Jinmao You[a, b]
Fang Zhu[a]
Wenchen Zhao[a]
Xian'en Zhao[b]
Yourui Suo[b]
Sujuan Liu[a]

[a] College of Chemistry Science, Qufu Normal University, Qufu, P.R. China
[b] Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining, P.R. China

Analysis of saturated free fatty acids from pollen by HPLC with fluorescence detection

A sensitive method for the determination of free fatty acids using 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl) ethyl-*p*-toluenesulfonate (ANITS) as tagging reagent with fluorescence detection has been developed. ANITS could easily and quickly label fatty acids in the presence of the K_2CO_3 catalyst at 90 °C for 40 min in *N*,*N*-dimethylformamide solvent. From the extracts of rape bee pollen samples, 20 free fatty acids were sensitively determined. Fatty acid derivatives were separated on a reversed-phase Eclipse XDB-C_8 column by HPLC in conjunction with gradient elution. The corresponding derivatives were identified by post-column APCI/MS in positive-ion detection mode. ANITS-fatty acid derivatives gave an intense molecular ion peak at *m/z* $[M+H]^+$; with MS/MS analysis, the collision-induced dissociation spectra of *m/z* $[M+H]^+$ produced the specific fragment ions at *m/z* $[M-345]^+$ and *m/z* 345.0 (here, *m/z* 345 is the core structural moiety of the ANITS molecule). The fluorescence excitation and emission wavelengths of the derivatives were $\lambda_{ex} = 250$ nm and $\lambda_{em} = 512$ nm, respectively. Linear correlation coefficients for all fatty acid derivatives are >0.9999. Detection limits, at a signal-to-noise ratio of 3 : 1, are 24.76–98.79 fmol for the labeled fatty acids.

Keywords: Rape bee pollen, fatty acids, 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl) ethyl-*p*-toluenesulfonate (ANITS), HPLC, fluorescence detection.

1 Introduction

Fatty acids are widely distributed in nature and important as nutritional substances and metabolites in living organisms. Many kinds of fatty acids play an important role in trace levels in the regulation of a variety of physiological and biological functions. The investigation of the composition of the fatty acids in rape bee pollen is of equal importance. Most fatty acids show neither natural absorption in the visible or UV regions nor fluorescence; thus, the detection of them at trace levels using absorptiometry is fairly difficult [1]. Therefore, derivatization of these analytes with labeling reagents has been widely adopted, since HPLC with UV or fluorescence detection has a higher sensitivity. The easily detectable fatty acid derivatives after methyl esterification, with GC or GC/MS, have been reported [2–4]. In contrast, with GC, use of HPLC allows the fatty acids to be converted to a large number of different derivatives. Derivatization can overcome some problems, such as tailing peaks and low detector sensitivity, by the formation of less polar compounds which can be more easily analyzed by LC. Therefore, derivatization of these analytes with labeling reagents, especially for the sensitive fluorescent detection, has been widely adopted. The reagents commonly used include coumarin-type derivatives [5–12]; diazomethane-type reagents such as 9-anthryldiazomethane (ADAM) [13, 14] and 1-pyrenyldiazomethane (PDAM) [15]; quinoxalinone derivatives [16–19]; benzofurazan-type reagents [20–22]; sulfonate ester reagents such as 2-(2-naphthoxy)-ethyl-2-(piperidino)-ethanesulfonate (NOEPES) [23], 2-(2,3-naphthalimido)-ethyl trifluoromethanesulfonate (NE-OTF) [24] and 2-(2,3-anthracenedicarboximido) ethyl trifluoromethanesulfonate (AE-OTF) [25]; benzohydrazide-type reagents such as 4-(1-methylphenanthro [9,10-*d*]imidazole-2-yl)benzohydrazide (MPIB-hydrazide) [26] and 4-(5,6-dimethoxy-2-benzimidazoyl)-benzohydrazide) (DMBI-hydrazide) [27], and so on. However, it has been reported that these reagents have limitations in their applications, such as short detection wavelengths, poor stability, low detection sensitivity, tedious analytical procedures, and serious interferences in the determination of biological samples [28].

The aims of the present work are: (1) to develop a new labeling reagent, 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl) ethyl-*p*-toluenesulfonate (ANITS), which allows the use of ■ pls check■ *N*,*N*-dimethylformamide (DMF) replacing crown ether and benzene or toluene as solvents to label fatty acids; (2) to develop a fast and simple technique for high extraction efficiency of fatty

Correspondence: You Jinmao, College of Chemistry Science, Qufu Normal University, Qufu, Shandong 273165, P.R. China. Phone: ■, Fax: +86 537 4456305, e-mail: jmyou6304@163.com

Research Paper

acids in rape bee pollen; different extraction methods such as ultrasound-assisted solvent extraction, distillation extraction and shaking extraction are tested. The optimal derivatization and extraction conditions are evaluated. HPLC separation coupled with atmospheric pressure chemical ionization (APCI)/MS identification for fatty acid derivatives is accomplished in combination with gradient elution on a reversed-phase Eclipse XDB-C_8 column. Linearity, detection limits and precision of the procedure are also determined. The proposed method can easily be applied to the determination of free fatty acids in bee pollen samples, with satisfactory results.

2 Materials and methods

2.1 HPLC instrumentation and conditions

The HPLC system used was from the Agilent HP 1100 series (Waldbronn, Germany) and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a fluorescence detector (FLD) (model G1321A). The mass spectrometer 1100 Series LC/MSD Trap-SL (ion trap) from Bruker Daltonik (Bremen, Germany) was equipped with an APCI source. The HPLC system was controlled by HP Chemstation software. The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1. Ion source conditions: APCI in positive-ion detection mode; nebulizer pressure, 60 psi; dry gas temperature, 350 °C; dry gas flow, 5.0 L/min; APCI Vap temperature, 350 °C; corona current, 4000 nA (pos); capillary voltage, 3500 V. Derivatives were separated on a reversed-phase Eclipse XDB-C_8 column (150 × 4.6 mm i.d, 5 μm; Agilent) by gradient elution. Eluent A was aqueous acetonitrile containing 30 mM ammonium formic acid (50%, vol/vol, pH 3.5); B was acetonitrile (100%). Before injection of the next sample, the column was equilibrated with eluent 95% A + 5% B. A linear 45-min gradient program was used: initial = 95% A + 5% B; 20 min = 30% A + 70% B; 45 min = 100% B (kept for 5 min). The flow rate was constant at 1.0 mL/min and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were set at λ_{ex} = 250 nm and λ_{em} = 512 nm, respectively. Fluorescence excitation and emission spectra of derivatives were obtained on a 650-10 S fluorescence spectrophotometer (Hitachi, Seisakusho, Tokyo, Japan). A Paratherm U_2 electronic water bath (Hitachi, Tokyo, Japan) was used to control the temperature for fluorescence scanning. Excitation and emission bandpass were both set at 15 nm. The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfield, IL, USA). Chromatographic peaks were identified by spiking the working standard with each individual fatty acid in turn and simultaneously confirmed by post-column mass spectrometry.

2.2 Chemicals

Saturated fatty acids (C_1–C_{20}) used as standards were of chromatographic grade and purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Spectroscopically pure acetonitrile was purchased from Germany (Merck, KGAa). DMF and dimethyl-sulfoxide (DMSO) were purchased from Jining Chemical Reagent Co. (Shandong, Jining, China) and treated with 5-Å molecular sieve, and then redistilled prior to use. Benzene, toluene, tetrahydrofuran (THF), methanol, potassium carbonate (K_2CO_3), pyridine and chloroform were of analytical grade and obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were also of analytical grade unless otherwise stated. Rape bee pollen samples were obtained from Northwest Plateau Institute of Biology, Chinese Academy of Sciences (Qinghai, Xining, China).

2.3 Synthesis of the labeling reagent ANITS

2.3.1 Synthesis of 2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazole

2-(Anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazole was synthesized as follows: 2,3-Diaminonaphthalene (5.3 g) and ethanol (200 mL) were fully mixed in a 500-mL round-bottom flask. A mixture of 9-anthraidehyde (7.2 g) and $NaHSO_3$ (3.7 g) in 80 mL ethanol was added dropwise with vigorous stirring. The contents were heated to reflux for 4 h with vigorous stirring. After cooling, the solution was concentrated using a rotary evaporator. The residue was poured into 500 mL water with vigorous stirring. The precipitated solid was recovered by filtration, washed with water and dried at room temperature for 48 h. The crude product was recrystallized twice from methanol/DMF mixed solvent (5 : 1, vol/vol) to afford yellow crystals; yield 89.4%.

2.3.2 Synthesis of 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl) ethanol

2-(Anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazole (6.0 g), ethylene carbonate (1.85 g), and KOH (0.2 g) were dissolved together in 80 mL DMF in a 250-mL round-bottom flask and rapidly heated to reflux for 2.5 h with vigorous stirring. After cooling, the contents were poured into 300 mL water with vigorous stirring. The precipitated solid

VCH Auftr.-Nr. 33663 Vers.: 6.06
S:/3d/vch/_EJLST/ej_07_03/fl_0224.3d
EJLST
1. Papierlauf Seite 2 – hg –
Mittwoch, den 14. 02. 07, 11 Uhr 22

was recovered by filtration, washed successively with water. The crude product was dried at room temperature and recrystallized twice from acetonitrile/DMF mixed solvent (5 : 1, vol/vol) to afford yellow crystals; yield 90.3%. m.p. >300 °C. Found, C 83.47, H 5.20, N 7.21; Calculated, C 83.48, H 5.19, N 7.21; IR (KBr): 3051.54 (-OH); 1519.28, 1452.29 (ph); 1402.85, 1350.58 (C-H); 1325.24, 1259.23, 1064.63 (C-O); 971.56, 845.71, 727.61, 605.87. MS: *m/z* 389.2 $[M+H]^+$.

2.3.3 Preparation of ANITS

To a solution of 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl) ethanol (2.72 g) in 50 mL pyridine (0 °C) in a 100-mL round-bottom flask, *p*-toluenesulfonyl chloride (5.34 g) was added in batches with vigorous stirring. After stirring at 0 °C for 6 h, the contents were kept at ambient temperature for another 4 h with stirring. The contents were transferred into ice water with vigorous stirring for 0.5 h; the precipitated solid was filtrated, washed with water and dried at ambient temperature for 48 h. The crude product was recrystallized twice from acetonitrile to give 2.73 g of slight yellow crystals; yield 71.8%. m.p. 207.6–209.6 °C. Found, C 75.24, H 4.82, N 5.16, S 5.90; Calculated, C 75.25, H 4.83, N 5.16, S 5.91; IR (KBr): 2976.62 (ph), 1658.17, 1630.94, 1619.52, 1595.77 (C=NR), 1526.01, 1445.29, 1433.55, 1400.23 (C-H), 1357.33 (-C-SO_2-), 1338.16, 1315.29, 1176.40 (ph-S-), 1092.69, 1047.67, 1013.95, 901.14, 858.93, 763.16, 742.82, 691.90. MS: *m/z* 543.2 $[M+H]^+$.

2.4 Preparation of standard solutions

The standard fatty acids for HPLC analysis at individual concentration of 1.0×10^{-4} mol/L were prepared by dilution of the corresponding stock solution (1.0×10^{-2} mol/L) with the mixed solvent of acetonitrile/DMF (9 : 1, vol/vol). ANITS solution (5.0×10^{-2} mol/L) was prepared by dissolving 271.0 mg ANITS in 10 mL DMF. The corresponding low concentration of derivatization reagent solution (5.0×10^{-3} mol/L) was obtained by diluting the stock solution with DMF. When not in use, all solutions were stored at 4 °C in a refrigerator until HPLC analysis.

2.5 Methods for extraction of free fatty acids in rape bee pollen samples

(1) Ultrasound-assisted extraction: To a 25-mL round-bottom flask, 0.2 g rape bee pollen and 5.0 mL chloroform were added. The flask was immersed in a sonicator water bath and the sample was sonicated for 20 min. The rape bee pollen was extracted three times. The contents were combined.

(2) Shaking extraction: The rape bee pollen (0.2 g) was extracted three times (20 min for each extraction) with 5.0 mL chloroform in a mechanical shaker and the extracts were combined.

(3) Distillation extraction: To a 25-mL round-bottom flask, 0.2 g rape bee pollen and 5.0 mL chloroform were added. The contents were rapidly heated to reflux for 20 min with vigorous stirring. After cooling, the chloroform was transferred into a 25-mL round-bottom flask. The distillation extraction was repeated three times with 5.0 mL chloroform and the extracts were combined.

(4) Distillation extraction with mixed solvent: To a 25-mL round-bottom flask, 0.2 g pulverized rape bee pollen and 5.0 mL mixed solvent of methanol and chloroform (1 : 1, vol/vol) were added. The contents were rapidly heated to reflux for 20 min with vigorous stirring. After cooling, the solvent was transferred into a 25-mL round-bottom flask. The extraction was repeated three times with 5.0 mL methanol/chloroform (1 : 1, vol/vol) and the extracts were combined.

To each extract mentioned above, 0.5 mL triethylamine was added and evaporated to dryness by a rotary vacuum evaporator at 60 °C. The residue was redissolved in 2.0 mL DMF and filtered through a 0.2-μm nylon membrane filter and stored at 4 °C until HPLC analysis.

2.6 Derivatization procedure

To a solution containing 50 μL of a standard fatty acid mixture in a 2-mL vial, 140 μL derivatization reagent solution, 10 mg K_2CO_3 and 210 μL DMF were added. The vial was sealed and allowed to react in a water bath at 90 °C with shaking in 5-min intervals for 40 min. After the reaction was completed, the mixture was taken to cool at room temperature. A 600-μL volume of acetonitrile solution (CH_3CN/DMF, 1 : 1, vol/vol) was added to dilute the derivatization solution. The diluted solution (10 μL) was injected directly into the chromatograph. The derivatization procedure is shown in Fig. 1.

2.7 Quantitative analysis

Quantitative conversion of fatty acids from the extracted rape bee pollen samples to their ANITS derivatives was guaranteed by using an excess of ANITS. All fatty acids were quantified in the rape bee pollen sample using the external standard method with detection at λ_{ex} = 250 nm and λ_{em} = 512 nm. The calibration curves for each ANITS-fatty acid derivative were obtained by linear regression, plotting peak area *versus* concentration.

VCH Auftr.-Nr. 33663 Vers.: 6.06
S:/3d/vch/_EJLST/ej_07_03/fl_0224.3d
EJLST
1. Papierlauf Seite 3 – hg –
Mittwoch, den 14. 02. 07, 11 Uhr 22

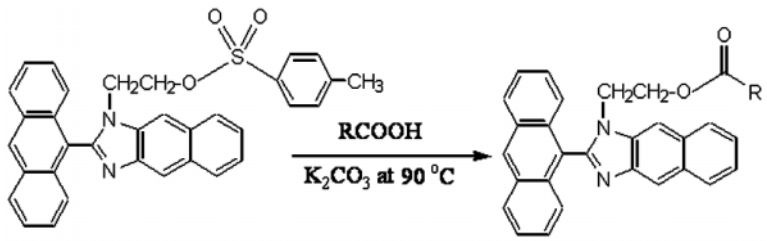

Fig. 1. Derivatization scheme of ANITS with fatty acids.

3 Results and discussion

3.1 Ultraviolet absorption of ANITS

2-(2-(Anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl) (ANI) derivatives are one of the most studied and important classes of photochromic molecules with a dual-signaling fluorescent molecule [29]. They exhibit interesting photochromic properties. For the determination of λ_{max}, and the molar absorption coefficients (ε) of ANI, a 1.5×10^{-5} mol/L acetonitrile solution was prepared. ANI showed high absorption efficiency in the UV range and exhibited two main absorption bands in the 200–400 nm range in acetonitrile. The maximum absorption bands were at 247 and 253 nm, respectively. The molar absorption coefficients were as follows: 2.10×10^5 L/mol/cm (247 nm), 2.31×10^5 L/mol/cm (253 nm). The maximum molar absorption coefficient (ε) was observed at 253 nm. The maximum UV responses did not exhibit obvious blue- or red-shift in the solvent system. With the concentration of acetonitrile >50% (vol/vol), the absorption intensities and maximum wavelengths remained basically constant. When the concentration of acetonitrile was <50% (vol/vol), no obvious change in the maximum absorption wavelength was observed; however, the absorption intensities decreased clearly with decreasing solvent concentration.

3.2 Fluorescence for ANITS and its derivatives

The excitation and emission spectra of the representative ANITS-C_7 derivative were collected using the scanning mode of the fluorescence detector. Maximum fluorescence responses of the ANITS-C_7 derivative were achieved at the excitation wavelength of 250 nm and the emission wavelength of 512 nm (no correction). The excitation and emission wavelengths in acetonitrile or methanol solution (0–100%) exhibited no obvious blue- or red-shift. The fluorescence intensity of the derivative is minimally quenched by inorganic anions such as sulfate, nitrate, and phosphate, organic anions such as bicarbonate and citrate, and monovalent and divalent cations that are abundant in biological fluids.

The fluorescence responses between ANITS itself and the corresponding derivatives were compared in acetonitrile. The results indicated that the fluorescence intensity of ANITS itself showed a dramatic quenching relative to that of its derivatives. This is probably due to the fact that the presence of sulfur atoms in the ANITS molecular core structure results in a significant quenching in emission intensity. The ratio $I_{em}/I'_{em} = 6.8 : 1$ was observed (here, I_{em} and I'_{em} were the emission responses of the derivative and ANITS itself, respectively). The steady-state fluorescence intensity of the representative ANITS-ester (C_{12}-ester) was investigated at different temperatures in acetonitrile. The temperature was tested in 10 °C increments from 35 to 75 °C. Emission spectra were recorded using the maximum excitation wavelength (250 nm) (profile not shown). The results indicated that the emission intensities decreased with increasing temperature, and the relative fluorescence intensity changed from 184 to 146 with the temperature increasing from 30 to 70 °C. Probably due to the loss of excited-state energy through hydrogen bonding or due to the protonation in strong hydrogen-bonding solvents, a corresponding decrease in emission intensity was observed ■ pls check■. A kinetic analysis of the fluorescence intensity of the representative ANITS-ester (C_{12}-ester) at the different temperatures (30–70 °C in 10 °C increments) led to a linear correlation between ln I_{em} and 1/*T*. (I_{em}: relative fluorescence intensity; *T*: thermodynamic temperature). The slopes of the working curves showed that the emission stabilization energies were 2.246 kcal/mol ($R^2 = 0.9988$, in acetonitrile).

3.3 Optimal derivatization

ANITS was stable in common organic solvents. After keeping the stock solution of ANITS in DMF at room temperature for 1 week, the corresponding derivatization yields for fatty acids were not obviously different ■ pls check■. Time course studies on the derivatization reaction of fatty acids with ANITS were investigated at 90 °C. As observed, the fluorescence responses reached the maximum at 40 min, indicating that the reagent rapidly reacted with fatty acids under these mild conditions to afford fluorescent derivatives. The derivatization yields

VCH Auftr.-Nr. 33663 Vers.: 6.06
S:/3d/vch/_EJLST/ej_07_03/fl_0224.3d
EJLST
1. Papierlauf Seite 4 – hg –
Mittwoch, den 14. 02. 07, 11 Uhr 22

were investigated with various solvent systems, such as acetonitrile, THF, DMF and DMSO. The results indicated that DMSO and DMF resulted in the most intense fluorescence responses. In addition, DMSO was used as the derivatization co-solvent in preference to other solvents, as it easily avoided the problem of precipitation of hydrophobic fatty acid derivatives. Although the responses in DMF were almost similar to those in DMSO, several serious interference peaks with DMSO as solvent were observed. A low detection response in THF solvent was observed, probably due to low solubility. In our experiments, DMF was finally chosen as the reaction co-solvent. Several basic catalysts including pyridine, 2-methylpyridine, triethylamine and 4-dimethylaminopyridine (DMAP), K_2CO_3, Na_2CO_3, $K_2C_2O_4$, $(CH_3)_4NCO_3$ and NaCN were evaluated for the derivatization. The results indicated that K_2CO_3 was the best basic catalyst and gave the highest detection responses. The effect of the added amount of K_2CO_3 on the derivatization was tested. Maximum and constant peak intensities could be attained with added amounts of K_2CO_3 >10 mg, and an excess of 20 mg K_2CO_3 did not significantly increase the detector responses. The effect of the reaction temperature on the derivatization was evaluated; the results indicated that the maximum and constant peak intensities for the derivatized fatty acids were obtained by the reaction of ANITS with the fatty acids at 90 °C for 40 min. Although NE-OTF and AE-OTF have been developed as sulfonate ester reagents for the determination of fatty acids, crown ether and potassium carbonate are usually used in the derivatization procedure with the toxic toluene as solvent, for which the derivatization solution is usually required to be treated prior to chromatographic analysis. With DMF solvent in our experiments, the detection responses were not remarkably different in the presence or absence of 18-crown-6. Constant fluorescence intensity was achieved with the addition of a six- to sevenfold molar reagent excess to total molar fatty acids, and further increasing the excess of reagent beyond this level had no significant effect on the yields. For an unknown concentration of sample, complete derivatization was guaranteed by using an excess of ANITS until constant peak intensity for detector responses.

3.4 Optimal extraction

Four methods for the extraction of fatty acids were evaluated by comparison of the fluorescence responses obtained by the analysis of the derivatized fatty acids from the extracted bee pollen samples. With only chloroform as extraction solvent, the analysis of the results obtained for ultrasound extraction shows that the extraction efficiency of the fatty acids was between the distillation extraction and the shaking extraction. The extraction yields for the saturated free fatty acids appeared to follow the pattern: distillation extraction (yield ~94%) > ultrasound extraction (yield ~91.4%) > shaking extraction (yield ~88.4%). With the mixed solvents as extraction reagent, maximal extraction yields close to 100% are observed with the distillation extraction using methanol/chloroform (vol/vol, 1:1). This was probably due to the fact that the solubility of the saturated free fatty acids in the mixed organic solvents was higher than that in chloroform alone. At the same time, the solubility of the saturated free fatty acids in hot organic solvents was higher than that at normal temperature. In most cases, relatively low extraction efficiency was observed for the shaking extraction. With various solvents as extraction solution, the highest extraction efficiency was observed using the mixed solvent methanol/chloroform (1 : 1, vol/vol). In general, the solubility of each tested representative fatty acid (C_{16}–C_{20}) in the solvents appeared to follow the pattern: methanol/chloroform (1 : 1, vol/vol) > chloroform > methanol > acetonitrile. The total extracted amount of fatty acids, using methanol/chloroform as extraction solvent in combination with the distillation method, was 1.06-fold higher than that obtained by the ultrasound extraction. Subsequently, all experiments in this study were performed by distillation extraction using the mixed methanol/chloroform (1 : 1, vol/vol) as extraction solvent. After the extraction solution was filtered, 2.0 mL triethylamine was added to the combined methanol/chloroform, to convert the free fatty acids to their corresponding salts. The methanol/chloroform solution was then evaporated to dryness with a stream of nitrogen gas. The residue was redissolved in 2.0 mL DMF, and the solution stored at 4 °C until HPLC analysis.

3.5 Chromatographic separation and MS/MS identification

The separation of fatty acid derivatives could be carried out by different columns such as BDS-C_{18}, ODS-C_{18} and so on; however, the separation of the fatty acid derivatives on the Eclipse XDB-C_8 column gave the best results. Therefore, an Eclipse XDB-C8 column (150 × 4.6 mm i.d, 5 μm; Agilent) was selected in conjunction with gradient elution; several programs were also investigated to ensure satisfactory HPLC separation within the shortest time. The gradient elution was carried out as described in the experimental section. The elution gave the best separation with the shortest retention values and the sharpest peaks. Under these conditions, all fatty acid derivatives from a real sample were separated with a good baseline resolution. The chromatogram of a complete baseline resolution for all fatty acid derivatives is shown in Fig. 2.

VCH Auftr.-Nr. 33663 Vers.: 6.06
S:/3d/vch/_EJLST/ej_07_03/fl_0224.3d
EJLST
1. Papierlauf Seite 5 – hg –
Mittwoch, den 14. 02. 07, 11 Uhr 22

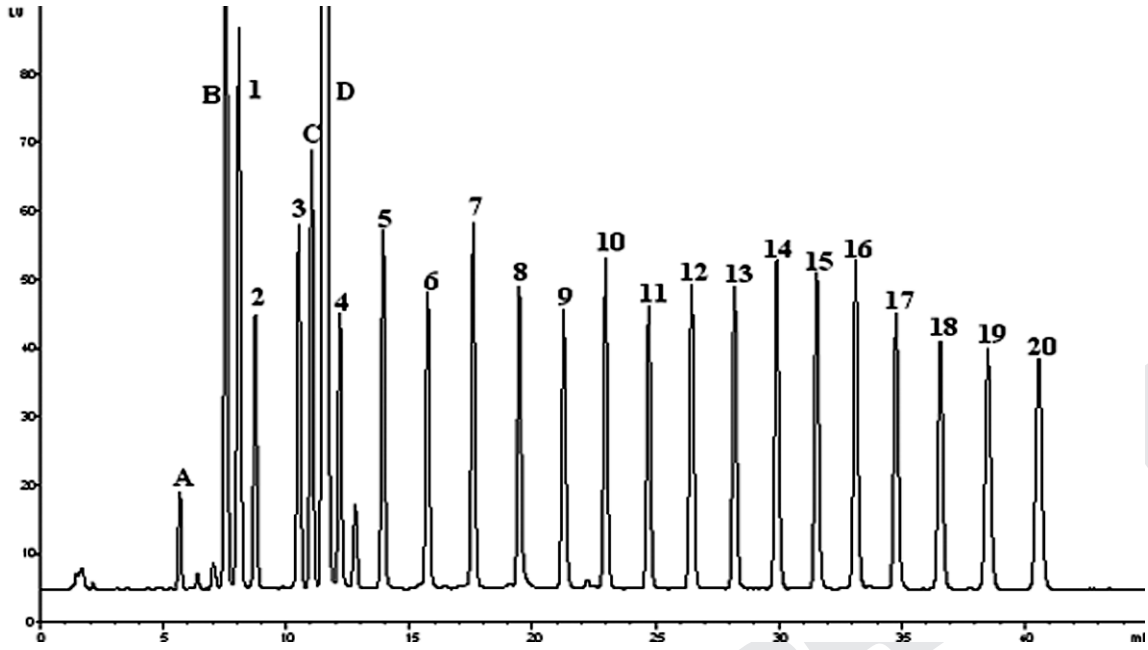

Fig. 2. Chromatogram of standard fatty acid derivatives (50 pmol). Chromatographic conditions: column temperature 30 °C; excitation wavelength λ_{ex} 250 nm; emission wavelength λ_{em} 520 nm; column 200 × 4.6 mm Hypersil BDS C_{18} (5 μm); flow rate 1.0 mL/min. Peaks: (1) formic acid; (2) acetic acid; (3) propionic acid; (4) butyric acid; (5) valeric acid; (6) hexanoic acid; (7) heptoic acid; (8) octoic acid; (9) pelargoic acid; (10) decoic acid; (11) undecanoic acid; (12) dodecanoic acid; (13) tridecanoic acid; (14) tetradecanoic acid; (15) pentadecanoic acid; (16) hexadecanoic acid; (17) heptadecanoic acid; (18) octadecanoic acid; (19) nonadecanoic acid; (20) arachidic acid; (A) 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl)-ethanol; (B) and (C) unidentified; (D) ANITS.

The ionization and fragmentation of the isolated ANITS-fatty acid derivatives were studied by mass spectrometry with APCI detection in positive-ion detection mode. As expected, the ANITS-fatty acid derivative produced an intense molecular ion peak at *m/z* $[M+H]^+$. When the derivatives were identified with ESI/MS detection, no ion current signals were observed in positive- or negative-ion detection mode; therefore, APCI/MS was selected for the identification of ANITS-derivatives of saturated free fatty acids. With MS/MS analysis of the fatty acid derivatives, the collision-induced dissociation spectra of *m/z* $[M+H]^+$ produced the specific fragment ions at *m/z* $[M+H–345]^+$ and *m/z* 345. The M' was the corresponding molecular mass of the fatty acids; the specific fragment ion *m/z* 345 was the corresponding protonated molecular core structure moiety. The specific fragment ion at *m/z* $[M+H–345]^+$ was the corresponding protonated fatty acid moiety. The selected reaction monitoring, based on the *m/z* $[M+H]^+ \rightarrow$ *m/z* $[M+H–345]^+$ and *m/z* 345 transitions, was specific for the fatty acid derivatives. There was no detectable signal from the blank water sample using this transition. Although other endogenous acidic compounds present in natural environmental samples were presumably coextracted and derivatized by the ANITS reagent, no interference was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ions in the *m/z* $[M+H–345]^+$ and *m/z* 345 transition. To reduce the disturbance from other unknown components present in the sample to a minimum, the gradient elution with HPLC for the separation and determination of derivatized fatty acids was an efficient method. In addition, ANITS did not react with esterified fatty acids such as triacylglycerols, since the esterified fatty acids could not dissociate into their free acid groups in anhydrous DMF solvent; therefore, the esterified fatty acids did not interfere with the derivatization of the free fatty acids. The characteristic fragment ion of *m/z* 345 (molecular core structure) came from the cleavage of the N-CH_2COO bond. With APCI in positive-ion detection mode, intense ion current signals for long-chain fatty acid derivatives should be attributed to the introduction of two weakly basic nitrogen atoms in the corresponding ANITS molecular core structure, resulting in high ionization efficiency. The MS/MS analysis and the corresponding cleavage mode for a representative ANITS-C_7 derivative are shown in Fig. 3a–c. All molecular ions $[M+H]^+$ and corresponding specific fragment ions for the C_1–C_{20} fatty acid derivatives are shown in Tab. 1.

VCH Auftr.-Nr. 33663 Vers.: 6.06
S:/3d/vch/_EJLST/ej_07_03/fl_0224.3d
EJLST
1. Papierlauf Seite 6 – hg –
Mittwoch, den 14. 02. 07, 11 Uhr 22

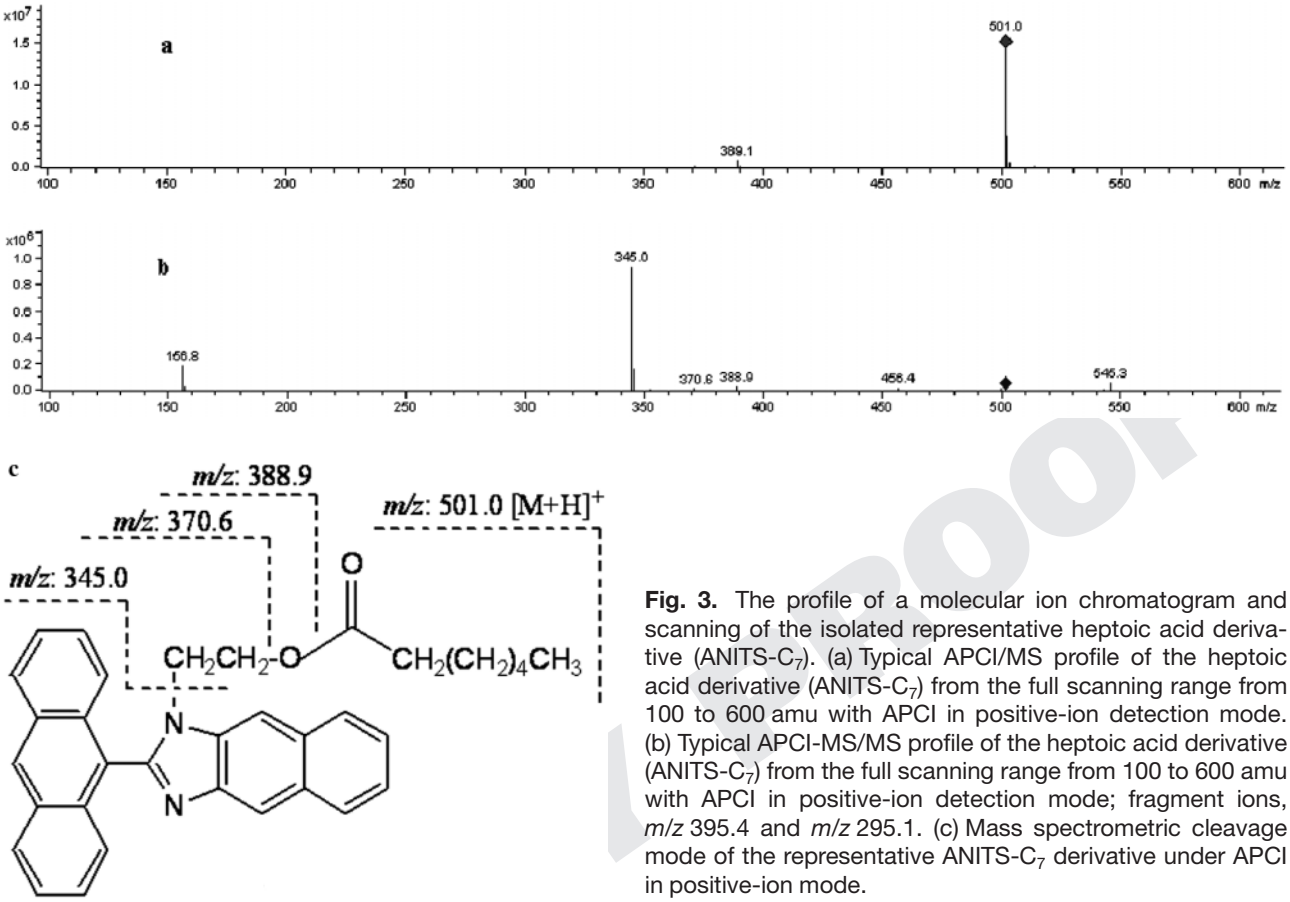

Fig. 3. The profile of a molecular ion chromatogram and scanning of the isolated representative heptoic acid derivative (ANITS-C_7). (a) Typical APCI/MS profile of the heptoic acid derivative (ANITS-C_7) from the full scanning range from 100 to 600 amu with APCI in positive-ion detection mode. (b) Typical APCI-MS/MS profile of the heptoic acid derivative (ANITS-C_7) from the full scanning range from 100 to 600 amu with APCI in positive-ion detection mode; fragment ions, *m/z* 395.4 and *m/z* 295.1. (c) Mass spectrometric cleavage mode of the representative ANITS-C_7 derivative under APCI in positive-ion mode.

Tab. 1. MS and MS/MS analysis.

Fatty acids	Molecular weight	Molecular weight of derivatives	$[M+H]^+$	MS/MS data	$[M+H-345]^+$
C_1	46	416	417.2	389.0, 371.0, 345.0	#
C_2	60	430	431.1	389.8, 370.9, 344.9	#
C_3	74	444	445.2	389.8, 371.1, 344.9	#
C_4	88	458	459.2	389.9, 372.0, 345.0	114.7
C_5	102	472	473.1	389.6, 371.1, 344.9	#
C_6	116	486	487.1	389.5, 372.0, 344.9	142.7
C_7	130	500	501.1	389.4, 371.3, 344.9	156.8
C_8	144	514	515.2	389.5, 371.6, 344.9	170.8
C_9	158	528	529.2	389.4, 371.8, 344.9	184.8
C_{10}	172	542	543.2	389.3, 370.9, 344.9	198.8
C_{11}	186	556	557.2	389.3, 370.9, 344.9	212.9
C_{12}	200	570	571.3	389.5, 370.6, 344.9	226.8
C_{13}	214	584	585.3	389.5, 370.5, 344.9	240.9
C_{14}	228	598	599.3	389.2, 370.9, 344.9	255.0
C_{15}	242	612	613.3	389.4, 371.9, 344.9	268.9
C_{16}	256	626	627.3	389.2, 371.0, 345.0	282.9
C_{17}	270	640	641.4	389.2, 371.0, 345.0	297.0
C_{18}	284	654	655.4	389.3, 371.9, 344.9	311.0
C_{19}	298	668	669.4	389.3, 370.8, 344.9	325.0
C_{20}	312	682	683.4	389.2, 370.9, 344.8	339.2

Data not shown.

VCH Auftr.-Nr. 33663 Vers.: 6.06
S:/3d/vch/_EJLST/ej_07_03/fl_0224.3d
EJLST
1. Papierlauf Seite 7 – hg –
Mittwoch, den 14. 02. 07, 11 Uhr 22

3.6 Repeatability, precision, calibration and detection limits

A standard solution containing C_1–C_{20} fatty acids (3.0×10^{-6} mol/L) was prepared and the method repeatability was examined; the corresponding injected amounts were 50 pmol. The relative standard deviations (RSD) of the peak areas and retention times varied from 0.16 to 0.91% and from 0.015 to 0.18%, respectively. The reproducibility of the method was evaluated by the determination of known concentrations of 20 fatty acids (concentrations are 50 and 100 ng/mL, respectively) in quintuplicate over 2 days. The relative standard deviations (RSD%) of the actual determined values were between 1.8 and 3.4%. Precision and accuracy: Six replicates ($n = 6$) at 0.1, 1.0, and 5.0 μmol/L of C_1–C_{20} fatty acids were used to make the low- to high-range concentrations. The mean interday accuracy ranged from 94.6 to 107.8%, with the largest mean %CV <7.8. The mean interday precision for all standards was <5.6% of the expected concentration. The recoveries were determined from values obtained following the actual analysis of the bee pollen as calculated from the calibration graph constructed by using the performed fatty acid derivatives. To two identical bee pollen samples, known amounts of the 20 above-mentioned fatty acids were added. The samples were treated according to the method as described in the text and derivatized with ANITS, and the analyses were carried out in duplicate. The experimental recoveries are in the range of 87.6–104.8%. Based on the optimum derivatization conditions, the linearities of the procedures were evaluated in the range of 9.766×10^{-3} to 20 μmol/L (injection volume 10 μL, corresponding to an injected amount from 200.0 pmol to 97.66 fmol, with a 2048-fold concentration range). The calibration graph was established with the peak area (y) *versus* fatty acid concentration (x: pmol, injected amount). All of the fatty acids were found to give excellent linear responses over this range, with correlation coefficients >0.9999. The data are shown in Tab. 2. The linear relationships for further higher concentrations were not tested due to over-linearity range. With 1.0 pmol injection for each derivatized fatty acid, the calculated detection limits (at a signal-to-noise ratio of 3 : 1) were from 24.76 to 98.79 fmol. With MS/APCI identification for the real sample, the comparison of the sensitivity between MS/APCI and fluorescence was investigated. With the injection of 50 pmol of each fatty acid derivative (the concentration of derivatized amines at 5.0×10^{-6} mol/L, 10 μL injection), the MS/APCI detection limits (at a signal-to-noise ratio of 3 : 1) were 0.32–16.74 pmol ■ check!■. The APCI/MS detection sensitivity could sufficiently satisfy the identification of fatty acid derivatives from the real extracted rape bee pollen samples.

Tab. 2. Linear regression equations, correlation coefficients, detection limits of fatty acid derivatives. The repeatability for peak area and retention time was determined by successive injection of 50 pmol derivatives ($n = 6$).

Fatty acid	Correlation coefficients	Detection limits [fmol]	Retention time RSD [%]	Peak area RSD [%]
C_1	0.9999	24.76	0.18	0.34
C_2	0.9999	49.86	0.16	0.82
C_3	0.9999	65.55	0.15	0.54
C_4	0.9999	75.21	0.067	0.52
C_5	0.9999	33.51	0.045	0.91
C_6	0.9999	86.48	0.022	0.63
C_7	0.9999	29.24	0.017	0.79
C_8	0.9999	65.55	0.015	0.46
C_9	0.9999	31.89	0.020	0.53
C_{10}	0.9999	43.39	0.027	0.74
C_{11}	0.9999	49.86	0.032	0.42
C_{12}	0.9999	65.55	0.038	0.37
C_{13}	0.9999	65.55	0.047	0.39
C_{14}	0.9999	49.86	0.058	0.45
C_{15}	0.9999	75.21	0.058	0.43
C_{16}	0.9999	43.39	0.057	0.56
C_{17}	0.9999	57.18	0.066	0.60
C_{18}	0.9999	26.70	0.080	0.55
C_{19}	0.9999	27.89	0.090	0.29
C_{20}	0.9999	98.79	0.090	0.16

3.7 Comparison of the derivatization conditions and detection limits with the reagents reported for fatty acids

Compared with 4-bromomethyl-7-methoxycoumarin (BrMMC) [30], 9-anthryldiazomethane (ADAM) [31], and 2-(2,3-naphthalimido)-ethyl trifluoromethane-sulfonate (NE-OTF) [24] (several of the applied fluorescent labeling reagents), ANITS exhibits the desired fluorescence properties superior to those of BrMMC, ADAM, and NE-OTF. The fluorescence excitation and emission wavelengths were examined in acetonitrile, methanol and water, which have been widely used as components of the mobile phase in reversed-phase LC. The maximal excitation and emission wavelengths are almost unchanged in these organic solutions. Derivatization of ANITS with fatty acids can be accomplished within 30–40 min at 90 °C and gives almost the theoretic yields of derivatives in DMF in the presence of the K_2CO_3 catalyst, which is comparable to the BrMMC derivatives. The derivatization with BrMMC is carried out at 60 °C in acetone or toluene solvent in the presence of K_2CO_3 and phase transfer agent (*e.g.* 18-crown-6). The detection sensitivity of the resulting esters is not only limited at approximate 10 pmol, but is also strongly affected by the chain lengths of the original free fatty acids [30]. Since the reaction is carried out in a non-

VCH Auftr.-Nr. 33663 Vers.: 6.06
S:/3d/vch/_EJLST/ej_07_03/fl_0224.3d
EJLST
1. Papierlauf Seite 8 – hg –
Mittwoch, den 14. 02. 07, 11 Uhr 22

aqueous medium including solid K_2CO_3, it seems to be fairly difficult to obtain the quantitative yield. Although NE-OTF has been developed as a sulfonate reagent for the determination of fatty acids with satisfactory results, with lower detection limits relative to that of ANITS, the derivatization reaction must be carried out in acetonitrile or toluene in the presence of K_2CO_3 and phase transfer agent (*e.g.* 18-crown-6). Before injection, the derivatization solution must be pretreated in order to remove the toluene and the phase transfer agent. The procedure is time-consuming. As observed, ANITS-derivatives are more photostable than ADAM- and BrMMC-derivatives. The derivatization solution of ANITS can be directly injected without pretreatment operation prior to HPLC. With ADAM derivatization, data reported previously indicated that the detection limit for fatty acids is ca. 100 pg/10 μL. ADAM cannot be stored for long periods as a solution, or even as a solid. The reagent often requires purification just before use with a suitable method such as column chromatography [32]. Therefore, ANITS is prospectively significant as a pre-column derivatizing reagent for fatty acid derivatization in terms of sensitivity and stability. It was shown that the quantification of fatty acids could be well done with the established method. The overall comparison of ANITS with other commonly used fluorescent labeling reagents for fatty acids in HPLC is given in Tab. 3. The detection limits of most reagents are higher than for ANITS, except NE-OTF. However, the labeling procedure of NE-OTF is time-consuming due to the pretreatment procedure. Considering the detection properties (such as wavelength, derivatization time, and detection limits) for the determination of fatty acids, ANITS is more advantageous than the other reagents. Current studies should further explore the derivatization of different fatty acid-containing compounds such as dicarboxylic acids and long-chain saturated and unsaturated fatty acids.

3.8 Analysis of samples

Rape bee pollen is promoted as a health food with a wide range of nutritional and therapeutic properties. It is used for its nutritional value in human diets. It is made up of natural flower pollen mixed with nectar and bee secretions and is rich in sugars, proteins, lipids, vitamins and flavonoids [33]. The lipid fraction of bee pollen includes many kinds of acidic compounds and a number of minor unidentified peaks. From the literature, it seems that, in general, the dominant fatty acids present in pollen are palmitic (C_{16}), oleic 18:1, linoleic 18:2 and linolenic 18:3 acids [29]. In this study, 20 free fatty acids from the extracts of rape bee pollen samples were determined by the sensitive fluorescence labeling reagent ANITS. The chromatogram for the analysis of saturated free fatty acids extracted from rape bee pollen samples (Qinghai, Xining, China) is shown in Fig. 4 (here, chromatograms of rape bee pollen from Xinjiang are not shown). As can be seen from Fig. 4, the saturated and unsaturated fatty acids are simultaneously separated; the saturated free fatty acids are identified by APCI/MS in positive-ion mode. Most unsaturated fatty acids are not identified; however, in this study, the major peaks for the unsaturated fatty acids in Fig. 4 are also identified by APCI/MS in positive-ion mode. They mainly include (E) eicosanonaenoic acid (20:9), (F) hexadecatrienoic (16:3), (G) octadecatrienoic acid (18:3), (H) octadecadienoic acid (18:2), and (I) octadecenoic acid (18:1). APCI/MS identification for the major unsaturated fatty acids is also shown in Tab. 4. Quantitative analyses for all unsaturated fatty acids were not carried out; the corresponding double bond position for the unsaturated fatty acids from (E) to (I) was not confirmed in this study; their structural validation and quantitative analysis are in progress in our laboratory ■ pls check■. The compositional data for the saturated free fatty acids are shown in Tab. 5. The results indicate that the main contents of the saturated free fatty acids in rape bee pollen are C_2 (38.28–48.32 μg/g), C_6 (5.787–7.453 μg/g), C_{14} (32.10–47.41 μg/g), C_{16} (136.0–174.3 μg/g), C_{18} (22.33–26.14 μg/g) and C_{20} (8.257–16.17 μg/g). It is demonstrated that the content of free fatty acids with an even carbon number in a series (C_1–C_{20} fatty acids) is higher than that of those with an odd carbon number. These data are of important value in order to obtain plentiful free fatty acids in bee pollen.

Tab. 3. Comparison of the derivatization conditions and detection limits of the reagents reported for fatty acids.

Reagent	$\lambda_{ex}/\lambda_{em}$	Reaction medium	Time	Temperature	Detection limits	Reference
BrMMC	346/395	Acetone or toluene K_2CO_3, 18-crown-6	30–60 min	60 °C	~10 pmol/10 μL	[30]
ADAM	255/415	THF	2–2.5 h	37 °C	~100pg/10 μL	[31]
NE-OTF	259/394	Acetone or toluene K_2CO_3, 18-crown-6	20–30 min	Room temperature	~1–4 fmol/10 μL	[24]
ANITS	250/512	Acetonitrile, DMF, K_2CO_3	30–40 min	90 °C	~24.76~98.79 fmol	This paper

VCH Auftr.-Nr. 33663 Vers.: 6.06
S:/3d/vch/_EJLST/ej_07_03/fl_0224.3d
EJLST
1. Papierlauf Seite 9 – hg –
Mittwoch, den 14. 02. 07, 11 Uhr 22

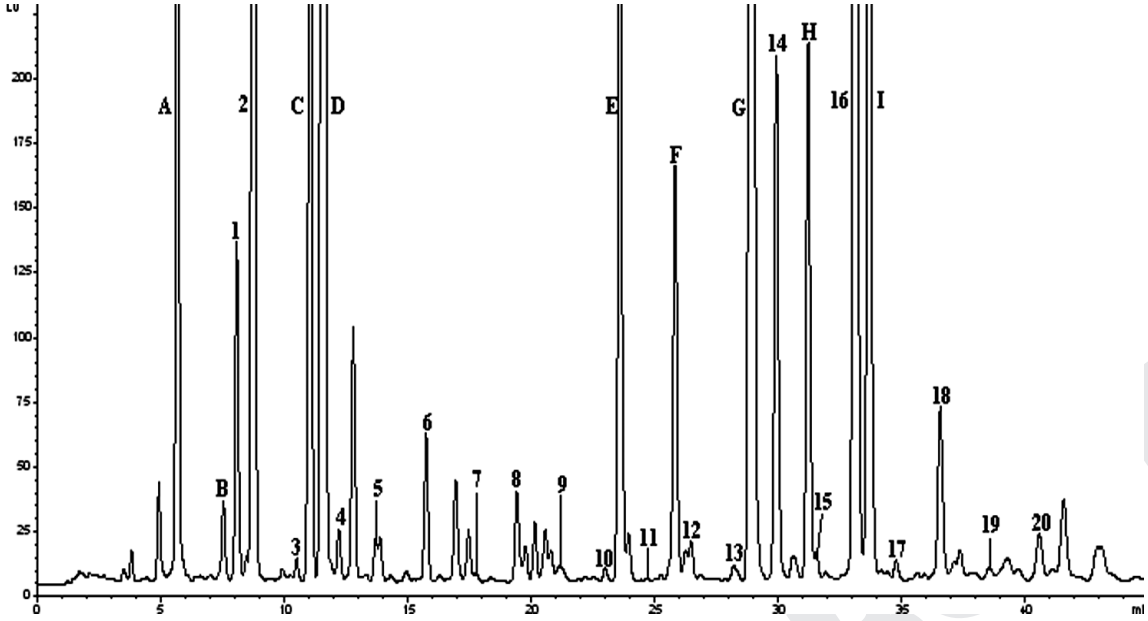

Fig. 4. Chromatogram of free fatty acids from rape bee pollen collected from QingHai. Chromatographic conditions and peaks 1–20 as in Fig. 2. (A) 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl)-ethanol; (B) and (C) unidentified; (D) ANITS; (E) eicosanonaenoic acid ($C_{20:9}$), (F) hexadecatrienoic ($C_{16:3}$), (G) octadecatrienoic acid ($C_{18:3}$), (H) octadecadienoic acid ($C_{18:2}$) and (I) octadecenoic acid ($C_{18:1}$). The major peaks from (E) to (I) were identified by APCI/MS; the corresponding double bond position was not confirmed.

Tab. 4. APCI/MS identification for major peaks of unsaturated fatty acids from real rape bee pollen samples.

Major unsaturated fatty acids	Molecular weight	Molecular weight of derivatives	$[M+H]^+$	MS/MS data
20:9	294	664	665.3	321.1, 344.9, 370.9, 388.8, 443.9, 484.9, 499.6, 538.8, 583.6, 607.0, 622.9, 647.1
16:3	250	620	621.2	277, 344.9, 370.7, 388.9, 456.4, 508.6, 525.1, 538.9
18:3	278	648	649.3	304.9, 344.9, 370.8, 389.3, 408.1, 442.9, 456.9, 568.8, 607.2
18:2	280	650	651.3	306.9, 344.9, 370.9, 389.3, 442.9, 567.1
18:1	282	652	653.4	309.0, 344.9, 371.1, 289.0, 494.5, 539.9

Tab. 5. Results of saturated free fatty acids from real samples (n = 3).

Fatty acid	Rape bee pollen QingHai [μg/g]	Rape bee pollen XinJiang [μg/g]	Fatty acid	Rape bee pollen QingHai [μg/g]	Rape bee pollen XinJiang [μg/g]
C_1	1.19	1.249	C_{11}	0.11	0.20
C_2	38.28	48.32	C_{12}	3.80	3.58
C_3	0.49	0.44	C_{13}	0.85	0.68
C_4	1.92	1.54	C_{14}	47.41	32.10
C_5	1.53	0.96	C_{15}	2.52	1.94
C_6	7.45	5.78	C_{16}	174.30	136.0
C_7	0.39	0.12	C_{17}	2.58	4.09
C_8	1.88	0.11	C_{18}	26.14	22.33
C_9	2.28	1.36	C_{19}	2.62	8.74
C_{10}	0.97	0.86	C_{20}	8.26	16.12

VCH Auftr.-Nr. 33663 Vers.: 6.06
S:/3d/vch/_EJLST/ej_07_03/fl_0224.3d
EJLST
1. Papierlauf Seite 10 – hg –
Mittwoch, den 14. 02. 07, 11 Uhr 22

4 Conclusions

The method described in this paper results in the separation of the free fatty acids obtained by distillation extraction with chloroform/methanol (1 : 1, vol/vol) as extraction solvent. The improved performance for the complete extraction of the fatty acids in bee pollen samples has been demonstrated in detail. The reagent ANITS for the labeling of the fatty acids was evaluated. The introduction of the 2-(2-(anthracen-10-yl)-1*H*-naphtho[2,3-*d*]imidazol-1-yl) functional group into the labeling reagent molecule dramatically augmented the *n*-π conjugation system and was favorable for the sensitive determination of free fatty acids with fluorescence detection. At the same time, the ANITS molecule contains two weakly basic nitrogen atoms in its molecular core structure that make it easily form more stable molecular ions and which produces a favorable result for the sensitive identification of ANITS-fatty acid derivatives under APCI/MS in positive-ion detection mode. The LC separation for the free fatty acid derivatives shows good repeatability. The reagent and its hydrolysis products do not interfere with the separation by gradient elution. The established method can also be applied to the determination of various drugs and plants containing free fatty acids.

Acknowledgment

This work was supported by the National Science Foundation under Grant No. 20075016.

References

[1] S. T. Ingalls, P. E. Minkler., C. L. Hoppel, E. Nordlander: Derivatization of carboxylic acids by reaction with 4'-bromophenacyl trifluoromethanesulfonate prior to determination by high-performance liquid chromatography. *J Chromatogr A.* 1984, **299**, 365–376.

[2] G. M. Mukwaya, D. F. Welch: Subgrouping of Pseudomonas cepacia by cellular fatty acid composition. *J Clin Microbiol.* 1989, **27**, 2640–2646.

[3] P. Verken, A. E. M. van Lint, A. H. B. Bootsma, H. Overmars, R. J. A. Wanders, A. van Gennip: Rapid stable isotope dilution analysis of very-long-chain fatty acids, pristanic acid and phytanic acid using gas chromatography-electron impact mass spectrometry. *J Chromatogr B.* 1998, **713**, 281–287.

[4] T. Řezanka: Identification of very long chain fatty acids by atmospheric pressure chemical ionization liquid chromatography-mass spectroscopy from green alga *Chlorella kessleri*. *J Sep Sci.* 2002, **25**, 1332–1336.

[5] S. Yoshida, S. Hirose, M. Iwamoto: Use of 4-bromomethyl-7-methoxycoumarin for derivatization of pyrimidine compounds in serum analysed by high-performance liquid chromatography with fluorimetric detection. *J Chromatogr B.* 1986, **383**, 61–68.

[6] J. H. Wolf, J. Korf: Improved automated precolumn derivatization reaction of fatty acids with bromomethylmethoxycoumarin as label. *J Chromatogr A.* 1990, **502**, 423–430.

[7] J. S. Yoo, V. L. MacGuffin: Determination of fatty acids in fish oil dietary supplements by capillary liquid chromatography with laser-induced fluorescence detection. *J Chromatogr A.* 1992, **627**, 87–96.

[8] W. Voelter, R. Huber, K. Zech: Fluorescence labelling in trace analysis of biological samples: Simultaneous determination of free fatty acids and related carboxylic compounds. *J Chromatogr A.* 1981, **217**, 491–507.

[9] K. Hayashi, J. Kawase, K. Yoshimura, K. Ara, K. Tsuji: Determination of trace levels of fatty acid metal salts by high-performance liquid chromatography with fluorescence prelabeling. *Anal Biochem.* 1984, **136**, 314–320.

[10] H. Tsuchiya, T. Hayashi, H. Naruse, N. Takagi: High-performance liquid chromatography of carboxylic acids using 4-bromomethyl-7-acetoxycoumarin as fluorescence reagent. *J Chromatogr A.* 1982, **234**, 121–130.

[11] A. Takadate, T. Masuda, C. Tajime, C. Murata, M. Irikura, S. Goya: 3-Bromoacetyl-7-methoxycoumarin as a new fluorescent derivatization reagent for carboxylic acids in high-performance liquid chromatography. *Anal Sci.* 1992, **8**, 663–668.

[12] A. Takadate, T. Masuda, C. Murata, C. Haratake, A. Isobe, M. Irikura, S. Goya: 3-Bromoacetyl-6,7-methylenedioxycoumarin as a highly reactive and sensitive fluorescence labeling reagent for fatty acids. *Anal Sci.* 1992, **8**, 695–698.

[13] Y. Yamauchi, T. Tomita, M. S. Hirai, T. Terano, Y. Tamura, S. Yoshida: High-performance liquid chromatographic analysis of arachidonic acid metabolites by pre-column derivatization using 9-anthryldiazomethane. *J Chromatogr A.* 1986, **357**, 199–205.

[14] T. Yoshida, A. Uetake, H. Yamaguchi, N. Nimura, T. Kinoshita: New preparation method for 9-anthryldiazomethane (ADAM) as a fluorescent labeling reagent for fatty acids and derivatives. *Anal Biochem.* 1988, **173**, 70–74.

[15] N. Nimura, T. Kinoshita, T. Yoshida, A. Uetake, C. Nakai: 1-Pyrenyldiazomethane as a fluorescent labeling reagent for liquid chromatographic determination of carboxylic acids. *Anal Chem.* 1988, **60**, 2067–2070.

[16] M. Yamaguchi, S. Hara, R. Matsunaga, M. Nakamura, Y. Ohkura: Highly sensitive fluorogenic reagent for carboxylic acid, 3-bromomethyl-6,7-dimethoxy-1-methyl-2-(1H)-quinoxalinone, in high performance liquid chromatography. *Anal Sci.* 1985, **1**, 295–296.

[17] M. Yamaguchi, S. Hara, R. Matsunaga, M. Nakamura, Y. Ohkura: 3-Bromomethyl-6,7-dimethoxy-1-methyl-2-(1H)-quinoxalinone as a new fluorescence derivatization reagent for carboxylic acids in high-performance liquid chromatography. *J Chromatogr A.* 1985, **346**, 227–236.

[18] M. Yamaguchi, K. Fukuda, S. Hara, M. Nakamura, Y. Ohkura: Fluorimetric high-performance liquid chromatography of prostaglandins and its application to their determination in human seminal fluid. *J Chromatogr B.* 1986, **380**, 257–265.

[19] T. Iwata, K. Inoue, M. Nakamura, M. Yamaguchi: Simple and highly sensitive determination of free fatty acids in human serum by high performance liquid chromatography with fluorescence detection. *Biomed Chromatogr.* 1992, **6**, 120–123.

[20] T. Toyo'oka, M. Ishibashi, Y. Takeda, K. Imai: 4-(Aminosulphonyl)-2,1,3-benzoxadiazole derivatives as pre-column fluorogenic tagging reagents for carboxylic acids in high-performance liquid chromatography. *Analyst* 1991, **116**, 609–613.

VCH Auftr.-Nr. 33663 Vers.: 6.06
S:/3d/vch/_EJLST/ej_07_03/fl_0224.3d
EJLST
1. Papierlauf Seite 11 – hg –
Mittwoch, den 14. 02. 07, 11 Uhr 22

[21] T. Toyo'oka, M. Ishibashi, Y. Takeda, K. Nakashima, S. Akiyama, S. Uzu, K. Imai: Precolumn fluorescence tagging reagent for carboxylic acids in high-performance liquid chromatography: 4-Substituted-7-aminoalkylamino-2,1,3-benzoxadiazoles. *J Chromatogr A.* 1991, **588**, 61–71.

[22] T. Toyo'oka, M. Ishibashi, T. Terao, K. Imai: Sensitive fluorometric detection of prostaglandins by high performance liquid chromatography after precolumn labelling with 4-(N,*N*-dimethylaminosulphonyl)-7-(1-piperazinyl)-2,1,3-benzoxadiazole (DBD-PZ). *Biomed Chromatogr.* 1992, **6**, 143–148.

[23] C.-Y. Lu, H.-L. Wu, S.-H. Chen: A fluorometric liquid chromatography for highly sensitive analysis of very long chain fatty acids as naphthoxyethyl derivatives. *Chromatographia.* 2000, **51**, 315–321.

[24] Y. Yasaka, M. Tanaka, T. Shono, T. Tetsumi, J. Katakawa: 2-(2,3-Naphthalimino)ethyl trifluoromethanesulphonate as a highly reactive ultraviolet and fluorescent labelling agent for the liquid chromatographic determination of carboxylic acids. *J Chromatogr A.* 1990, **508**, 133–140.

[25] K. Akasaka, H. Ohrui, H. Meguro: Determination of carboxylic acids by high-performance liquid chromatography with 2-(2,3-anthracenedicarboximido)ethyl trifluoromethanesulfonate as a highly sensitive fluorescent labelling reagent. *Analyst.* 1993, **118**, 765.

[26] T. Iwata, T. Hirose, M. Nakamura, M. Yamaguchi: 4-(1-Methylphenanthro-[9,10-d]-imidazol-2-yl)-benzohydrazide as derivatization reagent for carboxylic acids in high-performance liquid chromatography with conventional and laser-induced fluorescence detection. *Analyst.* 1994, **119**, 1747–1753.

[27] T. Iwata, M. Nakamura, M. Yamaguchi: 4-(5,6-Dimethoxy-2-benzimidazoyl)benzohydrazide as fluorescence derivatization reagent for carboxylic acids in high-performance liquid chromatography. *Anal Sci.* 1992, **8**, 889–892.

[28] T. Toyo'oka: Fluorescent tagging of physiologically important carboxylic acids, including fatty acids, for their detection in liquid chromatography. *Anal Chim Acta.* 2002, **465**, 111–130.

[29] R. H. Yang, K. A. Li, F. Liu, N. Li, F. L. Zhao, W. H. Chan: 3,3',5,5'-Tetramethyl-N-(9-anthrylmethyl)benzidine: A dual-signaling fluorescent reagent for optical sensing of aliphatic aldehydes. *Anal Chem.* 2003, **75**, 3908–3914.

[30] T. Toyo'oka: Fluorescent tagging of physiologically important carboxylic acids, including fatty acids, for their detection in liquid chromatography. *Anal Chim Acta.* 2002, **465**, 111–130.

[31] M. A. Quilliam, A. Gago-Martínez, J. A. Rodríguez-Vázquez: Improved method for preparation and use of 9-anthryldiazomethane for derivatization of hydroxycarboxylic acids. Application to diarrhetic shellfish poisoning toxins. *J Chromatogr A.* 1998, **807**, 229–239.

[32] Y. Yamauchi, T. Tomita, M. S. Hirai, T. Terano, Y. Tamura, S. Yoshida: High-performance liquid chromatographic analysis of arachidonic acid metabolites by pre-column derivatization using 9-anthryldiazomethane. *J Chromatogr A.* 1986, **357**, 199–205.

[33] H. Human, S. W. Nicolson: Nutritional content of fresh, bee-collected and stored pollen of Aloe greatheadii var. davyana (Asphodelaceae). *Phytochemistry*. 2006, **67**, 1486–1492.

[Received: October 12, 2006; accepted: January 19, 2007]

VCH Auftr.-Nr. 33663 Vers.: 6.06
S:/3d/vch/_EJLST/ej_07_03/fl_0224.3d
EJLST
1. Papierlauf Seite 12 – hg –
Mittwoch, den 14. 02. 07, 11 Uhr 22