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Journal of Chromatography A, 1061 (2004) 99–104
JOURNAL OF CHROMATOGRAPHY A
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Determination of 1-aminocyclopropane-1-carboxylic acid in apple extracts by capillary electrophoresis with laser-induced fluorescence detection

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Received 6 August 2004; received in revised form 18 October 2004; accepted 19 October 2004

Abstract

A rapid and sensitive method for the determination of 1-aminocyclopropane-1-carboxylic acid (ACC) in apple tissues has been described. This method is based on the derivatization of ACC with 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ), and separation and quantification of the resulting FQ–ACC derivative by capillary electrophoresis coupled to laser-induced fluorescence detection (CE–LIF). Our results indicated that ACC derivatized with FQ could be well separated from other interfering amino acids using 20 mM borate buffer (pH 9.35) containing 40 mM sodium dodecyl sulfate and 10 mM Brij 35. The linearity of ACC was determined in the range from 0.05 to 5 μM with a correlation of 0.9967. The concentration detection limit for ACC was 10 nM (signal-to-noise = 3). The sensitivity and selectivity of this described method allows the analysis of ACC in crude apple extracts without extra purification and enrichment procedure.

Keywords: Derivatization; 1-Aminocyclopropane-1-carboxylic acid; 3-(2-Furoyl)quinoline-2-carboxaldehyde; Micellar electrokinetic chromatography

1. Introduction

Ethylene as a phytohormone has been found in all higher plants, fungi, yeast and bacterial [1], and associated with many physiological processes, including fruit ripening, senescence, and abscission of plant organs. The major biosynthetic pathway of ethylene has been elucidated [2,3]. In this pathway, 1-aminocyclopropane-1-carboxylic acid (ACC) was identified as an important immediate precursor of ethylene. The level of ACC in plant tissues plays an important role in the biosynthesis of ethylene [4,5]. Thus, the quantitative analysis of ACC has been a central component of many studies into ethylene physiology.

The classical method is based on the gas chromatography (GC) analysis of ethylene, which was produced from oxidative ring cleavage of the cyclopropane ring [6]. The main drawback of this method is that the variable conversion efficiency of ACC to ethylene and poor anti-interfering capacity affect the accuracy of this method [7,8]. In order to overcome these shortcomings, several analytical techniques have been developed for the direct determination of ACC, such as GC–MS [9,10], HPLC–UV [9,11,12], HPLC–MS [13,14], HPLC–MS–MS [15]. Most recently, a GC–negative chemical ionization MS combination with solid-phase extraction (SPE) method has been reported for the determination of ACC using pentafluorobenzyl bromide as a derivatization reagent [16]. This method can allow the detection of ACC in samples with fresh masses of about 100 mg. Although the MS detections are very reliable and selective, plant matrices still need complicated and time-consuming sample-cleaning step prior to derivatization to ensure accurate quantitation of ACC. Furthermore, the expensive and complex instrumentation limit the application of these methodologies.

Because molecular structure of ACC contain both amino group and carboxylic acid group, ACC could be assayed by a number of analytical techniques developed for the analysis of amino acids. Capillary electrophoresis (CE) has now been

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doi:10.1016/j.chroma.2004.10.067

recognized as an important alternative method to analyze amino acids as the results of its high performance, high speed, and low sample consumption [17]. When laser-induced fluorescence detection (LIF) is introduced in CE procedure, the detection of attomole amounts is achieved [18]. Currently, LIF is one of the most sensitive detection methods available for CE. To our knowledge, there are no reports about the assay of ACC by CE.

The main problem of ACC assay by CE–LIF is that the ACC molecular structure does not have a fluorophore group suitable for LIF detection. Therefore, it is necessary to choose an appropriate fluorescent derivatization reagent for the analysis of ACC. Various dyes have been developed for the CE–LIF analysis of amino acids, such as fluorescein isothiocyanate (FTC) [19–23], DTAF [23–26], 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [27], naphthalene-2,3-dicarboxyaIdehyde (NDA) [28], and 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) [29], etc. Among those derivatization reagents, fluorescein analogues and FQ are more widely used in CE–LIF detection because they can yield fluorescent products with an excitation wavelength near 488 nm (main line) of the commercial argon-ion laser. As a fluorogenic derivatization reagent, FQ was found to yield cleaner chromatograms than other fluorescent-labeling reagents. It is, therefore, widely used for CE analysis of different amino compounds, including amino acids [30,31], biogenic amino [32], protein [33] and amino phospholipids [34,35].

In this work, a micellar electrokinetic chromatography (MEKC)–LIF detection method using FQ as a pre-column derivatization reagent was firstly developed for the analysis of ACC. The optimized conditions for separation of ACC were investigated, and two extraction methods of ACC were also compared. The developed method has been successfully employed to determine ACC in the crude extract of apple.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent grade, unless stated otherwise. Amino acids and ACC, purchased from Sigma (St. Luois, MO, USA), were prepared at a concentration of 0.05 M in deionized water and diluted to the required concentration. FQ and KCN were obtained from Molecular Probes (Eugene, OR, USA). 25 mM KCN was prepared with water. Stock solution of 10 mM FQ was prepared with methanol. A 10 μl of FQ solution was then placed into a 500 μl microcentrifuge tube and the solvent was removed under vacuum. The dried FQ aliquots were stored at −20 °C. Sodium borate and boric acid were from Shanghai Chemical Reagent Co. (Shanghai, China). Sodium dodecyl sulfate (SDS) and polyoxyethylene lauryl ether (Brij 35) were obtained form Sigma (St. Louis, MO, USA). Water purified by a Milli-Q system (Millipore, USA) was used for the preparation of all solutions. All solutions were filtered through a 0.45 μm pore-size membrane filter before use.

2.2. Apparatus

All CE experiments were conducted in a capillary electrophoresis system (Agilent technologies, model HP^{3D}CE, Palo Alto, CA, USA), equipped with a ZETALIF laser-induced fluorescence detector (Picometrics, Ramonville, France). The exciting light (488 nm) was provided by an argon laser source (20 mW). The fluorescence emission was collected at 560 nm filtered by a band pass filter and a notch filter was used to attenuate background radiation. The HP Chemstation software was used for system control, data collection and processing.

Uncoated fused-silica capillaries (Yongnian Optic Fiber Inc., He Bei, China) of 65 cm (50 cm effective length) × 50 μm i.d. × 365 μm o.d. were used. New capillaries were pre-treated with 1 M NaOH for 60 min and followed by water for 60 min at room temperature. At the beginning of each day, the capillary was washed by flushing 0.1 M NaOH solution and water for 5 min, respectively, followed by preconditioned with running buffer for 10 min. The above flushing cycle was repeated to ensure the separation reproducibility for each injection.

2.3. Derivatization procedure

According to the pervious derivatization method [31], a 5 μl aliquot of ACC stock solution prepared as above was transfer to a 500 μl vial containing 100 nmol dried FQ. Then 5 μl of 25 mM KCN and 5 μl methanol were added. The vial was capped, vortexed, and centrifuged briefly at 8000 × *g* to ensure that all the reactants were in the tip of the tube. The derivatization reaction was processed at 55 °C for 60 min. Finally, 10 μl water was added to stop the labeling reaction.

2.4. Capillary electrophoresis

The running buffer consisted of borate buffer (20 mM pH 9.35), SDS (40 mM) and Brij 35 (10 mM). Buffer solutions were prepared daily and filtered through a 0.45 μm membrane prior to use.

Sample introduction was performed by hydrodynamic injection at 50 mbar for 5 s. The calculated volume of the injection is approximately 5 nl. The applied voltage for the CE separation was typically 25 kV.

2.5. Sample preparation

Field-grown apples (*Malm pumila* Mill) were picked from local orchard at mature green stage and selected for uniformity of color and size and absence of defects. Wounding treatment of apple was performed by nicking apple with blade, and then the wounded apple was kept at room temperature for 24 h before use.

Two extraction methods were tested in this paper: (1) ACC extract was performed mainly according to Lizada and Yang's [6] procedure with minor modification: apple pericarp (0.5 g) was homogenized on ice with 5 ml of 5% sulfosalicylic acid, and the mash was kept in 4 °C overnight. Then, the mixture was centrifuged at 15,000 × *g* at 4 °C for 20 min. The supernatants were ultra-filtered through a 0.2 μm pore size membrane to remove any debris, and then kept at −20 °C. (2) Apple pericarp (0.5 g) was homogenized on ice with 5 ml of 80% (v/v) methanol. The mixture was kept overnight at 4 °C and then certificated at 15,000 × *g* at 4 °C for 20 min. The supernatant was collected without further purification and stored in −20 °C.

All extracts were directly derivatized with FQ using the same procedure for the derivatization of ACC standard. The recoveries of ACC were obtained by spiking the ACC standard in crude extracts of apple.

3. Result and discussion

3.1. Optimization of separation procedure

Due to the similar chemical properties and molecular structures, many amino acids with non-polar side chains (Ala, Gly, Val, He, Phe, Leu, Met, Tip) may interference the assay of ACC.

In this paper, the separation conditions of the 16 common amino acids and ACC were investigated in different separation mediums. It has been reported that MEKC was successfully used in the resolution of the FQ-amino acids [30]. Therefore, the MEKC mode was chosen for the further study.

Initially, 20 mM borate buffer containing 40 mM SDS was tried as the running buffer. However, as shown in Fig. 1A, the electrophoretic peaks of ACC and Ala were overlapped. Even if the concentrations of buffer or SDS were varied, the resolution between Ala and ACC has not been significantly improved.

Mixtures of different surfactants can effectively improve sensitivity and resolution. Lalljie and Sandra [23,24] developed a MEKC–LIF method for the separation of 19 DTAF derivatized amino acids by using a combination of mixed micelles (SDS–Brij 35) and the addition of organic modifiers. In this work, it was found that the addition of 10 mM Brij 35 in SDS–borate buffer resulted in the resolution of ACC and Ala (Fig. 1B). Furthermore, the addition of Brij 35 can enhance the detection sensitivity for the FQ-amino acids [31,36]. The similar results were also observed in our experiment (Fig. 1A and B). Additionally, higher concentration of Brij 35 (20 mM) caused the delay of migration time with peak broadening (Fig. 1C). The possible explanation is that high concentration of Brij 35 caused unstable high current in separation procedure, which would lead to the unstable analytical results. Therefore, 20 mM borate (pH 9.35) containing 40 mM SDS and 10 mM Brij 35 was chosen as the running buffer for further experiments.

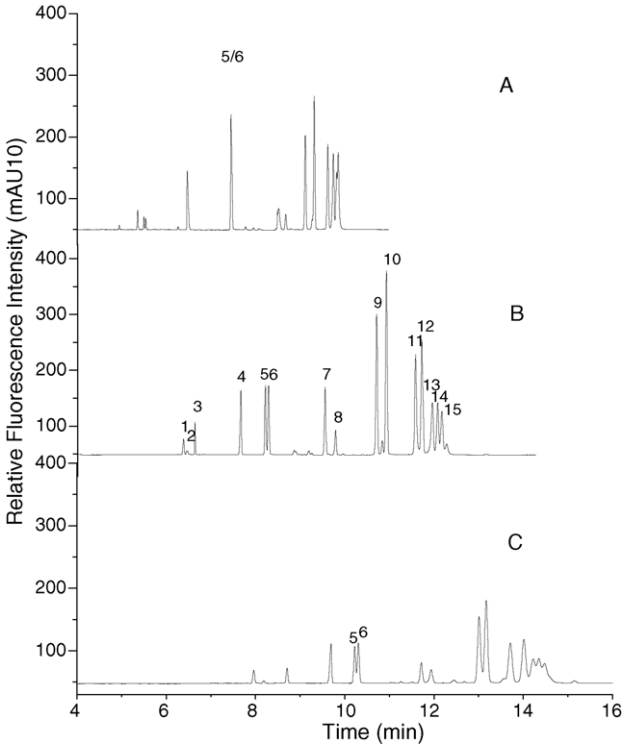

Fig. 1. Electropherograms obtained for separation of the 16 common amino acids and ACC in 20 mM borate buffer (pH 9.3) with different surfactant. (A) 40 mM SDS, (B) 40 mM SDS and 10 mM Brij 35, (C) 40 mM SDS and 20 mM Brij 35. Conditions: separation voltage: 25 kV; injection: 50 mbar for 5 s; temperature 25 °C. Amino acids concentrations: 1 μM. Peaks: (1) His, (2) Tyr, (3) Thr, (4) Gly, (5) ACC, (6) Ala, (7) Met, (8) Val, (9) Phe, (10) Leu/IIe, (11) Asn/Gln, (12) Trp, (13) Cys, (14) Glu, and (15) Asp.

3.2. Stability studies

The stability of the FQ–ACC derivative was studied at room temperature, 4, and −20 °C, respectively. When the derivative was placed in −20 °C for a week, No significant change (R.S.D. < 3.1%, $n = 5$) in the integrate peak area for ACC derivative was observed. However, there were 37% (R.S.D. < 7.2%, $n = 5$) and 13% (R.S.D. < 5%, $n = 5$) decreases in the peak area of ACC derivative over a period of a week at room temperature and 4 °C, respectively. Thus, the products of derivatization reaction should be stored in −20 °C to avoid the degradation of them.

3.3. Reproducibility, linearity and detection limits

The intra- and inter-day reproducibilities were determined by examining six sequential runs of 1 μM ACC standards solution for migration time and peak areas. The relative standard deviations (R.S.D.) of migration times in one day and between days were 2.1 and 4.7%, respectively. Additionally, the R.S.D. of peak areas in one day and between days were 2.6 and 5.9%, respectively. These data indicated that the CE–LIF method has a good reproducibility for ACC analysis.

Table 1
Comparison of the detection limits reported for ACC

Separation method	Sample treatment	Detection	Detection limit		Reference
			Concentration (M)	Mass	
GC	SPE	MS	2.5×10^{-8}	10 pg	[9]
	SPE	MS	1×10^{-8}	10 fmol	[16]
	No clean up	UV	5×10^{-8}	1 pmol	[11]
	SPE	UV	5×10^{-8}	1 pmol	[12]
HPLC	SPE	MS	1×10^{-7}	0.1 pmol	[13]
	No clean up	MS	1×10^{-6}	20 pmol	[14]
	SPE	MS	8×10^{-8}	0.8 pmol	[15]
CE	No clean up	LIF	1×10^{-8}	50 amol	This work

The quantitative analysis of ACC was performed by derivatization different amount of ACC standard in the range of 0.05–5 μM under optimum derivatization and separation conditions. The linearity was evaluated by plotting the peak area against ACC concentration, resulting in a correlation coefficient of 0.9967 and a slope of the regression of 1574 with an intercept of 37. The mass detection limit of ACC was 50 amol or 10 nM in terms of concentration (signal-to-noise = 3) and thus was better than the detection limits obtained by other reported methods including GC–MS, HPLC–UV, and HPLC–MS (Table 1).

3.4. Sample assay

The common extraction procedure of ACC from plant tissues involves the use of acidic water or aqueous alcoholic solution and extra purification and concentration step by solid-phase extraction. If the crude extracts were not clean up, ACC could be not detected or hardly quantified [16]. However, in this paper, the high sensitivity and selectivity of the developed CE–LIF method enable the direct determination of ACC in crude extracts without further purification and concentration. Additionally, different extraction solvents may seriously af-

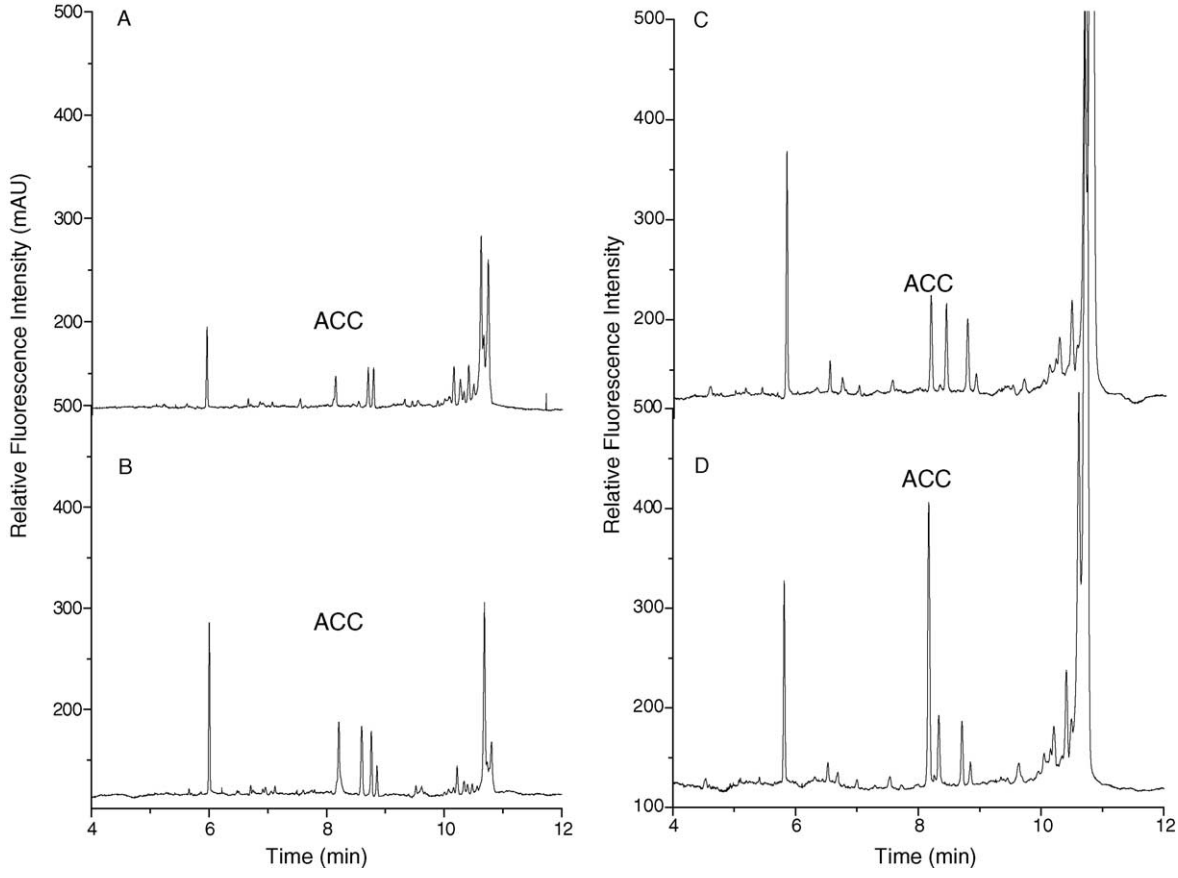

Fig. 2. Electropherograms of apple extracts using aqueous methanol (A) without wounding treatment, (B) with wounding treatment; and using 5% sulfosalicylic acid (C) without wounding treatment, (D) with wounding treatment. Conditions: running buffer consists of 20 mM borate buffer (pH 9.35), 40 mM SDS and 10 mM Brij 35, separation voltage: 25 kV; injection: 50 mbar for 5 s; temperature 25 °C.

Table 2
Analytical results of ACC in apple with FQ

ACC	Added (nmol/g)[a]	Found (nmol/g)	R.S.D. (%, $n = 5$)	Recovery (%)
Untreated apple	0	1.4	6.4	
	10	10.8	5.7	94.7
Wound apple	0	4.7	7.5	
	10	14.5	5.4	98.6

[a] g fresh mass.

fect the efficiency of extraction. In this work, the effect of sulfosalicylic acid and aqueous methanol on the extraction of ACC was also investigated (Fig. 2). The sulfosalicylic acid extraction method yielded 1.4 nmol/g fresh mass apple and the aqueous methanol only yielded 0.55 nmol/g fresh mass for the same sample, indicating that sulfosalicylic acid extraction method was more effective than that by aqueous methanol. Therefore, 5% sulfosalicylic acid was employed for the extraction of ACC in apple.

It has been reported that wounding treatment could lead to the increase of ACC in plant tissues [37]. As a preliminary application, the changes of ACC in apple without and with wounding treatment were also monitored by this CB–LIF method, respectively (Fig. 2C and D). It was found that the content of ACC in apple with wounding treatment increased about three times higher than that in apple without wounding treatment. The amount and recoveries of ACC in crude extracts of apple with wounding treatment or not are listed in Table 2. These results indicate that the assay of ACC in small amount of plant tissues can be well done by this developed method. The proposed method was directly used for the assay of ACC in crude extracts of apple without laborious time-consuming purification and concentration procedure compare to other protocols for the assay of ACC (Table 1).

4. Conclusion

A novel MEKC–LIF method for analysis of ACC after pre-column derivatization with FQ has been developed. The FQ–ACC derivative is stable, highly fluorescent and can be detected in a low concentration. The peak of ACC can be well resolved from other common amino acids using the borate–SDS–Brij 35 system. This simple, rapid and accurate procedure to determine ACC can allow the simultaneous analysis of many samples in a short time and represent several advantages in comparison with the conventional procedures involving complicated preparative steps and large amount of plant tissues.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (30230050).

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