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Review

Highly sensitive and selective derivatization-LC method for biomolecules based on fluorescence interactions and fluorous separations☆

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ABSTRACT

A fluorescence derivatization LC method is a powerful tool for the analysis with high sensitivity and selectivity of biological compounds. In this review, we introduce new types of fluorescence derivatization LC analysis methods. These are (1) detection-selective derivatization methods based on fluorescence interactions generated from fluorescently labeled analytes: excimer fluorescence derivatization and fluorescence resonance energy transfer (FRET) derivatization; (2) separation-selective derivatization methods using the fluorous separation technique: fluorous derivatization, F-trap fluorescence derivatization, and fluorous scavenging derivatization (FSD).

Contents

1. Introduction

A fluorescence derivatization LC method, which enables the femtomole-level detection of analytes, is a powerful tool for the analysis with high sensitivity and selectivity of biological compounds [1]. Thus far, various fluorescence derivatization reagents for functional groups such as amine, carboxylic acid, thiol and phenols have been reported. For

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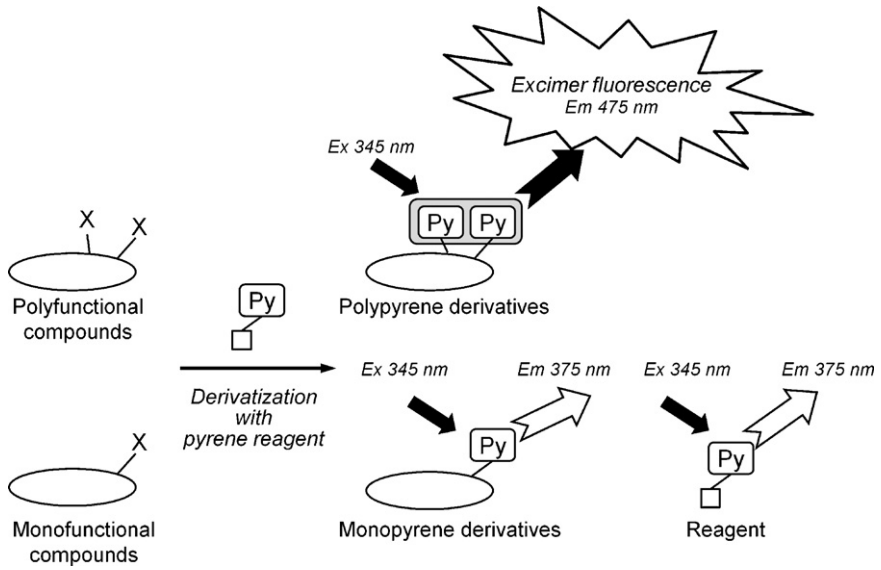

Fig. 1. General concept of intramolecular excimer-forming fluorescence derivatization with pyrene reagent.

instance, dansyl chloride [2,3], fluorescein isothiocyanate [4,5], 9-fluorenylmethyl chloroformate [3,6,7], 3-chlorocarbonyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalin (DMEQ-COCl) [8–11], and 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride [12–14] for amines, are commercially available and widely used. However, a major drawback associated with the use of most commercial fluorescence derivatization reagents is that large peaks appear in the chromatograms because of unreacted reagents. In contrast, fluorogenic derivatization reagents, which are themselves non-fluorescent but which react with amines to generate fluorescence, such as *o*-phthalaldehyde (OPA) with 2-mercaptoethanol [3], 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [15], 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) [16–18], and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ-Tag) [19–23], are supplied commercially. They are widely used for the highly sensitive analysis of amino biomolecules such as amino acids and peptides. However, the fluorescence properties (excitation and emission wavelengths, Stoke's shift and fluorescence quantum yields) and chemical stabilities of these reagents are not always suitable for the user's purpose. Although excess unreacted reagent will not fluoresce, it can influence the LC separation of the analytes. In addition, these reagents control the fluorescence quantum yields by means of their unique emission mechanism [24]; therefore, other fluorophores (for example, pyrene, coumarin, dansyl, fluorescein, and rhodamine) cannot be applied for this purpose.

Other researchers have reported fluorogenic derivatization reagents that recognize not functional groups but rather specific chemical structures. For example, benzylamine for 5-hydroxyindoles [25–27], 1,2-diphenylethylenediamine [28,29] and ethylenediamine [30–32] for catecholamines, 1,2-diamino-4,5-dimethoxybenzene [33,34] and 1,2-diamino-4,5-methylenedioxybenzene [35,36] for α-sialic acid are widely used in biological research and diagnosis.

In this review, we introduce new types of fluorescence derivatization LC analysis methods. These are (1) detection-selective derivatization methods based on fluorescence interactions generated from fluorescently labeled analytes: excimer fluorescence derivatization and fluorescence resonance energy transfer (FRET) derivatization; (2) separation-selective derivatization methods using fluorous separation technique: fluorous derivatization, F-trap fluorescence derivatization, and fluorous scavenging derivatization (FSD).

2. Detection-selective derivatization

2.1. Excimer fluorescence derivatization

When two pyrene fluorophores, which possess relatively long excited-state lifetimes (>100 ns), exist close together, the pyrenes frequently form excited-state dimers (excimers) with fluorescence emission at a longer wavelength (around 475 nm) than that of the monomers (around 375 nm) [37]. The excimer fluorescence can be divided into two categories: intermolecular and intramolecular. The former is observed only at high concentrations (>1 mM) whereas the latter is detectable even at low concentrations. These properties have been used to detect protein interactions [38–41], nucleic-acid hybridization [42], triple-helix formation [43], and phospholipase activity [44].

As shown in Fig. 1, Yamaguchi et al. have introduced the intramolecular excimer fluorescence phenomenon to derivatization LC analysis, making it easy to discriminate spectroscopically between mono-labeled and multiple-labeled fluorescent derivatives.

The analytes, derivatization reagents, real samples, and detection limits for the reported excimer fluorescence derivatization LC methods are summarized in Table 1. Detailed applications for several analytes are described as follows.

2.1.1. Analysis of polyamines, histamine, and amino acids

Nohta et al. [45] have introduced a novel approach for the highly selective fluorescence derivatization of polyamines. This method is based on an intramolecular excimer-forming fluorescence derivatization with a pyrene reagent, 4-(1-pyrene)butyric acid *N*-hydroxysuccinimide ester (PSE), followed by reversed-phase LC. Polyamines, which have two to four amino moieties in a molecule, were converted to the corresponding di-pyrene to tetrapyrene-labeled derivatives by reaction with PSE. The derivatives emitted intramolecular excimer fluorescence (450–520 nm), which can clearly be discriminated from the monomer fluorescence (360–420 nm) emitted from PSE, its hydrolysate and monopyrene-labeled derivatives of monoamines (Figs. 2 and 3). This method was applied to the determination of free and total polyamines (putrescine, cadaverine, spermidine, and spermine) in human urine [46].

Marks and Anderson [47] at the US Food and Drug Administration applied the method to the easy determination of putrescine

Table 1
Information about the analytes, derivatization reagents, real samples, and detection limits in the reported excimer fluorescence derivatization-LC methods.

Analyte	Derivatization reagent	Real sample	Detection limit	Others	Ref.
Polyamino compounds					
Polyamines	PSE	–	1–8 fmol/injection		[45]
Polyamines	PBC	Human urine	1.1–3.4 nM	Total and free polyamine analysis	[46]
Putrescine and cadaverine	PSE	Seafood	0.25 ppm		[47]
Polyamines	PSE	–	6–13 nM	MEKC-LIF analysis	[48]
Lysine and ornithine	PSE	Human urine	3.5–3.7 fmol/injection		[49]
Triethylenetetramine	PSE	Human and rabbit sera	0.13 nM 200 fmol/injection		[50]
Ethambutol	PBC	Rabbit serum	23 ng/ml 180 fmol/injection		[51]
Histamine	PSE	Human urine	0.5 fmol/injection		[52]
Histamine and histidine	PSE	–	2.3–3.8 fmol/injection		[53]
Histamine	PSE	Rat brain microdialysate	0.3 fmol/injection		[59]
Histamine	PSE	Rat plasma and tissue	0.18 nM Less than 1 mg tissue		[60]
Polycarboxylic compounds					
Dicarboxylic acids	PBH	–	1.3–22 fmol/injection		[67]
Dicarboxylic acids	PBH	Human urine	1.1–3.4 nM	From glutaric aciduria patients	[68]
Methylmalonic acid	PBH	Human urine	0.33 pmol/injection	From methylmaloic aciduria patients	[69,70]
Glutaric acid and 3-hydroxyglutraric acid	PBH	Human urine	0.4 μM (glutaric acid) 0.2 μM (3-hydroxyglutaric acid)	From glutaric aciduria type I patients Spectrofluorometric analysis	[71]
Acidic amino acids	PBH	–	21–460 fmol/injection		[72]
Polyphenolic compounds					
Bisphenols	PBC	Hot water extract from baby bottle	3.0–5.0 fmol/injection		[73]
Tetrabromobisphenol A	PBC	–	6.3 fmol/injection		[74]
Halogenated bisphenols	PBC	Spiked plasma			[75]
Polythiol compounds					
Dimercaprol and dithiothreitol	NPM	–	82–160 fmol/injection	Post-column derivatization	[76]
Dimercaprol, dithiothreitol, α-lipoic acid, α-lipoamide	PIAA	Human urine Commercial supplements	0.6–3.5 fmol/injection		[86]
Heterofunctional compounds					
Tyrosine and tyramine	PBC	Human urine	2.6–4.5 fmol/injection		[87]
Catecholamines and indoleamines	PBC	Human urine	0.11–0.51 μM 4.5–22.1 fmol/injection		[88]

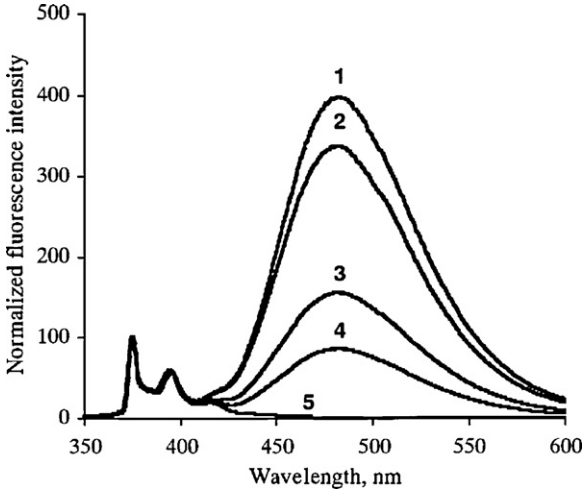

Fig. 2. Fluorescence emission spectra (excitation 345 nm) of the pyrene-labeled polyamines and *n*-decylamine; standard solutions (10 nmol/ml) of (1) putrescine, (2) cadaverine, (3) spermidine, (4) spermine, and (5) *n*-decylamine were treated according to the derivatization procedure, and the resulting derivatives were purified by LC. Each spectrum was normalized to the first peak at 375 nm.
From Ref. [45].

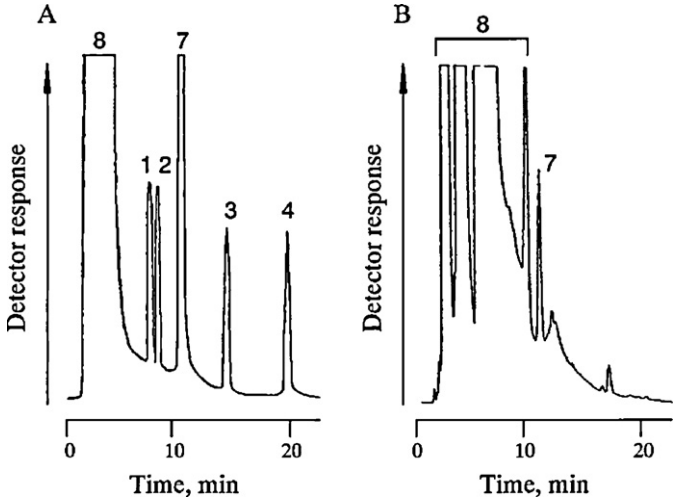

Fig. 3. Chromatograms obtained with a standard mixture of the polyamines. (A) Excimer and (B) monomer fluorescence intensities were monitored at emission wavelengths of 375 nm and 475 nm, respectively, with an excitation wavelength of 345 nm. A standard mixture of (1) 0.1 nmol/ml putrescine, (2) 0.1 nmol/ml cadaverine, (3) 0.5 nmol/ml spermidine, and (4) 1.0 nmol/ml spermine was treated according to the derivatization procedure, followed by LC. Peak 7 was due to PSE and peak 8 was due to other artifacts.
From Ref. [45].

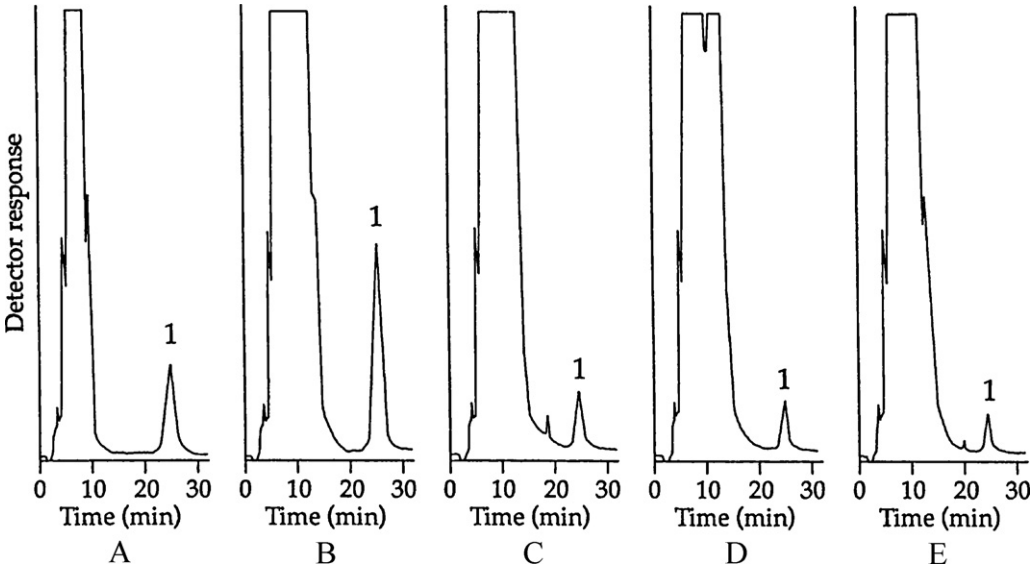

Fig. 4. Chromatograms of 10 μl of a standard solution containing 15 fmol histamine (A) and 10 μl microdialysis samples collected at basal conditions from the hypothalamus (B), prefrontal cortex (C), hippocampus (D), and striatum (E). The basal levels of histamine in the microdialysates from the hypothalamus, prefrontal cortex, hippocampus and striatum were 35.45 ± 4.56, 9.05 ± 1.56, 7.83 ± 0.86, and 6.54 ± 0.66 fmol per 10 μl, respectively. Peak 1 corresponds to histamine–PSE derivative. From Ref. [59].

and cadaverine in seafood (finfish and shellfish). It is also useful for screening for histamine in seafood. Paproski et al. [48] analyzed polypyrene-labeled polyamines not by LC fluorescence detection but by micellar electrokinetic chromatography (MEKC) with laser-induced fluorescence detection. The excimer-forming fluorescence derivatization technique for polyamines has been used for basic amino acids (ornithine and lysine [49]), polyamino pharmaceutical drugs (triethylenetetramine for Wilson's disease [50], and ethambutol for tuberculostatic agent [51]). It has been successfully applied to the monitoring of polyamino drugs in human or rabbit sera with a simple pretreatment (deproteinization with acetonitrile).

Using the above intramolecular excimer-forming fluorescence derivatization, Yoshitake et al. [52,53] with the Karolinska Institutet have developed a novel LC-determination method for histamine. Previously, LC analyses of histamine with fluorescence detection based on pre-column [54–56] and post-column [57,58] derivatization with OPA were widely used. These LC methods, however, are not very sensitive. Furthermore, OPA used as a fluorescence derivatization reagent is selective for primary amines but not for histamine. Thus, in the assay of biological samples, the methods require highly sophisticated clean-up procedures such as liquid–liquid or solid-phase extraction to eliminate interference from other endogenous primary monoamines. Therefore, biological and clinical fields require a simple, selective, and highly sensitive determination method for histamine. Histamine, which has two amino moieties in a molecule, was converted to a dipyrene-labeled derivative by PSE, and the derivative generated excimer fluorescence from the intramolecular dipyrene sites. Fig. 4A–E displays chromatograms of histamine standard solution (15 fmol per injection) and microdialysis samples from a rat hypothalamus, prefrontal cortex, hippocampus and striatum at basal conditions. Some other biogenic polyamino substances such as polyamines, basic amino acids, and lysine-containing peptides also reacted with PSE and yielded polypyrene derivatives. However, none of the derivatives was shown to interfere with the histamine–PSE peak.

Several microdialysis studies in rats and mice have demonstrated that acute stressors cause an increase in brain histamine levels [59]. Researchers also detected an immediate rise in extracellular histamine levels in the hypothalamus, prefrontal cortex, hippocampus, and striatum after exposure of rats to a 20-min forced swimming test. This method for the sensitive determination of histamine in microdialysis samples allows researchers to monitor *in vivo* the extracellular levels of histamine in the brain areas implicated in the affective behavior as well as the cognitive function. The method has been applied to determine histamine in rat plasma and tissue extracts [60]. The detection limit for histamine was 0.183 nM. This sensitivity allowed the determination of histamine in 10 μl of rat plasma or in extracts from less than 1 mg of tissue.

2.1.2. Analysis of polycarboxylic acid analysis

Dicarboxylic acids (glutaric acid, adipic acid, suberic acid, etc.) are produced from ω-oxidation of long-chain monocarboxylic acids to long-chain dicarboxylic acids, followed by β-oxidation [61]. Their levels in body fluids are significantly increased in patients with inborn errors of the fatty acid metabolism [62,63] including glutaric aciduria and other acetyl CoA dehydrogenase deficiencies, and with diabetic ketoacidosis [64,65]. Therefore, measurements of dicarboxylic acids are necessary for rapid diagnoses of and accurate therapies for metabolic disorders [66]. Nohta et al. have reported the LC [67] and spectrofluorometric [68] determination of dicarboxylic acids based on intramolecular excimer-forming fluorescence derivatization. The dicarboxylic acids were converted to the corresponding dipyrene-labeled derivatives by 4-(1-pyrene)butyric acid hydrazide (PBH) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and the derivatives generated excimer fluorescence from the intramolecular dipyrene sites. Fig. 5 illustrates fluorescence spectra obtained from the urine samples of a healthy person and a patient with glutaric aciduria type II. The spectrum for the healthy person (Fig. 5B) is almost the same as that of the reagent blank (Fig. 5C) whereas, that for the patient clearly shows excimer fluorescence (Fig. 5A) ascribable to dicarboxylic acids in the urine. Fig. 6 displays the chromatograms obtained for the same urine samples. As can be seen, the peaks of the dicarboxylic acids from the patient (Fig. 6B) are much higher than those from the healthy person (Fig. 6A). The estimated total amounts of the dicarboxylic acids obtained by spectrofluorometric measurement do not contradict the summation amounts of the LC determinations of glutaric acid, adipic acid, suberic acid, and sebacic acid. From these observations, the

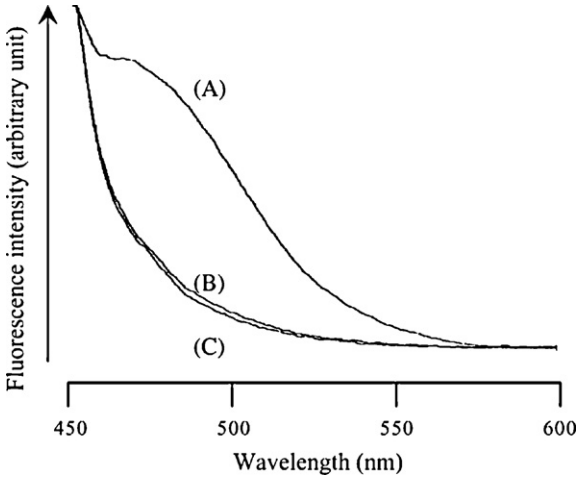

Fig. 5. Fluorescence emission spectra (Ex 345 nm) obtained from urine samples and reagent blank. Urine samples: (A) patient of glutaric aciduria type II; (B) healthy person; (C) reagent blank.
From Ref. [68].

spectrofluorometric method allows rapid measurement of the total amount of dicarboxylic acids in urine and is useful as a screening test for dicarboxylic aciduria.

Al-Dirbashi et al. [69–71] successfully applied this methodology to retrospective studies on a relatively large number of known methylmaloic aciduria cases [69,70] and previously diagnosed glutaric aciduria type I cases [71]. The excimer-forming fluorescence derivatization technique for polycarboxylic acids was also introduced to acidic amino acids: glutamic acid, aspartic acid and *N*-methyl-D-aspartic acid (NMDA) [72].

2.1.3. Analysis of polyphenols

Yoshida et al. [73–75] have described an intramolecular excimer-forming derivatization method for the determination of bisphenols, which contain two phenolic hydroxyl groups in a molecule, based on their one-step derivatization with 4-(1-pyrene)butanoyl chloride (PBC). This method was successfully applied to the determination of bisphenol A (BPA) in hot water in contact with commercially available baby bottle samples [73] and tetrabromobisphenol A [74,75], one of the most widely used flame retardants. Fig. 7 shows typical chromatograms obtained from the bottle samples. The peak components of peaks 1 and 2 were identified as the PBC derivatives of BPA and bisphenol F (internal standard), respectively. Baseline separations among the peaks for BPA and early-eluting blank peaks were attained in the excimer fluorescence detection (Fig. 7A). However, in monomer fluorescence detection, the BPA peak overlapped with the tailing of the blank peaks and could not be used for sensitive quantification (Fig. 7C). The limit of quantification of BPA in water sample was 0.24 pmol/100 ml (0.54 ppt), which corresponds to *ca.* 20 fmol on column per 20 μl injection volume. Six commercial baby bottles (four polycarbonate and two glass) were subjected to the migration test. The migration test was successfully repeated four times for each bottle. In the first migration test, BPA in polycarbonate bottles was found to migrate at 8–190 ppt, and the amount of migrated BPA decreased as the number of test repetitions increased. On the other hand, for glass bottles, no BPA could be detected in any of the tests (Fig. 7B).

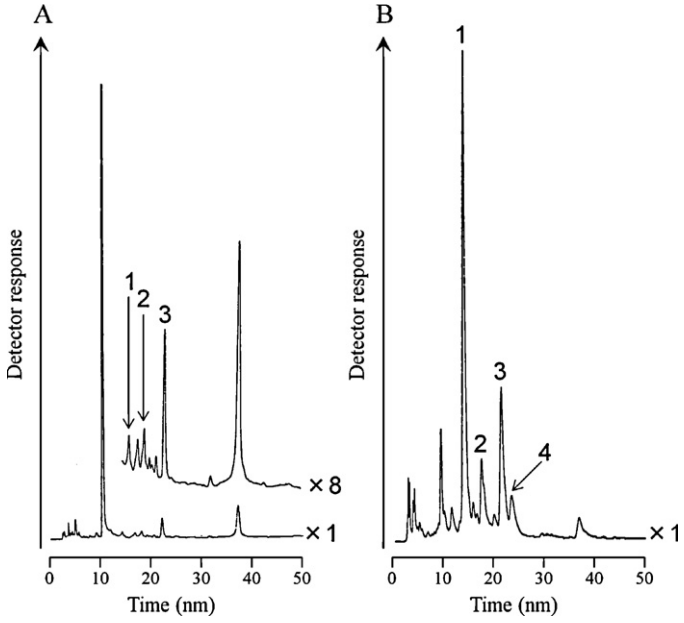

Fig. 6. Chromatograms obtained from urine samples of (A) healthy person and (B) patient with glutaric aciduria type II. Peaks: (1) adipic acid; (2) suberic acid; (3) glutaric acid; (4) sebacic acid; (others) reagent blanks and unknowns.
From Ref. [68].

2.1.4. Analysis of polythiols

A highly simple and selective method for the determination of polythiols based on LC with post-column excimer fluorescence derivatization using *N*-(1-pyrenyl)maleimide (NPM) as a derivatization reagent has been developed [76]. NPM is one of the most popular fluorogenic reagents for the LC analysis of thiols used in both post-column [77–80] and pre-column [81–85] reactions, but excimer fluorescence detection has not been carried out until now. Fig. 8 shows typical chromatograms obtained using a standard mixture of monothiols and polythiols with post-column derivatization [76]. Dimercaprol (BAL) and dithiothreitol (DTT) were used as model polythiols. When detected in the monomer fluorescence region, a good separation of BAL, DTT, and cysteine was achieved within 10 min on the ODS column (Fig. 8A). However, as shown in Fig. 8B, cysteine did not show any peak in the excimer fluorescence detection. Furthermore, cysteine-containing peptides, such as oxytocin, vasopressin, and vasotocin (with a disulfide structure within a molecule) showed respective peaks under the same LC system with excimer fluorescence detection, when they were reduced with tributylphosphine to dithiols before the LC injection.

An LC analysis of polythiols based on pre-column intramolecular excimer-forming fluorescence derivatization using *N*-(1-pyrene)iodoacetamide (PIAA) and *N*-(1-pyrenemethyl)iodoacetamide (PIAA) has also been developed [86]. This method was successfully applied to the selective determination of α-lipoic acid in commercial supplements and in human plasma.

2.1.5. Analysis of heterofunctional compounds

An intramolecular excimer-forming derivatization method for the determination of heterofunctional compounds, which have an amino and a phenolic hydroxyl group in a molecule, based on their one-step derivatization with PBC has been described [87,88]. This method was successfully applied to the determination of tyrosine and tyramine [87] and catecholamines and indoleamines [88] in human urine.

2.2. FRET derivatization

Fluorescence resonance energy transfer (FRET) is a non-radiative transfer of excited-state energy from an initially excited donor fluorophore to an acceptor one. Therefore, FRET results in

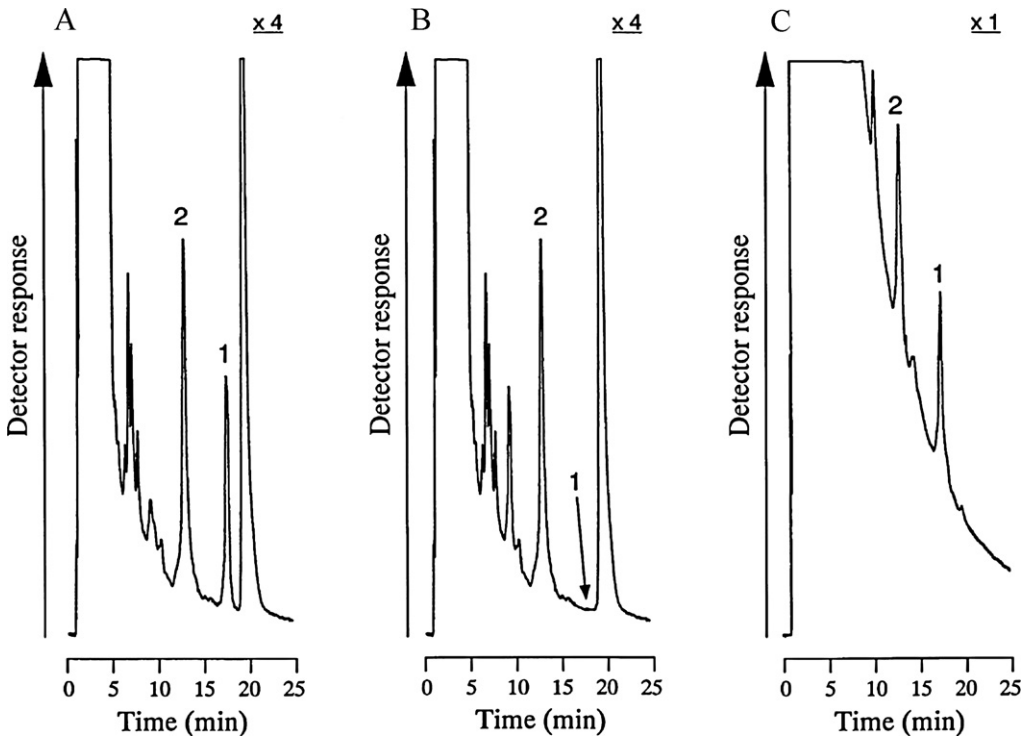

Fig. 7. Chromatograms obtained from water in contacted with baby bottles in: (A) and (B) excimer fluorescence region and (C) monomer fluorescence region. Bottle samples: (A) and (C) sample 2, polycarbonate, extract with first migration (bisphenol A, 89 ppt); (B) sample 5, glass, extract with first migration. Peaks: (1) bisphenol A; (2) bisphenol F (internal standard).
From Ref. [73].

quenching of the donor fluorescence and emission of the acceptor fluorescence. FRET occurs when the two fluorophores (donor and acceptor) exist in proximity and in a suitable orientation, and the emission spectrum of the donor overlaps well with the excitation spectrum of the acceptor. Thus, FRET has become a powerful tool for the monitoring of structural dynamics of nucleic acids [89,90] and proteins [91–93]. Recently, an internal FRET-based fluorescent dye, BigDye, has been applied effectively to the challenge of DNA sequencing [94]. To date however, FRET has not been applied to the derivatization LC analysis of low-molecular-weight biomolecules.

Yoshitake et al. [95] have successfully applied FRET to the highly sensitive LC analysis of native fluorescent bioamines (indoleamines and catecholamines). This method is based on the FRET-inducing derivatization of these amines and the subsequent detection of FRET from the native fluorescent bioamines to the derivatized fluorophore (Fig. 9).

To find the most effective reagent, they screened 15 amine-reactive pre-column fluorescence derivatization reagents for their induction of FRET in indoleamines and catecholamines in comparison with that of the non-fluorescent amino compound, isoleucine. From this screening result, OPA was found to be the most suitable reagent. Fig. 10 illustrates typical chromatograms obtained from the standard mixtures of indoleamines and isoleucine. The fluorescence intensities of indoleamines monitored at excitation/emission wavelengths of 280/445 nm (FRET detection; Fig. 10A) were more intense than the intensities of those monitored at 335/445 nm (conventional fluorescence detection; Fig. 10B). On the other hand, the fluorescence intensities of isoleucine and other amines that are not natively fluorescent monitored with FRET detection were weaker than the intensities of those monitored with conventional fluorescence detection. Fig. 11 illustrates the three-dimensional fluorescence emission chromatograms of tryptophan and isoleucine recorded at excitation wavelengths of 280 nm

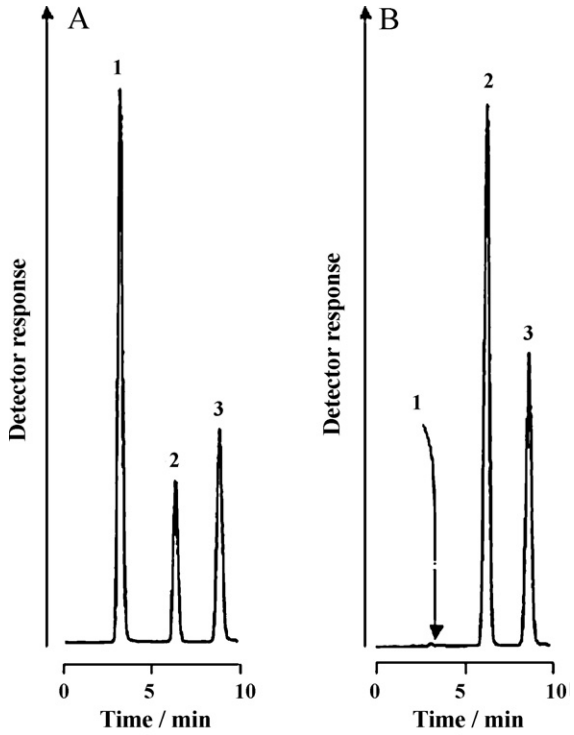

Fig. 8. Chromatograms obtained for a standard mixture of BAL, DTT, and cysteine (40 pmol each on column). Fluorescence detections (Ex/Em, nm): (A) monomer fluorescence (345/375); (B) excimer fluorescence (345/485). Peaks: 1, cysteine; 2, DTT; 3, BAL.
From Ref. [76].

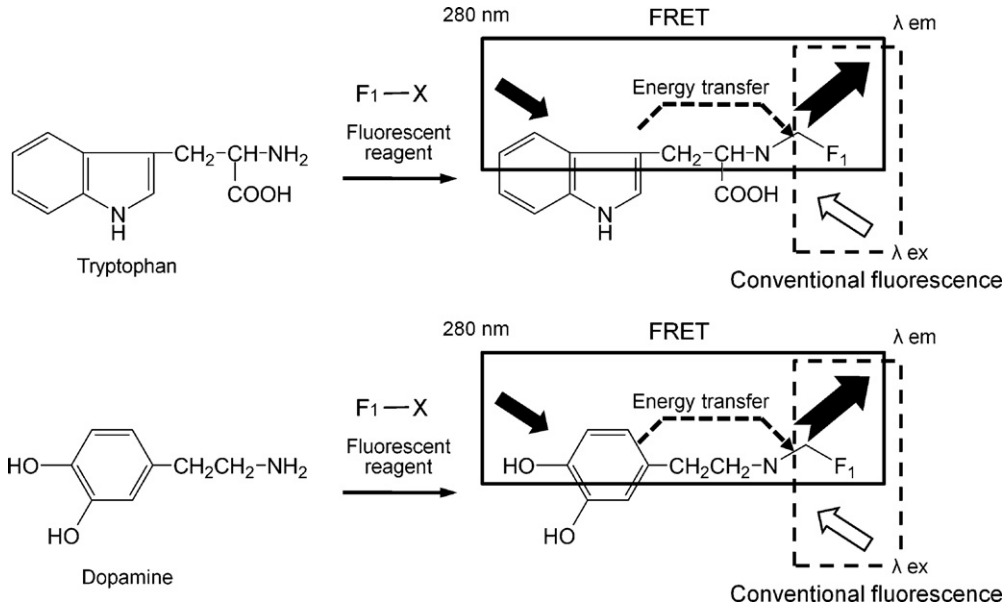

Fig. 9. FRET-inducing derivatization of tryptophan and dopamine.

(Fig. 11A) and 335 nm (Fig. 11B). The emission intensity of tryptophan (peak 1) was more intense than that of isoleucine at an excitation wavelength of 280 nm, but they were equally intense at 335 nm. The shape of the emission spectrum of tryptophan obtained at an excitation wavelength of 280 nm was the same as that obtained at 335 nm. Thus, FRET between the natively fluorescent moiety of tryptophan and the labeled OPA should occur when tryptophan is derivatized with OPA and the derivative is irradiated at the wavelength of the native fluorescent moiety.

This method was successfully applied to the determination of three indoleamines (tryptophan, serotonin, tyramine) in human urine. The fluorescence intensities of the three indoleamines monitored at an excitation wavelength of 280 nm were more intense than the intensities of those monitored at 335 nm. On the other hand, the other amines present in the urine gave weaker peaks at an excitation wavelength of 280 nm than they did at 335 nm. Thus, the FRET derivatization method was quite selective and also provided a simple chromatogram for biological analysis. For the routine analysis of simultaneous and continuous determination of indoleamines and catecholamines, online post-column derivatization with OPA and an intramolecular FRET detection system has also been reported [96].

The above method was also applied to the determination of tryptophan-containing peptides [97]. From the results of a screening test of FRET-forming derivatization reagents, OPA was selected as the most suitable intramolecular FRET-inducing derivatization reagent for native fluorescent Trp-containing peptides (osteostatin, delta sleep-inducing peptide (DSIP), Gly-Gly-Trp, Trp-Gly) and Gly-Gly-Cys. The fluorescence intensities of Trp-containing peptides monitored at an excitation/emission wavelength of 280/445 nm

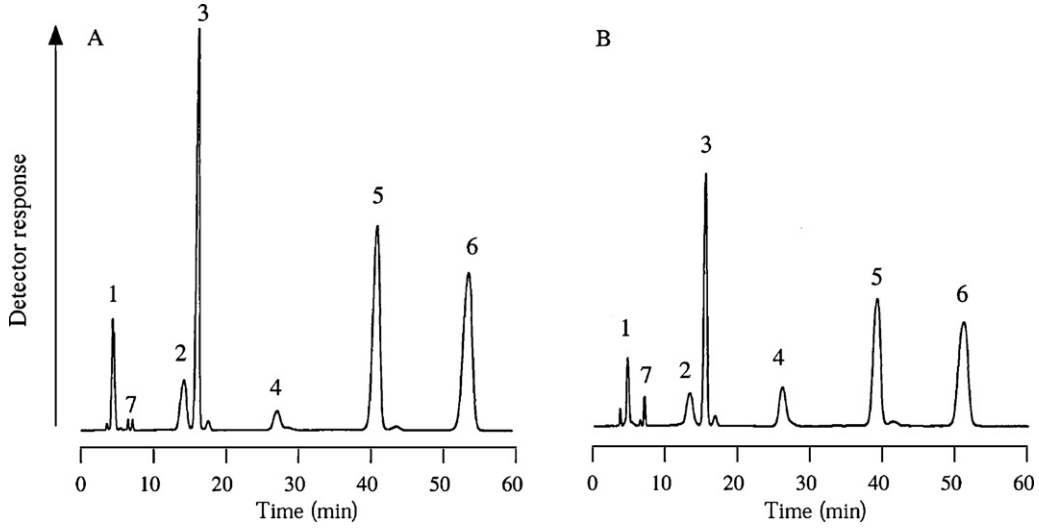

Fig. 10. Chromatograms obtained using a standard mixture of indoleamines and isoleucine (20 pmol each on the column). (A) FRET and (B) conventional fluorescence detections monitored at excitation wavelengths of 280 and 335 nm, respectively; emission wavelength, 445 nm. Peaks: 1, 5-hydroxytryptophan; 2, tryptophan; 3, serotonin; 4, isoleucine; 5, 5-methoxytryptophan; 6, tyramine; 7, unknown.
From Ref. [95].

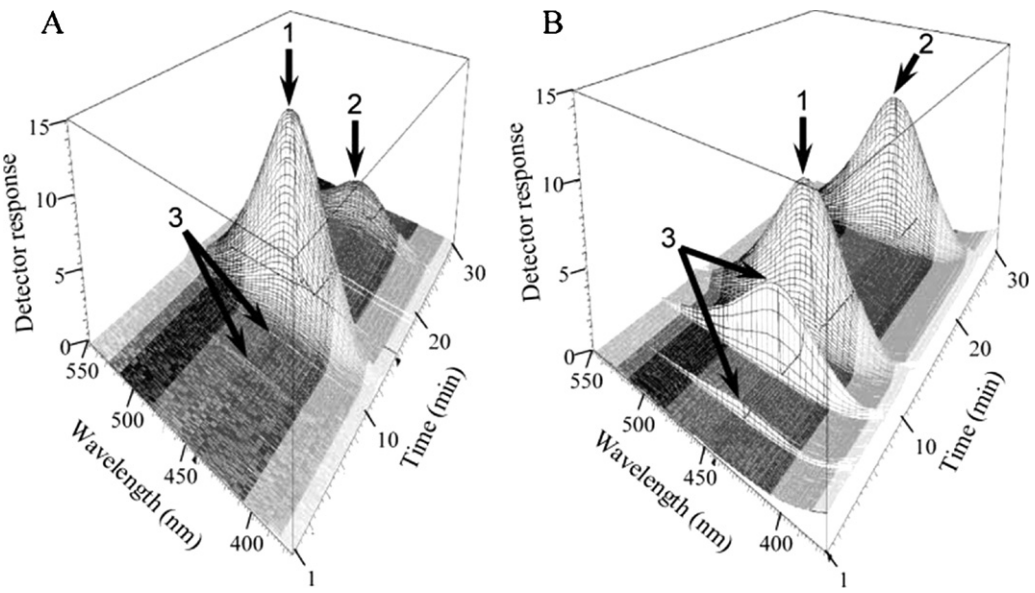

Fig. 11. Three-dimensional fluorescence emission chromatogram obtained using the OPA-labeled tryptophan and isoleucine samples (400 pmol each on the column) at excitation wavelengths of (A) 280 nm and (B) 335 nm. Peaks: 1, tryptophan; 2, isoleucine; 3, unknown.
From Ref. [95].

(FRET detection) were more intense than the intensities of those monitored at 335/445 nm (conventional fluorescence detection). On the other hand, the fluorescence intensities of Gly-Gly-Cys and reagent blanks monitored by FRET detection were weaker than the intensities of those monitored by conventional fluorescence detection.

Thus, this intramolecular FRET-inducing derivatization method permits the selective and sensitive determination of tryptophan containing peptides even if non-fluorescent amines are contained in the sample.

3. Separation-selective derivatization

3.1. Fluorous separation

The term *fluorous* means highly fluorinated or rich in fluorines, and the short perfluoroalkyl chain (C_4–C_{10}) is called a fluorous tag. Fluorous-tag-bound compounds are selectively trapped by fluorous solid-phase extraction (F-SPE). The F-SPE technique, which was first introduced by Curran et al. [98], has received much attention in recent years because of its ability to collect target molecules

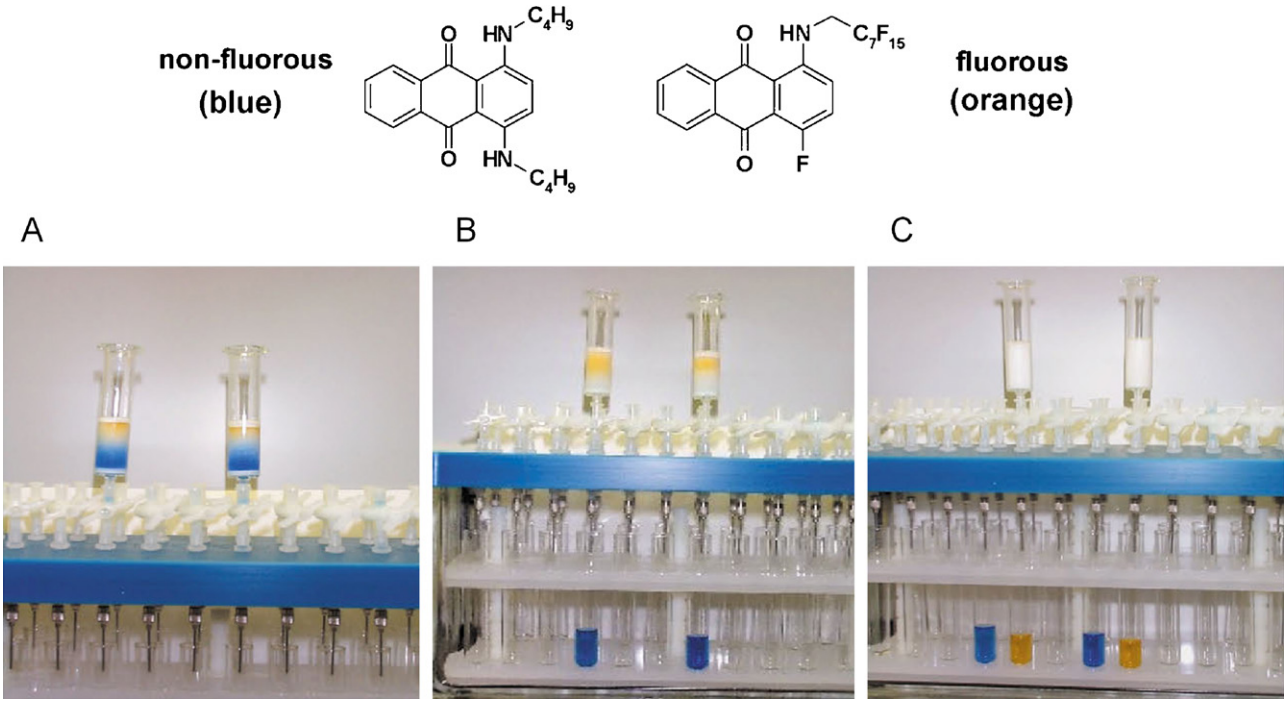

Fig. 12. F-SPE separation of non-fluorous (blue) and fluorous (orange) dye. (A) Elute with 80% aqueous methanol; (B) collect non-fluorous dye; (C) elute with methanol for fluorous dye. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
From Ref. [100].

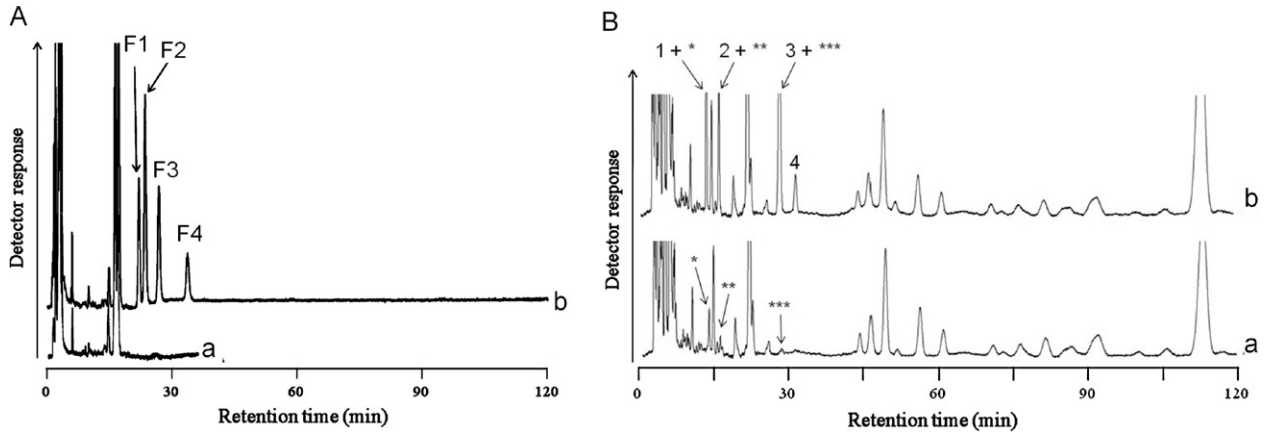

Fig. 13. Chromatograms obtained for plasma samples by (A) fluorous derivatization and fluorous-phase separation, (B) no derivatization and reversed-phase separation. (a) Drug-free plasma; (b) plasma spiked with native fluorescent drugs (1 nmol each/ml plasma) Peaks: F1, HFUA-labeled naproxen; F2, HFUA-labeled felbinac; F3, HFUA-labeled flurbiprofen; F4, HFUA-labeled etodolac; 1, naproxen; 2, felbinac; 3, flurbiprofen; 4, etodolac; others, endogenous native fluorescent compounds.
From Ref. [107].

selectively in solution [99–104]. The photographs in Fig. 12 show a mixture of non-fluorous (blue) and fluorous (orange) aminoanthraquinone dyes [100]. These two dyes can be separated on an LC column but not on an SPE cartridge charged with normal or reversed-phase silica gel. The non-fluorous blue dye is readily eluted with fluorophobic 80% aqueous methanol, while the fluorous dye is retained until it is washed with fluorophilic 100% methanol. Thus, many different types of organic and light-fluorous molecules can be separated using substantially the same method.

3.2. Fluorous derivatization

Brittain et al. [105] described a methodology for the effective enrichment and subsequent mass-spectrometric characterization of various subsets of peptides from highly complex mixtures of biological origin using fluorous derivatization and the F-SPE technique. Go et al. [106] applied a similar methodology to analyses of both a trypsin digest of proteins and amino acids in serum using desorption/ionization on silicon mass spectrometry (DIOS-MS).

Sakaguchi et al. [107] have applied fluorous derivatization to the LC-fluorescence analysis of native fluorescent compounds of biologically important metabolites (homovanillic acid, vanillylmandelic acid, and 5-hydroxyindole-3-acetic acid) and nonsteroidal anti-inflammatory drugs (NSAIDs) (naproxen, felbinac, flurbiprofen, and etodolac). In this study, 4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-heptadecafluoro-*n*-undecylamine (HFUA) was used as a derivatization reagent for carboxylic acids with a fluorous derivatization reagent, and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) [108,109] was used as an effective condensation reagent between HFUA and the analytes. Fluorous-labeled analytes could be retained specifically in commercial fluorous-phase LC columns and detected selectively. Fig. 13A shows typical chromatograms obtained for derivatized plasma samples. The components of peaks F1–F4 in Fig. 13A were identified as HFUA-labeled naproxen, felbinac, flurbiprofen, and etodolac, respectively. Only analytes can be retained intensely and specifically in the fluorous-phase LC column with fluorous separation. In contrast, Fig. 13B shows chromatograms obtained for the same plasma samples without fluorous derivatization using a reversed-phase LC column. Although the separation conditions are optimized, it was difficult to determine these pharmaceuticals because many

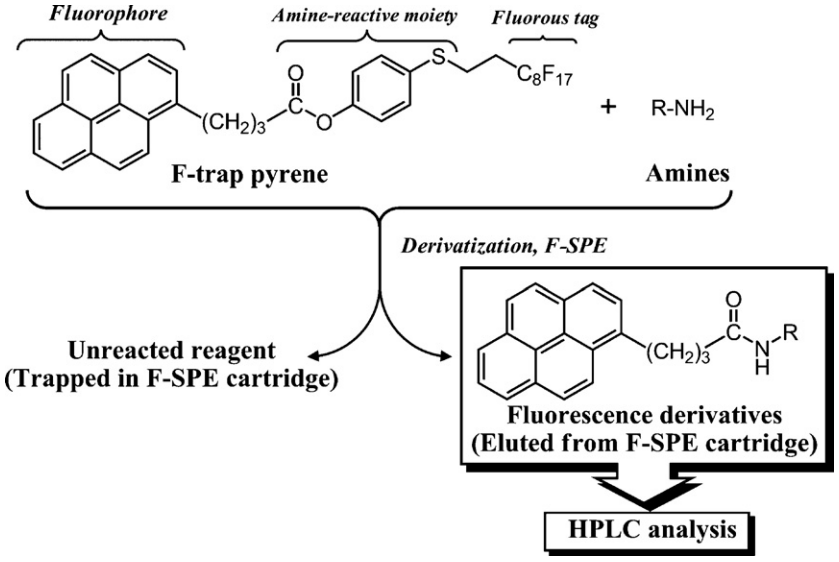

Fig. 14. Fluorescence derivatization of aliphatic amines using F-trap pyrene.

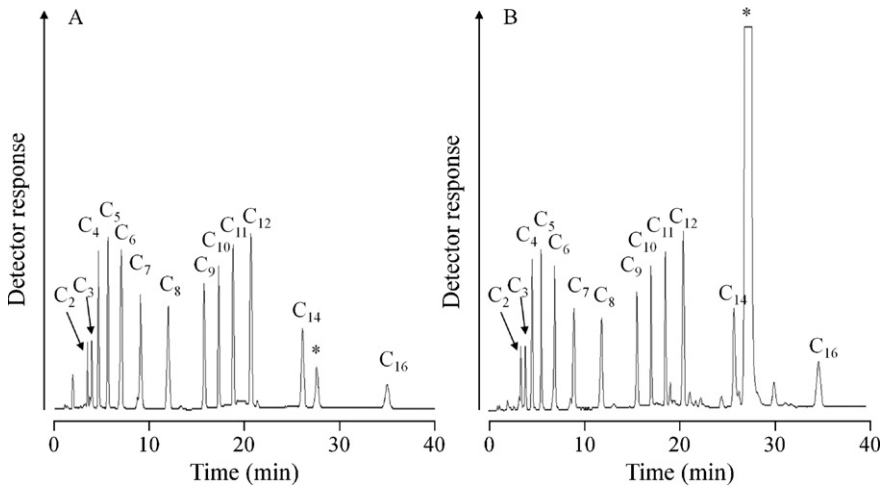

Fig. 15. Chromatograms of the pyrene derivatives of 13 aliphatic amines (A) with and (B) without F-SPE treatment (50 pmol each on column). Peaks: C_2, ethylamine; C_3, *n*-propylamine; C_4, *n*-butylamine; C_5, *n*-pentylamine; C_6, *n*-hexylamine; C_7, *n*-heptylamine; C_8, *n*-octylamine; C_9, *n*-nonylamine; C_{10}, *n*-decylamine; C_{12}, *n*-dodecylamine; C_{14}, *n*-tetradecylamine; C_{16}, *n*-hexadecylamine; *F-trap pyrene.
From Ref. [110].

disturbance peaks appeared at their retention times. Therefore, a combination of fluorous derivatization and LC separation with a fluorous stationary phase results in a simple and effective method for the determination of native fluorescent carboxylic acids in a short time.

3.3. F-trap fluorescence derivatization

We have developed a novel pre-column fluorescence derivatization reagent, F-trap pyrene, for use in the LC analysis of biological amines, and the use of this reagent does not result in the large peaks due to unreacted reagents [110,111]. Fig. 14 shows the structure of F-trap pyrene and its fluorescence derivatization reaction scheme for amine analysis. This reagent consists of a fluorescent pyrene moiety, an amine-reactive Marshall linker, and a fluorous tag. When the reagent reacts with aliphatic amines and amino acids to give fluorescent derivatives, the fluorous tag in the reagent is simultaneously eliminated. Therefore, excess unreacted reagents in the derivatization reaction solution still have the fluorous tag and could be removed by F-SPE selectively before the LC analysis.

Fig. 15A presents a typical chromatogram obtained from a standard mixture of 13 aliphatic (C_2–C_{16}) amines. In this chromatogram, a small peak of the unreacted F-trap pyrene at around 28 min was observed, but this peak did not interfere with the separation and quantification of the amines. For comparison, a diluent of the reaction mixture without F-SPE treatment was injected into the same LC system (Fig. 15B). In contrast to Fig. 15A, the unreacted reagent peak was significant and disturbed the analysis of *n*-tetradecylamine (C_{14}). Although the F-trap pyrene exhibited similar elution behavior to those of the C_{14} and C_{16} derivatives in reversed-phase LC, it was selectively removed by F-SPE treatment before the LC analysis. From these results, the removal criteria for the unreacted reagent are determined not by hydrophobicity, but by fluorophilicity.

The proposed derivatization method was also applied to the analysis of amino acids. F-trap pyrene could become a powerful tool in proteomics and metabolomics research for amine analysis demanding high sensitivity and comprehensive detection.

3.4. Fluorous scavenging derivatization

An FSD method for reagent-peak-free LC-fluorescence analysis of carboxylic acids has also been developed (Fig. 16) [112]. In this method, 1-pyrenemethylamine (PMA) was used as an alkylamine-type fluorescence derivatization reagent, EDC and 1-hydroxy-1*H*-benzotriazole (HOBt) were used as condensation reagents, 2-(perfluorooctyl)ethyl isocyanate (PFOEI) was used as a fluorous scavenging reagent, and a handmade micro F-SPE spin column was used as a trapping column to remove excess unreacted reagent.

Fig. 17A shows a typical chromatogram obtained using a standard mixture of eight linear aliphatic carboxylic (C_1–C_8) acids. In this chromatogram, a small peak of unreacted PMA was observed at 3.3 min. However, the peak did not interfere with the separation and quantification of the acids. For comparison, a diluent of the reaction mixture without F-SPE treatment was injected into the same LC system (Fig. 17B). The fluorous-tagged PMA peak was selectively removed by micro F-SPE treatment before the LC analysis. Therefore, the removal criteria for the fluorous-tagged PMA are determined not by hydrophobicity but by fluorophilicity. Fig. 17C shows a chromatogram of PMA-carboxylic acid derivatives without fluorous tagging and F-SPE treatment. In contrast to chromatograms Fig. 17A and B, the peaks of the PMA derivatives of the short-chain (C_1–C_4) acids are obscured by a large peak from the excess unreacted PMA. Furthermore, a peak derived from an unknown reaction byproduct overlapped with that of C_8 (Fig. 17C). Removal rates of the reagent and the average recoveries

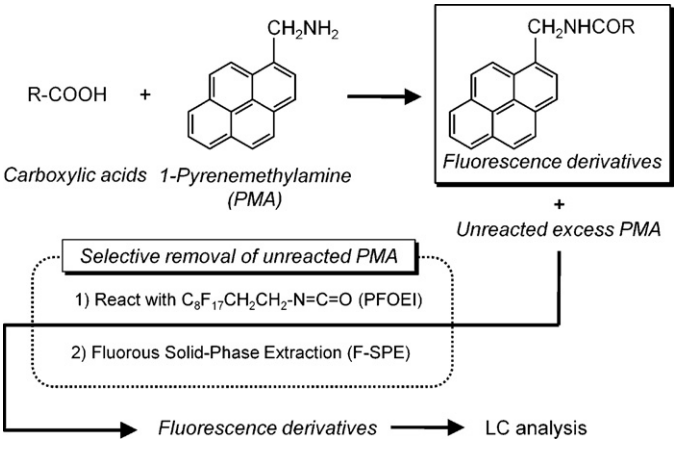

Fig. 16. Fluorous scavenging derivatization of carboxylic acids using PMA and PFOEI.

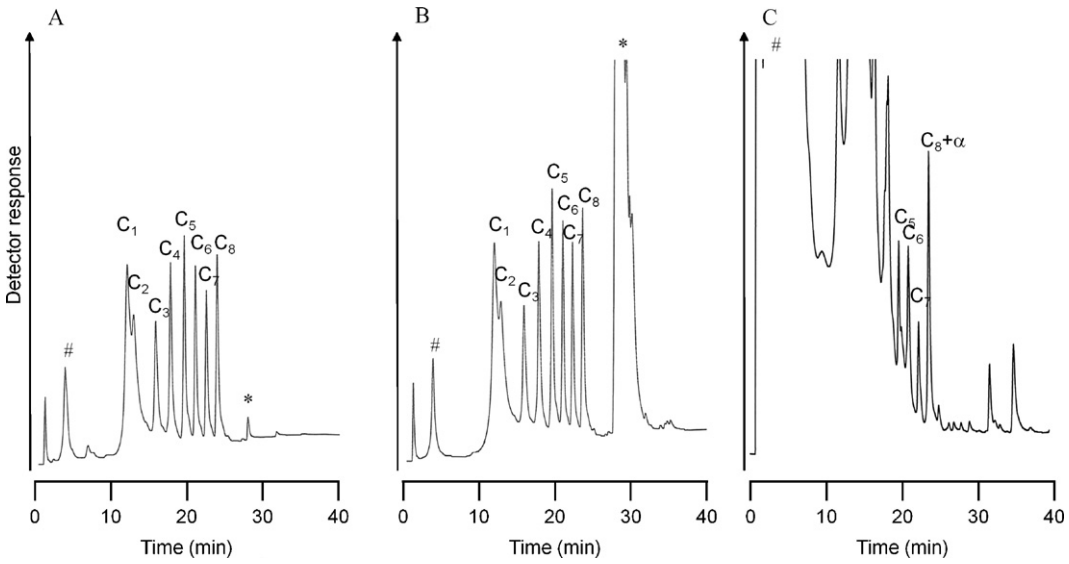

Fig. 17. Chromatograms of the PMA derivatives of 8 carboxylic acids (3.1 pmol each on column) with FSD (A), treated with fluorous tagging reaction (B), and without FSD (C). Peaks: C_1, formic acid; C_2, acetic acid; C_3, *n*-propionic acid; C_4, *n*-butyric acid; C_5, *n*-valeric acid; C_6, *n*-hexanoic acid; C_7, *n*-heptanoic acid; C_8, *n*-octanoic acid; #PMA; *fluorous-tagged PMA. Other peaks are reaction byproducts and unknowns.
From Ref. [112].

of the carboxylic acid derivatives obtained by F-SPE with different fluorophobic elutions were calculated. By combining fluorophilic (methanol or acetonitrile) and fluorophobic (water) eluents, the micro F-SPE spin column selectively trapped more than 99.9% of the unreacted reagent.

From these results, the FSD method is useful for the comprehensive and sensitive LC analysis of carboxylic acids, especially highly polar carboxylic acids. The proposed method was applied to the analysis of highly polar carboxylic acids such as α-keto acids and tricarboxylic acid cycle intermediates (Fig. 18). All the fluorescence peaks of the corresponding PMA derivatives and only small peaks of PMA or the fluorous-tagged PMA were observed. All the dicarboxylic and tricarboxylic acids examined, except for fumaric acid and oxaloacetic acid, were changed into the corresponding polypyrene derivatives and produced excimer fluorescence as described in Section 2.1.2. These polypyrene derivatives were strongly retained in the ODS column and their peaks were detected after 33 min with the emission of excimer fluorescence. For comparison, diluents of the reaction mixture without F-SPE treatment (Fig. 18B) or without fluorous tagging and F-SPE treat-

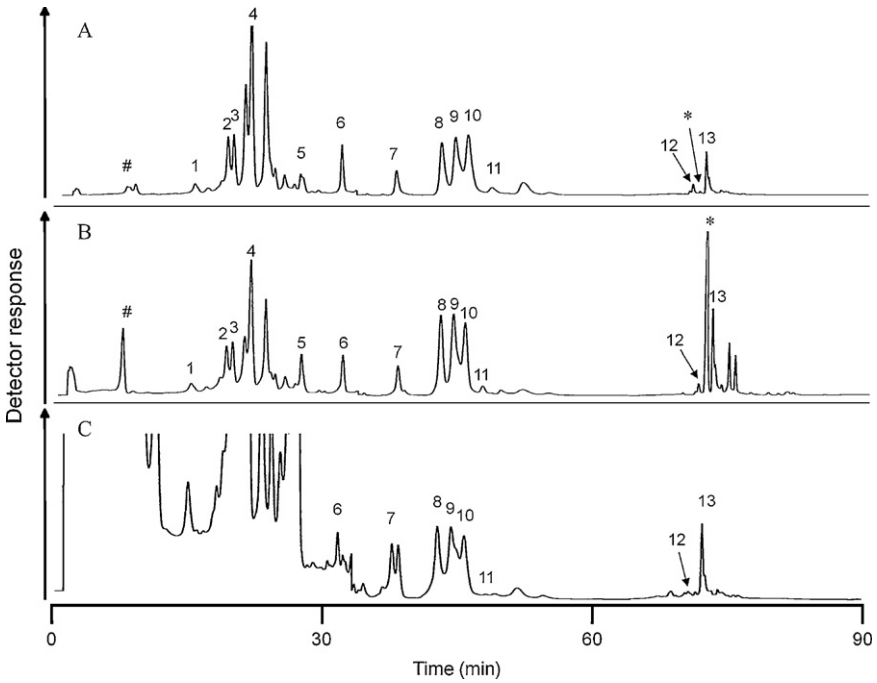

Fig. 18. Chromatograms of the PMA derivatives of α-keto acids and tricarboxylic acid cycle metabolites with FSD (A), treated with fluorous tagging reaction (B), and without FSD (C). The acid amounts were 1.3 pmol (peaks 4 and 6), 2.6 pmol (peaks 2, 3, and 7), 5.2 pmol (peaks 1, 5, 8–10, 12, and 13), and 10.4 pmol (peak 11). Peaks: 1, fumaric acid; 2, pyroglutamic acid; 3, glycolic acid; 4, lactic acid; 5, oxaloacetic acid and pyruvic acid; 6, 3-phenyllactic acid; 7, tartaric acid; 8, malic acid; 9, succinic acid; 10, 2-oxoglutaric acid; 11, malonic acid; 12, isocitric acid; 13, citric acid; #PMA; *fluorous-tagged PMA. Other peaks are reaction byproducts and unknowns.
From Ref. [112].

ment (Fig. 18C) were injected into the same LC system. In Fig. 18B, a strong fluorous-tagged-reagent peak was detected between the peaks of isocitric acid and citric acid and disturbed the analysis of these acids. In Fig. 18C, peaks of the PMA derivatives of highly polar monocarboxylic acids, except for 3-phenyllactic acid, were fully buried in the excess unreacted PMA peaks and were not detected in the chromatogram.

These results show that the FSD method is suitable for the highly sensitive and comprehensive LC analysis of biologically important metabolites.

4. Conclusions

In this review, we have described new fluorescence derivatization LC analysis methods: detection-selective derivatization methods based on fluorescence interactions and separation-selective derivatization methods using the fluorous separation technique. These methods possess unique properties different from those of conventional derivatization LC methods, and they can be used for the highly sensitive and selective analysis of many biomolecules. Because of the recent progress in LC–MS and LC–MS/MS instruments, it has become possible to detect target analytes sensitively and directly without derivatization. However, derivatization-LC methods are largely unaffected by various matrix components and are still useful for many analyses in complicated clinical, biological, environmental and industrial applications because of their high sensitivity, selectivity, and robustness. We believe that all the methods introduced could become powerful tools in these areas.

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