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Quantitative analysis of 1-aminocyclopropane-1-carboxylic acid by liquid chromatography coupled to electrospray tandem mass spectrometry

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Abstract

Liquid chromatography coupled to electrospray tandem mass spectrometry was investigated as a method for the analysis of 1-aminocyclopropane-1-carboxylic acid (ACC) in plant tissues. Isotopically labelled ACC ([$^2H_4$]ACC) was used as an internal standard. After solid-phase extraction, samples were derivatized with phenyl isothiocyanate and the obtained phenylthiohydantoin compound was analyzed by multiple reaction monitoring of the appropriate $[MH]^+$ to product ion transitions (219→98 and 223→102). Optimal conditions for positive ion electrospray tandem mass spectrometry showed a linear response within a concentration range of 0.1–100 pmol per 10 μl injected, and between cold and deuterated ACC within a range of 1:50 to 50:1. A detection limit was observed of 0.1 pmol injected on column. The high specificity of detection allows the analysis of endogenous ACC levels in plant extracts without excessive purification. Despite the complex nature of the plant matrices, chromatographic conditions were developed under which a complete baseline separation with interfering compounds was obtained within an analysis time of 12 min. © 1997 Elsevier Science B.V.

Keywords: Plant hormones; Aminocyclopropanecarboxylic acid; Carboxylic acids

1. Introduction

The plant hormone ethylene is involved in several plant developmental processes such as fruit ripening, senescence, abscission of plant parts, etc. [1]. Using flow-through systems, the analysis of ethylene in whole plants is straightforward [2,3], but assignment of ethylene synthesis to certain (detached) plant parts by direct analysis of ethylene is difficult since its synthesis is stimulated by wounding and desiccation. The level of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene [4], is still accepted as rate limiting for the production of the hormone and its concentration is determined by the activity of the enzyme ACC-synthase. However, it can not be excluded that the activity of ACC-oxidase, i.e. the enzyme responsible for the conversion of ACC into ethylene, also has a regulatory role in the production of the hormone.

In order to assign ethylene synthesis to plant parts in which the direct analysis of the hormone is difficult and to gain knowledge about ACC-oxidase as a possible rate-limiting factor in the ethylene biosynthetic pathway, plant physiologists are com-

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mitted to the analysis of ACC. Therefore, reliable techniques for the analysis of this molecule in biological samples are of the utmost importance. Since 1979, ACC was usually measured indirectly by chemical conversion to ethylene, followed by gas chromatographic analysis [5,6]. However, it has been shown that this procedure can lead to either under- [6–8] or overestimation [9,10] of ACC levels. Furthermore, the sensitivity of the assay has been shown to be strongly affected by various interfering compounds [11]. This problem was solved when techniques were developed to determine ACC in a direct way, e.g.: GC–MS analysis of the 2,4-dinitrophenylmethylester [12] or isotope dilution GC–MS of the phthalimidomethylester [7] of ACC; HPLC with on-line UV detection after derivatization with either *o*-phthaldialdehyde [13] or phenylisothiocyanate [8], and LC–thermospray MS of the phenylthiohydantoin (PTH) compound of ACC [10].

Although the mass spectral detections are very reliable, plant matrices usually still need very time-consuming purification steps prior to derivatization to ensure accurate quantitation of ACC. In this paper we present a novel technique for the analysis of ACC, based on (+) electrospray tandem mass spectrometry of the PTH compound of ACC. Due to its high specificity, the method is very fast, requiring only a short solid-phase extraction and derivatization procedure prior to LC–MS quantitation.

2. Experimental

2.1. 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 (HPLC grade), ethanol (HPLC grade), trifluoroacetic acid (TFA), triethylamine and ammonium acetate were obtained from Merck (Darmstadt, Germany). RP-C_{18} solid-phase cartridges were from Varian (Harbor City, CA, USA), DEAE-Sephadex A-25 from Pharmacia (Uppsala, Sweden) and Alumina-A cartridges from Waters (Milford, MA, USA). Water was purified by reversed osmosis with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Standard samples

Standard samples were obtained after derivatization of ACC with PITC as described earlier [10]. The amount of ACC necessary (see below) was placed in small reaction tubes (5×0.5 cm I.D.). All dilutions were made in 80% methanol and the volume in the tubes never exceeded 50 μl. After evaporation in a Speedvac concentrator, the samples were redissolved in 40 μl of a mixture containing [ethanol–water–triethylamine (TEA)–PITC (2:1:1:1, v/v)] and reaction was allowed to proceed at room temperature for 20 min. After drying, 50 μl of 40% trifluoroacetic acid (TFA) was added and the reaction mixture was heated at 90°C for 1 h. The samples were dried again and stored at −20°C prior to LC–MS analysis.

A stock of PTH-ACC was obtained in this way by derivatizing 5000 pmol of ACC. This stock was used for full-scan recordings and production of samples for linearity plots.

For the calibration curve, mixtures were made by spiking 500 pmol ACC with 10, 25, 50, 100, 250 and 500 pmol [2,2,3,3-$^2H_4$]ACC in small glass tubes or vice versa. All these mixtures were derivatized separately.

2.3. Plant material

Nicotiana tabacum cv. Petit Havana SR1 seeds were surface-disinfected and germinated on water-agar plates. The plants were individually transferred to pots (d=15 cm) containing glass beads (d=0.5 cm) and Hoagland nutrient solution [13] when they were about 5 cm tall. 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 average temperature of 25°C. At the age of eight weeks the leaves were cut off and leaves, stem and roots were weighed individually and immediately frozen in liquid nitrogen. The samples ranged in mass between 0.1 and 3 g. They were stored at −20°C until extraction.

2.4. Extraction and purification

Isolation of ACC from plant material was done as

described earlier [10] with some minor modifications.

Between 0.1 and 3 g frozen material was homogenised in 80% methanol, after addition of 1000 pmol of [$^2H_4$]ACC as an internal standard and kept at −20°C for 1 h. The slurry was centrifuged at 24 000 *g* for 20 min and half of the supernatant was brought in glass tubes and dried (Speedvac) for future analysis of ACC conjugates. The remainder was applied to a C_{18} column. The effluent was diluted with water till 50% methanol and immediately applied to a DEAE-Sephadex A-25 column (formate form, 3.0×1.5 cm I.D.), under which an Alumina A column, prerinsed with 50% methanol was coupled. The Alumina column was eluted with 10 ml of a solution containing [methanol–water–formic acid (80:14:6, v/v)] and the eluate was evaporated under vacuum and stored at −20°C.

The amount of ACC conjugates was measured indirectly by gaseous acid hydrolysis [14,15]. For this purpose, the glass tubes were brought into a vacuum glass container, together with 250 μl of 6 *M* HCl at the bottom of the container and reaction was allowed to proceed at 100°C for 2 h. The samples were then resuspended in 50% methanol, applied to a DEAE-Sephadex column and further treated as described for free ACC. The amount of ACC conjugates was calculated by subtraction of free ACC from the amount of total ACC obtained after hydrolysis.

The samples were derivatized with PITC as described for the standard samples and kept at −20°C prior to LC–MS analysis.

2.5. Liquid chromatography–mass spectrometry of PTH-ACC

Samples were injected into the HPLC system, connected to a Quatro II mass spectrometer (Micromass) equipped with an electrospray interface (LC–ES-MS–MS).

The HPLC equipment consisted of a Kontron 325 solvent delivery pump, a Kontron 465 autosampler and a C_8 reversed-phase column (Merck, LiChrospher 60 RP Select B; 5 μm; 125 mm×4 mm). The mobile phase [methanol–ammonium acetate 0.01 *M* (40:60, v/v)] flow-rate was 0.8 ml min^{-1}. Using a post-column split of 1/20 the effluent was introduced into the electrospray source (source temp 80°C, capillary voltage +3.5 kV, cone voltage 30 V). Full-scan spectra were recorded by scanning a mass range of 50–300 u at 300 u s^{-1}. Collision-activated dissociation (CAD) spectra of $[MH]^+$ were obtained at a collision energy of 20 eV and a P_{ar} of 5×10^{-3} mbar. Quantitation was done by multiple reaction monitoring (MRM) of $[MH]^+$ (dwell time 0.2 s) and the product ion of both PTH-ACC and [$^2H_4$]PTH-ACC.

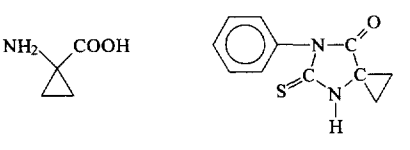
For plant matrices, mass spectrometric conditions were the same as described above, but a stepwise gradient was used: [methanol–ammonium acetate (40:60, v/v)] for 5 min, followed by [methanol–ammonium acetate (80:20, v/v)] for 5 min. The system was allowed to equilibrate under the initial conditions before the next sample was injected.

Data were processed by Masslynx software.

3. Results and discussion

3.1. Qualitative aspects of LC–MS–MS of PTH-ACC

Given the low molecular mass of ACC (M_r=101), LC–MS of genuine ACC is difficult since the protonated molecule at *m/z* 102 is hard to detect amongst the intense background signals. Therefore, ACC was derivatized with PITC as described in Section 2 in order to obtain the PTH compound of ACC (Fig. 1). Fig. 2 shows the full-scan spectrum of 500 pmol PTH-ACC (loop injection) under the mass spectral conditions described above. The base peak is found at *m/z* 219, corresponding to the protonated molecule $[MH]^+$. Using a collision energy of 20 eV and a P_{ar} of 5×10^{-3} mbar in the collision cell, an optimal yield of the product ion at *m/z* 98 was



ACC PTH-ACC

Fig. 1. Structure of ACC (M_r=101) and the phenylthiohydantoin compound of ACC (PTH-ACC, M_r=218), obtained after derivatization with PITC as described in Section 2.

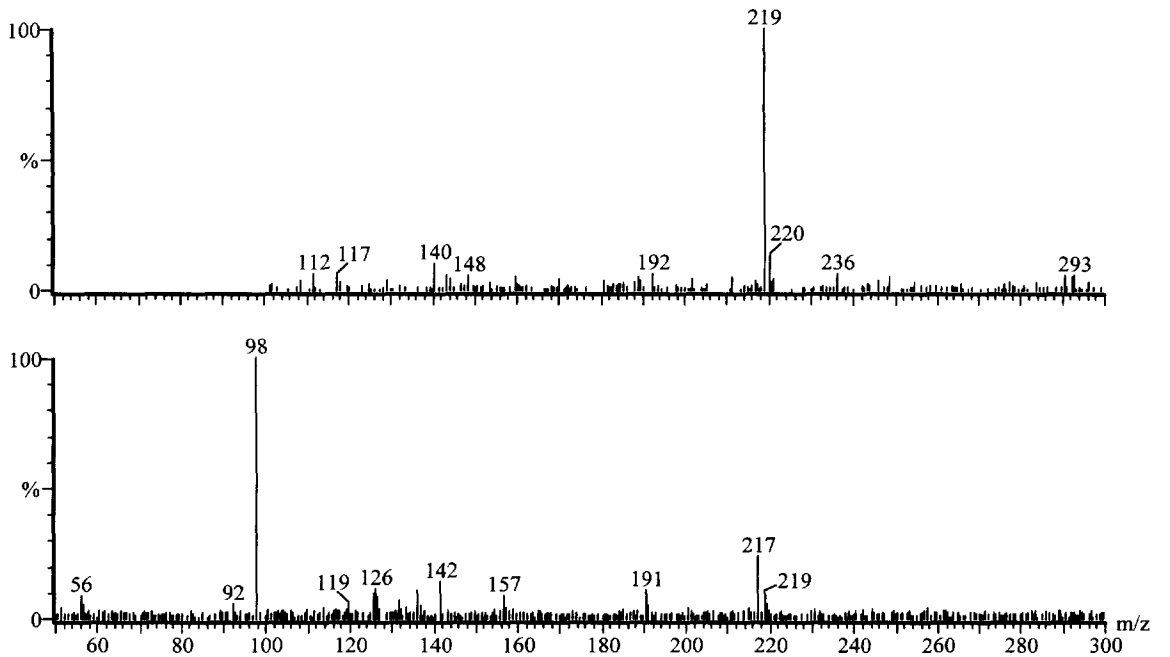


Fig. 2. Top, full-scan spectrum of PTH-ACC in (ES)+[source temperature 80°C, capillary voltage 3.5 kV, cone voltage 30 V]. *m/z* 219 corresponds to $[MH]^+$; bottom, product ion scan of *m/z* 219 using collision-activated dissociation (collision energy 20 eV, P_{ar} 5×10^{-3} mbar). Results are obtained by flow injection analysis of 500 pmol PTH-ACC.

obtained (relative intensity=100%) (Fig. 2). Under these conditions about 10% of the precursor ion remained unaffected. Other settings resulted in either insufficient degradation of the precursor ion or further degradation to several other ions of lower *m/z* value (results not shown). Therefore, the described settings were chosen for quantification.

Fig. 3 shows the reconstructed chromatogram of the $[MH]^+$ to product ion transition (219→98), obtained after on-column injection of 50 pmol PTH-ACC and using the chromatographic conditions (isocratic run) as described in Section 2. Under these conditions, PTH-ACC has a retention time of 4.7 min.

3.2. Quantitative aspects of LC–MS–MS of PTH-ACC

3.2.1. Linearity

In order to determine the linearity of the response of the product ion at *m/z* 98 versus the injected amount of PTH-ACC, different concentrations were injected and analysed by LC–ES-MS–MS under multiple reaction monitoring (MRM) of (219→98). This was done by injecting 10 μl of different concentrations (10^{-4} *M*–10^{-8} *M*) on column using the chromatographic conditions described in Section 2 (isocratic run). All measurements were done in triplicate and data were processed by Masslynx software. After logarithmic transformation of the results, linearity was obtained between 0.1 pmol and 100 pmol injected on column [$x=(0.998\pm0.008)y+(2.693\pm0.024)$, $n=12$, $R^2=0.9997$]. The minimum detectable amount injected was 0.1 pmol at a signal-to-noise ratio of at least 5:1.

3.2.2. Quantitation using deuterated ACC

Deuterated ACC (1-amino-[2,2,3,3-$^2H_4$]ACC) was used as an internal standard for quantitation purposes. In order to determine the linearity of response between cold and deuterated ACC, a calibration curve was made up 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 (unlabelled:labelled ACC). These mixtures were derivatized as described above and analysed by

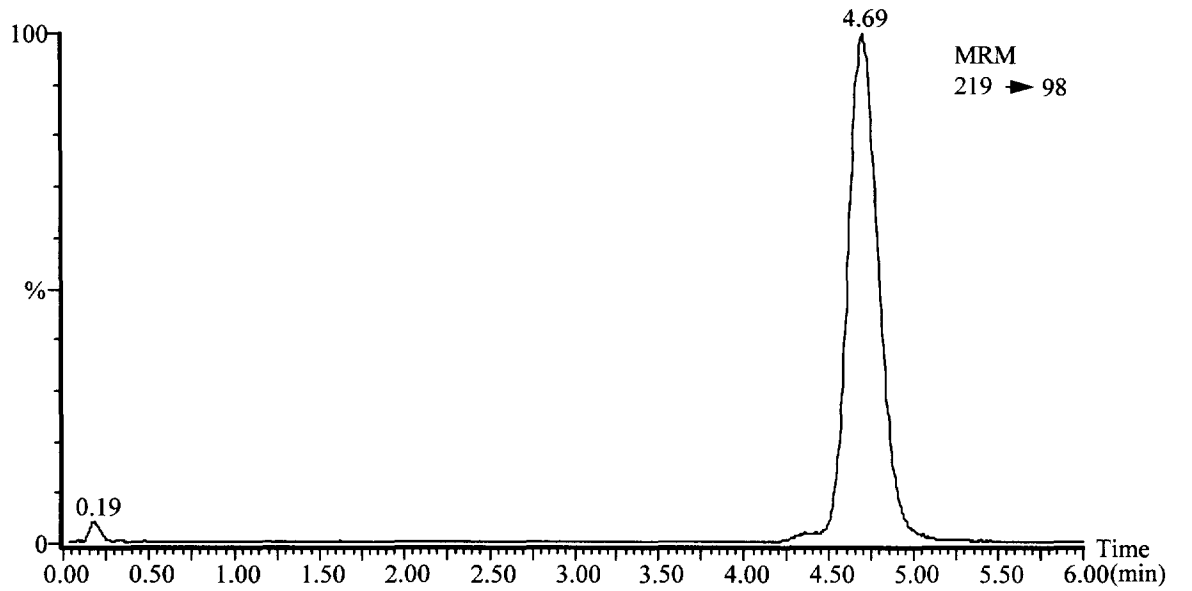


Fig. 3. Reconstructed chromatogram of the $MH]^+$ to product ion transition (219→98), obtained after injection of 50 pmol PTH-ACC. Column, RP-C_8 (Merck, LiChrospher 60 RP Select B, 5 μm, 125×4 mm I.D.); mobile phase, methanol–0.1 *M* ammonium acetate (40:60, v/v); flow-rate, 0.8 ml min^{-1}.

MRM of (219→98) and (223→102) after injection on column (10 μl) of between 0.1 and 100 pmol of each compound. All measurements were done in triplicate.

After logarithmic transformation of the results a significant linear fit was obtained within the investigated range [$x=(0.994\pm0.008)y-(0.008\pm0.043)$, $n=33$, $R^2=0.998$]. With this knowledge, the ACC content of biological samples can easily be quantified based on the ratio of cold and deuterated compounds, without the necessity of recalculation by means of a calibration curve.

3.3. Application to plant samples

In order to evaluate (+)ES LC–MS–MS as a method for the analysis of plant samples, *Nicotiana tabacum* cv. Petit Havana SR1 leaves, stems and roots were extracted with 80% methanol and ACC was purified using solid-phase extraction as described in Section 2. After derivatization of the purified sample with PITC, half of the sample (10 μl) was injected on column using the chromatographic conditions described in Section 2 and the eluting peak was monitored by MRM of (223→102) and (219→98). Using [methanol–ammonium acetate (40:60, v/v)], PTH-ACC (retention time 4.7 min) was sufficiently separated from the bulk of more polar contaminants which eluted earlier (full-scan chromatogram in Fig. 4). Indeed, co-elution of the polar contaminants with PTH-ACC must be avoided in order to circumvent a serious reduction in sensitivity (up to 50% reduction has been observed) which would result in a poor detection of PTH-ACC.

Regarding the high specificity of MRM, we were surprised to detect some additional compounds by the transitions (223→102) and (219→98). These products were not identical to PTH-ACC, since (i) they eluted later, and (ii) no co-elution between peaks detected by the transitions 223→102 and 219→98 was observed for these compounds, while the labeled ACC was added at the start of the extraction. Furthermore, the full-scan spectra of these (unknown) compounds turned out to be different from those of either PTH-ACC or [$^2H_4$]-PTH-ACC (results not shown). In order to avoid interference with the detection of PTH-ACC and/or contamination of the column, a rinsing step at 80% methanol from 5 to 10 min was used, resulting in elution of these compounds before 10 min. After calibration to

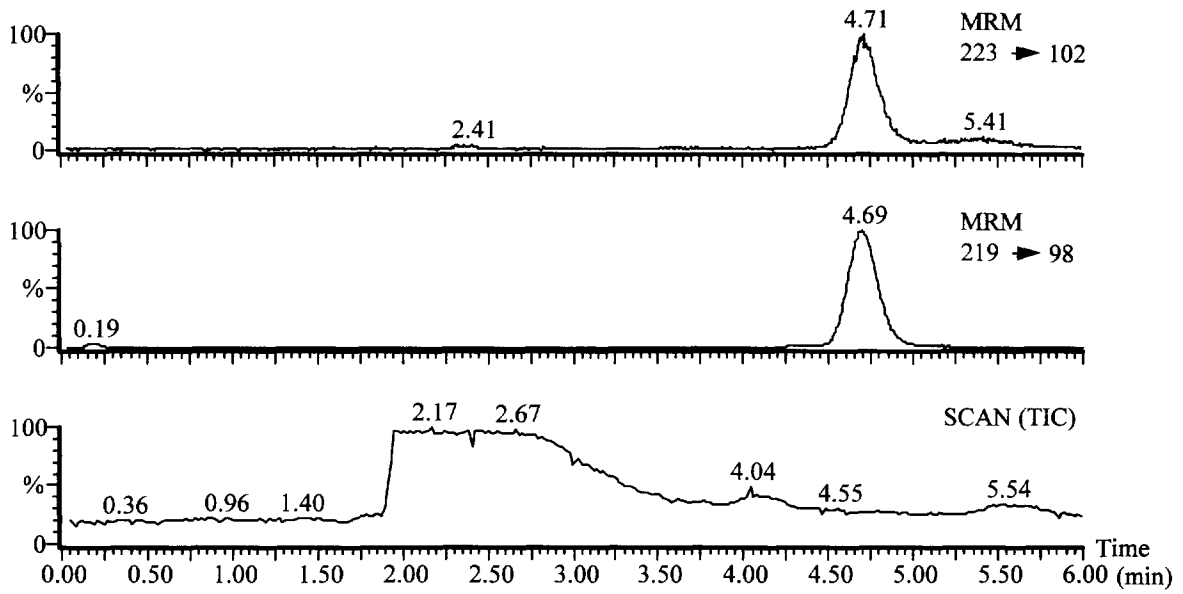


Fig. 4. Chromatogram of a plant extract, after solid-phase extraction and derivatization with PITC. Column, RP-C_8 (Merck, LiChrospher 60 RP Select B, 5 μm, 125×4 mm I.D.); mobile phase, methanol–0.1 *M* ammonium acetate (40:60, v/v) for 5 min, followed by methanol–0.1 *M* ammonium acetate (80:20, v/v) for 5 min; flow-rate, 0.8 ml min^{-1}. Top and centre, MRM chromatogram of $[MH]^+$ and the product ions of both $[^2H_4]$PTH-ACC and PTH-ACC (223→102 and 219→98, respectively); bottom, total ion chromatogram (TIC) as a result of scanning a mass range of 50–300 u at 300 u s^{-1}. PTH-ACC elutes at 4.7 min.

the original solvent composition, the system was ready for injection of the next sample after 12 min. During analysis of numerous tobacco samples, no additional interfering compounds have been detected.

Fig. 5 shows levels of both free and conjugated ACC in *Nicotiana tabacum* leaves, stems and roots obtained with the presented technique. The highest concentrations of both ACC and conjugated ACC were observed in the apex and the first (youngest) leaf and concentrations tended to decrease as the leaves matured. These results fit very well with the observation that the highest rates of ethylene production are usually found in young expanding leaves [16]. Higher ACC concentrations were again observed in the lower part of the stem and in the roots. In these parts, the ratio between conjugated and free ACC appeared to be much higher as compared to the upper parts of the plant, which might be due to poor aeration in the nutrient solution. Indeed, Dong et al. [17] showed that wheat plants react to water logging by an initial increase in the synthesis of both ACC and ethylene, followed by a decrease in ethylene biosynthesis after a few days due to induction of *n*-malonyltransferase. These differences appeared to be most marked in the lower parts of the plants.

4. General conclusion

ACC is a very small molecule (M_r=101) and therefore the analysis by LC–MS of the genuine molecule in biological samples is difficult owing to the presence of intense background signals from the eluant and eventually from other co-eluting low-molecular-mass compounds. Derivatization of ACC with PITC is easy and has already been shown to be applicable to LC–thermospray MS analysis, with a detection limit of 10 pmol injected on column [10]. This technique however still required a preparative HPLC run to separate PTH-ACC from other derivatized amino acids and other interfering compounds. Electrospray mass spectrometry is much more promising in terms of sensitivity and ruggedness. Indeed, in the case of LC–ES-MS–MS a detection limit of

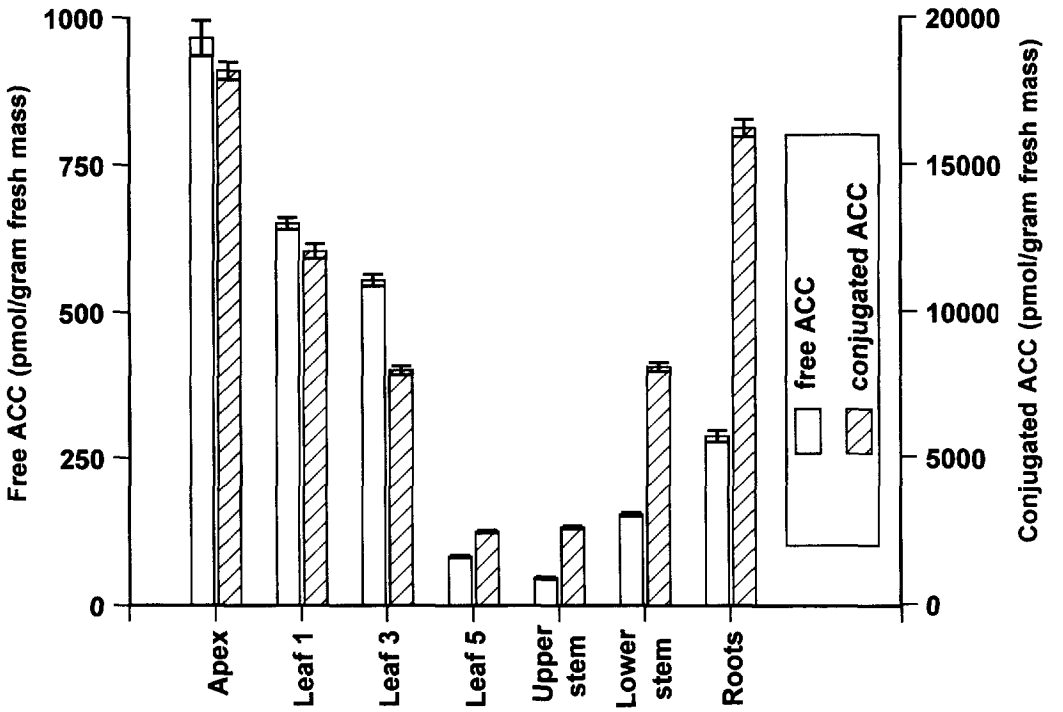


Fig. 5. Levels of free and conjugated ACC in leaves, stems and roots of *Nicotiana tabacum*. Leaf 1 corresponds to the first leaf under the apex which could be easily separated. Results are the average of three replicates±S.D.

0.1 pmol injected on column was obtained, which is a factor 100 fold better than for LC–thermospray MS, while the concentrations of endogenous ACC observed in tobacco leaves measured by LC–ES-MS–MS were comparable to those measured by LC–thermospray MS [10]. Furthermore, we have shown that MRM of (219→98) and (223→102) is sufficiently specific and hence permits the elimination of the time-consuming preparative HPLC run prior to LC–MS analysis, allowing a high sample throughput. Moreover, the observation of a linear relationship between concentration and response permits quantitation of PTH-ACC within a concentration range usually found in biological samples.

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