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Application of a Fluorescent Derivatization Reagent 9-Chloromethyl Anthracene on Determination of Carboxylic Acids by HPLC

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A simple and sensitive high-performance liquid chromatography (HPLC) method is proposed for the analysis of some carboxylic acids in food samples and the environment. The use of 9-chloromethyl anthracene as a fluorescence-labeling reagent has been investigated. The derivatization reagent reacts with unitary carboxylic acids and tetrabutylammonium bromide as a catalyst within 50 min in acetonitrile to give esters, which can be separated by HPLC employing fluorescence detection at $\lambda_{ex} = 365$ and $\lambda_{em} = 410$ nm. The optimum conditions for derivatization, fluorescence detection and chromatographic separation are established. The method shows good sensitivity, with a detection limit from 0.18 to 2.53 pmol, and good linearity between 1–250 nmol/mL of each analyte. The practical applicability of the method was demonstrated by analyzing samples that were spiked with the acid standards, environment and food samples.

Introduction

Carboxylic acids are widely distributed in nature. Various carboxylic acids include unitary acids as formic acid, acetic acid and benzoic acid (1–3) and binary acids as oxalic acid, succnic acid and citric acid (4–6), which play an important physical and sapor role at trace levels in the fields of environment and industrial food chemistry. The isolation and quantitation of these compounds are of widespread interest. Because binary acids are much more sensitive than unitary acids for their ultraviolet properties (2, 4), a reliable and sensitive monitoring of unitary carboxylic acid substances is crucial for environmental pollution control, industrial applications and food nutrition.

The common methods for determination of unitary carboxylic acids are the titration technique and colorimetric analysis (7–8). These methods have poor sensitivity and common interference, which have reduced their applications. In recent years, electrochemical methods such as ion chromatography (IC) (9) and high-performance capillary electrophoresis (HPCE) (10) have been introduced for the analysis of carboxylic acids because of their high chromatographic efficiency. However, the low detection limit and complex purification procedures have limited their usage. Gas chromatography (GC) coupled with various kinds of detectors, such as flame ionization detection and electrochemical detection, provides total analysis of multiple components with highly sensitive and selective determination by a single chromatographic run (11–12). Because carboxylic acids have low pH value and high polarity, a prior derivatization step is necessary to increase the volatility and sensitivity.

Compared with GC and capillary electrophoresis (CE), high-performance liquid chromatography (HPLC) is a good alternative technique that has been used for the analysis of a wide variety of samples, including carboxylic acids (13). Generally, many unitary carboxylic acids do not exhibit efficient detection properties, such as absorption in ultraviolet-visible (UV-vis) regions, luminescence, fluorescence (FL), chemiluminescence (CL) and redox activity. Therefore, pre-chromatographic derivatization is necessary to increase sensitivity and selectivity.

The FL detection method, which is based on the fluorescence derivatization reagent having a fluorophore group and a reactive functional group for carboxylic acids, is known as one of the most efficient and sensitive systems. Some derivatization reagents, such as 9-(2-hydroxyethyl)-carbazole (14), 5-(4-pyridyl)-2-tiophenmethanol (15) 2-(4-hydrazinocarbonyl-phenyl)-4,5-diphenylimidazole (16), 6,7-dimethoxy-1-methyl-2-oxo-1,2-dihydroquinoxalin-3-yl- propionohydrazide (DMEQ-hydrazine) (17), 5-amino-4-sulfanylphthalhydrazide (ASPH) (18) and 2-aminooxy-N-[3-(5-dimethylamino)-naphthalene-1-sulfonylamino]propylacetamide (19) have been used to make FL derivatives of carboxylic acids. However, reliable derivatization procedures based on novel labeling reagents that offer high sensitivity, high selectivity and good FL properties with good stabilities of the reagents and the derivatives are still needed.

The aim of this paper was to perform an analytical study on the carboxylic acids in environmental samples and food samples. 9-Chloromethyl anthracene was used as the pre-column derivatization agent to convert carboxylic acids to its derivatives and analyze them using HPLC with FL detection, with the purpose of obtaining the best resolution of peaks and detector selectivity than with GC methods, and better sensitivity than that achieved with HPLC–UV-vis detection.

Experimental

Instrumentation

The HPLC system was from the HP 1100 series (Agilent, St. Louis, MO) and consisted of an on-line vacuum degasser (model G1322A), a quaternary pump (model G1312A), a thermostated column compartment (model G1316A), an FL detector (FLD) (model G1321A) and an injector. HP Chemstation software was used to control the operation, data collection and manipulation of all chromatographic systems.

Chemicals

Labeling agent 9-chloromethyl anthracene (97% purity, Merck, Darmstadt, Germany) was prepared at concentrations of 1 and 10 μmol/mL in acetonitrile. Tetrabutylammonium bromide
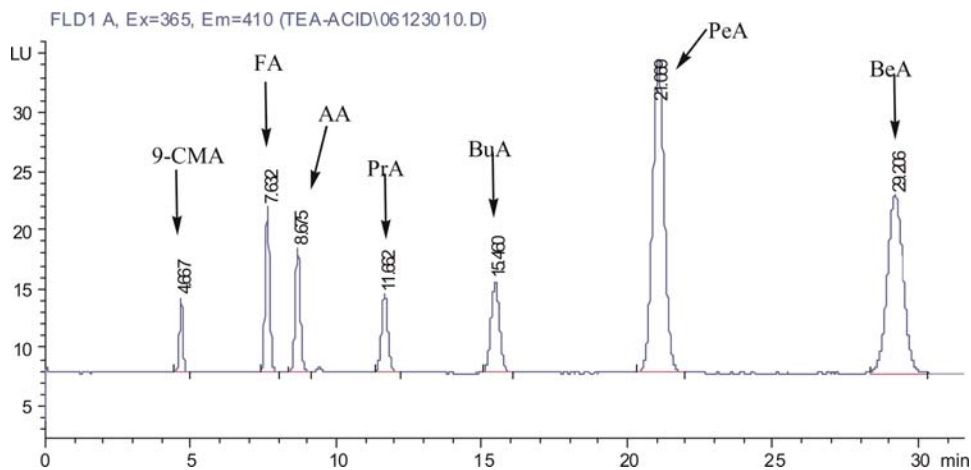
Figure 1. Chromatogram of acid derivatives with fluorescent detection.

(98% purity) and triethanolamine (97% purity) were purchased from Aldrich. Tetrabutylammonium bromide (20 μmol/mL) solution in acetonitrile and 5% triethanolamine solution in acetone were prepared. These solutions were stored at 4°C in refrigerator before use. Standard carboxylic acids as formic acid, acetic acid, propionic acid, butyric acid, pentanoic acid and benzoic acid were of analytical grade and purchased from Guangzhou Chemical Reagent Company. Acetonitrile (HPLC grade, Merck, Darmstadt, Germany) was used as solvent and mobile phase. Water was purified on a Milli-Q system. All other reagents and organic solvents were of analytical grade.

Chromatographic condition

HPLC separation of carboxylic acid derivatives was performed on a Hewlett Packard RP-18 column (200 mm × 4.6 mm, 5 mm) with the column temperature set at 30°C. The mobile phase with 64% acetonitrile and 36% water was used and flow rate was set constant at 1.0 mL/min. The injection volume was 2mL for each sample. The selected excitation and emission wavelengths were set at λ_{ex} 365 and λ_{em} 410 nm, respectively.

Preparations of calibration and quality control samples

Standard solutions of formic, acetic, propionic, butyric, pentanoic and benzoic acid (1 μmol/mL) were made by dissolution of the appropriate amount of each carboxylic acid in acetonitrile. Briefly, 1–250 μL of every standard carboxylic acid (1 μmol/mL) were used in derivatization to prepare the solution curves, with final concentrations of 1, 5, 25, 100, 200, and 250 nmol/mL detected on HPLC. Quality control (QC) solutions were used for QC preparation, with each carboxylic acid concentration of low (5 nmol/mL), medium (50 nmol/mL), and high (200 nmol/mL). When not in use, the solutions were stored at 4°C in a refrigerator.

Sample preparation and derivatization procedure

Samples of mixed carboxylic acids standard solution were directly derivatized. Carboxylic acid spiked samples and biological samples as juice and soil were prepared as follows.

The samples (0.2 g or 0.5 mL) were dissolved and diluted to 5 mL with water and filtered with a 0.45 μm membrane to remove the impurities, and then adjusted to pH 2.0 with 1 mol/mL hydrochloric acid solution. To 0.2 mL of the filtrate, 0.3 mL of ethyl acetate was added and vortexed at high speed for 3 min. The procedure was repeated three times and the supernatant solvent was collected. The organic layer was evaporated to dryness in a flask under a stream of nitrogen at 40°C after adding 20 μL of triethanolamine solution. To the residue, 50 μL of 9-CMA solution (1 or 10 μmol/mL) was added, followed by successively added tetrabutylammonium bromide solution (20 μmol/mL). To the flask was added 0.5 mL acetonitrile and allowed to stand at 75°C for 50 min in a thermostatic water-bath in the dark to facilitate derivatization. The reaction solution was cooled and diluted to 1 mL for HPLC analysis.

Results and Discussions

Analysis efficiency

A chromatogram of carboxylic acid derivatives with fluorescent detection was shown (Figure 1). According to the chromatogram, six acid derivatives were well separated. The retention time of each acid derivative and relative standard deviation (RSD%) of retention time and peak area were studied (Table I).

Method validation

Linearity and detection limits

The standard curve range was 1–250 nmol/mL for the final concentration of carboxylic acid derivatives. Each 2 μL was injected for HPLC analysis. Six calibrations were obtained by plotting the peak area ratio of the analytes (Y) against the corresponding concentrations (X). Excellent linearity was achieved in these specified concentration ranges with the correlation coefficients (r) greater than 0.9981. The calibration curves (Table II) were suitable for the quantification of carboxylic acid derivatives during the intra-day and inter-day validations.

Table I
Retention Time, RSD of Retention Time and Peak Area of Carboxylic Acid Derivatives

Carboxylic acids	Retention time (tr/min)	Retention time (RSD %)	Peak area (RSD %)
Formic Acid (FA)	7.632	2.44	1.241
Acetic Acid (AA)	8.675	0.93	1.922
Propionic Acid (PrA)	11.662	1.22	2.376
Butyric Acid (BuA)	15.460	1.39	1.235
Pentanoic Acid (PeA)	29.206	0.73	1.462
Benzoic Acid (BeA)	21.069	1.64	2.094

Table II
Linear Regression Equations and Correlation Coefficient Data of Acid Derivatives

Carboxylic acids	$Y = A \times X + B$	correlation coefficients (r)	Detection limits (pmol)	LOD(mg/L)
FA	$Y = 17.49X — 28.13$	0.9994	2.53	1.49
AA	$Y = 29.98X — 46.00$	0.9995	1.82	10.91
PrA	$Y = 25.21X — 2.29$	0.9993	1.28	12.51
BuA	$Y = 11.70X + 1.21$	0.9989	0.83	–
PeA	$Y = 15.16X + 190.01$	0.9981	0.56	–
BeA	$Y = 19.99X — 27.14$	0.9994	0.18	0.18

Detection limits are an important consideration when the components of biological matrices are analyzed; in particular, they are present at low or trace concentrations. The detection limits for each standard carboxylic acid derivative (at a signal-to-noise ratio = 3:1) are from 0.05 to 0.10 pmol; as for the biological samples, the detection limits were from 0.18 to 2.53 pmol, as shown in Table II. Compared to those methods using HPLC–UV-vis detection (nmol/mL) (1, 2), the proposed method offers lower detection limits.

Stability and selectivity

In this study, the stability of the labeling agent 9-chloromethyl anthracene and the labeled carboxylic acids were investigated. Anhydrous acetonitrile solution of 9-CMA was stored at 4°C in a refrigerator, the derivatization yields for acids showed no obvious difference in four weeks. The labeled carboxylic acids with different concentrations of low (5 nmol/mL), medium (50 nmol/mL) and high (200 nmol/mL) were analyzed. The corresponding derivative for further HPLC analysis showed normalized peak areas varying less than 3.0%.

Biological samples are complex, containing other organic acids such as oxalic acid, succinic acid, fumaric acid, citric acid, tartaric acid, butenedioic acid and (o-, p-) phthalic acid. The influence of these binary acids during derivatization reaction and HPLC analysis were studied. The chromatography spectrum suggested that binary acids hardly reacted with 9-CMA and had no effect on HPLC analysis.

Accuracy and precision

The accuracy and precision of the method were assessed by analyzing the low, medium and high QC samples (5, 50 and 200 nmol/mL). The samples were prepared, derivatized and HPLC analyzed as described previously. Five repeated analyses at each concentration were performed. Table III summarizes

Table III
Results of Recovery and Precision Test ($n = 5$)

Carboxylic acids	Low QC (5 nmol/ml)		Medium QC (50 nmol/ml)		High QC (200 nmol/ml)	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
FA	77.8	4.28	83.4	3.77	87.2	3.24
AA	87.2	3.74	91.1	3.45	93.4	2.43
PrA	81.6	3.08	83.9	3.49	86.2	3.02
BuA	79.2	3.36	84.6	2.96	86.8	2.86
PeA	84.9	3.59	88.5	3.18	89.3	2.73
BeA	88.2	2.64	90.6	2.51	92.0	2.16

the precision and accuracy of carboxylic acid derivatives on each concentration by calculating recovery (RE%) and RSD%.

Optimization of the method

Extraction efficiency

Selection of the extraction solvent. Each carboxylic acid solution (0.5mL, 1 μmol/mL) was mixed and diluted to 2 mL with water. Every 0.5mL of the mixed solution was used for study on the extraction effect of different solvent. Ether, ethyl acetate and n-hexane were employed as the extraction solvents and HPLC analyzed after derivatization. The results indicated that ethyl acetate was found to be the best choice of the three solvents.

Optimization of the extraction pH. The effect of the pH on extraction was studied by varying the pH of sample solvent during extraction. Different pH of 1.0, 2.0, 2.5, 3.0 and 4.0 were investigated. With parallel derivatization and HPLC analysis, the yield reached maximum at pH 2.0 suggested that the optimum pH for the extraction of carboxylic acids at room temperature is 2.0.

Optimitization of derivatization

It is clear that 9-CMA is a very useful label for the analysis of carboxylic acids. However, prior to developing a separation procedure for the analysis of carboxylic acids using 9-CMA derivatives, the factors that influence the derivatization were investigated. The derivatization was thoroughly optimized with respect to derivatization solvent, the ratio of the label concentration of 9-CMA, reaction time and temperature and catalyst concentration.

Effect of derivatization solvent. Several types of solvents were also evaluated in this study for the optimum derivatization, including n-hexane, acetonitrile, THF and acetone. Acetonitrile and THF were similar to achieve satisfactory derivatization yields. As for the stability of 9-CMA and carboxylic acid derivatives in acetonitrile, the results converged at acetonitrile as the optimal derivatization solvent.

Effect of 9-CMA concentration. The concentration of 9-CMA is critical for the labeling reaction, and the effect of 9-CMA concentration on the derivatization yields was studied. Fifty microliters of each carboxylic acid solution (1 μmol/mL) were mixed and reacted with 9-CMA (10 μmol/mL) by varying the ratio of the label concentration to that of the acids (9-CMA/acids) in the range of 1/1 to 10/1. The yield reached maximum at a ratio of 9-CMA/acids of 5/1, after which it
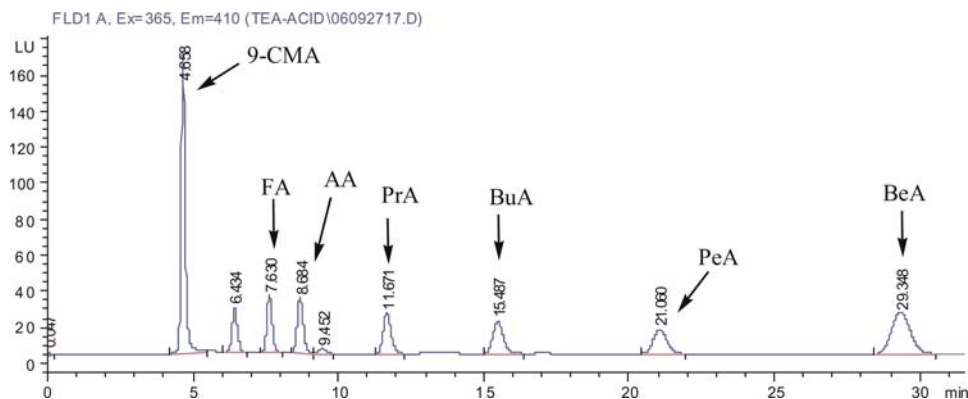
Figure 2. Chromatogram of a soil sample.

become constant. However, a ratio of 10/1 is used in this study to establish an excess of reagent.

Effect of reaction temperature and time. The effect of temperature on derivatization was tested in acetonitrile solvent containing 50 μL of each carboxylic acid solution (1 μmol/mL) with 50 μL 9-CMA (10 μmol/mL) from 50 to 100°C. The results indicated a remarkable increase in detection response with increasing temperature that reached maximum at 75°C. At temperatures higher than 90°C, a slight decrease in detection response was observed. This is probably because higher derivatization temperature results in some by-products. With reaction temperature at 75°C, the maximum detection response of every acid derivative was reached after 40 min. The response remained constant from 40 to 70 min. However, taking both derivatization time and by-product into consideration, derivatization temperature and time were set at 75°C for 50 min.

Effect of catalyst. The derivatization reactions of carboxylic acids with 9-CMA are usually accelerated in the presence of phase transfer catalyst, and the catalyst experiment indicated that tetrabutylammonium bromide with the concentration > 1.0% accelerated the derivatization.

HPLC chromatographic separation

Influence of mobile phase. Mobile phase composition is one of the most important chromatographic factors affecting the separation because of the small variability in the types of the stationary phase available. The effect of changing the percentage of acetonitrile with water was investigated. Detection responses of carboxylic acid derivatives were sensitive with slight variety with the acetonitrile concentration from 45 to 70% on the separation. The best compromise for a high signal intensity and separation quality was achieved by using a mobile phase containing 64% acetonitrile and 36% water.

Influence of mobile phase flow-rate. The flow rate was studied between 0.5 and 1.2 mL/min. At low flow rates below 1.0 mL/min, retention times were increased; however, very broad peaks were obtained. At flow rates higher than 1.0 mL/min, the change in the chromatogram features was not significant; therefore, a flow rate of 1.0 mL/min was used for the HPLC analysis.

Selection of fluorescent detection wavelength. The choice of the excitation and the emission wavelengths is essential to obtain good sensitivity and repeatability in FL methods.

Table IV
Results of the Soil Sample

Carboxylic acids	FA	AA	PrA	BuA	PeA	BeA
Concentration (nmol/mL)	8.46	6.32	4.85	2.11	1.14	0.82

The carboxylic acid of its 9-CMA derivative has their maximum excitation wavelength in acetonitrile medium at 256 and 365 nm. Considering the selectivity, 365 nm was prepared. The maximum emission wavelength was found to be 410 nm from the emission spectrum obtained by exciting the analysts at 365 nm.

Application to samples

Various samples were collected, including soils from a garden, mud from a country road, slurry from a river bed and juice samples. IC (Dionex DX-600 IC system) showed no response signal of carboxylic acid. Each sample preparation was performed as described previously. HPLC analysis found the existence of acid, which indicated the application of the method for carboxylic acid analysis. Figure 2 shows the chromatogram of one of the samples, with the results shown in Table IV.

Conclusions

A highly sensitive method for the simultaneous determination of organic acids in biological samples was developed and validated using liquid–liquid extraction, derivatization and HPLC separation with FL detection. Good separation, linearity and relative recoveries were achieved. The use of chemical derivatization of 9-chloromethyl anthracene significantly enhanced the detection sensitivity. This validated method can be applied for organic acid analysis in environmental samples and food samples.

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