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Determination of 1-aminocyclopropane-1-carboxylic acid and its structural analogue by liquid chromatography and ion spray tandem mass spectrometry

K. Petritis[a], V. Dourtoglou[b], C. Elfakir[a,*], M. Dreux[a]

[a]*Institut de Chimie Organique et Analytique (ICOA), CNRS UPRES-A, Université d'Orléans, B.P. 6759, Orléans Cedex 2, France*
[b]*VIORYL Chemical & Agricultural Industry, S.A., Viltanioti 36, 14564 K. Kifisia, Athens, Greece*

Abstract

Liquid chromatography coupled to ion spray tandem mass spectrometry was developed as a method for the simultaneous analysis of the amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) and its structural analogue, cyclopropane-1,1-dicarboxylic acid (CDA). ACC and CDA fragmentation as well as optimization of MS parameters were investigated in positive ion mode. In selective reaction monitoring mode the protonated molecule $[M+H]^+$ was selected as parent ion for both ACC and CDA, while the immonium ion from ACC and the $[M+H-H_2O]^+$ ion from CDA were selected, respectively, as product ions. In spite of the high selectivity of MS/MS among the 20 protein amino acids potentially present with ACC and CDA in the plant material analyzed, Glu and Thr can interfere with the signal of ACC. As a result, their chromatographic separation is necessary. This was achieved in less than 4 min by ion-pair reversed-phase chromatography with nonafluoropentanoic acid as ion-pair reagent. A linear response within a concentration range of 1–5 mg l^{-1} was observed for this LC method and the detection limit was found to be 20 pmol for ACC and 150 pmol for CDA (using a 20-μl loop). This methodology was successfully applied to the detection of ACC in apple tissue.

Keywords: Apples; Food analysis; Aminocyclopropanecarboxylic acid; Amino acids; Cyclopropanedicarboxylic acid; Carboxylic acids

1. Introduction

Ethylene is a biologically active molecule which regulates many aspects of plant growth development, senescence and fruit ripening [1]. The major biochemical pathway for the production of ethylene [2–5] is the enzymatic oxidation of 1-aminocyclopropane-1-carboxylic acid (ACC). Consequently the ACC level in plant tissue is of the greatest importance. The inhibition of ethylene by environmentally friendly inhibitors in order to manipulate ripening/senescence in horticultural crops is of considerable commercial importance in agriculture (reduction of producer losses, less frequent harvesting, reduced spoilage throughout the distribution chain, etc). Recently, a new group of chemical ACC oxidase inhibitors which are structural analogues of ACC have been identified, and several derivatives have been synthesized and tested in model systems. Among them, cyclopropane-1,1-dicarboxylic acid (CDA) has shown the strongest inhibition of ethylene production [6,7]. In order to continue experiments concerning the in vivo inhibition of ethylene pro-

*Corresponding author.
E-mail address: claire.elfakir@univ-orleans.fr (C. Elfakir).

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duction, an analytical method has to be developed for the simultaneous determination of ACC and CDA in biological matrices.

CDA analysis has never been investigated, whereas several analytical methods for ACC determination have been published. Early workers [8,9] achieved the indirect determination of ACC by its chemical conversion to ethylene. However, these methods led to the under- [10] or overestimation [11] of ACC levels in plant material due to the presence of various interfering compounds [10–12]. More recently, derivatized ACC was determined by means of GC–MS [10,13] or LC–UV [14–16]. Furthermore, the liquid chromatography–mass spectrometry (LC–MS) and tandem MS (MS–MS) of ACC phenylthiohydantoin derivatives have been performed using thermospray (TSP) [17] and electrospray (ES) [18] as ion source, respectively. However, all the above techniques cannot be used for the simultaneous determination of ACC and CDA as CDA does not contain an amino group in its structure, and consequently cannot be derivatized with the specific reagents used. Moreover, alternative methods for protein amino acid analysis without derivatization step have been proposed by LC–evaporative light-scattering detection (ELSD) [19,20], or LC–ion spray (ISP) MS [20,21] or LC–ISP-MS–MS [22].

In this paper, we present the simultaneous analysis of the underivatized amino acid ACC and its structural analogue CDA under modified chromatographic conditions to those previously described for underivatized amino acid analysis by LC–ISP-MS–MS detection [22]. A first approach for ACC and CDA detection in apple samples is investigated.

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) were obtained from J.T. Baker (Noisy le Sec, France). Nonafluoropentanoic acid (NFPA) was purchased from Aldrich (St Quentin-Fallavier, France). ACC and CDA were purchased from Sigma (St. Louis, MO, USA). Deionized (18 MΩ) water, using an Elgastat UHQ II system (Elga, Antony, France), was used for the preparation of amino acid and ion-pairing reagent solutions.

2.2. Apparatus

LC–ISP-MS–MS was carried out using a Perkin-Elmer (Toronto, Canada) model LC-200 binary pump and a Perkin-Elmer Sciex (Forster City, CA, USA) API 300 mass spectrometer triple quadrupole with IonSpray as ion source. The mass spectrometer was operated in positive ion mode. Nitrogen was used as curtain and collision gas. After optimization of MS parameters state files were as follows; NEB=7, CUR=7, CAD=1, I.S.=5000, OR=19, RNG=190, Q0=−5, IQ1=−6, ST=−10, RO1=−6, IQ2=−15, RO2=−23, IQ3=−35, RO3=−25, DF=−400, CEM=2100. Quad 1: 30 (0.010), 100 (0.050), 1000 (0.400), 2000 (0.742). Quad 3: 10 (0.008), 100 (0.035), 1000 (0.285), 2000 (0.530). The NEB=7 (nebulizer gas) corresponds to a flow-rate of 0.95 l min^{-1} and the CUR=7 (curtain gas) corresponds to a flow-rate of 1.02 l min^{-1}. The selective reaction monitoring (SRM) mode was used to monitor the parent and product ions. The dwell time was set at 500 ms and the pause time was 5.0 ms. Injections were done by a Perkin-Elmer series 200 autosampler (Toronto, Canada) fitted with a 20-μl loop. For the study of MS parameters a Harvard Model 22 syringe pump was used to infuse the ACC and CDA solutions in the MS system at a flow-rate of 5 μl min^{-1}.

Separation was carried out on a Purospher RP-18e 125×4 mm I.D. (Merck, Darmstadt, Germany) column fitted with its precolumn. Flow-rate was 1 ml min^{-1}. For LC–MS–MS, a split 1/40 was used to avoid too high a flow-rate in the ion source. Chromatographic separation was carried out under isocratic conditions.The mobile phase was prepared by dissolving 2 m*M* NFPA in a water–acetonitrile (90:10) mixture.

Column equilibration was monitored using a Vydac conductivity meter (Wescan, Santa Clara, USA) model 6000 CD as previously described [19,20].

Before use of the Purospher column, 30 ml MeOH, then 30 ml ACN, 30 ml THF, then 30 ml

MeOH were percolated through the column for surface regeneration.

2.3. Sample preparation

'Golden Delicious' apples were purchased from the local market. A total of 100 g of apple (cut into four to five pieces) was juiced using a mixer. Then, 5 ml of juice were mixed with 10 ml of 5% sulfosalicylic acid. The mixture was then centrifuged at 10 000 rpm for 15 min; 1 ml of the supernatant was used for the analysis.

3. Results and discussion

Fig. 1 shows the chemical structure of ACC and CDA. Previous LC–MS studies [18] have shown that because of the low molecular mass of underivatized ACC (M_r=101) its protonated molecule at m/z 102 is hard to detect among the intense background signals. In LC–ISP-MS–MS, it has been noted that for all the protein amino acids, in positive ionization mode, the protonated molecular ion $[M+H]^+$ was the more abundant and the intensity of the $[M+H]^+$ signal depends on the collisionally induced dissociation (CID) MS fragmentation which occurs through the curtain gas of the interface. The relative abundances of these fragments are strongly dependent on the orifice (OR) and the focusing ring (RNG) voltage adjustments [21].

3.1. Optimization of MS parameters

Optimization of MS parameters took place in two steps. First the parameters responsible for the extraction of the molecular ion [orifice voltage (OR) and focusing ring voltage (RNG)] were optimized. Then, the optimum OR and RNG values (inducing the most abundant $[M+H]^+$) were used in order to determine the collisionally activate dissociation (CAD) fragments of the $[M+H]^+$ ion as well as the optimum collision energy as described previously [22].

Fig. 1. Molecular structure of ACC and CDA.

Fig. 2a–d shows the evolution of the molecular ion of ACC and CDA and their major CID fragment in relation to OR and RNG voltage values. Low voltages for OR and RNG gave low abundance of the parent ion due to insufficient ion extraction. High values of OR and RNG voltage gave low abundance of the $[M+H]^+$ due to high CID fragmentations. The intermediate values OR=20 V and RNG=200 V were the optimum for the CDA, whereas OR=18 V and RNG=190 V were the optimum for ACC. Consequently the intermediate values of OR=19 V and RNG=190 V were used for further studies.

Then, the mass spectrometry was set in product-ion-scan by selecting the $[M+H]^+$ as parent ion and scanning in a mass area from 10 to $[M+H+3]^+$ mass units for determination of the collisionally activated dissociation (CAD) fragments (product-ions). The above proceeding was repeated 3 times for each of the collision energies: 10, 20, 30 eV. Table 1 sums up all the product ions observed. The immonium ion $[H_2N=CH\text{-}R]^+$ was the most abundant CAD fragment for ACC as for all the α-aliphatic protein amino acids [22]. For CDA, the elimination of one or two molecules of water gave the most abundant CDA fragments.

The molecular ion of ACC and CDA as well as their highest abundant CAD fragments were then monitored in relation to collision energy in order to select the product ion that will be selecting in SRM conditions as well as the optimum collision energy value. Fig. 2e,f depicts the relative fragment abundances versus the collision energy (in the range 12–40 eV) for ACC and CDA. It appears that an increase in the collision energy (increase in internal energy of $[M+H]^+$) involves a decrease in the corresponding $[M+H]^+$ ion with the resulting formation of the CAD fragments. By increasing the collision energy, the CAD ion abundance passes through a maximum and the optimum of the breakdown graph corresponds, respectively, to a collision energy value equal to 20 eV for ACC and to 16 eV for CDA. Therefore, an intermediate collision energy value equal to 18 eV was fixed as a satisfactory

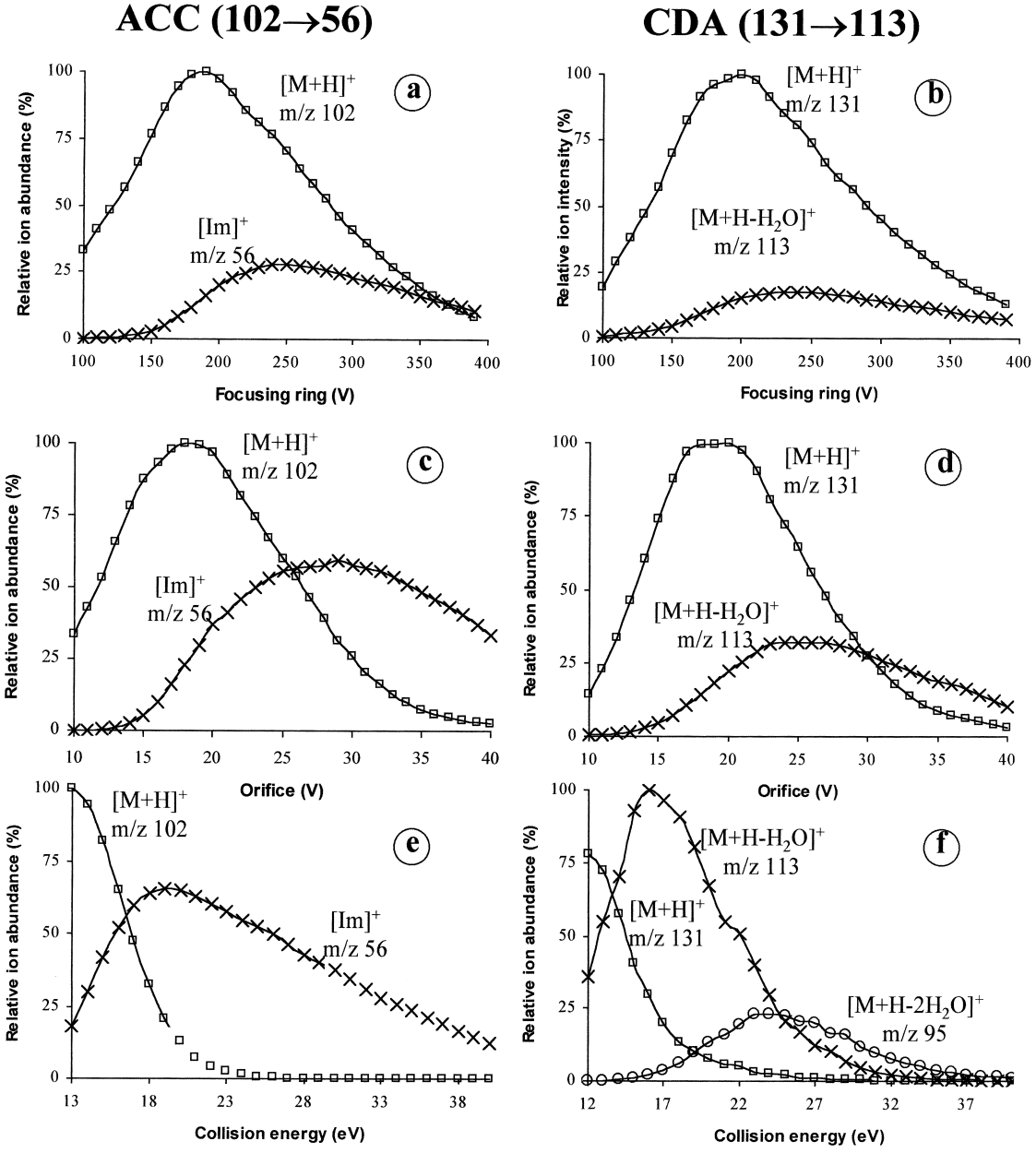

Fig. 2. Optimization of MS parameters (OR, RNG and collision energy voltage) for ACC and CDA ion intensity. (a–d) Collisionally induced dissociation (CID); (e–f) collisionally activated dissociation (CAD).

compromise for further studies. Under these MS tandem conditions ACC can be specifically detected with a combination of m/z 102→56 and CDA with a combination of m/z (131→113).

3.2. Matrix interferences, liquid chromatography, linearity and application to apple tissue

Generally, when the analysis of a specific amino

Table 1
m/z values of CAD fragments of ACC, CDA, Thr and Glu in product-ion-scan mode from 10 to 30 eV of collision energy[a]

Compound	m/z			
	$[M+H]^+$	$[M+H-H_2O]^+$	$[Im]^+$	Others
ACC	102		56	28
CDA	131	113		95, 53, 45, 43, 41
Thr	120	102*	74	56*, 84
Glu	148	130	102*	84, 56*, 41

[a] Underlined values correspond to parent and product ion. Asterisks correspond to mass transitions interfering with ACC transitions. $[Im]^+=[H_2N=CH\text{-}R]^+$ where R is the residue of the amino acid.

acid in a biological material is required, none of the available extraction protocols are selective enough and lead to the extraction of the totality of the amino acids (with exception the highly hydrophobic amino acids). Furthermore, due to the characteristic fragmentation of these molecules, naturally occurring amino acids may interfere with ACC and/or CDA. For the above reason a mixture of the 20 underivatized protein amino acids (UPAAs) were analyzed under the chromatographic conditions previously described [22], by monitoring the specific transitions of ACC and CDA. Among the 20 UPAAs, only Glu and Thr induced identical transitions (102→56) with ACC. Furthermore, none of the protein amino acids gave identical transitions with CDA (131→113). Table 1 sums up the CAD fragmentation of Glu and Thr in addition to the CAD fragmentation of ACC and CDA. It can be seen that CID and CAD fragmentations of Thr and Glu induced identical transitions with ACC. Such m/z values have been previously reported [23,24] using different ionization conditions.

In order to eliminate the potential interference of Glu and Thr with ACC, a C_{18} Purospher column was used with mobile phase containing 2 m*M* NFPA as ion pairing reagent in a water–acetonitrile (90:10) mixture. At the above isocratic chromatographic conditions Thr and Glu were co-eluted but are separated from ACC.

Fig. 3a shows the selective reaction monitoring (SRM) of the ACC and CDA as well as the extracted ion current (XIC) of ACC and CDA. The detection limits were found to be 20 pmol for ACC and 150 pmol for CDA (using a 20-μl loop). The higher detection limits for CDA in relation to ACC can be explained by the absence of an easily protonated amino group (for CDA). The limit of detection obtained for ACC is of the same order of magnitude with the detection limit of derivatized ACC obtained with LC–TSP-MS [17] and is about 100-fold higher than the detection limit of derivatized ACC obtained with LC–ISP-MS–MS [18]. The reasons for the relatively high detection limits have been discussed previously [22]. In order to determine the linearity of the method, standard concentrations of 1, 2, 3, 4, 5 mg l^{-1} were injected and analysed by LC–ISP-MS–MS under SRM of (102→56) and (131→113). All measurements (x (concentration at mg l^{-1}) versus y (response of the product ion given by its peak area)) were done in triplicate. Acceptable linearity was obtained for ACC ($y=5882x+596$, $r^2=0.999$) and CDA ($y=1610x-189$, $r^2=0.991$).

Fig. 3b shows the XIC of ACC from an apple sample. The first peak corresponds to the interference of Glu and Thr which are co-eluted. The second peak corresponds to ACC. CDA was not detected as it does not exist naturally in the plants and no other compound interferes with its masses (131→113).

4. Conclusion

A preliminary evaluation of a new analytical method has been developed for the simultaneous analysis of underivatized ACC and CDA, by LC–ISP-MS–MS. Because of the high specificity of tandem mass spectrometry, the need for a total separation of the protein amino acids, ACC and CDA is not necessary for their determination. Among the 20 protein underivatized amino acids Glu and Thr have shown interferences with the ACC signal. Consequently, they have been separated by the

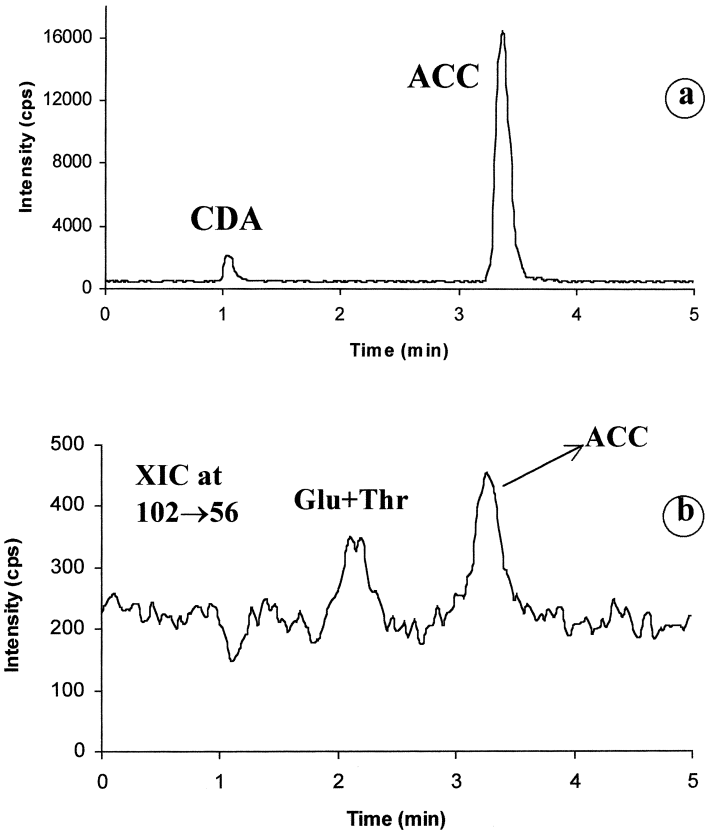

Fig. 3. LC–ISP-MS–MS isocratic analysis of the ACC and CDA in standard mixture and in an apple sample. Flow rate, 1 ml min^{-1}; split 1/40; MS parameters, see Section 2. (a) Selective reaction monitoring of 10 mg l^{-1} of ACC (102→56) and CDA (131→113). (b) Extracted ion current of ACC, monitoring at m/z 102→56 of an apple sample.

appropriate chromatographic system. Furthermore, ACC has been successfully detected in apple juice after a simple pretreatment of the sample before its analysis.

The future of this work is to develop a suitable extraction protocol, and if necessary preconcentration of ACC and CDA, depending on the plant tissue that will be chosen for the in vivo experiments.

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