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Liquid chromatographic–mass spectrometric determination of 1-aminocyclopropane-1-carboxylic acid in tobacco

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ABSTRACT

Liquid chromatography–thermospray mass spectrometry was investigated as a method for determination of 1-aminocyclopropane-1-carboxylic acid (ACC) in *Nicotiana tabacum* cv. Petit Havana SR1. A stable isotope analogue of ACC ([$^2H_4$]ACC) was used as an internal standard. The internal standard was added to the plant samples prior to extraction. After solid-phase extraction both ACC and [$^2H_4$]ACC were derivatized with phenyl isothiocyanate (PITC) to the corresponding phenylthiohydantoin compounds PTH-ACC and [$^2H_4$]PTH-ACC. Selected ion monitoring of the protonated molecules $[MH]^+$ of m/z 219 and 223 was used for quantification. The method is sensitive and highly specific for the determination of ACC in plant extracts. This in contrast with the assay Lizada and Yang, which in several instances overestimated ACC in tobacco, probably owing to the presence of one or more interfering compounds.

INTRODUCTION

1-Aminocyclopropane-1-carboxylic acid (ACC) is accepted as the immediate precursor of the plant hormone ethylene [1] (Fig. 1). Not only because the availability of ACC is the most limiting step in the production of ethylene, but also because of the inducing activity of auxins on ACC-synthase, plant physiologists are more and more in need of an accurate method to de-

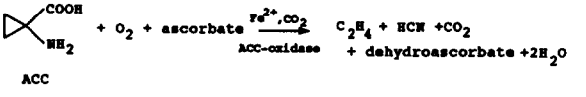

Fig. 1. Stoichiometry of ACC oxidation to ethylene by the enzyme ACC oxidase [2].

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termine ACC. For several years, the most commonly used techniques for ACC determination were based on the oxidative conversion of ACC to ethylene, followed by gas chromatographic (GC) analysis [3,4]. The main disadvantage of these techniques is that they are indirect and therefore not very specific.

More recently, some groups have been working on the development of more specific methods to determine ACC, *i.e.*, to detect ACC itself. Savidge *et al.* [5] identified ACC in Compressionwood (*Pinus contorta*) by GC–MS analysis of the 2,4-dinitrophenylmethyl ester of ACC. No attempt at quantification was made, however. McGaw *et al.* [6] determined the phthalimidomethyl ester of ACC by GC–MS. Although very sensitive, the method is very tedious, requiring several HPLC steps prior to GC–MS.

As ACC is in fact an amino acid, some groups have concentrated on the known techniques for amino acid analysis. Miller *et al.* [7] determined ACC in plasma and brain of mice by HPLC after derivatization with *o*-phthaldialdehyde and Lanneluc-Sanson *et al.* [8] used reversed phase HPLC on-line with UV detection for the determination of the phenylthiocarbamyl derivative of ACC (PTC-ACC). As some plant hormones are present in low (pmol/g fresh weight) concentrations, sensitive qualitative and quantitative techniques are needed for their measurement. Determination of underivatized ACC by combined liquid chromatography–mass spectrometry (LC–MS) using a thermospray interface (TSP) is difficult owing to the low molecular mass (101) of ACC, the protonated molecule [MH^+] of which is hard to detect amongst the intense background signals. Therefore, ACC was derivatized to the corresponding phenylthiohydantoin compound, which under LC–TSP-MS conditions produces a very intense protonated molecule at m/z 219 (relative intensity 100%). Further, the derivatives showed excellent chromatographic behaviour under conditions usually employed for LC–TSP-MS.

In this paper we present the quantitative results obtained for the determination of ACC in *Nicotiana tabacum* cv. Petit Havana SR1 by using selected ion monitoring on the ions at m/z 219 and 223 (interval time = 20 ms). The method is very specific but is less tedious than previously described GC–MS methods [5,6].

EXPERIMENTAL

Materials

ACC, [$^2H_4$]ACC (1-amino-[2,2,3,3-$^2H_4$]cyclopropane-1-carboxylic acid) and phenyl isothiocyanate (PITC) were purchased from Sigma (St. Louis, MO, USA). Methanol (gradient grade), ethanol (gradient grade), ammonia (25% solution), acetic acid (98%), trifluoroacetic acid (TFA), triethylamine and ammonium acetate were obtained from Merck (Darmstadt, Germany). RP-C_{18} solid-phase cartridges were obtained from Analytichem International (Harbor City, CA, USA) and Sephadex ion-exchange resins from Pharmacia (Uppsala, Sweden). [^{14}C]ACC was purchased from NEN (France) and hydrochloric acid (38%) from RPL (Leuven, Belgium). Water was purified by reversed osmosis with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Standard samples

Standard solutions were prepared in deionized water containing 1, 10 and 100 ng/μl ACC or [$^2H_4$]ACC. From these solutions, standard mixtures for the calibration graph were prepared by spiking 500 ng of ACC with 10, 25, 50, 100 and 250 ng of [$^2H_4$]ACC in small glass tubes (5 × 0.5 cm I.D.) or *vice versa*, in such a way that the final volume never exceeded 20 μl. After evaporation under vacuum, the standards were derivatized as described for the plant samples and analysed by LC–TSP-MS, using only one tenth of the mixture for every injection.

Plant samples

Production of plant material. Nicotiana tabacum cv. Petit Havana SR1 seeds were brought to germination in trays with soil. The trays were covered with perforated plastic foil until the plants were about 1 cm high. When they were about 5 cm tall, the plants were individually transferred to plastic pots (d = 15 cm).

The plants were germinated and grown under a light regime of 18 h/6 h (light/darkness), with a light intensity of 200 μmol s^{-1} m^{-2}, a relative

humidity of 85% and an average temperature of 25°C. At the age of 10 weeks (*ca.* twelve-leaf stage) the leaves were cut off, weighed individually and immediately frozen in liquid nitrogen. They were stored at −20°C prior to extraction.

Isolation of 1-aminocyclopropane-1-carboxylic acid. Approximately 1 g of frozen plant material was homogenized in 80% methanol, after addition of 250 ng of [$^2H_4$]ACC and 250 Bq of [^{14}C]ACC (specific radioactivity = 840 Bq/nmol) as internal standards, and kept at −20°C for 1 h during extraction. The slurry was centrifuged at 14 000 *g* for 20 min and the supernatant was diluted to 50% methanol by adding water and applied to a Bond Elut C_{18} column (2 ml). This column was immediately coupled to a column of anion-exchange resin (DEAE-Sephadex A-25, formate form, 3.0 × 1.5 cm I.D.). The last effluent was titrated to pH 2–2.5 with 1 *M* HCl and applied to a cation-exchange column (SP-Sephadex C-25, H^+ form, 3.0 × 1.5 cm I.D.). After consecutive washings with 3 m*M* HCl and water, the latter was eluted with 5% ammonia solution (5 ml). The eluate was evaporated under vacuum. One aliquot (half) was concentrated in small glass tubes prior to derivatization, while the other part was kept for analysis by Lizada and Yang's method [3].

Determination of ACC by GC. For the determination of ACC by GC the method of Lizada and Yang [3] was followed. The oxidation efficiency of ACC was monitored by spiking 100-μl aliquots of the sample with different amounts of ACC (except for the blank, to which no ACC was added). Three different concentrations of ACC were used per sample, and every measurement was carried out in duplicate. The total amount of ACC in the sample was calculated by means of linear regression. The amount of [$^2H_4$]ACC, added as an internal standard for LC–MS analysis, was subtracted from this value, to yield the initial amount of native ACC.

Derivatization with phenyl isothiocyanate. The synthesis of PTH-ACC was carried out according to Hewick *et al.* [9], with a slight change in TFA concentration and cyclization temperature in order to obtain a 100% conversion of PTC-ACC to PTH-ACC (Fig. 2). To test-tubes, 20 μl of

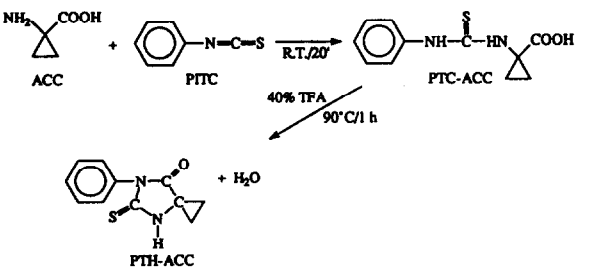

Fig. 2. Derivatization procedure of 1-aminocyclopropane-1-carboxylic acid (ACC) with phenyl isothiocyanate (PITC), resulting in the phenylthiohydantoin compound of ACC (PTH-ACC).

the derivatization solution [ethanol–water–triethylamine–phenyl isothiocyanate (7:1:1:1)] were added, and the reaction was allowed to proceed for 20 min at room temperature. After evaporation under vacuum, 50 μl of 40% TFA were added to the tubes and they were left to react at 90°C for 1 h. The reaction mixtures were evaporated to dryness under vacuum and the tubes were kept at −20°C prior to HPLC.

Preparative HPLC of PTH-amino acids. A preparative HPLC step was used to separate and isolate PTH-ACC from other PTH-amino acids and interfering products. This was done with a Spectra-Physics HPLC system, equipped with a 100-μl loop and an Alltech C_{18} (3 μm) cartridge (100 × 4.6 mm I.D.), on-line with an Applied Biosystems multi-wavelength UV detector, set at 260 nm. The mobile phase [methanol–water–acetic acid (40:59.5:0.5, v/v/v)] flow-rate was 0.5 ml/min. The eluted PTH-ACC (retention time 13 min, detected by ^{14}C analysis) was dried under vacuum and stored at −20°C for analysis by LC–TSP-MS.

Liquid chromatography–thermospray mass spectrometry of PTH-ACC

The PTH-ACC samples were injected into the HPLC system, connected to a VG TRIO-2000 mass spectrometer equipped with a thermospray interface. The HPLC system used consisted of a Waters 600-MS pump, a Waters 700 Satellite WISP autoinjector and an Alltech C_{18} (3 μm) column (100 × 4.6 mm I.D.). The mobile phase [methanol–0.1 *M* ammonium acetate (50:50, v/v)] flow-rate was 0.8 ml/min. Under these conditions, PTH-ACC had a retention time of

3.7–3.8 min. Tuning of the capillary temperature (210°C), source temperature (250°C) and the voltage on the repeller electrode (200 V) was done while introducing a standard sample in order to obtain maximum sensitivity.

Mass spectra were recorded by scanning a mass range of 150–400 u in 1 s. To improve the sensitivity, selected ion monitoring (SIM) was used for quantification. The protonated molecules at m/z 219 for PTH-ACC and m/z 223 for [$^2H_4$]PTH-ACC were monitored in the SIM chromatograms, using a dwell time of 100 ms and an interchannel delay of 20 ms.

For the quantification of the samples, the corresponding peak areas in these SIM chromatograms were used. The areas were calculated by manually selecting the start and end points of the peaks and by using Unix V6 software.

RESULTS AND DISCUSSION

Qualitative aspects of PTH-ACC analysis by LC–TSP-MS

In order to evaluate LC–TSP-MS for the determination of PTH-ACC in tobacco, a crude reaction mixture of PTH-ACC was analysed. This reaction mixture was obtained by first derivatizing pure ACC in ethanol–water–triethylamine–PITC (7:1:1:1) for 20 min at room temperature to the corresponding phenylthiocarbamyl compound. By subsequent treatment of the dried mixture with 40% TFA for 1 h at 90°C the corresponding PTH derivative was obtained. After evaporation under vacuum this mixture was analysed on an Alltech C_{18} column (3 μm, 100 × 4.6 mm I.D.) using methanol–0.1 *M* ammonium acetate (50:50, v/v) as the mobile phase at a flow-rate of 0.8 ml/min. The total ion current (TIC) chromatogram, together with the reconstructed mass chromatogram for m/z 219 and the UV chromatogram at 260 nm, is depicted in Fig. 3. As can be seen PTH-ACC elutes at 3.9 min.

The mass spectrum of PTH-ACC is shown in Fig. 4 together with that for the corresponding [$^2H_4$]PTH-ACC. The latter compound was obtained by an analogous derivatization procedure and analysed under the same conditions as mentioned above. The protonated molecules at

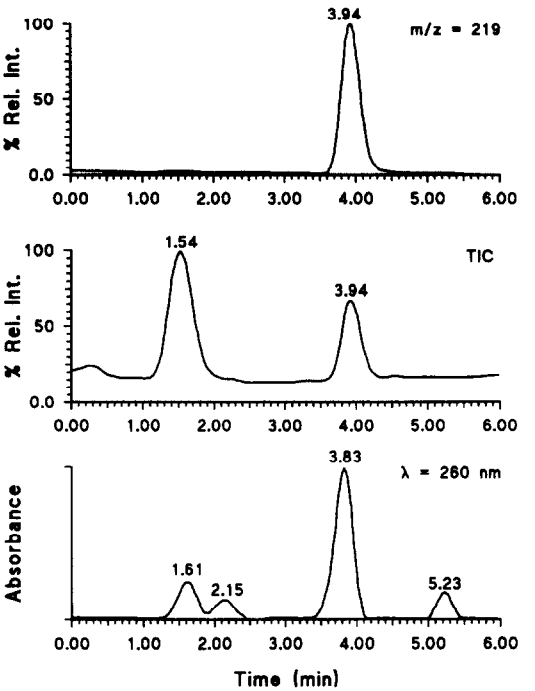

Fig. 3. Chromatograms (total scanning) of 100 ng of PTH-ACC. Column, Alltech C_{18} (3 μm, 100 × 4.6 mm I.D.); mobile phase, methanol–0.1 *M* ammonium acetate (50:50, v/v); flow-rate, 0.8 ml/min. Top, reconstructed mass chromatogram for m/z 219, corresponding to the protonated molecule (MH^+) of PTH-ACC; centre, TIC chromatogram (150–400 u in 1 s); bottom, UV trace at 260 nm. Rel. Int. = Relative intensity.

m/z 219 and 223 are intense and m/z 219 is absent in the mass spectrum of the deuterated internal standard. These features will allow the selective and highly sensitive measurement of ACC in plant material by LC–TSP-MS.

With this knowledge, several plant samples were analysed for the presence of ACC. For this purpose *Nicotiana tabacum* cv. Petit Havana SR1 leaf tissue was homogenized in 80% methanol after addition of known amounts of [^{14}C]ACC and [$^2H_4$]ACC as internal standards. After extraction for 1 h at −20°C, the ACC in the supernatants was concentrated by solid-phase extraction. The dried samples were derivatized as described for the standard mixtures. Prior to LC–TSP-MS, these samples were applied to an Alltech C_{18} column (3 μm, 100 × 4.6 mm I.D.) using methanol–water–acetic acid (40:59.5:0.5,

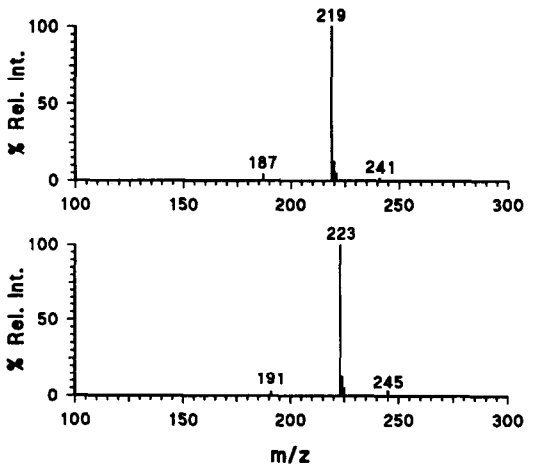

Fig. 4. Top, TSP mass spectrum of the PTH-ACC peak (retention time 3.9 min) in Fig. 3; bottom, mass spectrum of [$^2H_4$]PTH-ACC peak, obtained by an analogous procedure as for PTH-ACC. In both instances 100 ng of sample were injected.

v/v/v) as the mobile phase at a flow-rate of 0.5 ml/min. This supplementary step was used to isolate PTH-ACC from other PTH-amino acids and interfering compounds. The isolated PTH-ACC compound was analysed by LC–TSP-MS. The corresponding TIC chromatogram is shown in Fig. 5, together with the reconstructed mass chromatograms for the ions at *m/z* 219 and *m/z* 223. The PTH-ACC compound elutes at 3.8 min.

The corresponding mass spectrum is depicted in Fig. 6, where the base peak corresponds to *m/z* 207. This ion is due to another compound, however, eluting at 3.4 min (reconstructed mass chromatogram shown in Fig. 5). No contribution due to either *m/z* 219 or *m/z* 223 is expected. Compared with the standard chromatogram in Fig. 3, the analysis of the plant material shows an extra compound (*m/z* 223 at retention time 2.87 min). However, there is no contribution to $m/z = 219$. Moreover, because of the isotope dilution principle, this peak cannot be due to [$^2H_4$]PTH-ACC, but has to be due to another compound, only separated from PTH-ACC under LC–TSP-MS conditions. Therefore, this peak does not interfere with the determination of PTH-ACC. Hence LC–TSP-MS is a highly specific method for the determination of PTH-ACC.

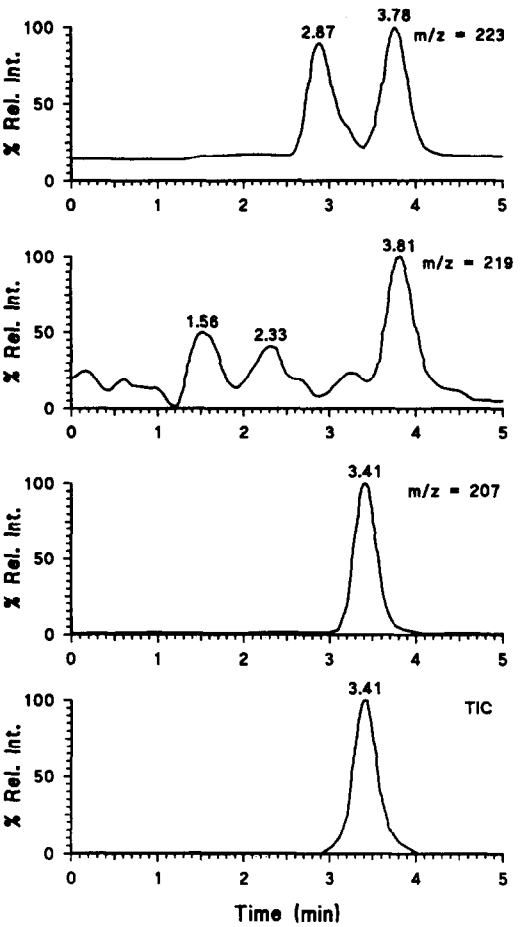

Fig. 5. Chromatograms (total scanning) of a plant sample, after preparative HPLC. Same column and mobile phase as for the standards. From top to bottom: reconstructed mass chromatograms for *m/z* 223, 219 and 207, respectively, and the TIC chromatogram. PTH-ACC elutes at 3.8 min. The peaks at *m/z* 223 and 219 correspond to *ca.* 20 and 10 ng, respectively.

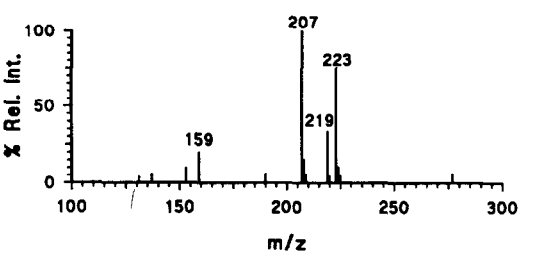

Fig. 6. TSP mass spectrum of the PTH-ACC peak (retention time 3.8 min) in Fig. 5.

Quantitative aspects of PTH-ACC analysis by LC–TSP-MS

In order to obtain the highest sensitivity possible, SIM of m/z 219 and 223 was used for the determination of PTH-ACC in both standard mixtures and unknowns. A calibration graph was obtained by spiking ACC with different amounts of [$^2H_4$]ACC and *vice versa*, resulting in a range of concentration ratios from 50:1 to 1:50 (H:^{2}H). Each concentration ratio was made up four times. These mixtures were derivatized as stated earlier and analysed by LC–TSP-MS. The area to area ratio (m/z = 219/223) was plotted against the molar ratio. The resulting calibration graph is shown in Fig. 7. For the higher ratios (H:^{2}H = 2:1 to 50:1) the inverse values were plotted. In this way, every molar ratio on the calibration graph corresponds to eight different area ratios. The regression equation for the calibration graph was $y = 0.965x + 0.004$ and the correlation coefficient was 0.99989.

With this knowledge, several plant samples were analysed by both LC–TSP-MS-SIM and the method of Lizada and Yang [3]. For this purpose, *Nicotiana tabacum* cv. Petit Havana SR1 leaf tissue was extracted with 80% methanol, after addition of known amounts of [^{14}C]ACC and [$^2H_4$]ACC as internal standards. After centrifugation and solid-phase extraction of the supernatant, one aliquot (half) of the sample was derivatized with PITC and the resulting PTH-ACC was isolated by means of a preparative HPLC step and finally determined by LC–TSP-MS using the conditions mentioned above. A typical SIM chromatogram for m/z 219 and 223 of a plant sample is depicted in Fig. 8, together with the UV trace at 260 nm. Peak areas were obtained by manually selecting the start and end points and using the Unix V6 software to calculate the areas. The initial amount of ACC in the sample was calculated by means of the regression line, using the following equation

$$\text{mol H} = (\text{area H/area } ^2\text{H} - 0.004) \cdot (\text{mol } ^2\text{H}/0.9654)$$

where (mol ^{2}H) is the amount of deuterated ACC (in moles), initially added as internal standard.

The other part of the sample was analyzed by means of the method of Lizada and Yang [3]. The amount of [$^2H_4$]ACC added as internal standard for LC–MS was subtracted from the results, yielding the initial amount of native ACC.

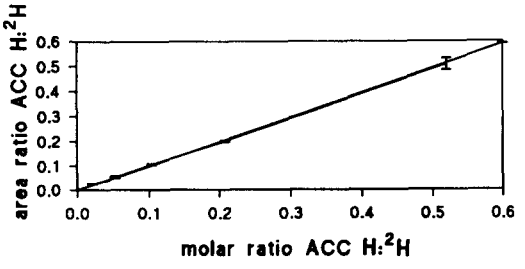

Fig. 7. Calibration graph for PTH-ACC, obtained by plotting the (m/z 219)/(m/z 223) area ratio against the injected PTH-ACC/[$^2H_4$]PTH-ACC ratio. Areas were obtained by selected ion monitoring on m/z 219 and 223. Equation: $y = 0.965x + 0.004$. Correlation coefficient = 0.99989.

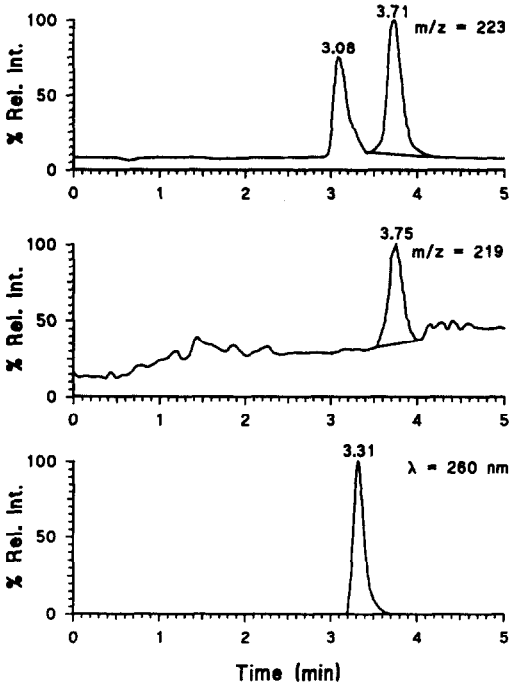

Fig. 8. Chromatogram of a plant sample (selected ion monitoring) for m/z 219 (MH^+ PTH-ACC) and m/z 223 (MH^+ [$^2H_4$]PTH-ACC), together with the UV trace at 260 nm. Conditions are the same as for the standards. The peaks at m/z 223 and 219 correspond to *ca.* 20 and 10 ng, respectively.

TABLE I

COMPARISON OF LC–TSP-MS DETERMINATION OF ACC WITH THE METHOD OF LIZADA AND YANG [3] IN THREE *NICOTIANA TABACUM* LEAF SAMPLES

Examples chosen are arbitrary (see text).

Sample No.	Endogenous ACC individual values (nmol/g fresh weight)	
	LC–MS	Lizada and Yang
1	1.46; 1.22	1.73; 1.39
2	0.63; 0.41	2.85; 1.93
3	0.73; 0.55	3.25; 2.91

Table I shows a comparison of the two methods for three tobacco leaf samples. Examples are arbitrarily chosen from a range of comparable results and, in the context of this paper, they have no physiological relevance. The values obtained from the oxidative conversion of ACC to ethylene are in several instances higher than those obtained by our method. These results are somewhat in contradiction with the reports by McGaw *et al.* [6] and Lanneluc-Sanson *et al.* [8], who compared Lizada and Yang's method with GC–MS of the phthalimido-ACC methyl ester of ACC and reversed-phase HPLC of PTC-ACC, respectively. Both groups observed an underestimation of the ACC content by Lizada and Yang's method, possibly owing to losses of ethylene during sampling.

On the other hand, it is well known that some substances may interfere with the assay according to Lizada and Yang [3]. In addition to overestimation, an underestimation of ACC can occur with this method [10,11]. For instance, treatment of senescing carnation flowers with α-aminoxyacetic acid, an inhibitor of ACC synthase, results in the production of a factor that gives rise to ethylene in the Lizada and Yang assay [10]. It is not unlikely that in tobacco one or more naturally occurring compounds are present that give rise to an overestimation of ACC owing to chemical oxidation. We can conclude that LC–TSP-MS of the phenylthiohydantoin derivative of ACC is a sensitive (detection limit = 10 pmol) and highly specific method for its determination. As ACC is usually present in plants in nanomolar concentrations, the method can be applied for the routine determination of ACC in plant tissues.

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