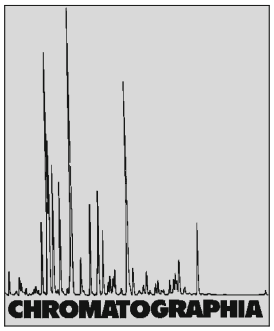
Determination of Fatty Acids (C_1 – C_{10}) from Bryophytes and Pteridophytes



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Abstract

A simple and mild method for the determination of fatty acids (C_1 – C_{10}) based on a condensation reaction using 7-aminonaphthalene-1,3-disulfonic acid (ANDSA) as labeling reagent with capillary zone electrophoresis has been developed. The detection was performed with a diode array detector at 254 nm. A 58.5 cm × 50 μm i.d. (50 cm effective length) untreated fused-silica capillary was used. To optimize the separation conditions, the background electrolyte concentration, column temperature, voltage and other factors were evaluated. The optimal separation conditions were as follows: 30 mmol L^{-1} borate buffer (pH 9.5), 15 mmol L^{-1} β-CD, temperature at 20 °C, pressure 50 mbar and injection time 8 s. Under the established conditions, 10 fatty acid derivatives could be well-separated within 17 min. The linearity was in the range of 0.07–5.0 μmol L^{-1}. Detection limits (at a signal-to-noise ratio of 3) were in the range of 0.027–0.042 μmol L^{-1}. The fatty acids from the extracted *Funaria* Hedw. and *Selaginella* samples were determined with satisfactory results.

Keywords

Capillary zone electrophoresis
Pre-column derivation
Fatty acids
Funaria Hedw. and *Selaginella*

Introduction

Bryophytes and pteridophytes are two large groups of spore producing land plants. The phytochemistry of bryophytes and pteridophytes has been neglected for a long time because they are very small and difficult to collect in large amounts as pure samples. However, the bryophytes and pteridophytes have been used as medicinal plants to cure cuts, external wounds, bacteriosis, pulmonary tuberculosis, neurasthenia, pneumonia, neurasthenia, etc. It is estimated that 80% of the bryophytes and pteridophyte plants show sharp, acrid, and unpleasant taste that are not damaged by insect, bacteria, snails, and mammals [1]. Although many chemical components have been elucidated in these plants such as lipophilic terpenoids [2, 3] acetogenins [4] and bisbibenzyl compounds [5] literature reports on fatty acids in bryophyte and pteridophyte plants are relatively poor. Fatty acids play physiologically important roles at trace levels in the regulation of a variety of physiological and biological functions. The investigation of the composition of fatty acids in bryophyte and pteridophyte plants is of equal importance. Although most fatty acids give no natural absorption in UV region, they can be easily detected by their fatty acid derivatives obtained by methyl esterification with GC or GC–MS [6, 7] and exhibit excellent sensitivity. Other methods including LC, micro-column LC [8, 9] and capillary electrophoresis [10, 11] have also been reported. However, the detection of fatty acids is mainly performed by indirect ultraviolet detection in capillary electrophoresis technology [12], and results in some shortcomings, such as low sensitivity, narrow linear range, serious background

interference and baseline drift, etc. At present, popular methods for the determination of fatty acids are pre-column and post-column derivatization with UV or fluorescence detection. Several common UV and fluorescent derivatization reagents [13, 14] have also been fully developed. In spite of the popularity of these pre-column methods, there have also been many reports describing various shortcomings in applications, such as high solvent consumption, time-consuming and tedious analysis. In our previous studies [15], long-chain fatty acids (LCFAs > C20) from the extracted bryophyte plants were determined by LC with fluorescent detection using 1-[2-(*p*-toluenesulfonate)ethyl]-2-phenylimidazole-[4,5-*f*]-9,10-phenanthrene (TSPP) as tagging reagent. However, the determination of short-chain fatty acids from the extracted *Funaria* Hedw. and *Selaginella* samples by capillary zone electrophoresis had no reports.

In this study, the principal goal is to develop a rapid method for the determination of short-chain fatty acids by capillary zone electrophoresis. The short-chain fatty acids are easily derivatized to their acid amides using ANDSA as labeling reagent in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl). The condensation reaction gives higher yields of the desired fatty acid amides. Corresponding fatty acid amides can be efficiently separated by capillary electrophoresis with a good baseline resolution. The optimal separation conditions, such as background electrolyte concentration, column temperature, and voltage are investigated. Linearity and detection limits are also evaluated. At the same time, the applications for the determination of short-chain fatty acids (C_1-C_{10}) from *Funaria* Hedw. and *Selaginella* samples have been reported. The suitability of the developed method for the analysis of actual samples is satisfactory.

Experimental

Instruments and Reagents

HP-3D capillary electrophoresis instrument (Agilent Technologies, Palo Alto, CA, USA), and capillary column: 58.5 cm × 50 μm i.d. (50 cm effective length) (Hebei Yongnian Ruifeng Co. Ltd, Shandong, China) were employed. Fatty acids ($C_1 - C_{10}$) standards were purchased from Jining Chemical Reagent Co. (Shandong, China). 7-Amino-1,3-naphthalene disulfonic acid (99.8%) was from BoDi Chemical Limited Co (Tianjin, China). EDC.HCl was from Sigma (St. Louis, MO, USA). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA).

Preparation of Standard Solutions

Individual stock solutions of fatty acids were prepared in acetonitrile. The standard fatty acids for CE analysis at an individual concentration of $5.0' 10^{-4}$ mol L^{-1} were prepared by diluting the corresponding stock solutions ($1.0' 10^{-3}$ mol L^{-1}) of each fatty acid with acetonitrile. ANDSA solution ($5.0' 10^{-4}$ mol L^{-1}) was prepared by dissolving 3.03 mg ANDSA in 2.0 mL of phosphate buffer (pH 3.0). EDC.HCl solution ($1.0' 10^{-3}$ mol L^{-1}) was prepared by dissolving 3.83 mg of EDC.HCl in 2.0 mL of acetonitrile. When not in use, all standards were stored at 4 °C.

Derivatization of Standard Solution

25 μL acetonitrile solution of fatty acids was added in a vial, to which 75 μL ANDSA phosphate buffer (pH 3.0) and 150 μL of EDC.HCl acetonitrile solution were then added. The solution was allowed to stand for 15 min at 60 °C in a water bath. The derivatization solution was directly injected into the CE system for the separation.

Pretreatment of Samples

The freshly obtained *Funaria* Hedw. and *Selaginella* samples were washed with water and dried at room temperature. To a 25 mL round-bottom flask, the pulverized *Funaria* Hedw. (0.1022 g) and 10 mL chloroform were added. The contents were sonicated for 30 min and allowed to stand for 24 h and then filtered. The residue was again washed with chloroform (5.0 mL). The combined chloroform layers were saturated with ammonia and evaporated to dryness under a stream of nitrogen gas, and the residues were re-dissolved in DMF and stored at 4 °C until derivatization analysis. The *Selaginella* sample was treated according to the same method as described above.

Separation Conditions

A new capillary column was activated by washing consecutively with 0.1 mol L^{-1} sodium hydroxide (30 min) and deionized water (30 min). At the beginning of each working day, the capillary was prewashed with 0.1 mol L^{-1} NaOH for 10 min and running buffer for 20 min. Before each analysis, the capillary was consecutively rinsed with 0.1 mol L^{-1} NaOH (2 min), water (1 min) and running buffer (5 min). Sample was loaded onto the column by pressure injection for 8 s at 50 mbar.

Results and Discussion

Optimization for Derivatization Reaction

As observed, molecular structure of ANDSA contained an amino function group; it had the same condensation reaction with a carboxylic function group of fatty acids as the PPIA for amine derivatization previously reported in our experiment [16]. Derivatization of ANDSA with fatty acids could be achieved within 10 min at 60 °C. The detector responses of ANDSA-derivatives increased with increasing amounts of reagent concentrations. The maximal yields close to 100% were observed with a 3.0-fold molar reagent excess to total molar fatty acids, increasing the excess of reagent beyond this level had no significant effect on yields. With as little as a 3.0-fold molar excess of reagent to total molar fatty acids, the derivatization of fatty acids was incomplete and obviously resulted in low detection

responses. To an unknown concentration of sample, complete derivatization was guaranteed by using a large excess of ANDSA until constant peak intensity for detector responses.

EDC. HCl as reaction coupling reagents was investigated. Reaction was carried out at 60 °C for 10 min with $5.0' 10^{-4}$ mol L^{-1} of 10 fatty acids in the presence of various amounts of EDC.HCl. The results indicated that detector responses for the derivatized fatty acids increased with increasing amounts of EDC.HCl. The constant peak intensity could be achieved with a six-fold molar EDC.HCl excess to total molar fatty acids, increasing the excess of EDC.HCl beyond this level had no significant effect on yields. The labeled derivatives exhibited high stability and were enough to be efficiently analyzed by CE 48 h.

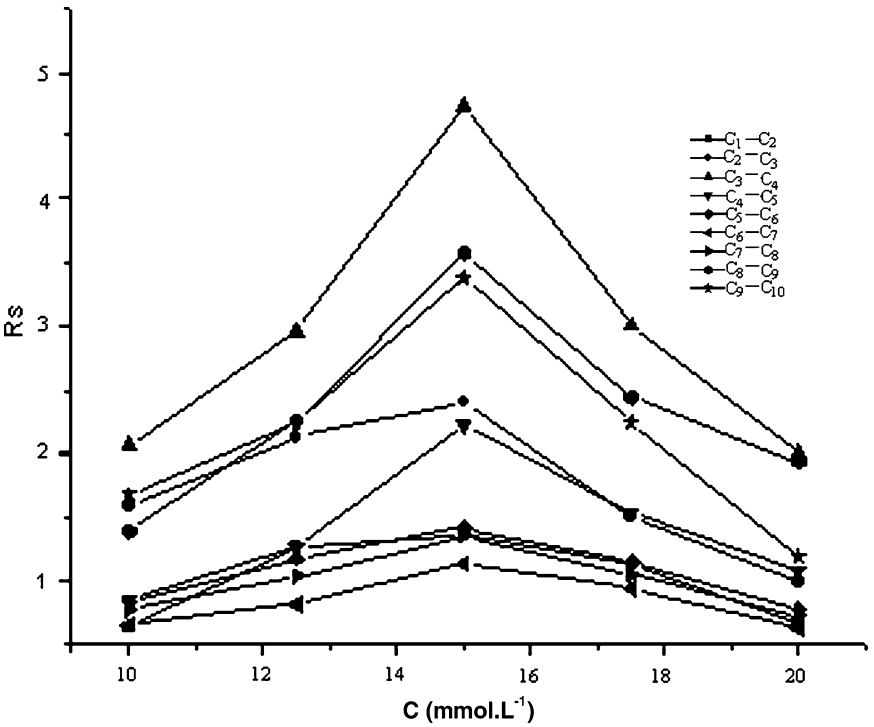

Fig. 1. Effects of β-CD concentration on resolution (conditions: temperature, 20 °C; voltage, 18 kV.) $\mathbf{C_1}$ = formic acid; $\mathbf{C_2}$ = acetic acid; $\mathbf{C_3}$ = propionic acid; $\mathbf{C_4}$ = butyric acid; $\mathbf{C_5}$ = valeric acid; $\mathbf{C_6}$ = hexanoic acid; $\mathbf{C_7}$ = heptanoic acid; $\mathbf{C_8}$ = octanoic acid; $\mathbf{C_9}$ = pelargonic acid and $\mathbf{C_{10}}$ = decanoic acid

Optimization of CZE Separation

Effect of Buffer pH and Concentration on Separation

The effects of buffer pH on separation in this experiment were investigated in the pH range of 8.0 – 10.0. At pH < 9.0, most of the fatty acids were resolved with the exception of C_6 and C_7 (partially co-eluted). With pH > 10.0, the pairs of C_1 and C_2, C_6 and C_7 could not completely achieve the baseline resolution. With pH 9.5, 10 fatty acid derivatives could be obtained with a good baseline resolution.

Several types of buffers, including carbonate buffers, phosphate buffers and borate buffers, at concentrations of 20–40 mmol L^{-1} (pH 9.5) were also examined to compare the separation efficiency and peak-to-peak resolutions of the ANDSA-fatty acid derivatives. The results indicated that a good separation for fatty acid derivatives was obtained using borate buffer. Several different borate buffers at concentrations of 20, 25, 30, 35 and 40 mmol L^{-1} have been examined. It was found that the resolution of each fatty acid derivative was very sensitive to borate buffer concentrations. An increase of borate buffer concentration from 20 to 30 mmol L^{-1} resulted in great improvement to the separation efficiency for fatty acid derivatives, while the elution order of each fatty acid derivative was kept constant. An increase of buffer concentration from 30 to 40 mmol L^{-1} resulted in a relatively long migration time and low resolution. To obtain highest separation efficiency with the shorter migration time, the borate buffer concentration was selected at 30 mmol L^{-1}.

Effects of β-CD on the Separation

The separation was achieved by the addition of β-CD to the background electrolyte. Comparing borate electrolyte containing β-CD with pure borate electrolyte (30 mmol L^{-1}, pH 9.5), an obvious increase in resolution with increasing concentration of β-CD from 10 to 15 mmol L^{-1} was observed (Fig. 1). A gradual increase of β-CD concentration beyond 15 mmol L^{-1} resulted in a relatively long migration time and low separation efficiency. The increasing migration times with increasing β-CD concentrations are due to complexation and increase in buffer viscosity. In this study, the selected β-CD concentration was 15 mmol L^{-1}.

Effects of Temperature and Voltage on the Separation

The effects of the temperature on the resolution of ANSDA derivatives were investigated. The results indicated that the resolution of ANDSA derivatives slightly increased with increasing temperature from 10 to 30 °C. To decrease Joule heating, capillary working temperature was set at 20 °C. In addition, migration time and resolution of ANSDA derivatives decreased obviously with increasing working potential from 15 to 35 kV. In this study, 18 kV working voltage was selected. On the basis of the tested results, the optimal conditions for the separation of ANSDA derivatives were set at: 30 mmol L^{-1} borate buffer (pH 9.5), 30 mmol L^{-1} β-CD, working voltage at 18 kV, temperature at 20 °C,

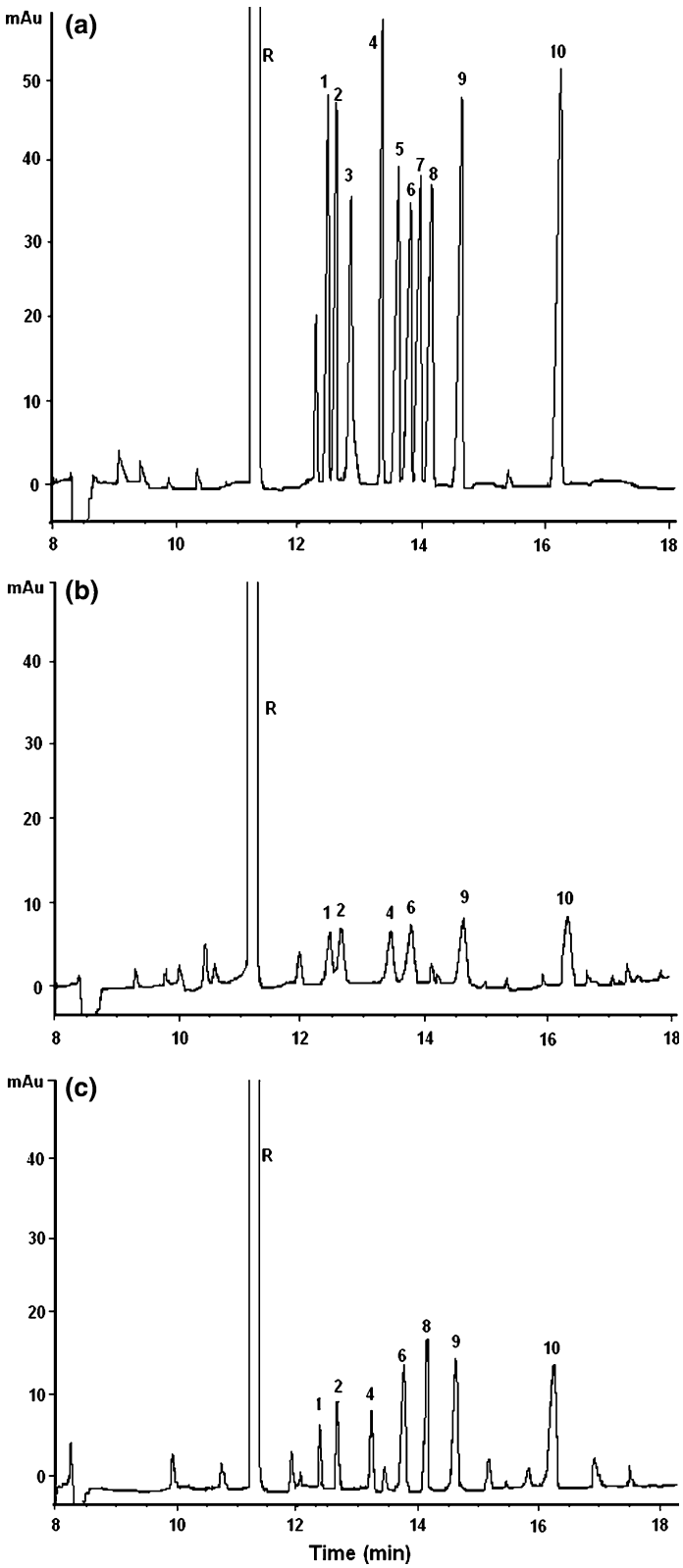

Fig. 2. Electropherograms of a mixture standard solution (**a**), *Funaria* Hedw. sample (**b**) and *Selaginella* sample (**c**). **1** = formic acid; **2** = acetic acid; **3** = propionic acid; **4** = butyric acid; **5** = valeric acid; **6** = hexanoic acid; **7** = heptanoic acid; **8** = octanoic acid; **9** = pelargonic acid; **10** = decanoic acid; **R** = 7-amino-1,3-naphthalene disulfonic acid

injection pressure 50 mbar and injection time 8 s. Figure 2a shows the separation of ANSDA derivatives in CZE mode under the conditions proposed.

Method Validation

The linearity was established with serial analysis of standard ANSDA derivatives at the concentration range of 0.07 – 5.0 μmol L^{-1}. All ANSDA derivatives were found to give an excellent linear response over this range investigated, with correlation coefficients of >0.9991 (Table 1). The calculated detection limits for each derivatized fatty acid were from 0.027 to 0.042 μmol L^{-1} (at a signal-to-noise ratio = 3:1). The repeatabilities of the migration time and peak area were estimated by making repetitive injection of a standard derivative under the selected conditions ($n = 5$). The relative standard deviations of the migration time and peak area were from 0.34 to 0.52% and from 1.36 to 1.94%, respectively.

Two actual samples of *Funaria* Hedw. and *Selaginella* were, respectively, spiked with standard fatty acids and used to validate the accuracy of this method. The recoveries for 10 fatty acid derivatives were in the range of 96.8 – 102.1% with a relative standard deviation from 1.1 to 3.4%. The results indicated that the method was sufficiently stable for the simultaneous determination of fatty acid derivatives.

Applications

The typical chromatogram for the determination of fatty acids obtained from the extracted *Funaria* Hedw. and *Selaginella* samples is shown in Fig. 2b, c. The qualitative analysis was performed by a standard addition method and migration time for each analyte, respectively. The results indicated that *Funaria* Hedw. sample contained six fatty acids (C_1, C_2, C_4, C_6, C_9, and C_{10}) and *Selaginella* sample contained seven fatty acids (C_1, C_2, C_4, C_6, C_8, C_9, and C_{10}). The contents of fatty acids are shown in Table 2.

Table 1. Linear regression equation, correlation coefficient, linear range and detection limits of fatty acids

Fatty acids	Linear regression equation	Correlation coefficient	Linear range (μmol L^{-1})	Detection limit (μmol L^{-1})	Migration time RSD (%)	Peak area RSD (%)
C_1	$Y = 1.10 + 32.75x$	0.9997	0.07–5	0.032	0.42	1.73
C_2	$Y = 2.45 + 29.76x$	0.9988	0.07–5	0.033	0.49	1.94
C_3	$Y = 0.28 + 34.91x$	0.9992	0.16–5	0.039	0.41	1.62
C_4	$Y = 0.77 + 35.79x$	0.9985	0.07–5	0.027	0.37	1.48
C_5	$Y = 2.91 + 35.17x$	0.9983	0.16–5	0.039	0.52	1.91
C_6	$Y = 0.01 + 34.26x$	0.9995	0.16–5	0.042	0.34	1.36
C_7	$Y = -1.37 + 36.28x$	0.9991	0.07–5	0.037	0.47	1.83
C_8	$Y = 0.42 + 36.07x$	0.9996	0.07–5	0.038	0.44	1.81
C_9	$Y = 3.58 + 44.31x$	0.9992	0.07–5	0.031	0.52	1.94
C_{10}	$Y = 1.57 + 58.04x$	0.9998	0.07–5	0.028	0.39	1.53

Table 2. The contents of fatty acids, recoveries and relative standard deviations for fatty acids spiked in the *Funaria* Hedw. and *Selaginella* ($n = 3$)

Fatty acids	*Funaria* Hedw.					*Selaginella*				
	Contents (μg g^{-1})	Added (μg g^{-1})	Found (μg g^{-1})	Recovery (%)	RSD (%)	Contents (μg g^{-1})	Added (μg g^{-1})	Found (μg g^{-1})	Recovery (%)	RSD (%)
C_1	2.13	9.2	11.4	101.2	1.8	2.06	9.2	11.1	97.9	3.4
C_2	2.78	12.0	14.9	100.8	2.1	2.93	12.0	14.6	97.3	2.8
C_3	[a]	14.8	14.4	97.2	2.4	[a]	14.8	15.0	101.2	1.7
C_4	1.77	17.6	19.3	99.7	1.7	1.91	17.6	19.4	99.1	2.1
C_5	[a]	20.4	20.2	99.2	2.4	[a]	20.4	19.7	96.7	2.6
C_6	2.44	23.2	26.1	102.1	1.4	4.28	23.2	27.6	100.7	1.3
C_7	[a]	26.0	25.2	96.9	1.1	[a]	26.0	26.5	101.9	1.7
C_8	[a]	28.8	28.6	99.2	3.1	3.99	28.8	32.6	99.2	2.3
C_9	2.05	31.6	33.8	100.6	1.6	3.30	31.6	33.9	96.8	2.2
C_{10}	1.80	34.4	36.6	101.1	2.1	2.64	34.4	36.9	99.7	1.7

[a] Not detectable or below detection limits

Conclusion

The proposed method offers a number of advantages: (i) Derivatization of fatty acids using ANSDA as labeling reagent is relatively simple; (ii) Derivatives can obtain a sensitive CZE-DAD detection. The described method shows good correlation for the quantitative analysis of fatty acids from *Funaria* Hedw. and *Selaginella* samples. The proposed method has been successfully applied to the determination of free fatty acids in real samples with satisfactory results. Current studies should further explore the separation of long-chain fatty acids by improving the CE mode or CZE operational conditions.

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