.98	11.60*	20.87	28.31**	26.18	-	5.06	-
16	8.39	20.79*	4.64	22.38	14.81	20.59	5.30	3.10
17	24.60	23.35*	2.19	6.79	16.17	25.69	1.22	-

\*: the partially separated peak integrals are listed in Table 2

\*\* : this value represents the sum of the fatty acids **5** and **9**, which could not be separated in this experiment.

A comparison of the data, but without statistical analysis, shows some striking features: (i) the two unsaturated fatty acids lauric acid (**1**) and arachidic acid (**13**) appear only in a few samples and there only in low concentrations; arachidic acids can be identified only in comedons. (ii) Lauric acid could be detected preferentially on the skin of older test persons (> 50 years). (iii) The fatty acids **2 + 6 + 7** appear mainly in the sebum samples of young test persons (< 30 years); here, the concentrations of these fatty acids is 1.5 to 2-times higher than in older test persons. From Table 2 it can be seen that among young test persons the sum of the fatty acids **7 + 6** (sapienic acid and palmitoleic acid) is double as high as the concentration of myristic acid (**2**). (iv) Oleic acid (**9**) dominates in all cases, followed by **7 + 6**.

## 2.2 The Chromatographic Mobility of Fatty Acid Phenacyl Esters as a Function of Physico-chemical Properties of the Parent Fatty Acids

The isolation of an unknown lipid component (e.g. from a sebum sample) is indispensable for its structural elucidation. The latter is usually performed by a combination of mass spectrometry and high resolution NMR spectroscopy. Using these methods detailed information concerning the position and

geometry of double bonds as well as ramification points can be obtained. Especially problematic for the structural analysis of fatty acids is their variety of isomers which often cannot be separated sufficiently. Therefore, each chemical and physico-chemical information is highly valuable in order to confine the number of conceivable structures prior to a mass- or NMR spectrometric analysis.

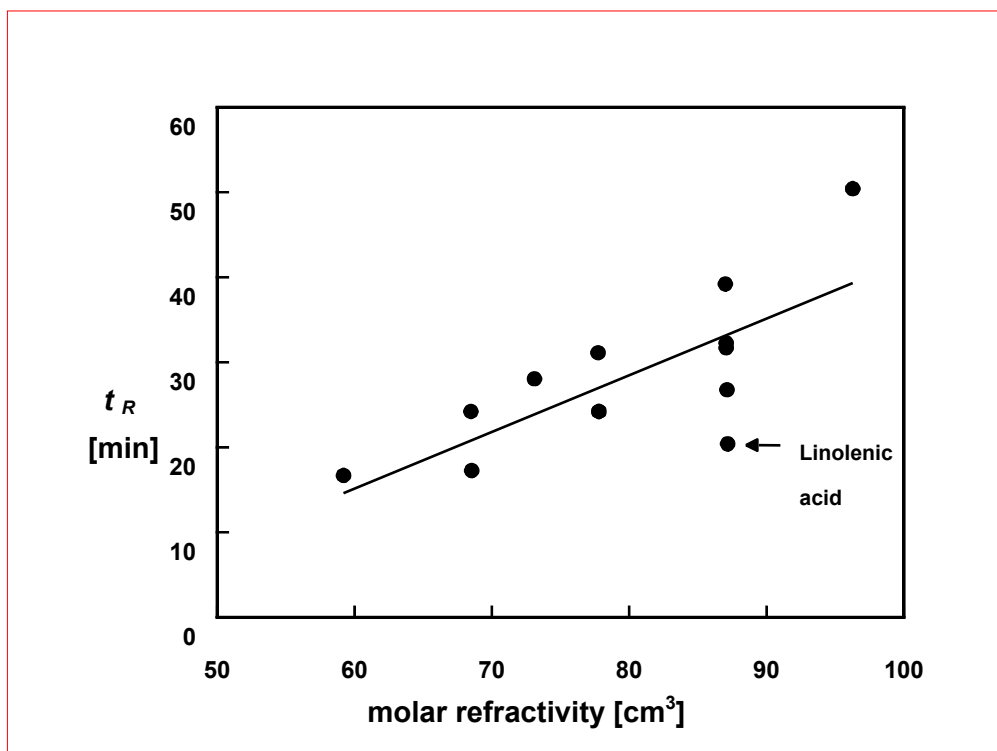
Natural fatty acids are built up biosynthetically by successive addition of C-2 or C-3 carbon units to the growing lipid chain. The so obtained fatty acids exhibit a more or less different mobility in RP-18 HPLC. This implies that certain additive and constitutive properties of the fatty acids may be comprised by increments and can be calculated within limits. These calculable properties may be empirically correlated with the corresponding HPLC retention times of appropriate fatty acid derivatives such as phenacyl esters. Vice versa it should be possible to reason from the chromatographic mobility of an unknown fatty acid ester on calculated additive and constitutive parameters and to limit its constitution and configuration. (e.g. the (Z)- and (E)-configuration at a C=C double bond).

**Table 2.** Spectra of the Fatty acids **2** and **7 + 6** Taken From 10 Test Persons.

Sample	<b>2</b>	<b>7 + 6</b>
1	12.80	29.38
9	9.75	32.34
10	8.52	34.06
11	11.87	29.10
12	10.44	35.80
13	9.82	34.58
14	11.85	22.15
15	5.80	5.80
16	7.48	13.31
17	8.04	15.31

In the following we demonstrate that between the  $R_E$  values of the fatty acid phenacyl esters studied and (i) the molar refractivity, (ii) the molar volume, (iii) the parachor, (iv) the density, (v) the polarizability, and (vi) the  $\log P$  value fairly good linear correlations exist (Figures 2a-f). The molecular parameters were calculated from the corresponding increments (central atom and neighboring sphere considering the order and aromaticity of bonds) using the program suite ChemSketch (version 8.0, provided by Advanced Chemistry Developments Inc., Toronto, Canada; <http://www.acdlabs.com>). The best correlation ( $r = 0.948$ ) was found between the  $R_E$  and the  $\log P$  values (Figure 2f). Figures 2a-f show RP-18 HPLC retention times of fatty acid phenacyl esters as a function of their physico-chemical properties.

**Figure 2a.** RP-18 HPLC Retention times of fatty acid phenacyl esters as a function of their molar refractivity.



**Figure 2b.** RP-18 HPLC Retention times of fatty acid phenacyl esters as a function of their molar volume.

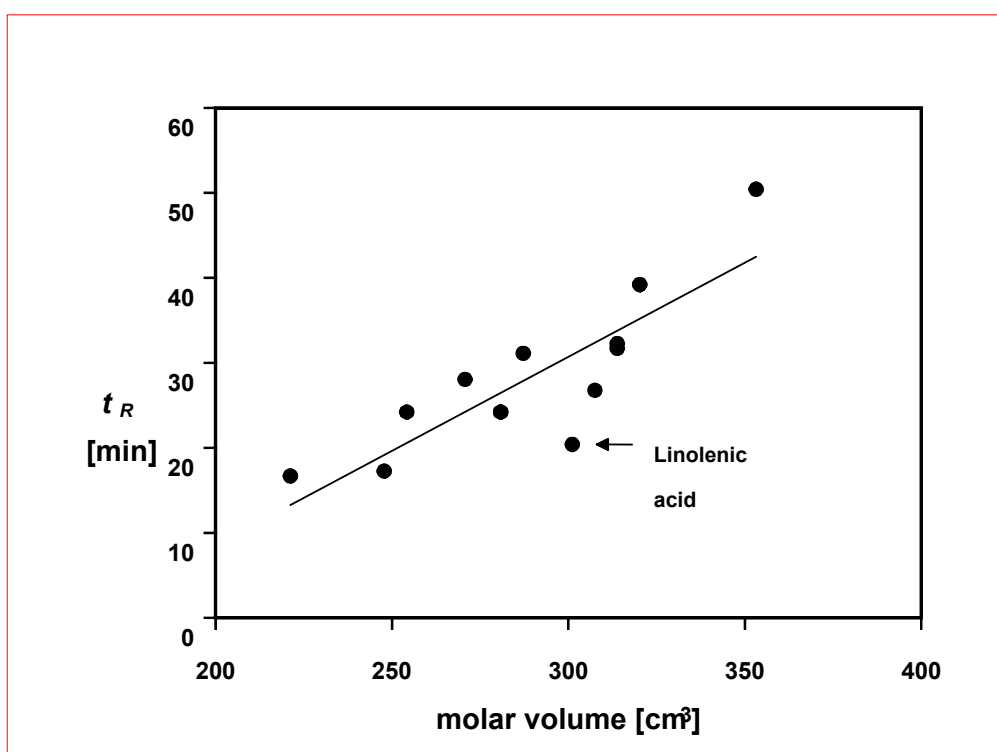


Figure 2c. RP-18 HPLC Retention times of fatty acid phenacyl esters as a function of their parachor.

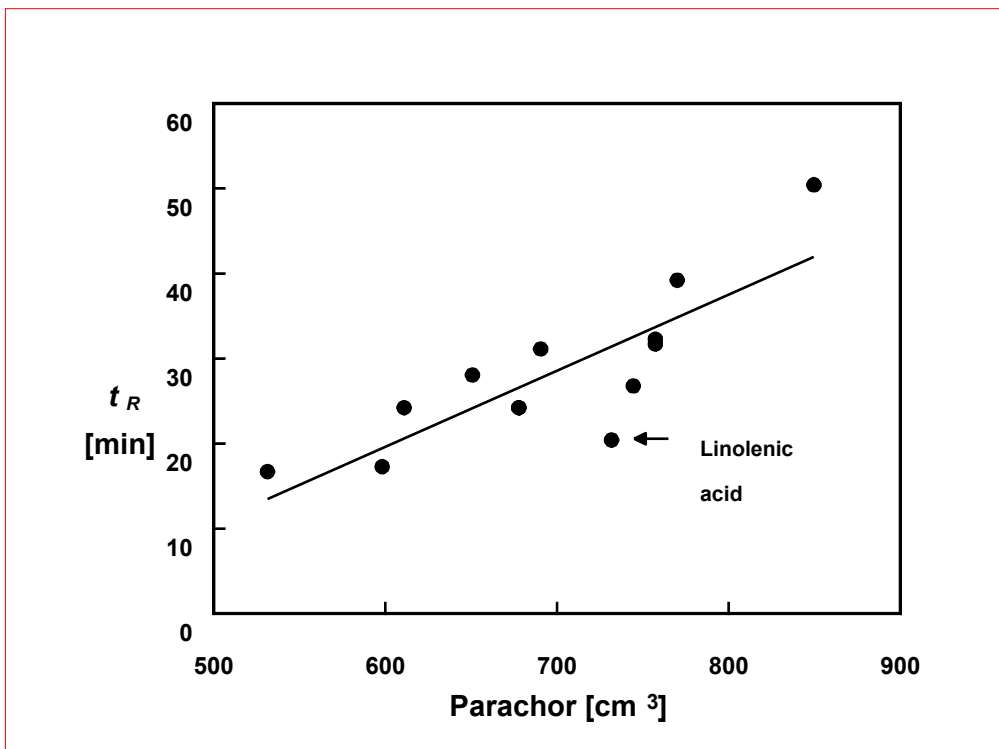
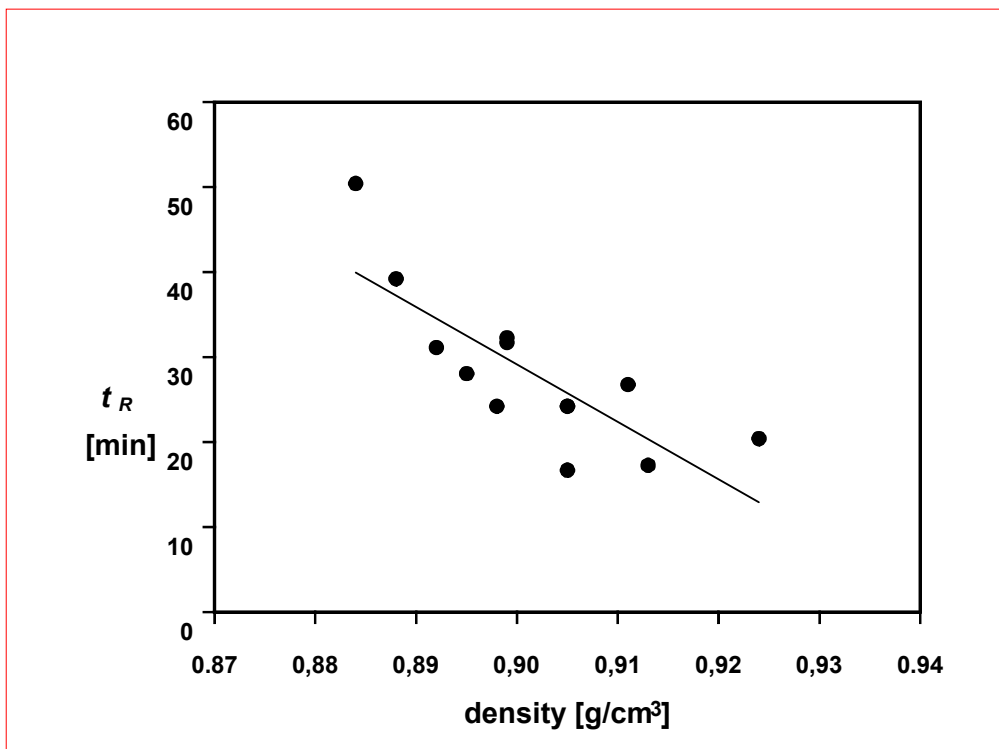
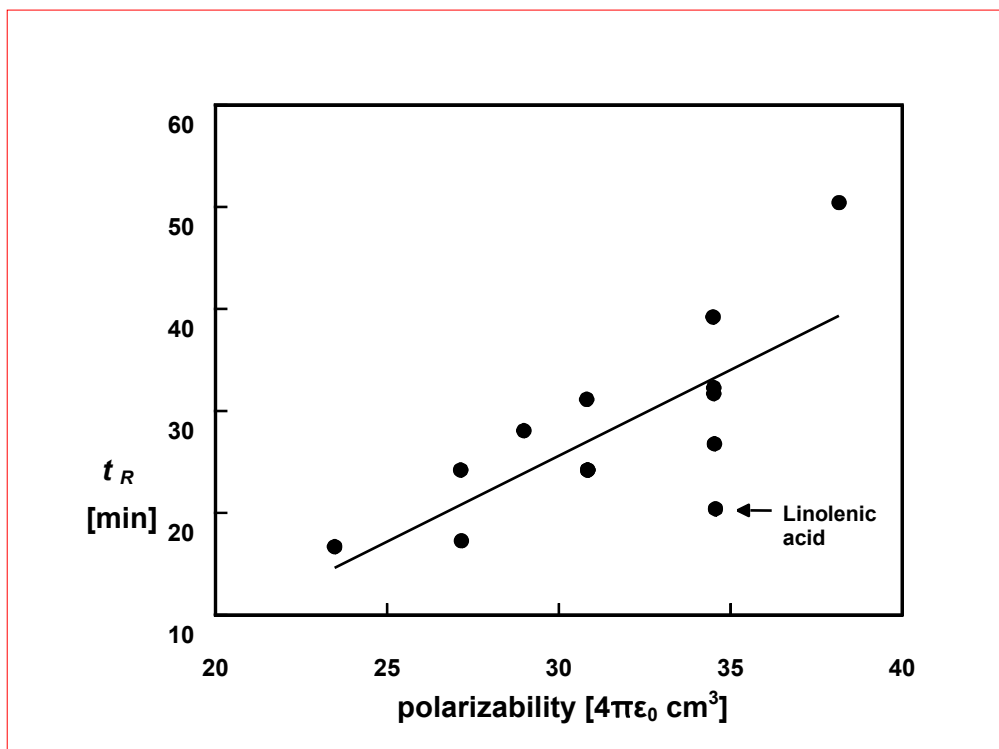


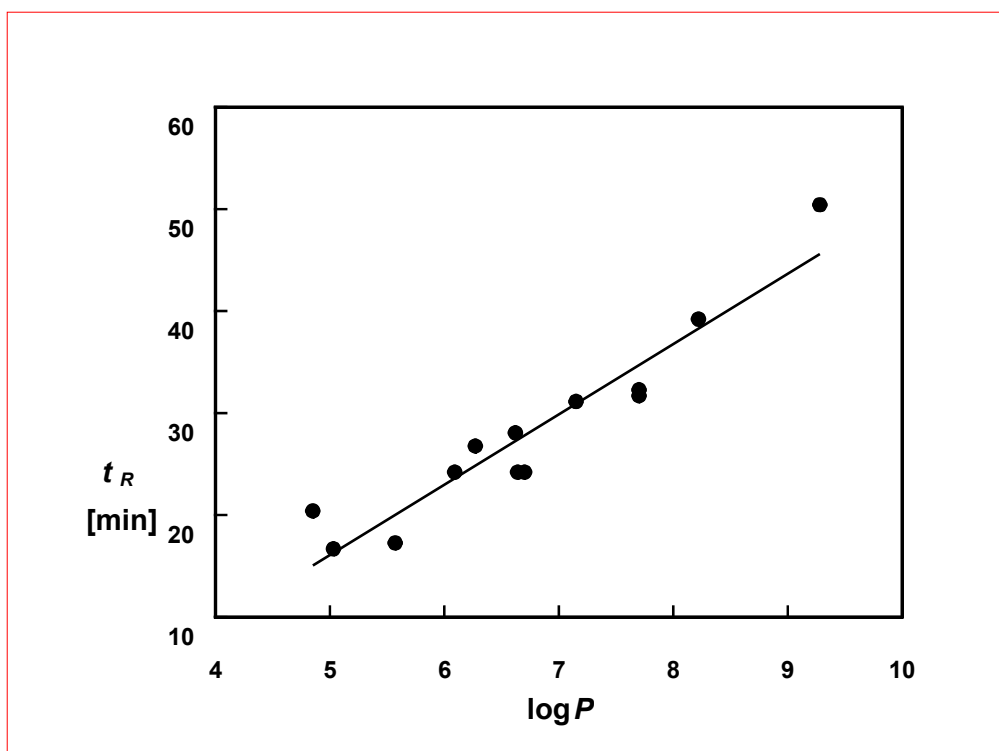
Figure 2d. RP-18 HPLC Retention times of fatty acid phenacyl esters as a function of their density.



**Figure 2e.** RP-18 HPLC Retention times of fatty acid phenacyl esters as a function of their polarizability.



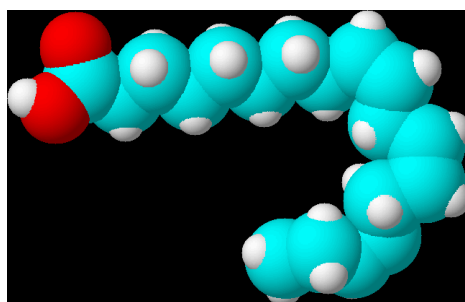
**Figure 2f.** RP-18 HPLC Retention times of fatty acid phenacyl esters as a function of their  $\log P$  values.





In all other cases the values for linolenic acid deviate significantly from the linear relationships. This might be traced back to its rigid, hook-like structure (Figure 3) being different from all other fatty acids studied and which might hamper a “snaking-in” and binding to the stationary reversed phase of the adsorbent. This leads obviously to a higher chromatographic mobility as expected.

**Figure 3.** 3D-Optimized structure of linolenic acid.



Using the interrelation between the chromatographic mobilities ( $R_E$ ) of fatty acid phenacyl esters and calculated physico-chemical parameters such as  $\log P$  and others a structural limitation of a non-identified fatty acid from natural sources should be possible.

### 3. Experimental Section

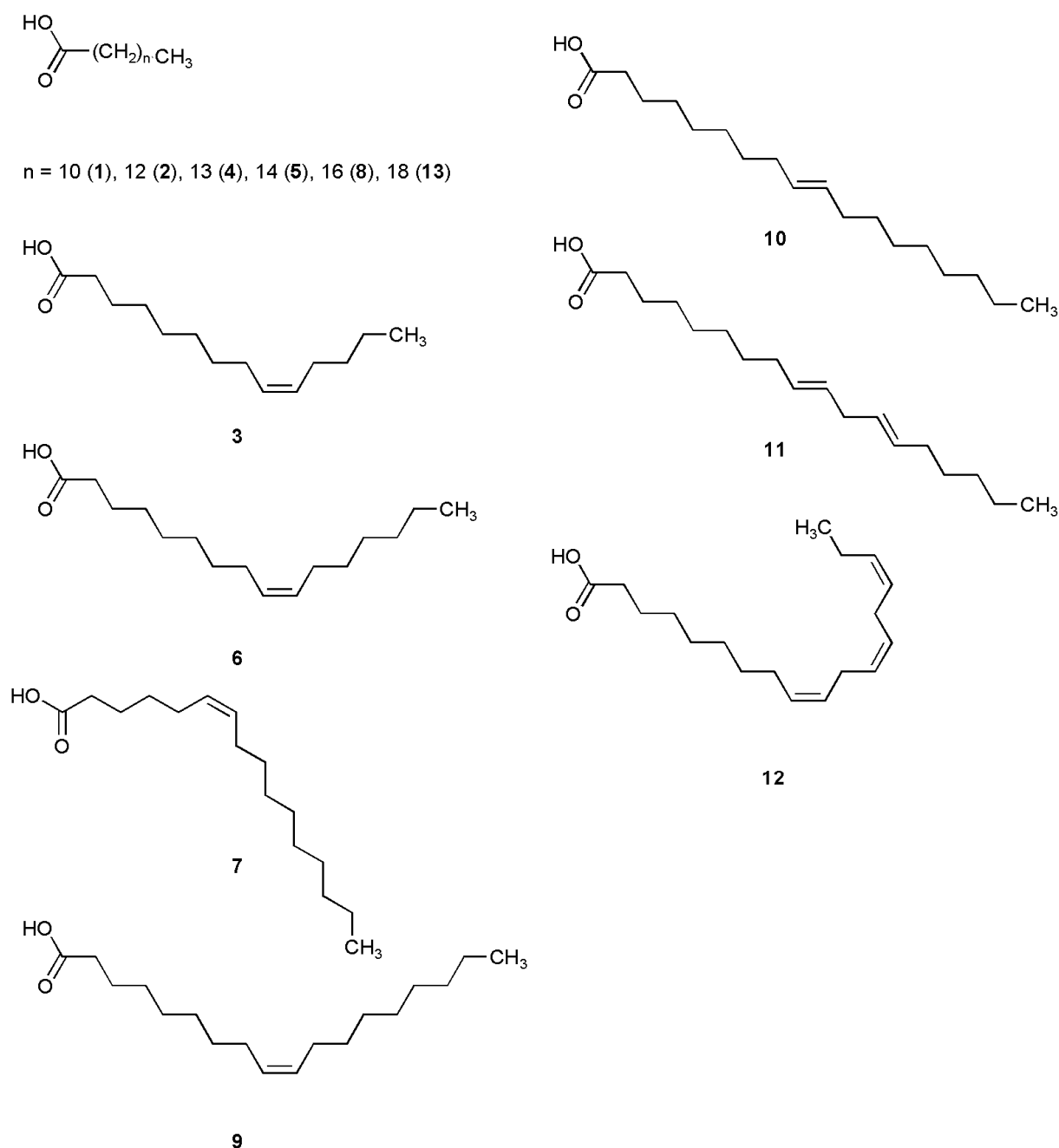
#### 3.1 Equipments and Reagents

HPLC Separation: HPLC was carried out on a 250 x 5 mm RP-18 column, LiChrospher 100, 5  $\mu\text{m}$  (Merck, Darmstadt, Germany) using an HPLC apparatus consisting of a gradient pump (Pharmacia LKB, model 2249, Pharmacia, Sweden), a Rheodyne injection system (Rheodyne, California, USA), a UV-VIS detector (Hewlett Packard, HP 3090) and an integrator (Hewlett Packard, HP 3395). Each chromatographic run was performed using the following solvent systems and the following gradient: A: 0.1 % (v/v) trifluoroacetic acid in bi-distilled  $\text{H}_2\text{O}$ ; B: 0.1 % (v/v) trifluoroacetic acid in acetonitrile. Gradient: 0 min: 80 % B in A; 0-30 min: 80 – 100 % B in A; 30 – 50 min: 100 % B; 50-55 min: 100-80 % B in A. Detector wavelength: 254 nm. Flow rate: 1 ml/min. Prior to each sample injection the column was equilibrated by purging (20 min) with 80 % B in A. Reagents: All solvents and reagents were of analytical grade and were purchased from Merck (Merck, Darmstadt, Germany) or Riedel de-Haen (Riedel de-Haen, Seelze, Germany). Acetonitril (MeCN, Multisolvent, p.A.) was purchased from Scharlau Chemie (Scharlau Chemie S.A., La Jota, Barcelona, Spain). For the derivatization of the fatty acid samples the following pre-prepared reagent was used: Phenacyl-8 (= 0.1 mmol/ml phenacyl bromide, 0.005 mmol/ml 18-crown-6 in MeCN (Pierce, Rockford, Illinois, USA). Table 3 lists the various fatty acids used for standardization as well as their chromatographic mobilities ( $R_E$ ) under the conditions as described above. Compounds **1** – **3**, **5** and **10** – **13** were purchased from Applied Science Laboratories (State College, Penna, USA), compounds **4**, **6**, and **9** from Sigma-Aldrich (Germany), compound **8** from Fluka (Fluka, Buchs, Switzerland); all were of analytical grade (p.A). Compound **7** was obtained from a  $\text{CO}_2$  extract from an oil raffinate.

## 3.2 Preparation and Derivatization of Fatty Acid Standards

The fatty acids (10  $\mu$ l, each, of 10 mM solutions in MeOH) were mixed with 10  $\mu$ l of an aqueous  $\text{KHCO}_3$  solution (40 mM). After mixing with a Vortex mixer the solutions were dried overnight in a desiccator under vacuo. Subsequently, each fatty acid was mixed with 20  $\mu$ l of Phenacyl-8 (= 0.1 mmol/ml phenacyl bromide, 0.005 mmol/ml 18-crown-6 as phase transfer catalyst) in MeCN and agitated for 30 min at 80°C in a thermomixer (Eppendorf, model 5436) [9]. The samples (5  $\mu$ l, each) were cooled to room temp., and mixed with a solution (55  $\mu$ l) consisting of 0.1 M trifluoroacetic acid (2 ml) and MeCN (8 ml). The resulting solution (60  $\mu$ l, each) were directly injected into the HPLC [10].

## Formula scheme



**Table 3.** Formula Numbers, Trivial Names, Systematic Names and Chromatographic Mobility ( $R_E$  value) of Phenacyl Esters of Fatty Acid Standards.

Formula number, trivial name, systematic name	$R_E$ [min]
1, lauric acid, dodecanoic acid	16.7
2, myristic acid, tetradecanoic acid	24.22
3, myristoleic acid, $\Delta^9$ -tetradecenoic acid	17.28
4, pentadecanoic acid	28.07
5, palmitic acid, hexadecanoic acid	31.14
6, palmitoleic acid, (Z)- $\Delta^9$ -hexadecenoic acid	24.22
7, sapienic acid, (Z)- $\Delta^6$ -hexadecenoic acid	24.22
8, stearic acid, octadecanoic acid	39.22
9, oleic acid, (Z)- $\Delta^9$ -octadecenoic acid	31.71
10, elaidic acid, (E)- $\Delta^9$ -octadecenoic acid	32.29
11, linolelaidic acid, (9E,12E)- $\Delta^9,\Delta^{12}$ -octadecadienoic acid	26.78
12, linolenic acid, (9Z,12Z,15Z)- $\Delta^9,\Delta^{12},\Delta^{15}$ -octadecatrienoic acid	20.42
13, arachidic acid, eicosanoic acid	50.43

### 3.3 Physico-chemical Properties of Fatty Acids

The following molecular parameters [11] were calculated from the corresponding increments (central atom and neighboring sphere considering the order and aromaticity of bonds) using the program suite ChemSketch (version 8.0, provided by Advanced Chemistry Developments Inc., Toronto, Canada; <http://www.acdlabs.com>): (i) molar refractivity, (ii) molar volume, (iii) parachor, (iv) density, (v) polarizability, and (vi)  $\log P$  value. The parameters are defined as follows: molar refractivity (unit,  $\text{cm}^3$ )  $R_M = [n^2 - 1/n^2 + 2] M/\rho$  ( $n$ : refraction index at 589 nm;  $M$ : molecular weight;  $\rho$ : density); molar volume of an organic compound (unit  $\text{cm}^3$ )  $V_s$  (measured at the boiling point at 1 atm) is calculated additive from the volumes of the elements and constitutive contributions depending on the kind of bonding of these elements; the parachor [12] (unit  $\text{cm}^3$ )  $P = \sigma^{1/4} M/(\rho_l - \rho_g)$  ( $\sigma$ , surface tension;  $\rho_l$ , density in liquid state;  $\rho_g$ , density in gaseous state) considers upon calculation of the molar volume that the latter is smaller than the calculated value due to the surface tension. The molecular polarizability  $\alpha_m$  (unit,  $4\pi\epsilon_0 \text{cm}^3$ ) represents a measure for the induction of a dipole moment.  $\log P$  (dimensionless) is the partition quotient of a compound between octan-1-ol and water [13].

### 3.4 Collection of Human Skin Surface Sebum Samples

For verification of the method established 17 skin lipid samples were collected from test persons, 21 to 65 years in age within a period of 1 week. Table 4 summarizes age, sex, collection area, and the amounts of totally isolated skin lipids. None of the test persons suffered from obvious skin diseases.

However, the volunteers were not seen by a dermatologist. None of them had used any skin cosmetics or crèmes 24 h before sample collection. No washing procedures with syndets, soap or even water had been applied; in the case of the entrance 15 the sebum was retrieved from the comedon by squeezing.

**Table 4.** Amounts of Collected Lipids from 17 Test Persons.

No. of sample	Age (y)	Sex (m or f)	Collection area	Total amount of lipid [mg]
1	24	F	Forehead, nose	3.1
2	22	F	Forehead, nose	3.1
3	24	M	Forehead, nose	4.6
4	23	M	Forehead, nose	8.6
5	21	F	Forehead, nose	5.7
6	27	M	Forehead, nose	13.1
7	25	F	Forehead, nose	4.5
8	25	M	Forehead, nose	6.2
9	23	F	forehead	7.1
10	26	M	forehead	3.8
11	50	M	forehead	5.8
12	63	M	comedon (back)	6.7
13	63	M	Nose, left wing	2.5
14	63	M	Nose, right wing	3.7
15	63	M	Comedon (cheek)	1.2
16	65	F	Nose	1.7
17	65	F	Sternum	3.4

Samples were collected in the morning after sebum secretion overnight using fat-free blotting paper strips (size: 1 x 5 cm) which were wiped three times over the collection area [14]. In case of dry skin the strip was soaked in ethanol prior to wiping. A dry skin – as defined here – showed a slight form of seborrhea but no atopic eczema. For the various factors which affect skin sebum secretion and skin greasiness see [14] After the smear test the skin lipids were washed from the paper strips into 25 ml flasks using 5 ml of acetone, each, in several portions. The solvent was evaporated on a water bath (ca. 35°C). The residues were again dissolved in few drops of acetone, transferred into Sarstedt vials (Sarstedt, Nümbrecht, Germany) and again lyophilized to dryness in a SpeedVac concentrator (Savant).

### 3.5 Preparation of the Samples Ready for Use in RP-18 HPLC

For the hydrolysis of triglycerides to the corresponding sebum samples (1-17) were added 0.5 M ethanolic KOH (0.5 ml) and ethanol (0.5 ml). The solutions were irradiated in a microwave oven (350 W) for 2 min and then stored overnight at room temperature [15]. Then, the samples were neutralized by addition of 1N aq. HCl (ca. 0.2 ml) and diluted by addition of H<sub>2</sub>O. From these solutions the fatty acids were extracted with n-hexane (2 ml). The organic layers were separated, evaporated and transferred into the phenacyl esters as described above for the fatty acid standards. The hydrolysis of the triglycerides was monitored by qualitative TLC (silica gel 60 F 254 plates, Merck) using a solvent system consisting of CH<sub>2</sub>Cl<sub>2</sub> (9.6 ml), acetone (0.4 ml), glacial acetic acid (0.05 ml). As reference compounds were used triolein (10 mg in 1 ml of n-hexane) and oleic acid (10 mM in MeOH).

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