ELSEVIER

Journal of Chromatography B, 671 (1995) 91–112

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

# Use of derivatization to improve the chromatographic properties and detection selectivity of physiologically important carboxylic acids

Toshimasa Toyo'oka

*Department of Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422, Japan*

**Abstract**

In this review, tagging techniques with reagents used for ultraviolet-visible (UV-Vis), fluorescent (FL), chemiluminescent (CL) and electrochemical detection (ED) for higher carboxylic acids in HPLC are evaluated in terms of the tagging reactions, handling, flexibility, stability of the reagents and the corresponding derivatives, sensitivity and selectivity. Emphasis is given to the applications of these tagging techniques to biologically important carboxylic acids of relatively high molecular mass including free fatty acids, prostaglandins, leukotrienes and thromboxanes etc. Some typical examples are described. Although RIA and GC–MS are powerful techniques for the highly sensitive determination of carboxylic acids, tagging for these techniques is not included in this review because recent progress in tagging methods has been mainly concerned with HPLC detection.

## Contents



## List of abbreviations

| | |
|---|---|
| AA | Arachidonic acid |
| ABD-AE | 4-(Aminosulfonyl)-7-(2-amino-ethylamino)-2,1,3-benzoxadiazole |
| ABD-APy | (+)- and (−)-4-(Aminosulfonyl)- |

0378-4347/95/$29.00 
*SSDI* 0378-4347(94)00559-1

| | |
|---|---|
| | 7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole |
| ABD-CD | 4-(Aminosulfonyl)-7-(5-aminopentylamino)-2,1,3-benzoxadiazole |
| ABD-PZ | 4-(Aminosulfonyl)-7-(1-piperazinyl)-2,1,3-benzoxadiazole |
| ABEI | N-(4-Aminobutyl)-N-ethylisoluminol |
| ABMI | N-(4-Aminobutyl)-N-methylisoluminol |
| ADAM | 9-Anthryldiazomethane |
| AP | *p*-Aminophenol |
| APMB | (−)-2-[4-(1-Aminoethyl)phenyl]benzoxazole |
| BAN | α-Bromo-2′-acetonaphthone |
| Benzotriazole derivatives | 2-(*p*-Aminomethylphenyl)-N,N-dimethyl-2H-benzotrizolyl-5-amine |
| | 2-[*p*-(2-Aminoethyl)phenyl]-N,N-dimethyl-2H-benzotrizolyl-5-amine |
| | 2-(*p*-Aminomethylphenyl)-2H-benzotrizolyl-5-amine |
| BMPI | 2-Bromo-1-methylpyridinium iodide |
| BPB | *p*-Bromophenacyl bromide |
| BrADMFe | 3-Bromoacetyl-1,1′-dimethylferrocene |
| BrAFe | 3-Bromoacetylferrocene |
| BrAMC | 3-Bromoacetyl-7-methoxycoumarin |
| BrAMDC | 3-Bromoacetyl-6,7-methylenedioxycoumarin |
| BrDMEQ | 3-Bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone |
| BrMAC | 4-Bromomethyl-7-acetoxycoumarin |
| BrMDMC | 4-Bromomethyl-6,7-dimethoxycoumarin |
| BrMMC | 4-Bromomethyl-7-methoxycoumarin |
| BrMMEQ | 3-Bromomethyl-6,7-methylenedioxy-1-methyl-2(1H)-quinoxalinone |
| CE | Capillary electrophoresis |
| CL | Chemiluminescence |
| CZE | Capillary zone electrophoresis |
| DAP | 9,10-Diaminophenanthrene |
| DBD-APy | (+)- and (−)-4-(N,N-Dimethylminosulfonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole |
| DBD-CD | 4-(N,N-Dimethylminosulfonyl)-7-(5-aminopentylamino)-2,1,3-benzoxadiazole |
| DBD-PZ | 4-(N,N-Dimethylminosulfonyl)-7-(1-piperazinyl)-2,1,3-benzoxadiazole |
| DCC | Dicyclohexylcarbodiimide |
| DEPC | Diethyl phosphorocyanidate |
| DICI | N,N′-Diisopropyl-O-(7-methoxycoumarin-4-yl)methylisourea |
| DIPEA | N,N-Diisopropylethylamine |
| DMA | 2,4-Dimethoxyaniline |
| DMBI-Hz | 4-(5,6-Dimethoxy-2-benzimidazoyl)benzohydrazide |
| DMEQ-Hz | 6,7-Dimethoxy-1-methyl-2(1H)-quinoxalinone-3-propionylcarboxylic acid hydrazide |
| DNBC | 3,5-Dinitrobenzoyl chloride |
| DNPH | 2,4-Dinitrophenylhydrazine |
| DNPI | 3,5-Dinitrophenyl isocyanate |
| DNS-Hz | 5-(Dimethylamino)naphthalene-1-sulfonhydrazide |
| DNS-PZ | 5-(Dimethylamino)-1-naphthalenesulfonyl-semipiperazide |
| DPDS | 2,2′-Dipyridyl disulfide |
| ED | Electrochemical detection |
| EDC | 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride |
| ESR | Electron spin resonance |
| FA | Fatty acid |
| FFA | Free fatty acid |
| FL | Fluorescence |
| HA | Hydroxamic acid |
| HCPI | 2-(4-Hydrazinocarbonylphenyl)-4,5-diphenylimidazole |
| HETE | Hydroxyeicosatetraenoic acid |
| HMA | 9-(Hydroxymethyl)anthracene |
| Kriptofix 222 | 4,7,13,16,21,24-Hexaoxa-1,10-diazabicyclo-8,8,8-hexacosane |
| L-LeuBOX | 2-[4-(L-Leucyl)aminophenyl]-6-methoxybenzoxazole |
| LIF | Laser-induced fluorescence |
| LT | Leukotriene |
| Luminarin-4 | Quinolizinocoumarin |

| | |
|---|---|
| LX | Lipoxine |
| MDC | Monodansyl cadaverine |
| MEKC | Micellar electrokinetic chromatography |
| NB | Nitrosobenzene |
| NBD-APy | (+)- and (−)-4-Nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole |
| NBD-CD | 4-Nitro-7-(5-aminopentylamino)-2,1,3-benzoxadiazole |
| NBD-PZ | 4-Nitro-7-(1-piperazinyl)-2,1,3-benzoxadiazole |
| NE-OTf | 2-(2,3-Naphthalimino)ethyl trifluoromethanesulfonate |
| NPA | 2-Bromo-2′-nitroacetophenone |
| NPB | *p*-Nitrophenacyl bromide |
| NPH | 2-Nitrophenylhydrazine |
| PB | Phenacylbromide (2-bromoacetophenone) |
| PBr | *p*-(9-Anthroyloxy)phenacyl bromide (panacylbromide) |
| PDAM | 1-Pyrenyldiazomethane |
| PDC | Pyridinium dichromate |
| S(−)-PEAS | (−)-Phenylethylamine |
| PG | Prostaglandin |
| D-PgBOX | 2-[4-(D-Phenylglycyl)aminophenyl]-6-methoxybenzoxazole |
| L-PheBOX | 2-[4-(L-Phenylalanyl)aminophenyl]-6-methoxybenzoxazole |
| PNBA | *p*-Nitrobenzyloxyamine hydrochloride |
| PPB | *p*-Phenylphenacyl bromide |
| PS-NB | Poly(4-nitrobenzyl *p*-styrenesulfonate) |
| PS-NE | Poly[2-(1-naphthyl)ethyl *p*-styrenesulfonate)] |
| PS-PE | Poly[(2-phthalimino)ethyl *p*-styrenesulfonate] |
| RIA | Radioimmunoassay |
| SDS | Sodium dodecyl sulfate |
| Synperonic NP-12 | Polyoxyethylene(12)nonylphenyl ether |
| TDeABr | Tetrakis(decyl)ammonium bromide |
| TEA | Triethylamine |
| TPP | Triphenylphosphine |
| TX | Thromboxane |

## 1. Introduction

Carboxylic acids are widely distributed in nature and important as nutritional substrates and metabolites in living organisms. Many kinds of AA metabolites, including HETEs, LTs, TXs and PGs, which are commonly known as eicosanoids, are present in biological specimens, and play physiologically important roles at trace levels in the regulation of a variety of physiological and biological functions. Therefore, there is a widespread interest in the isolation and quantitation of these compounds. It has not been possible to accurately estimate PG and TX levels in biological tissues because of their presence in extremely low quantities. Thus, a highly sensitive and simple method for the determination of the metabolites is required for biological and biomedical investigations. The detection of these compounds at trace levels using absorptiometry is fairly difficult because of their weak absorption in the UV-Vis region. Furthermore, the interference by endogenous substances in real samples causes difficulties in measurement. This necessitated the use of sensitive techniques for measurement of these substances. Sophisticated and time-consuming methods, such as RIA and GC–MS, are used to detect picogram levels of these eicosanoids in tissues. Although RIA offers excellent sensitivity in the picogram range, this method is not suitable for the analysis of multiple components in a single experiment. While total analysis of multi-components with highly sensitive detection is possible by GC–MS, the method requires complex pretreatments including multi-step derivatization. Therefore, there was a need for more convenient methods that are simple and reliable.

Since many of biologically important carboxylic acids do not exhibit efficient absorption (UV-Vis), luminescent properties (FL and CL) or electrochemical (EC) activity, chemical derivatization with a suitable tagging reagent can be used to confer these properties. Thus, detection of the substances can be enhanced. Derivatization methods are classified into two categories, i.e. labelling of substances prior to column separation and on-line post-column derivatiza-

tion of substances in the column eluates. In this review, attention is mainly focused on the pre-column tagging techniques for UV-Vis, FL, CL and EC detection of the carboxylic functional group in a variety of higher carboxylic acids in biological samples. The scope of this review is limited to recent advances in tagging techniques for carboxylic acids involving FFAs, PGs, LTs and TXs for highly sensitive and selective detection by HPLC. Some typical examples of the use of those reagents for the determination of higher carboxylic acids, FFAs, PGs, LTs and TXs, in biological samples are also described. Derivatization for GC is not included in this review because most of the recent advances have been directed at HPLC detection.

## 2. Derivatization and application

A major part of modern analytical problem solving deals with the trace level detection of contaminants in biological, biomedical, food and environmental samples. In the analysis of these samples, chromatographic techniques play a predominant role. Even with efficient separations coupled with sophisticated detection techniques, development of adequate selectivity and/or sensitive detection can be challenging for many samples. In such cases, special attention has to be devoted to derivatization or conversion of the analyte(s) to improve detection sensitivity and/or selectivity. Sample pretreatment is also another important aspect to consider to avoid interference due to endogenous materials.

Although GC methods with derivatization are used extensively for carboxylic acid analyses, there are disadvantages, particularly with respect to heat-labile compounds. In order to overcome some of these shortcomings, a number of HPLC methods have been introduced. These methods usually offer insufficient resolution of the biologically important carboxylic acids because the separation and detection without labelling are neither selective nor sensitive, due to lack of suitable chromophores in the target carboxylic acids and interference of endogenous materials in the samples. Furthermore, absorbance of underivatized compounds near 200 nm cannot be recommended because of the adsorbance of the mobile phase. Impurities in organic solvents are especially undesirable in gradient elution. In order to increase the sensitivity and selectivity of detection, a number of UV-absorbing, fluorescent, chemiluminescent and electrochemically active derivatives have been prepared.

### 2.1. UV-Vis detection

Tagging of carboxylic acids with reagents that afford chromophores having UV and Vis bands has become quite common in the past two decades. Many reports concerning the use of such reagents have appeared. The application of these reagents to higher carboxylic acids is summarized in Table 1. Among those reagents, PB and substituted PBs have been widely accepted for the determination of FFAs. They are easily prepared in quantitative yields, and permit nanogram levels of detection of FAs. The tagging reaction with these reagents is usually performed in organic solvents containing an alkaline reagent (KOH or $K_2CO_3$) with catalysis with crown ethers to produce UV-absorbing derivatives. These types of reagents have been successfully used for the determination of FAs in biological samples, e.g. human red blood cells [1], rat adipose tissue and blood vessel walls [2]. Fig. 1 shows chromatograms of FFAs labelled with PB [2]. Since FAs are found throughout the living body at high concentrations, the determination is relatively easy. When sensitive detection at the femtomole level in real samples is required, extensive pretreatment is necessary to obtain adequate results because the selectivity of the methods referenced above is fairly low due to detection in the UV region around 250 nm.

*Cis* and *trans* isomers of unsaturated FAs, which are converted into phenacyl esters with PB, were separated by silver-ion chromatography [3]. The technique is useful for separating ester derivatives of geometrical isomers of FAs. A stable ion-exchange column loaded with silver ions is useful for the isolation of FAs containing *trans* double bonds. This technique has been applied to natural fats, margarines, cooking fats,

Table 1
UV-Vis tagging reagents and their application to real samples

| Reagent | Catalyst | Wavelength (nm) | Sample and treatment | Reference |
|---|---|---|---|---|
| PB | TEA | 242 | FAs in rat adipose tissue, blood vessel wall, boiling water bath 5 min. Separon SGX $C_{18}$ | 2 |
| PB | TEA | 242 | *cis* and *trans* unsaturated FAs from hydrogenated soybean oil HPLC in silver-ion mode | 3 |
| PB | TEA | 242 | FAs from frog retinal lipids, boiling water, 5 min, Supelco LC-18 | 26 |
| BPB | 18-crown-6 | 254 | FFAs and bounded FAs in human red blood cell, 80°C, 20 min, RP-18 | 1 |
| BPB | 18-crown-6 | 245 | low-molecular-mass FAs in industrial emission, 80–90°C, 15 min Develosil ODS-3 | 4 |
| BPB | 18-crown-6 + KOH | 254 | $\omega$- and ($\omega$-1)-hydroxy-FAs by liver microsome, 80–100°C, 15 min $\mu$-Porasil | 7 |
| BPB | Synperonic NP-12 TDeABr | 254 | Saturated long-chain FAs, 60°C, 20 min in dark | 25 |
| BPB | 18-crown-6 + KOH | 254 256 | Halogen adducts of FAs, 80°C, 60 min, Kaseisorb ODS | 6 |
| BPB, PPB | Kriptofix 222 | 288 | FFAs in baltic sea water, 80°C, 15 min | 5 |
| BPB, NPB | DIPEA | 254 | PGs formed from in vitro synthetase incubation, 45°C, 2.5–3.0 h $\mu$-Bondapak $C_{18}$ | 10 |
| PPB | Kriptofix 222 | 288 | FFAs in baltic sea water, 85°C, 20 min, microbore-HPLC | 8 |
| NPB | DIPEA | 254 | Covalently bound FAs, 65°C, 15 min, Ultrasphere ODS | 9 |
| BAN | DIPEA | 254 | PGs by silver-modified normal-phase HPLC, 45°C, 1 h | 12 |
| BAN | DIPEA | 254 | $PGA_1$ and $PGB_1$, RT, 90 min, LiChrosorb Si-100 | 11 |
| NPH | EDC + pyridine | 230 | FAs in L-$\alpha$-phosphatidylcholine or in fat and oil, 60°C, 20 min, YMC-C8 | 13 |
| NPH | EDC + pyridine | 230 | Short-chain FAs in serum, 60°C, 20 min, YMC-C8 | 14 |
| NPH | EDC + NaOH | 550 | Post-column reaction, non-ionic surfactants of FAs esters, 70°C and 100°C | 15 |
| PDC | | 229, 230 | PGs oxidized to 15-oxo-PGs, microbore $C_{18}$ | 16 |
| PDC | | 230 | PGs in human seminal fluids, oxidized to 15-oxo-PGs, microbore $C_{18}$ | 17 |
| HAs | $CH_3ONa$ | 213 | FA profiles of lipids, Nucleosil $C_{18}$ | 18 |
| PS-NB | 18-crown-6 + $K_2CO_3$ | 254 | FAs, 47°C, 30 min, TSK gel ODS-80TM | 20 |
| PS-PE | 18-crown-6 + $K_2CO_3$ | 221 | FAs, 85°C, 2 h, TSK gel ODS-80TM | 20 |
| DNPI | Pyridine | 226 | Hydroxy-FA-methyl esters, enantioseparation with chiral capillary column | 21 |
| *S*(−)-PEA | TEA | 214 | Benzidene-PGs, RT, 15 min, Zorbax CN, Zorbax TMS, CSP columns | 22 |

and hydrogenated oils (e.g. soybean, rapeseed and fish). Determination of FA vapors in industrial emissions [4], the composition of FAs in particulate matter from the Baltic Sea [5], halogen adducts of FAs [6], and hydroxylated metabolites of FAs in liver microsomes [7] have

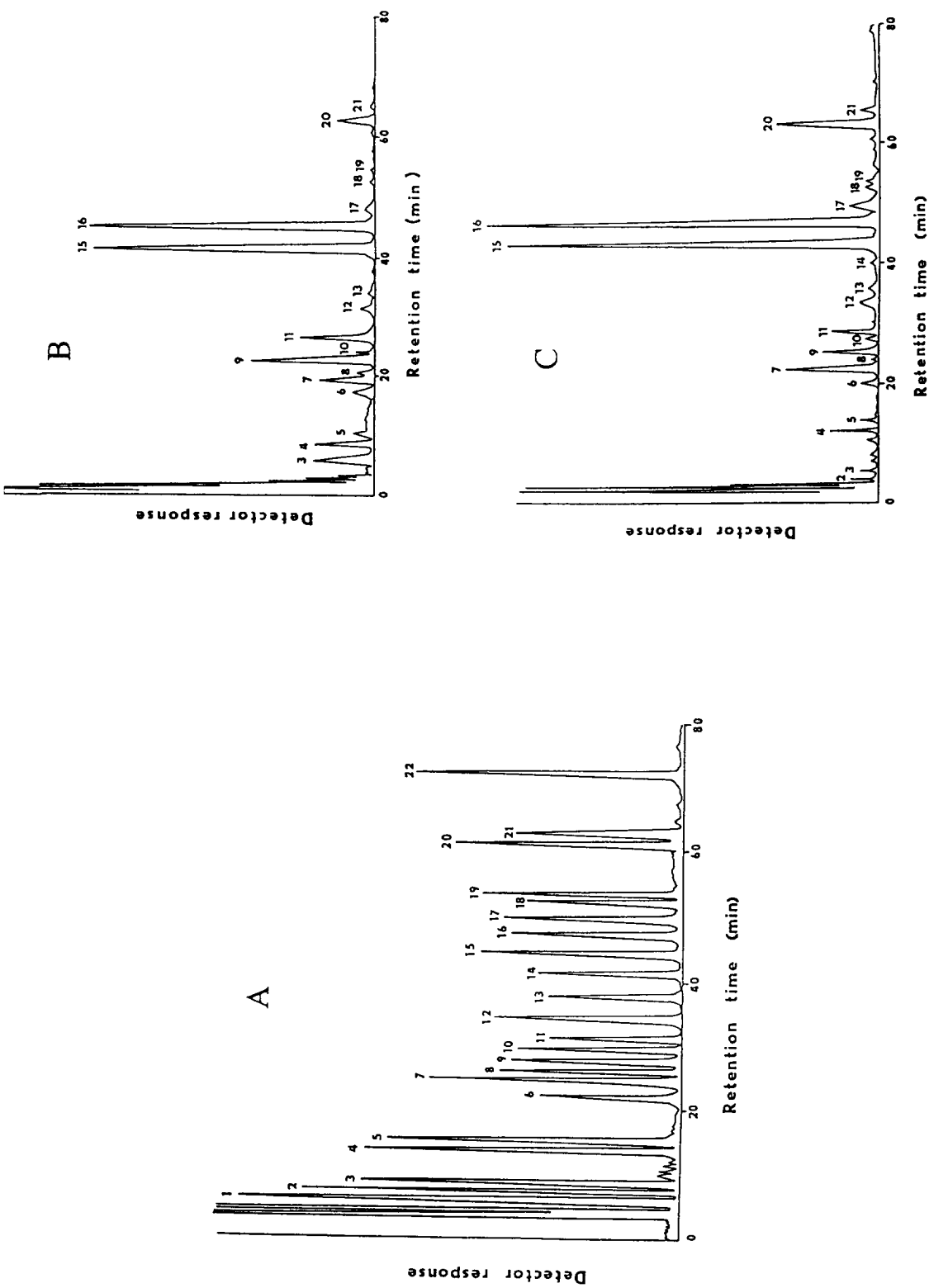



Fig. 1. HPLC separation of FA phenacyl esters. Chromatograms: (A) standard mixture; (B) adipose tissue of a rat fed diet A (cereal based); (C) blood vessel wall of a rat fed diet A. Peaks: 1 = $C_{6:0}$; 2 = $C_{8:0}$; 3 = $C_{10:0}$; 4 = $C_{12:0}$; 5 = $C_{14:1}$; 6 = $C_{18:3}$; 7 = $C_{14:0}$; 8 = $C_{22:6}$; 9 = $C_{16:1}$; 10 = $C_{20:4}$; 11 = $C_{18:2cc}$; 12 = $C_{15:0}$; 13 = $C_{18:2tt}$; 14 = $C_{20:3}$; 15 = $C_{16:0}$; 16 = $C_{18:1c}$; 17 = $C_{18:1t}$; 18 = $C_{20:2}$; 19 = $C_{17:0}$; 20 = $C_{18:0}$; 21 = $C_{20:1}$; 22 = $C_{22:1}$. Column, Separon SGX $C_{18}$ (250 × 4 mm I.D., 5 μm) at 40°C; gradient elution, methanol–acetonitrile–water (40:40.5:19.5, v/v, 0 min; 81.5:0:18.5, v/v, 25–27 min; 90:0:10, v/v, 70 min; 100:0:0, 90 min); detection, UV absorbance at 242 nm; flow-rate, 1.0 ml/min. Reprinted with permission from Ref. [2].

also been reported as analyzed by other applications of this type of reagent. PPB [8] and NPB [9] are similar types of reagents. The chromatographic separation of FFAs in sea water on a microbore column after labelling with PPB is shown in Fig. 2. Almost all reports on this type of reagents describe the analysis of FAs. One exception is the determination of PGs in seminal vesicles [10]. However, the detection potential was not very good. To improve the detectability, BAN, which is similar to PB, is employed for the determination of PGs by conversion of the carboxylic acid to a naphthacyl ester. The PG derivatives were better resolved than the phenacyl derivatives due to lower polarity [11] and detectability is better at 254 nm because of the high molar absorptivity ($\epsilon$) of ca. 37 000 [12].

Miwa and Yamamoto [13,14] demonstrated that FAs converted into NPH could be separated by reversed-phase HPLC with methanol–water as the eluent. The NPH method permits the direct derivatization of saponified samples of fats and oils in the presence of EDC without the need for extraction steps [13,14]. The detection limits with UV detection at 230 nm are in the range of 200–400 fmol per injection [14]. It is possible to develop a violet color with the hydrazide derivatives, which is detected at 550 nm, in strong alkaline medium with a post-column reactor [15].

PGs oxidized with PDC were detected at high sensitivity (0.15 pmol) by using a photometric detector equipped with a cadmium UV emission source. The resulting 15-oxo-PGs have a UV

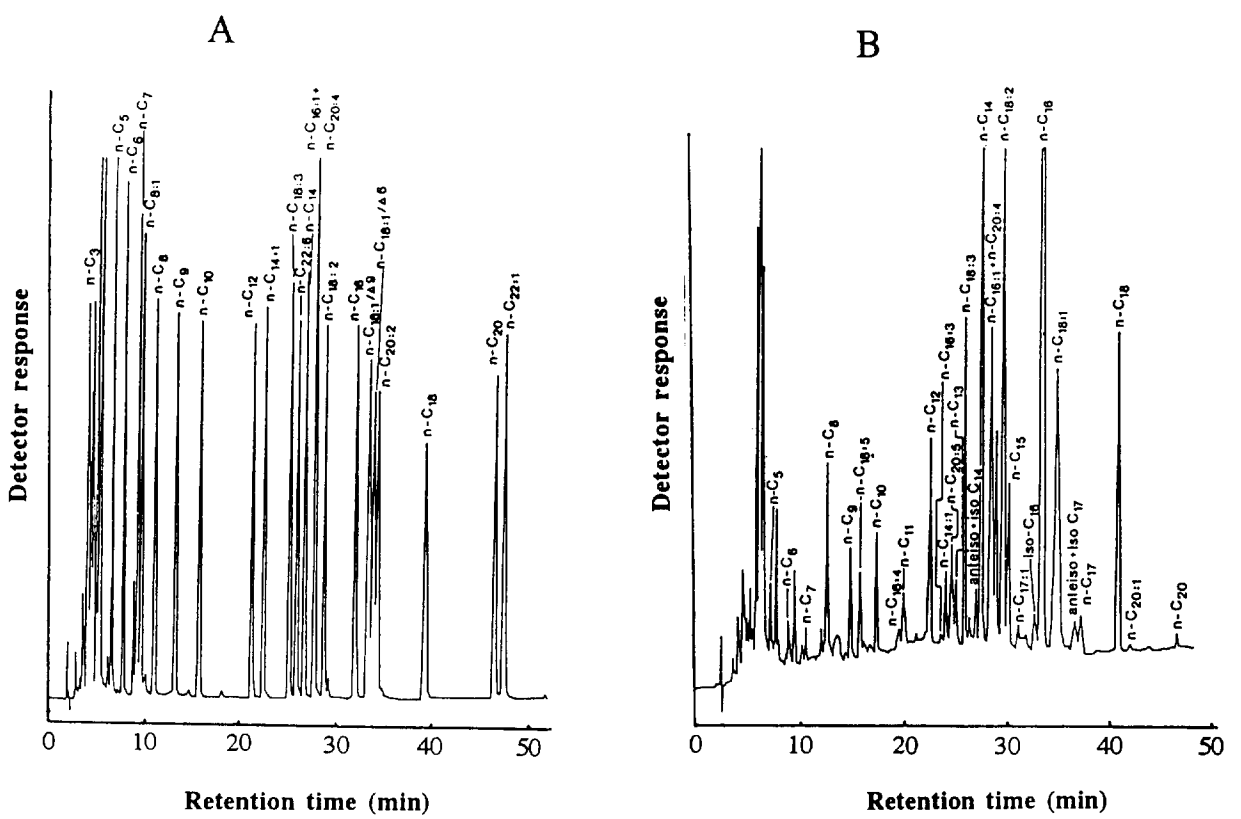



Fig. 2. Microbore HPLC chromatograms of FA phenylphenacyl esters. Chromatograms: (A) standard mixture (100 pmol each); (B) sea water. Column, Supersphere RP-8 (250 × 1.6 mm I.D., 4 $\mu$m); eluent, acetonitrile–water; gradient, 80–82% acetonitrile (0–10 min), 82–90% acetonitrile (10–27 min), 90–93% acetonitrile (27–32 min), 93% acetonitrile (32–47 min), 90–100% acetonitrile (47–62 min) and 100% acetonitrile (62–79 min). Reprinted with permission from Ref. [8].

absorption maxima at ca. 230 nm, which is the emission maximum of cadmium [16]. This oxidation method was applied to human seminal fluids [17] for the determination of PGs. Analyses of FAs as their hydroxamic acids [18] and monoalkanoylamides [19] have been also reported.

Recently, polymeric reagents, PS-NB and PS-PE, have been developed for the analysis of FAs [20]. These reagents are easily eliminated from the resulting derivatives by filtration or centrifugation [20].

After tagging with DNPI, the chromatographic separation of 2-hydroxy-FA enantiomers was performed on a chiral capillary column [21]. As another example of chiral separation, the determination of enantiomeric purity of a benzindene-PG was carried out after diastereomer formation with a chiral tagging reagent, *S*(−)-PEA [22].

Since LTs and TXs absorb in the UV region, it is possible to determine non-labelled LTs and TXs in human serum [23] or granulocytes [24] by HPLC with UV detection.

### 2.2. FL detection

HPLC with FL detection has emerged as one of the most powerful means for trace determination due to its high sensitivity; however, relatively few chemical compounds exhibit native fluorescence. In order to make the method useful for a much wider range of analytes, derivatization or chemical transformation of the analytes with various reagents have been carried out.

Recent efforts have been focused on new tagging reagents because fluorometry is both sensitive and selective. An increasing number of papers dealing with the development of novel reagents and fluorescence labels have appeared. Native fluorescent compounds in real samples usually emit in the relatively short wavelength region of 300–400 nm. As a result, the potential for interference by other naturally occurring is always present. Hence, the fluorescence properties of labelled derivatives can be exploited even with naturally fluorescent compounds because the derivatives have better chromatographic and fluorescence properties. Many fluorogenic tagging reagents reported for the carboxyl functional group are currently used for the analysis of higher carboxylic acids. Generally, excellent tagging reagents exhibit the following characteristics: (1) the reagent and its hydrolysate show no fluorescence, (2) the reagent should react with target compounds selectively and rapidly, and (3) the resulting derivatives should be stable and preferably have fluorescence properties in the longer wavelength region to avoid interference by native substances in samples. Another detection mode like chemiluminescence, involving LIF instead of FL, can also be used, but depends on the fluorescent properties of the resulting derivatives. Since only few ideal reagents have been reported to this day, the development of novel tagging reagents is still required in spite of the many already existing reagents. Their applications to higher carboxylic acids in real samples and wavelengths for maximal fluorescence of the resulting derivatives are listed in Table 2.

Although a large number of reagents have been described, most reactions must be carried out in non-aqueous media, because water inhibits most of the reactions. Many applications utilizing reagents having a coumarin structure, such as BrMMC and BrMAC, have been reported. BrMMC was synthesized by Baker and Collins in 1949 [27]. It was first reported by Dünges as a tagging reagent at the end of 1977 [28]. The applicability of the reagent has been demonstrated for a great variety of compounds including aliphatic and aromatic carboxylic acids, herbicides, barbiturates, drugs, PGs, etc.

The tagging reaction of carboxylic acids with BrMMC [29–32] proceeds in aprotic solvents such as acetone and acetonitrile in the presence of a base catalyst (e.g. $K_2CO_3$) and a phase-transfer agent (e.g. 18-crown-6). The reaction mechanism is given in Fig. 3. Carboxylic acid forms the potassium salt under reflux. The salt is dissolved with the crown ether in the aprotic solvent, and then reacts with the BrMMC reagent.

FAs are frequently derivatized with BrMMC. Acids in serum or plasma [33–37], rat brain

Table 2
FL tagging reagents and their application to real samples

| Reagent | Catalyst | Wavelength (nm) | | Sample and treatment | Reference |
|---|---|---|---|---|---|
| | | ex. | em. | | |
| BrMMC | 18-crown-6 + $K_2CO_3$ | 325 | >398 | FAs in rat blood, sonication for 30 min, Chromspher $C_{18}$ | 31 |
| BrMMC | dibenzo-18-crown-6 + $KHCO_3$ | 325 (He–Cd laser) | 420 | FAs in fish oil dietary supplements, 37°C, 6 h in dark capillary column | 32 |
| BrMMC | 18-crown-6 + $K_2CO_3$ | 320 | >370 | Oxirane carboxilic acids in serum, 60°C, 30 min in dark, RP-8 | 36 |
| BrMMC | 18-crown-6 + KOH | 360 | 410 | FAs, reflux, 25 min in dark, reversed-phase HPLC | 39 |
| BrMMC | EDTA-3K + 18-crown-6 | 328 | 380 | FA metal salts in river water, 70°C, 30 min, Deverosil $C_8$ | 41 |
| BrMMC | 18-crown-6 + $K_2CO_3$ | 330 | 410 | $TXB_2$ in human platelets, 70°C, 30 min, LiChrosorb 100 Diol | 45 |
| BrMAC | dibenzo-18-crown-6 + $KHCO_3$ | 365 | 460 | PGs in human seminal fluid, 80°C, 1 h in the dark LiChrosorb RP-18 | 47 |
| BrMAC | dibenzo-18-crown-6 + $KHCO_3$ | 365 | 460 | FFAs in healthy human blood and diabetic patients, 50°C, 30 min in the dark, LiChrosorb RP-18 | 48 |
| BrAMC | Duolite A-375 (anion-exchange resin) | 365 | 417 | FFAs in human plasma of normal and diabetes, RT, 30 min Spelco LC-18 | 50 |
| BrAMDC | 18-crown-6 + $KHCO_3$ | 388 | 475 | FFAs in human blood plasma, RT, 30 min, Unisil Pack $5C_{18}$-250A | 51 |
| PBr | TEA | 254 | 415 | $TXB_2$ in human platelets, 45°C, 30 min, LiChrosorb 100 Diol | 45 |
| PBr | TEA | 375 | 470 | PGs in human peripheral blood adherent cell, 37°C, 3 h, Nova-Pak $C_{18}$ | 57 |
| PBr | TEA | 365 | >475 | Myocardial PGs and TXs, 40°C, 2 h, Bondapak $C_{18}$ | 58 |
| PBr | 18-crown-6 + TEA + cesium fluoride | 249 | >413 | PGs in epithelial cell and fibroblast of rat mammary gland, 21°C, 18 h, Zorbax-Sil | 59 |
| PBr | TEA | 360 | >419 | PGs and TXs in whole blood of healthy volunteer, LiChrocart Superspher 100-RP-18. | 60 |

(Continued on p. 100)

Table 2 *(Continued)*

| Reagent | Catalyst | Wavelength (nm) | | Sample and treatment | Reference |
|---|---|---|---|---|---|
| | | ex. | em. | | |
| ADAM | | 365 | 412 | PGs in incubation mixture of rat pleural cell, RT over night, reversed-phase ODS columns | 64 |
| ADAM | | 350 | 412 | PGs and TXs in human plasma, RT, Hitachi ODS | 66 |
| ADAM | | 365 | 412 | Icosapentaenoic acid and arachidonic acid in fish and plankton living in sea area, RT, Zorbax $C_8$ | 67 |
| ADAM | | 365 | 412 | FAs in butterfat without saponification, RT, 1 h, LiChrosorb RP-18 | 70 |
| BrDMEQ | 18-crown-6 + $K_2CO_3$ | 370 | 455 | FFAs in serum from normal subject and patient, 50°C, 20 min in the dark, YMC-Pack $C_8$ | 74 |
| BrDMEQ | 18-crown-6 + $KHCO_3$ | 370 | 455 | PGs in human seminal fluid, 50°C, 15 min in the dark, YMC-Pak $C_8$ | 75 |
| BrMMEQ | 18-crown-6 + $KHCO_3$ | 370 | 455 | AA metabolites in stimulated leukocyte, 50°C, 15 min in the dark, Inertsil ODS | 77 |
| DMEQ-Hz | EDC + pyridine | 360 | 435 | FFAs in human serum from healty persons, 37°C, 10 min, YMC-Pack $C_8$ | 79 |
| HPCI | EDC + pyridine | 335 | 455 | Saturated FFAs in normal human serum, RT, 45 min, Shim-pack CLC-ODS | 81 |
| DNS-Hz | HCl | 365 | 505 | PGs in rat tissues and human urine, 40°C, 30 min, Fine pack SIL $C_{18}$ | 82 |
| DNS-PZ | DCC | 350 | 530 | FFAs in human blood plasma, RT, 30 min, Hitachi $C_{18}$ 3056 | 86 |
| MDC | DEPC | 340 | 518 | FFAs in rabbit plasma, RT, 15 min, TSK-gel ODS 80TM | 85 |
| NE-OTf | 18-crown-6 + KF | 259 | 394 | Carboxylic acids in mouse brain, RT, 10 min, Chemcosorb $5C_{18}$ | 97 |

homogenates [33,38], vegetable oils [39,40], river water [41], lake sediments [42], and atomospheric particulate matter [43] were analyzed by HPLC after labelling with the reagent. Recently, femtomole level detection of BrMMC-labelled FAs in fish oil dietary supplements was achieved by using a high-efficiency capillary column (240 000 theoretical plates), combined with

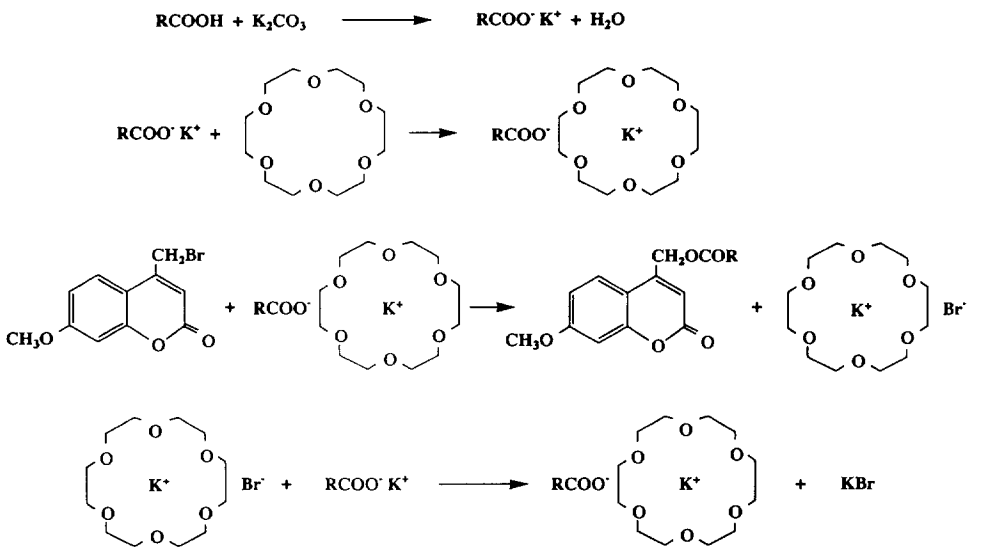



Fig. 3. Reaction scheme of base-catalyzed derivatization reaction in the presence of crown ether.

helium–cadmium laser detection at 420 nm (excitation at 325 nm) (Fig. 4) [32]. However, only few applications of the reagent to PGs and TXs etc. were developed [44,45].

Tsuchiya et al. [46] reported the use of BrMAC, a similar coumarin-type reagent, for the labelling of carboxylic acids. From the study of fluorescence intensities of several coumarin derivatives, it was discovered that the fluorescence quantum yields are greatly affected by the substituent group at the 7-position of the coumarin structure. According to that study, an electron donating group such as the hydroxyl group tends to enhance the fluorescence intensity. Based on these results, the ester derivatives separated by reversed-phase chromatography were hydrolyzed to 4-methyl-7-hydroxycoumarin with an alkaline solution (0.1 *M* borate buffer, pH 11) in a reaction coil (10 m × 0.5 mm I.D. at 50°C). The fluorescence intensities obtained from this post-column detection system did not change with the acetonitrile concentration in the eluent. Detection limits of 10 fmol were attained in this system. The usefulness of the method was demonstrated by the determination of PGs in human seminal fluids [47], and FFAs in normal human blood and blood from diabetic patients (Fig. 5) [48]. Kelly et al. [49] also utilized this technique, with minor modifications of the method, for the analysis of endogenous carboxylic acids including AA metabolites.

Recently, Takadate et al. developed BrAMC [50] and BrAMDC [51] for the tagging of carboxylic acids. The tagging reaction with these reagents proceeds at room temperature in the presence of $KHCO_3$ or an anion-exchange resin. The reactivity is greater than that of the other 4-bromomethylcoumarin reagents.

Other coumarin-type reagents, 4-bromomethyl-6,7-dimethoxycoumarin (BrMDC) [52], 4-bromomethyl-6,7-methylenedioxycoumarin (BrMDOC) [53], 4-bromomethyl-7,8-benzocoumarin (BrBC), 4-diazomethyl-7-methoxycoumarin (DMC), 7-(diethylamino)-coumarin-3-carbohydrazide (DCCH) and 7-(diethylamino)-3-{4-[(iodoacetyl)amino]phenyl}-4-methylcoumarin (DCIA) [54] have been developed. However, these reagents have not been used for the analysis of higher carboxylic acids such as FFAs and PGs.

Although esters derived from PBr are UV absorbent [55,56], highly sensitive detection at the picogram range is possible with fluorometry (excitation at 375 nm, emission at 470 nm) [57–62]. The methods utilizing this reagent have been used for the determination of $TXB_2$ and pros-

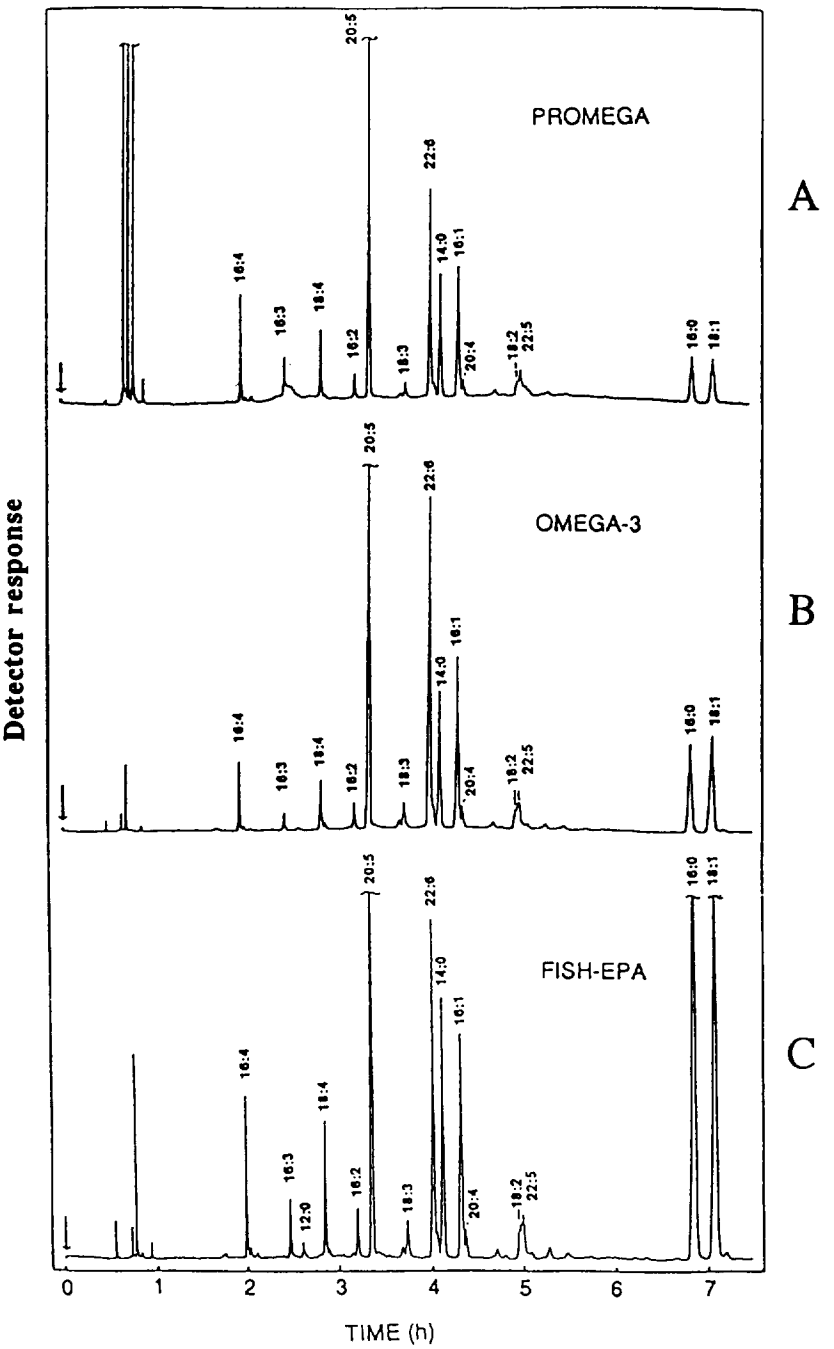



Fig. 4. He–Cd LIF detection of FAs constituents labelled with BrMMC in fish oil dietary supplement of Promega Pearls, Omega-3, and Fish-EPA. Column, Micro-Pak SP-18 (152 cm × 200 μm I.D., 3 μm) at 30°C; LIF detection, excitation at 325 nm, emission at 420 nm; mobile phase, methanol–acetonitrile–water (90:2:8, v/v); flow-rate, 0.75 μl/min. Reprinted with permission from Ref. [32].

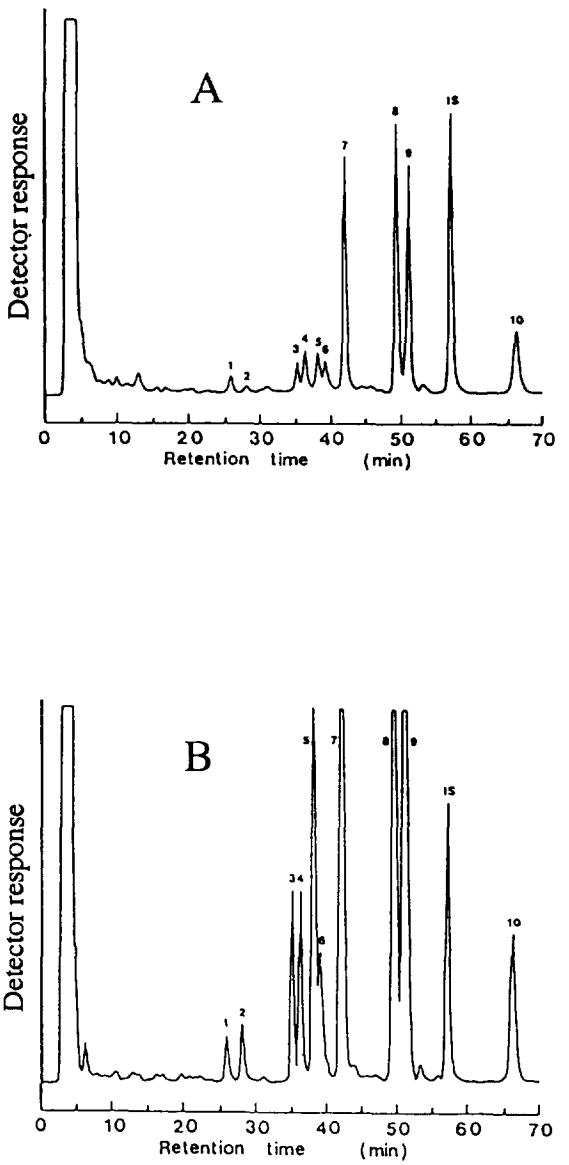



Fig. 5. Chromatograms obtained from human plasma samples. Chromatograms: (A) normal subject; (B) diabetic patient. Peaks: 1 = $C_{12:0}$; 2 = $C_{14:1}$; 3 = $C_{18:3}$; 4 = $C_{14:0}$; 5 = $C_{20:4}$; 6 = $C_{16:1}$; 7 = $C_{18:2}$; 8 = $C_{16:0}$; 9 = $C_{18:1}$; 10 = $C_{18:0}$ (I.S). Column, Lichrosorb RP-18 (250 × 4.0 mm I.D., 5 $\mu$m) at 40°C; mixing coil temperature, 50°C; mobile phase: first solvent, methanol–acetonitrile–water (35:35:30, v/v), second solvent, 90% aqueous methanol (the gradient was prepared by adding the second solvent to the first solvent); mobile phase flow-rate, 1.2 ml/min; flow-rate of alkaline solution for hydrolysis, 0.4 ml/min; FL detection, excitation at 365 nm, emission at 460 nm. Reprinted with permission from Ref. [48].

tacyclin metabolites (6-ketoPGF$_{1\alpha}$, 6-keto-PGE$_1$ and 13,14-dihydro-6,15-diketo-PGF$_{1\alpha}$) in tissue culture medium by reversed-phase LC [56]. Subpicogram detection of PGs and TXs in myocardial perfusates is possible with a high-gain photomultiplier and a xenon–mercury arc lamp (Fig. 6) [58]. PGs in epithelial cells and fibroblasts of the rat mammary gland were also assayed with fluorescence detection by normal-phase LC using a silica-gel column [59]. Since the tagging with PBr is essentially the same as with PB, it requires a catalyst and a crown ether as phase-transfer reagent.

ADAM [63–70] is an another important label for fluorescence detection of higher carboxylic acids. This reagent reacts with the carboxylic acid functional group under mild conditions at room temperature without a catalyst. Unfortunately, the reagent is apt to be contaminated with impurities and decomposition products. ADAM can not be stored for long periods as a solution, or even as a solid. Therefore, the reagent often requires purification just prior to use with a suitable method such as column chromatography [63]. To solve this problem, PDAM is prepared as a more stable aryldiazoalkane [71]. PDAM readily reacts with carboxylic acids at room temperature without a catalyst to form an intensely fluorescent ester (excitation at 340 nm, emission at 395 nm) [71]. Pre-column derivatization with ADAM has been applied to the determination of AA metabolites produced by washed human platelets and rat peritoneal macrophages (Fig. 7) [63]. PGs and TXs in the exudate of rat pleurisy induced by phorbol myristate acetate [64] and in human plasma [66], unsaturated FAs in ovary and testis in fishes and plankton [67] and FA in butterfat without saponification [70] have been studied with this reagent.

Yamaguchi and co-workers [72–79] have developed several quinoxalinone derivatives (e.g. BrDMEQ, BrMMEQ, and DMEQ-Hz) for carboxylic acid labelling. According to the reports, low femtomole detection is achieved by the methods using these reagents [74–77]. However, the conditions for tagging with BrDMEQ or BrMMEQ are not suitable for unstable com-

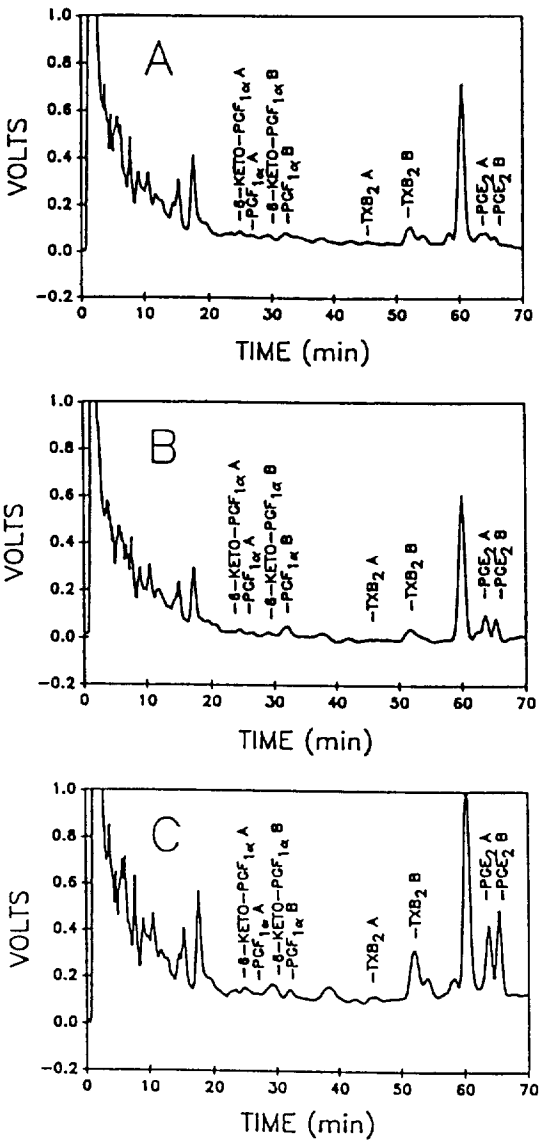



Fig. 6. Separation of panacyl derivatives of prostaglandins and thromboxane from perfusates of isolated rat hearts. Chromatograms: (A) baseline perfusate; (B) after 60 min of ischemia; (C) after 60 min of reperfusion. Column, Ultrasphere $C_{18}$ (7.5 cm × 4.6 mm I.D., 3 μm); FL detection, excitation at 365 nm, emission cut-off filter. Reprinted with permission from Ref. [58].

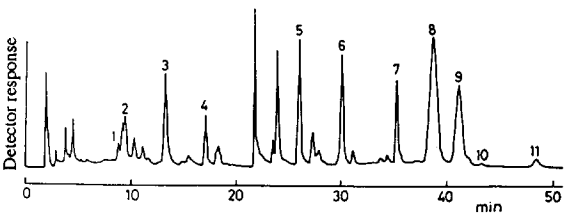



Fig. 7. Normal-phase chromatography of ADAM-derivatized AA metabolites produced in stimulated rat peritoneal macrophages. Peaks: 1 = 12-HETE; 2 = 15-HETE; 3 = HHT; 4 = 5-HETE; 5 = $PGB_2$ (I.S.); 6 = $LTB_4$; 7 = $PGD_2$; 8 = 6-keto-$PGF_{1\alpha}$; 9 = $TXB_2$; 10 = $PGE_2$; 11 = $PGF_{2\alpha}$. Column, PG-Pack B (25 × 0.46 cm I.D.); eluents: (A) isooctane–ethyl acetate–acetic acid (93:7:1, v/v), (B) isooctane–ethyl acetate–ethanol–acetic acid (80:15:4:2, v/v); stepwise elution: eluent A for 12 min, then eluent A–B (1:1, v/v) for 15 min and eluent B for 20 min; FL detection, excitation at 365 nm, emission at 412 nm; flow-rate, 1.2 ml/min. Reprinted with permission from Ref. [63].

pounds because of the relatively drastic reaction conditions. In contrast, hydrazide reagents are easily labelled in the presence of a condensation catalytic medium such as EDC/pyridine [78,79]. The FAs in the serum are directly tagged with DMEQ-Hz without chloroform extraction [79]. The FFAs in human serum [74] and PGs in human seminal fluid [75] were pre-labelled with BrDMEQ, separated on reversed-phase columns, and detected at the low-femtomole level (Fig. 8). FFAs in human serum [79] and $LTB_4$ in leukocytes [77] were also determined with DMEQ-Hz and BrMMEQ, respectively.

DMBI-Hz which has a benzimidazole moiety [80] and HCPI which has a lophine skeleton [81] were recently reported for the detection of FFAs. DNS-Hz [82,83] is a well-known hydrazide reagent. The reagent reacts with PGs in the presence of TEA as a catalyst to produce the corresponding hydrazone derivatives [83]. $PGE_2$ in rat organ and human urine are determined with a method utilizing DNS-Hz [82]. Other DNS derivatives for the analysis of carboxylic acids, DNS-cadaverine [84,85] and DNS-PZ [86] have been reported. Although these amine-type reagents require a catalyst such as DCC and DEPC which activates the carboxylic acid functional group, the reaction proceeds at room temperature in non-protic solvents like chloroform and DMF. DNS-PZ and DNS-cadaverine

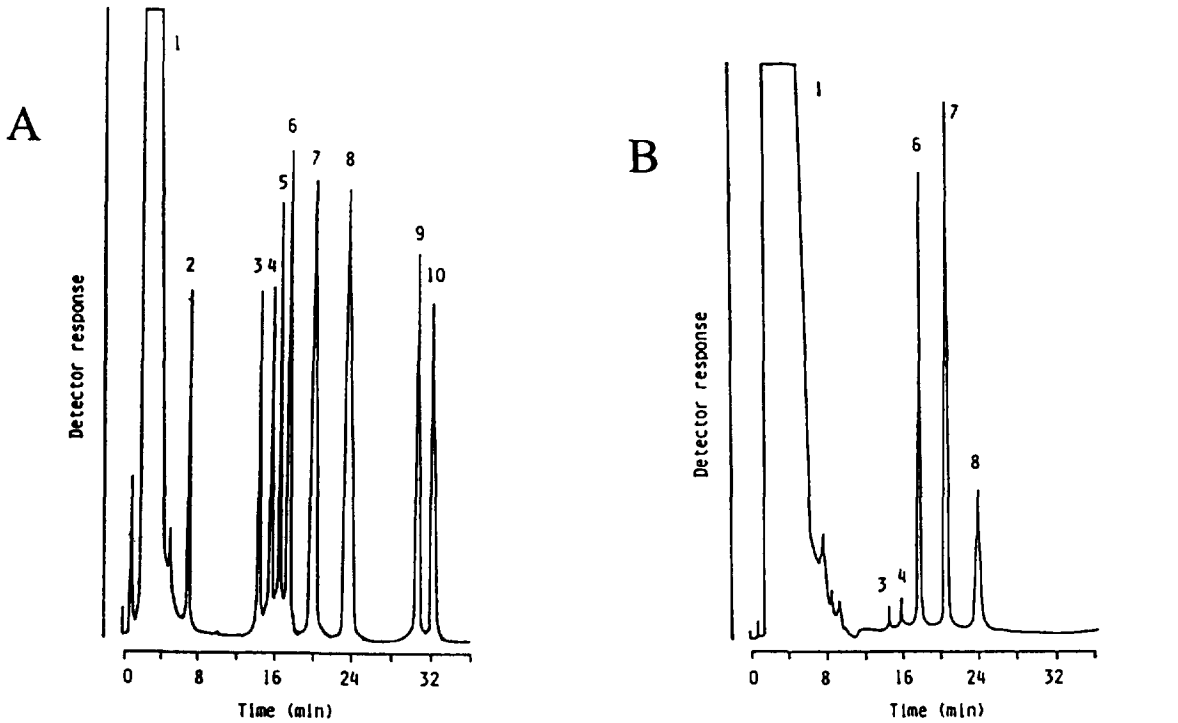



Fig. 8. Chromatograms of DMEQ-derivatives of PGs. (A) Standard mixture; (B) human seminal fluid. Peaks: 1 = BrDMEQ; 2 = 6-keto-$PGF_{1\alpha}$; 3 = $PGF_{2\alpha}$; 4 = $PGF_{1\alpha}$; 5 = $PGD_2$; 6 = $PGE_2$; 7 = $PGE_1$; 8 = 16-methyl-$PGF_{1\alpha}$; 9 = $PGA_2$ and $PGB_2$; 10 = $PGA_2$ and $PGB_2$. Column, YMC Pak $C_8$ (150 × 6 mm I.D., 10 μm) at RT; Eluents: (1) acetonitrile–methanol–water (35:10:55, v/v), (2) acetonitrile–methanol–water (35:30:35, v/v); Stepwise elution, eluent 1 for 26 min and eluent 2 for 10 min; FL detection, excitation at 370 nm, emission at 455 nm; flow-rate, 2.0 ml/min. Reprinted with permission from Ref. [75].

were used for the analyses of FFAs in human blood serum [86] and in rabbit blood plasma [85].

As another type of reagent, Toyo'oka et al. [87–89] reported the use of benzofurazan derivatives, e.g. NBD-PZ, NBD-CD, DBD-PZ, NBD-CD, ABD-ED and ABD-PZ. Among these reagents, DBD-PZ and DBD-CD react with FFAs [88] and PGs [89] in the presence of a condensation reagents such as DPDS/TPP and DEPC under mild conditions at room temperature to form the corresponding amide derivatives.

Chiral reagents (NBD-APy and DBD-APy) which have an asymmetric center in their structure have been developed for resolution of enantiomers of carboxylic acids [90–92]. This is based upon diastereomer formation with the reagents before HPLC separation. The method has been utilized for the resolution of N-acetyl amino acids and anti-inflammatory drugs such as naproxen and ibuprofen. However, there are no reports of resolution of optically active higher carboxylic acids with these reagents. The resulting derivatives with NBD-APy provide sensitive detection not only with conventional FL but also with LIF, while the DBD-APy derivatives are also sensitive in the peroxyoxalate CL method.

DAP [93], DICI [94], HMA [95], benzotriazole derivatives [96] and NE-OTf [97] are also reported as tagging reagents for the carboxyl functional group. Of these reagents, only NE-OTf has been utilized with a real sample, i.e. the determination of FFAs in mouse brain (Fig. 9) [97]. Although the derivatization requires a base

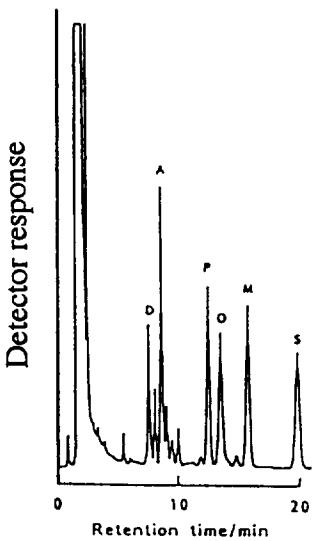



Fig. 9. Chromatogram of carboxylic acids in mouse brain. Peaks: D = docosahexaenoic acid; A = arachidonic acid, P = palmitic acid, O = oleic acid, M = margaric acid (I.S.), S = stearic acid. Column, Chemucosorb $5C_8$ (150 × 4.6 mm I.D., 5 μm); mobile phase, methanol–water (87:13, v/v); FL detection, excitation at 259 nm, emission at 394 nm; flow-rate, 1.0 ml/min. Reprinted with permission from Ref. [97].

(KF) and a crown ether (18-crown-6), the reaction conditions at room temperature for 10 min in acetonitrile are milder than those used for bromomethyl-type reagents such as BrMMC and BrDMEQ. Tanaka et al. [98] reported a fluorescent polymeric reagent (PS-NE) which is similar to PS-NB and PS-PE and which can be used as UV label.

Ohkura et al. [99] reviewed the FL derivatization methods for biologically important compounds including organic acids. This paper is helpful for the fluorescence analysis of organic compounds having various functional groups.

### 2.3. EC detection

EC detection is widely used for compounds that are readily reduced or oxidized to provide highly selective and sensitive methods for HPLC. Optimal detection is achieved with active compounds that are easily analyzed at low applied potential, because of the relatively low background current. Since elimination of reducible substances in eluents is usually difficult, determination by HPLC–ED in the oxidative mode is recommended. Furthermore, removal of oxygen dissolved in the eluents is very important in order to obtain a high signal-to-noise ratio in the reductive mode. Some AA metabolites such as LTs and LXs are electrochemically active [100,101]. However, ED methods with oxidation of LTs [101] and LXs [100] not only require applied potentials higher than + 1.2 V, they also give only limited sensitivity. $LTB_4$, 20-OH-$LTB_4$ [101], $LXA_4$ and $LXB_4$ [100] in human polymorphonuclear granulocytes were determined by HPLC–ED methods. Derivatization to an electrochemically active form is performed prior to HPLC separation to obtain highly sensitive detection. The tagging reagents for ED are rather few in number, e.g. AP [102], DMA [103], ferrocene derivatives [104], DNBC, NPA, PNBA and DNPH [105]. The tagging procedures of the carboxyl functional group are essentially the same as those used for UV or FL labelling reagents. Beck et al. [105] evaluated three aromatics, NPA, PNBA and DNPH, as tagging reagents for the detection of PGs by HPLC–ED. Methods utilizing these reagents require potentials more negative than −0.9 V, however, response plateaus were not observed. Among the three, DNPH was the best and PNBA was the least suitable. Generally, the detection limits with amperometric detection are not as good as with FL detection. The utility of the procedures was demonstrated by the detection of PGs in human urine and plasma [105]. Although ED of aromatic compounds is usually done in the reductive mode, detection in the oxidative mode is often possible [106]. Detection in the reductive mode (−0.7 V) of LT-dinitrobenzoyl esters is better than in the oxidative mode at + 1.15 V [106]. This method was applied to the detection of LTs in plasma, and nasal and bronchial fluids of asthma patients [106].

Compounds having phenol and catechol structures are easily detected with low oxidation potentials. Based on this, Ikenoya et al. [102] have developed AP for EC detection of carboxylic acids. The reagent reacts with carboxylic acids in the presence of BMPI and TEA to

produce the corresponding amide derivatives. The resulting derivatives are detected in the anodic mode as the *p*-benzoquinone compounds. The applied potential of more than + 0.7 V gives a stable response. The method was used for the determination of FFAs in guinea pig plasma and bile acids in human bile [102]. In a similar way, DMA was used for the determination of PGs [103]. The detection limit on the column was ca. 50 pg.

Shimada et al. [104] reported ferrocene derivatives (BrAFe and BrADMFe) as unique reagents. The reagents react with FAs in the presence of 18-crown-6 and KF. The excellent properties of these reagents are that the resulting derivatives are more easily oxidized at + 0.6 V than the other derivatives ( > + 1.0 V). High sensitivity at 0.5 pmol is another advantage of the method with BrADMFe [104]. The proposed method was applied to the determination of FAs in human serum (Fig. 10).

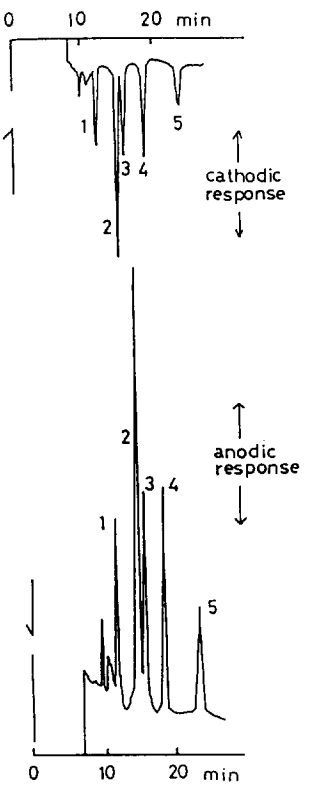



Fig. 10. Chromatogram of labelled FAs in human serum. Peaks: 1 = $C_{18:2}$; 2 = $C_{16}$; 3 = $C_{18:1}$; 4 = $C_{17}$ (I.S.); 5 = $C_{18}$. Column, TSK gel ODS-80TM (15 × 0.4 cm I.D., 5 μm) at RT; eluent, 0.1 *M* $NaClO_4$ in methanol–water (13:1, v/v); flow-rate, 1.0 ml/min; EC detection, upstream electrode + 0.6 V, downstream electrode + 0.2 V. Reprinted with permission from Ref. [104].

## 2.4. CL detection

The detection of trace amounts of organic compounds by a CL reaction is a potentially very sensitive technique. Since the CL reaction emits light without excitation by a light source, interference from this light source, often encountered in fluorescence analysis, is not problem. Therefore, dynamic ranges for detection up to three orders of magnitude can be theoretically achieved by the CL reaction system. Fluorescent compounds having a low excitation energy are usually used in chemiluminometry.

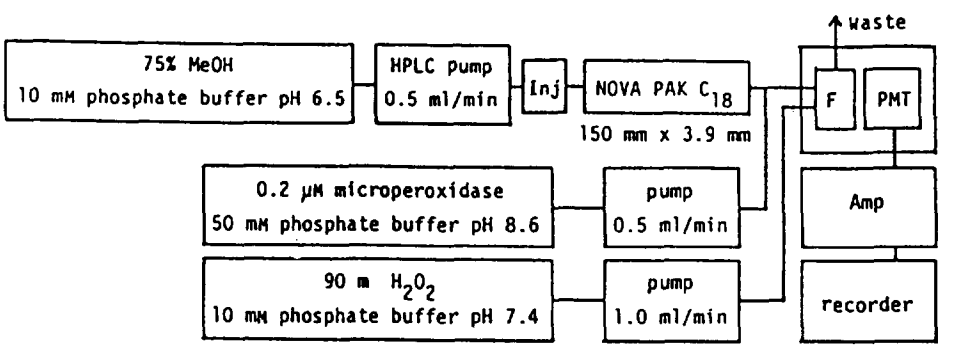
It is possible to determine FFAs by utilizing specific enzymes, such as acyl-CoA synthetase (ACS) and acyl-CoA oxidase (ACO) [107–109]. FFA is activated by ACS to acyl-CoA in the presence of ATP and CoASH. The acyl-CoA is converted to 2,3-trans-enoyl-CoA and hydrogen peroxide ($H_2O_2$) by ACO. The resulting $H_2O_2$ is detected with spectrophotometry, fluorometry and luminometry (CL and bioluminescence). In the case of CL, the $H_2O_2$ reacts with chemiluminogenic reagents such as luminol [109] and isoluminol [107,108] to yield the corresponding emitter in the presence of a peroxidase catalyst such as horse-radish peroxidase (HRP). However, it is generally difficult to obtain picogram detection with a method based upon enzyme reactions.

Tagging with a chemiluminogenic reagent before the HPLC separation can also be employed. The method is based upon the pre-labelling of the carboxyl functional group with a chemiluminogenic reagent and then conversion to an emitter with co-oxidation systems such as $H_2O_2$–potassium hexacyanoferrate(III) [110] or $H_2O_2$–peroxidase [111,112] in alkaline solution. Luminol derivatives are commonly used for this purpose. Karatani [113] reported the CL quantum efficiency ($\phi_{CL}$) of luminol and its analogs in

an $H_2O_2$–$K_2S_2O_8$ oxidation system in $Na_2CO_3$ solution. ABEI and ABMI produce higher quantum yields than other derivatives such as isoluminol. FFAs pre-labelled with ABEI were detected with post-column CL using an $H_2O_2$–$K_3Fe(CN)_6$ reaction system as detector after reversed-phase HPLC [110]. Eicosapentaenoic acid (EPA) and some FFAs in human serum were determined with a similar method (Fig. 11) [111]. Femtomole detection of carboxylic acids is achieved with these methods. Tod et al. [114] reported that luminarin-4, which is another type of tagging reagent, reacts with carboxylic acids in the presence of activation reagents such as N-hydroxysuccinimide (NOHS) and DCC. The lower limit of CL detection with the oxalate ester and $H_2O_2$ with the $PGE_2$ derivative is 32 fmol [114]. The derivatives obtained with DBD-CD, DBD-PZ [88] and DBD-APy [115] are sensitively detected with an oxalate ester–$H_2O_2$ CL reaction system. Since low fmol detection of carboxylic acids such as anti-inflammatory drugs was realized with the CL reaction system, the proposed method may be applicable to the detection of higher carboxylic acids in biological samples.

### 2.5. Other detection techniques

Kusaka and Ikeda [116,117] have developed a liquid chromatographic–mass spectrometric method with an atomospheric-pressure chemical-ionization (LC–APCI-MS) interface system for analyzing amide FAs derivatives, i.e. anilide




HPLC System with Chemiluminescence Detector

Amp. amplifier; F, flow cell; Inj, injector; PMT, photomultiplier.

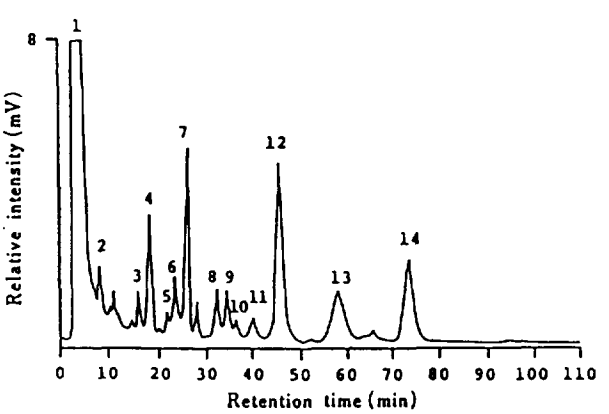



Fig. 11. Chromatogram of FA in human serum. Peaks: 1 = ABEI; 2 = lauric acid; 3 = myristic acid; 4 = linolenic acid; 5 = eicosapentaenoic acid; 6 = palmitoleic acid; 7 = unknown; 8 = linoleic acid; 9 = arachidonic acid; 10 = docosahexaenoic acid; 11 = dihomo-r-linoleic acid; 12 = palmitic acid; 13 = oleic acid; 14 = margaric acid. Reprinted with permission from Ref. [111].

[116], N-*n*-propylamide [117]. This method was applied to the detection of various kinds of hydroxy- and non-hydroxy-FAs in rat brain [117]. With respect to the sensitivity, 10 pg N-*n*-propylpalmitamide and 25 pg palmitanilide are detectable by the method [117]. Similarly, some hydroperoxy-FAs split from photo-oxidized lecithin after treatment with phospholipase $A_2$ were detected and identified with the LC–APCI-MS system [118]. In this case, 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (BrMB) [119] was used as the tagging reagent for the FAs. An important aspect of the MS method is the capability to distinguish the detailed structures of the compounds of interest. This is difficult or impossible to achieve with spectrophotometric and chromatographic methods, which are widely employed in the analysis of common FAs. Although structural identification is performed in many cases by GC–MS, specific compounds like hydroperoxy-FAs are too labile to be analyzed by GC. On the other hand, the applicability of LC–MS methods is limited because of difficulties such as restrictions on the buffer salts and solvents that can be used as eluents.

Another unique technique was described by Sugata et al. [120] who used an HPLC–ESR method for the determination of radical species derived from polyunsaturated FAs upon reaction with lipoxygenase. This method is based upon HPLC analysis of spin adducts of nitrosobenzene with the radical species. However, the method is not as sensitive (ca. 1500-fold) as UV detection at 300 nm. The identification of the radical species of linoleic, linolenic and arachidonic acids, and the comparison of the sensitivities was done by combining UV and ESR detection.

## 3. Conclusions

The present review focuses on tagging methods which are useful for the determination of biologically important carboxylic acids in real samples. An other elegant review concerning the labelling of carboxyl functional groups has been written by Yasaka and Tanaka [121]. As described in these reviews, many tagging reagents for UV-Vis, FL, EC and CL detection require catalysts such as crown ether and EDC. Since halogenomethyl-type reagents decompose readily in aqueous reaction media, considerable care must be exercised to avoid moisture. Thus, it is more difficult to obtain accurate and reproducible results. The determination of ultra-trace quantities at the picogram level of higher carboxylic acids, such as PGs in blood, is still difficult even with the sensitive and selective detection methods reviewed in this report. Consequently, work must continue on the development of procedures for this important class of compounds.

High-performance capillary electrophoresis (HPCE) is another attractive means of separation of multi-components. CE methods have been used for the analysis of ionic compounds in the past two decades [122]. CE has become a popular technique for separating various organic compounds including low-molecular-mass and high-molecular-mass compounds. In this respect the development of fused-silica capillary columns has been very beneficial. Furthermore, MEKC as proposed by Terabe et al. [123] extends the research of CE. Various compounds, including neutral substances, are efficiently analyzed by MEKC using detergents such as SDS and cholesterols [124,125]. The advantages of HPCE are the rapid analysis of multi-components and the high separation potential. Moreover, ultra-trace analysis at the attomole ~ zeptomole level are possible with LIF and MS detection. However, CE currently has the following disadvantages: (1) low repeatability and reproducibility of the amounts injected and of the migration times, (2) necessity to use a high concentration of the analytes (usually in the $\mu M$ order) caused by the small injection volume (usually nl ~ $\mu$l), and (3) complicated separation mechanism, etc. Although carboxylic acids can be determined with CZE [126,127], application to real samples is limited due to interference from endogenous components and the low sensitivity in UV detection. Both the interference and the low sensitivity may be reduced by utilizing derivatization and MEKC. Although HPCE with various separation modes, such as CZE and MEKC, is still inferior

for the determination of carboxyl compounds, the method probably will become a common separation technique when the instrumentation and detection method are improved.

## Acknowledgement

The author thanks Dr. C.R. Warner, Food and Drug Administration in Washington DC, for reviewing the manuscript.

## References

[1] G.J. Engelmann, E.L. Esmans and F.C. Alderweireldt, *J. Chromatogr.*, 432 (1988) 29.
[2] T. Haniš, M. Smrž, P. Klír, K. Macek, J. Klíma, J. Baše and Z. Deyl, *J. Chromatogr.*, 452 (1988) 443.
[3] W.W. Christie and G.H. McG. Breckenridge, *J. Chromatogr.*, 469 (1989) 261.
[4] K. Kuwata and S. Tanaka, *J. Chromatogr.*, 455 (1988) 425.
[5] C. Osterroht, *Chromatographia*, 23 (1987) 419.
[6] Y. Suzuki and H. Koizumi, *Bunseki Kagaku*, 38 (1989) 718.
[7] T. Aoyama and R. Sato, *Anal. Biochem.*, 170 (1988) 73.
[8] C. Osterroht, *Fresenius J. Anal. Chem.*, 345 (1993) 773.
[9] F.E. Callahan, H.A. Norman, T. Srinath, J.B. St. John, R. Dhar and A.K. Mattoo, *Anal. Biochem.*, 183 (1989) 220.
[10] F.A. Fitzpatrick, *Anal. Chem.*, 48 (1976) 499.
[11] P.H. Zoutendam, P.B. Bowman, J.L. Rumph and T.M. Ryan, *J. Chromatogr.*, 283 (1984) 281.
[12] L.D. Kissinger and R.H. Robins, *J. Chromatogr.*, 321 (1985) 353.
[13] H. Miwa and M. Yamamoto, *J. Chromatogr.*, 351 (1986) 275.
[14] H. Miwa and M. Yamamoto, *J. Chromatogr.*, 421 (1987) 33.
[15] Y. Kondoh, A. Yamada and S. Takano, *J. Chromatogr.*, 541 (1991) 431.
[16] J. Doehl and T. Greibrokk, *J. Chromatogr.*, 349 (1985) 431.
[17] J. Doehl and T. Greibrokk, *J. Chromatogr.*, 529 (1990) 21.
[18] G. Gutnikov and J.R. Streng, *J. Chromatogr.*, 587 (1991) 292.
[19] A.A. Ben-Bassat and T. Wasserman, *J. Liq. Chromatogr.*, 10 (1987) 2939.
[20] Y. Yasaka, M. Tanaka, T. Matsumoto, K. Funazo and T. Shono, *Anal. Sci.*, 5 (1989) 611.
[21] T. Takagi and Y. Itabashi, *J. Chromatogr. Sci.*, 27 (1989) 574.
[22] C.P. Clark, B.G. Snider and P.B. Bowman, *J. Chromatogr.*, 408 (1987) 275.
[23] K. Nyyssönen, K. Seppänen and J.T. Salonen, *J. Chromatogr.*, 612 (1993) 27.
[24] D. Steinhilber, T. Herrmann and H.J. Roth, *J. Chromatogr.*, 493 (1989) 361.
[25] I. Zamir, *J. Chromatogr.*, 586 (1991) 347.
[26] H. Chen and R.E. Anderson, *J. Chromatogr.*, 578 (1992) 124.
[27] W. Baker and C.B. Collis, *J. Chem. Soc.*, (1949) s12.
[28] W. Dünges, *UV Spectr. Group Bull.*, 5 (1977) 38.
[29] S. Yoshida, S. Hirose and M. Iwamoto, *J. Chromatogr.*, 383 (1986) 61.
[30] J.B.F. Lloyd, *J. Chromatogr.*, 178 (1979) 249.
[31] J.H. Wolf and J. Korf, *J. Chromatogr.*, 502 (1990) 423.
[32] J.S. Yoo and V.L. McGuffin, *J. Chromatogr.*, 627 (1992) 87.
[33] J.H. Wolf and J. Korf, *J. Chromatogr.*, 436 (1988) 437.
[34] J.H. Wolf, L. Veenma vd Duin and J. Kolf, *J. Chromatogr.*, 487 (1989) 496.
[35] F.A.L. Horst, G.G. Eikelboom and J.J.M. Holthuis, *J. Chromatogr.*, 456 (1988) 191.
[36] W. Voelter, R. Huber and K. Zech, *J. Chromatogr.*, 217 (1981) 491.
[37] H. Cisse, R. Farinotti, S. Kirkiacharian and A. Dauphin, *J. Chromatogr.*, 225 (1981) 509.
[38] J.H. Wolf, L. Veenma, V. Duin and J. Kolf, *J. Pharm. Pharmacol.*, 43 (1991) 101.
[39] S. Lam and E. Grushka, *J. Chromatogr.*, 158 (1978) 207.
[40] V.L. McGuffin and R.N. Zare, *Appl. Spectrom.*, 39 (1985) 847.
[41] K. Hayashi, J. Kawase, K. Yoshimura, K. Ara and K. Tsuji, *Anal. Biochem.*, 136 (1984) 314.
[42] K.A. Hordijk and T.E. Cappenberg, *Appl. Environ. Microbiol.*, 46 (1983) 361.
[43] W. Elbert, S. Breitenbach, A. Neftel and J. Hahn, *J. Chromatogr.*, 328 (1985) 111.
[44] R. Wintersteiger and H. Juan, *Prostaglandins Leukotriens Med.*, 14 (1984) 25.
[45] R.B. Gueddour, M. Matt and A. Nicolas, *Anal. Lett.*, 26 (1993) 429.
[46] H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, *J. Chromatogr.*, 234 (1982) 121.
[47] H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, *J. Chromatogr.*, 231 (1982) 247.
[48] H. Tsuchiya, T. Hayashi, M. Sato, M. Tatsumi and N. Takagi, *J. Chromatogr.*, 309 (1984) 43.
[49] R.A. Kelly, D.S. O'Hara and V. Kelley, *J. Chromatogr.*, 416 (1987) 247.
[50] A. Takadate, T. Masuda, C. Tajima, C. Murata, M. Irikura and S. Goya, *Anal. Sci.*, 8 (1992) 663.
[51] A. Takadate, T. Masuda, C. Murata, C. Haratake, A. Isobe, M. Irikura and S. Goya, *Anal. Sci.*, 8 (1992) 695.

[52] R. Farinotti, P. Siard, J. Bourson, S. Kirkiacharian, B. Valeur and G. Mahuzier, *J. Chromatogr.*, 269 (1983) 81.
[53] H. Nakamura and Y. Kawahara, *J. Chromatogr.*, 478 (1989) 149.
[54] S. Yoshida, K. Urakami, M. Kito, S. Takeshima and S. Hirose, *Bunseki Kagaku*, 39 (1990) 261.
[55] R.H. Pullen, J.A. Howell and J.W. Cox, Prostaglandins, *Leukotrienes and Medicine*, 29 (1987) 205.
[56] W.D. Watkins and M.B. Peterson, *Anal. Biochem.*, 125 (1982) 30.
[57] J.S. Hawkes, M.J. James and L.G. Cleland, *Prostaglandins*, 42 (1991) 355.
[58] G.A. Cordis and D.K. Das, *J. Chromatogr.*, 536 (1991) 309.
[59] C.E. Morreal, D.K. Sinha, C.J. White and D.T. Nemoto, *J. Chromatogr.*, 345 (1985) 380.
[60] W. Engels, M.A.F. Kamps, P.J.M.R. Lemmens, G.J. Van Der Vusse and R.S. Reneman, *J. Chromatogr.*, 427 (1988) 209.
[61] T.A. Stein, L. Angus, E. Borrero, L.J. Auguste and L. Wise, *J. Chromatogr.*, 395 (1987) 591.
[62] J.W. Cox and R.H. Pullen, *Anal. Chem.*, 56 (1984) 1866.
[63] Y. Yamaguchi, T. Tomita, M. Senda, A. Hirai, T. Terano, Y. Tamura and S. Yoshida, *J. Chromatogr.*, 357 (1986) 199.
[64] K. Kiyomiya, K. Yamaki, N. Nimura, T. Kinoshita and S. Oh-ishi, *Prostaglandins*, 31 (1986) 71.
[65] T. Yoshida, A. Uetaka, H. Yamaguchi, N. Nimura and T. Kinoshita, *Anal. Biochem.*, 173 (1988) 70.
[66] K. Tohyama, S. Manabe, H. Yanagisawa and O. Wada, *Jpn. J. Hygiene*, 42 (1988) 1064.
[67] N. Ichinose, S. Abe, K. Adachi and R. Kato, *Nihon Kagakukaishi*, (1986) 1011.
[68] M. Hatsumi, S. Kimata and K. Hirosawa, *J. Chromatogr.*, 253 (1982) 271.
[69] S.A. Barker, J.A. Monti, S.T. Christian, F. Benington and R.D. Morin, *Anal. Biochem.*, 107 (1980) 116.
[70] N. Nimura and T. Kinoshita, *Anal. Lett.*, 13 (1980) 191.
[71] N. Nimura, T. Kinoshita, T. Yoshida, A. Uetake and C. Nakai, *Anal. Chem.*, 60 (1988) 2067.
[72] M. Yamaguchi, S. Hara, R. Matsunaga, M. Nakamura and Y. Ohkura, *Anal. Sci.*, 1 (1985) 295.
[73] M. Yamaguchi, S. Hara, R. Matsunaga, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 346 (1985) 227.
[74] M. Yamaguchi, R. Matsunaga, S. Hara, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 375 (1986) 27.
[75] M. Yamaguchi, K. Fukuda, S. Hara, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 380 (1986) 257.
[76] M. Yamaguchi, O. Takehiro, S. Hara, M. Nakamura and Y. Ohkura, *Chem. Pharm. Bull.*, 36 (1988) 2263.
[77] M. Yamaguchi, O. Takehiro, J. Ishida and M. Nakamura, *Chem. Pharm. Bull.*, 37 (1989) 2846.
[78] M. Yamaguchi, T. Iwata, K. Inoue, S. Hara and M. Nakamura, *Analyst*, 115 (1990) 1363.
[79] T. Iwata, K. Inoue, M. Nakamura and M. Yamaguchi, *Biomed. Chromatogr.*, 6 (1992) 120.
[80] T. Iwata, M. Nakamura and M. Yamaguchi, *Anal. Sci.*, 8 (1992) 889.
[81] K. Nakashima, Y. Taguchi, N. Kuroda and S. Akiyama, *J. Chromatogr.*, 619 (1993) 1.
[82] K. Yamada, M. Onodera and Y. Aizawa, *J. Pharmacol. Methods*, 9 (1983) 93.
[83] V.V. Bezuglov, N.M. Gretskaya, G.S. Kogteva, E.M. Manevich, E.M. Koroleva, B.G. Belen'kii, Y.E. Lille and L.D. Bergel'son, *Dokl. Akad. Nauk SSSR*, 308 (1989) 231.
[84] Y.-M. Lee, H. Nakamura and T. Nakajima, *Anal. Sci.*, 5 (1989) 209.
[85] Y.-M. Lee, H. Nakamura and T. Nakajima, *Anal. Sci.*, 5 (1989) 681.
[86] I. Yanagisawa, M. Yamane and T. Urayama, *J. Chromatogr.*, 345 (1985) 229.
[87] T. Toyo'oka, M. Ishibashi, Y. Takeda and K. Imai, *Analyst*, 116 (1991) 609.
[88] T. Toyo'oka, M. Ishibashi, Y. Takeda, K. Nakashima, S. Akiyama, S. Uzu and K. Imai, *J. Chromatogr.*, 588 (1991) 61.
[89] T. Toyo'oka, M. Ishibashi, T. Terao and K. Imai, *Biomed. Chromatogr.*, 6 (1992) 143.
[90] T. Toyo'oka, M. Ishibashi and T. Terao, *Analyst*, 117 (1992) 727.
[91] T. Toyo'oka, M. Ishibashi and T. Terao, *J. Chromatogr.*, 625 (1992) 357.
[92] T. Toyo'oka, M. Ishibashi and T. Terao, *Anal. Chim. Acta*, 278 (1993) 71.
[93] J.B.F. Lloyd, *J. Chromatogr.*, 189 (1980) 359.
[94] S. Goya, A. Takadate and H. Fujino, *Yakugaku Zasshi*, 102 (1982) 63.
[95] H. Lingeman, A. Hulshoff, W.J.M. Underberg and F.B.J.M. Offermann, *J. Chromatogr.*, 290 (1984) 215.
[96] S. Narita and T. Kitagawa, *Chem. Pharm. Bull.*, 37 (1989) 831.
[97] Y. Yasaka, M. Tanaka, T. Shono, T. Tetsumi and J. Katakawa, *J. Chromatogr.*, 508 (1990) 133.
[98] M. Tanaka, Y. Yasaka, K. Funazo and T. Shono, *Fresenius Z. Anal. Chem.*, 335 (1989) 311.
[99] Y. Ohkura, M. Kai and H. Nohta, *J. Chromatogr. B*, 659 (1994) 85.
[100] T. Herrmann, D. Steinhilber, J. Knospe and H.J. Roth, *J. Chromatogr.*, 428 (1988) 237.
[101] T. Herrmann, D. Steinhilber and H.J. Roth, *J. Chromatogr.*, 416 (1987) 170.
[102] S. Ikenoya, O. Hiroshima, M. Ohmae and K. Kawabe, *Chem. Pharm. Bull.*, 28 (1980) 2941.
[103] J. Knospe, D. Steinhilber, T. Herrmann and H.J. Roth, *J. Chromatogr.*, 442 (1988) 444.
[104] K. Shimada, C. Sakayori and T. Nambara, *J. Liq. Chromatogr.*, 10 (1987) 2177.
[105] G.M. Beck, D.A. Roston and B. Jaselskis, *Talanta*, 36 (1989) 373.
[106] S. Steffenrud and H. Salari, *Biomed. Chromatogr.*, 3 (1989) 5.

[107] Y. Uji, Y. Sakai, M. Shiraki, A. Noma, M. Maeda, A. Tsuji and H. Okabe, *Bunseki Kagaku*, 37 (1988) 322.
[108] Y. Uji, H. Okabe, Y. Sakai, A. Noma, M. Maeda and A. Tsuji, *Clin. Chem.*, 35 (1989) 325.
[109] B.M.A. Näslund, K. Bernström, A. Lundin and P. Arner, *J. Biolumin. Chemilumin.*, 3 (1989) 115.
[110] T. Kawasaki, M. Maeda and A. Tsuji, *J. Chromatogr.*, 328 (1985) 121.
[111] H. Yuki, Y. Azuma, N. Maeda and H. Kawasaki, *Chem. Pharm. Bull.*, 36 (1988) 1905.
[112] H. Karatani, J. Takano, S. Morishita, M. Yoshida and M. Sato, *Bunseki Kagaku*, (1989) 59.
[113] H. Karatani, *Bull. Chem. Soc. Jpn.*, 60 (1987) 2023.
[114] M. Tod, M. Prevot, J. Chalom, R. Farinotti and G. Mahuzier, *J. Chromatogr.*, 542 (1991) 295.
[115] T. Toyo'oka, M. Ishibashi and T. Terao, *J. Chromatogr.*, 627 (1992) 75.
[116] T. Kusaka, M. Ikeda, H. Nakano and Y. Numajiri, *J. Biochem.*, 104 (1988) 495.
[117] M. Ikeda and T. Kusaka, *J. Chromatogr.*, 575 (1992) 197.
[118] T. Kusaka and M. Ikeda, *J. Chromatogr.*, 639 (1993) 165.
[119] A. Nakanishi, H. Naganuma, J. Kondo, K. Watanabe, K. Hirano, T. Kawasaki and Y. Kawahara, *J. Chromatogr.*, 591 (1992) 159.
[120] R. Sugata, H. Iwahashi, T. Ishii and R. Kido, *J. Chromatogr.*, 487 (1989) 9.
[121] Y. Yasaka and M. Tanaka, *J. Chromatogr. B*, 659 (1994) 139.
[122] F.E.P. Mikkers, F.M. Everaerts and Th.P.E.M. Verheggen, *J. Chromatogr.*, 169 (1979) 11.
[123] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 111.
[124] S. Michaelsen, P. Mϕller and H. Sϕrensen, *J. Chromatogr. A*, 680 (1994) 299.
[125] K. Otsuka, K. Karuhara, M. Higashimori and S. Terabe, *J. Chromatogr. A*, 680 (1994) 317.
[126] A. Hiraoka, J. Akai, I. Tominaga, M. Hattori, H. Sasaki and T. Arato, *J. Chromatogr. A*, 680 (1994) 243.
[127] M. Shirao, R. Furuta, S. Suzuki, H. Nakazawa, S. Fujita and T. Maruyama, *J. Chromatogr. A*, 680 (1994) 247.