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Extraction and quantitative analysis of 1-aminocyclopropane-1-carboxylic acid in plant tissue by gas chromatography coupled to mass spectrometry

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Abstract

We developed a new method for the determination of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) using quantitative GC–negative chemical ionisation MS as a detection and quantification system, in combination with isotope dilution using [$^2H_4$]ACC and an off-line solid-phase extraction. By derivatisation with pentafluorobenzyl bromide, ACC could easily be detected with m/z 280 being the most abundant ion. Determination of this component resulted in a detection limit of 10 fmol and a linear fit in the 100 fmol–100 pmol range. The combination of a rapid, high yield purification method with a stable derivatisation procedure and a sensitive detection method allowed the detection of ACC in samples as low as 100 mg fresh mass.

Keywords: Plant material; *Brassica napus*; *Arabidopsis thaliana*; Aminocyclopropanecarboxylic acid; Carboxylic acids

1. Introduction

1-Aminocyclopropane-1-carboxylic acid (ACC) is the immediate precursor of the plant hormone ethylene [1]. Despite being a precursor, determination of its levels can be very informative for several reasons. At a biochemical level, analysis of the ACC content can give information on the ethylene metabolism thereby allowing investigation of the relationship between ethylene and the components controlling its metabolism. Furthermore, Bradford and Yang [2] proposed that transport of ACC is involved in the regulation of plant responses to flooding. As ACC is not always converted to ethylene at its site of production, ACC quantification at the organ level can thus give some information on inter-organ signalling. Finally, the determination of ethylene production by organs is also difficult, as detached organs will produce ethylene in response to wounding and desiccation. Consequently the analysis of its immediate precursor ACC can be a solution [3].

Many techniques for the determination of ACC have been published. The oldest method is based on the oxidative conversion of the extracted ACC to ethylene followed by GC analysis [4]. Despite lower accuracy [5], the technique is still commonly used. Later, techniques were developed for direct detection

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of ACC. Therefore, ACC is first derivatised to more easily detectable compounds. Savidge et al. [6] and McGaw et al. [7], respectively, synthesised the 2,4-dinitrophenylmethyl and the *o*-phthaldialdehyde ester of ACC before characterisation by GC–MS. However, while the former was non-quantitative, the latter required several preparative HPLC steps. Other groups applied methods developed for amino acid analysis to ACC. HPLC was used for both analysis of the *o*-phtaldialdehyde [8] and the phenylthiocarbamyl derivatives [9]. However, these techniques are limited due to their detection by UV. Chauvaux et al. determined the phenylthiohydanthion compound of ACC by LC–thermospray-MS [10] and LC–positive electrospray (ES^+)-MS/MS [11]. The technique requires a laborious derivatisation procedure. Moreover on column focussing of this derivative is not possible implying that further downscaling to micro and capillary LC–MS, as was described for auxins and cytokinins [12], is not possible [13]. More recently, GC coupled to a nitrogen–phosphorus detector was used for investigating the ACC transport in tomato [14]. Petritis et al. [15] were the first to report the analysis of ACC without derivatisation: ACC was concentrated and separated from interfering amino acids by ion-pair reversed-phase chromatography and detected by tandem mass spectrometry in positive electrospray mode. A drawback is however the detection limit of ~20 pmol. Penrose et al. [16] reported a modified WatersACCQ·Tag amino acid analysis method for quantification of ACC in root extracts of canola seedlings. As no internal reference was used, the amount of ACC was quantified by using an external ACC standard curve that was linear between 1 and 25 pmol.

In order to investigate ACC housekeeping in a broad spectrum of different types of plants and tissues, we developed a novel technique of ACC extraction and analysis. Pentafluorobenzyl bromide (PFBBr) allowed sensitive detection of ACC using GC–MS in negative chemical ionisation mode. Combining solid-phase extraction and an isocratic GC run at low temperature, the specificity of MS determination increased. This way, laborious purification and off-line HPLC purification steps could be omitted, enabling the simultaneous handling of many samples.

2. Experimental

2.1. Materials

ACC, [$^2H_4$]ACC and PFBBr were purchased from Sigma (St. Louis, MO, USA). Phenylisothiocyanate (PITC) was from Pierce (Rockford, IL, USA) and trifluoroacetic acid was from Janssen Chemica (Geel, Belgium). Methanol (HPLC grade), ethyl acetate and acetone (Pestiscan) were obtained from LabScan (Dublin, Ireland) and triethylamine from BDH (Poole, UK). Water was purified by reversed osmosis with a Milli-Q water purification system (Millipore, Bedford, MA, USA). RP-C_{18} and strong cation-exchange (Extract-Clean, 200 mg) solid-phase cartridges were obtained from, respectively, Varian (Middelburg, The Netherlands) and Alltech (Lokeren, Belgium).

2.2. Standard samples

Solutions of ACC ranging between 10^{-5} and 10^{-8} *M* and of [$^2H_4$]ACC 10^{-7} *M* were prepared in 80% aqueous MeOH. A 10-μl sample of each ACC dilution was spiked with 1 μl of [$^2H_4$]ACC. After drying under nitrogen, samples were derivatised as described for plant samples and dissolved in 10 μl 100% MeOH of which 1 μl was injected on GC–MS.

2.3. Plant material

Brassica napus L. plants were grown in an unheated glasshouse during the period March–July. Sieved seeds (1.7–2.0-mm diameter fraction) of the spring-sown *B. napus* cultivar N90-740 were sown in John Innes No. 1 compost. The 14-day-old seedlings were grown in pots containing ~1000 ml compost and started to flower ~5 weeks later. On one occasion during flowering, the most recently opened flower (anthesis) was labelled. Then 6 weeks after anthesis, pod wall material was harvested.

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col.) seeds were sterilised by soaking in 30% bleach for 15 min with agitation and rinsed five times in deionised water. Seeds were germinated on

Murashige and Skoog medium, composed of Murashige and Skoog salts and vitamins as indicated by the producer (Duchefa, Haarlem, The Netherlands), 3% sucrose (Duchefa) and 0.8% agar (Sigma). Plants were grown for 7 days at 24 °C with 16-h light (60 μmol photons m^{-2} s^{-1})/8-h dark regime. Whole seedlings were harvested, weighed and immediately frozen in liquid nitrogen. Samples were stored at −70 °C until analysis.

2.4. Extraction and purification of ACC

2.4.1. Purification of 1-aminocyclopropane-1-carboxylic acid

Approximately 200 mg of plant material was ground in liquid nitrogen with mortar and pestle, transferred into 80% aqueous MeOH and extracted overnight at −20 °C. [$^2H_4$]ACC (200 pmol) was added if samples were used for quantification purposes. After centrifugation (20 000 rpm, 15 min, 4 °C), the supernatant was applied to a C_{18} cartridge. The effluent was dried to the water phase, brought to pH 2 using 0.01 *M* HCl and purified over a strong cation-exchange resin (Extract-Clean, 200 mg) as described by Persson and Näsholm [17]. After rinsing the cartridges with a water–MeOH (1:8) solution, ACC was eluted by 2×1 ml of 4 *M* NH_4OH, dried under nitrogen at 60 °C (Zymark Turbovap LV) and stored at −20 °C until further analysis.

2.4.2. Derivatisation with pentafluorobenzyl bromide

Samples were transferred to derivatisation vials by 80% aqueous MeOH, dried under nitrogen, and dissolved in 60 μl acetone. Samples of triethylamine (4 μl) and pentafluorobenzyl bromide (10 μl) were added after which the tubes were incubated at 60 °C for 15 min (Reacti-ThermIII heating module, Pierce).

The derivatised samples were further purified by liquid–liquid extraction (ethyl acetate–water). The ethyl acetate fraction was transferred to GC injection vials and dried. Before injection on GC, samples were dissolved in 10 μl 100% MeOH.

2.5. Gas chromatography–chemical ionisation mass spectrometry of PFB-bis-ACC

The ACC-bis-PFB samples were injected by a HP6890 series injector on a GC system (HP 5890 series II) connected to a VG TRIO-2000 mass spectrometer using a chemical ionisation interface. A 1-μl sample was injected with an injector temperature gradient (Gerstel-cis3, Analytical Applications, Brielle, The Netherlands) from 150 to 325 °C in 2 min. The GC column used was from Varian (CP-SIL 5CB, Low Bleed/MS, 15 m, 0.25 mm I.D., film thickness 0.25 μm) using He (99.995%, Air Liquide) as carrier gas at a flow rate of 1.5 ml/min. The oven gradient used for routine analysis was 11 min at 150 °C followed by a linear gradient to 300 °C in 7.5 min, finished by 1 min at 300 °C. ACC-bis-PFB eluted at a retention time of 10.85 min.

Ionisation (V=31 eV) took place in negative chemical ionisation mode (CI^-) by means of CH_4 as an ionising gas at a pressure of $1 \cdot 10^{-4}$ mbar and a source temperature of 160 °C. Mass spectra were recorded by scanning a mass range of 70–470 u in 1.2 s. To improve the sensitivity, selected ion monitoring (SIM) was used for quantification. The de-protonated molecules at m/z 280 for ACC-PFB and m/z 284 for [$^2H_4$]ACC-PFB were monitored in the SIM chromatograms, using a dwell time of 80 ms and a interchannel delay of 20 ms. The chromatograms obtained were processed by means of Masslynx 3.2 software (Micromass, UK). Concentrations could be calculated following the principles of isotope dilution and expressed in pmol per gram fresh mass. The detection limit was set at a signal-to-noise ratio of 3. The detection limit of biological samples was determined taking into account this signal-to-noise ratio in combination with the sample-specific recovery calculated using the [$^2H_4$]ACC tracer.

2.6. PITC derivatisation and LC–ES^+-MS/MS quantification of PTH-ACC

As a control of the technique presented here, *Brassica* and *Arabidopsis* samples were also analysed on LC–ES^+-MS/MS after successive deri-

vatisation in a PITC and trifluoroacetic acid solution according to Chauvaux et al. [11].

3. Results and discussion

3.1. Qualitative analysis of ACC-bis-PFB by GC–CI^--MS

Given the low-molecular mass of ACC (M_r=101), GC–MS of genuine ACC is difficult since the ionised molecule would be hard to detect amongst the intense background signals. Therefore, ACC was derivatised before injection on GC–MS. In accordance with Netting and Duffield [18] who described a PFBBr derivatisation procedure for the amino acids leucine and tyrosine, ACC derivatisation by PFBBr was tested. During this reaction under strong alkaline conditions, the amino acid is de-protonated at the carboxyl and amino terminal groups (Fig. 1). PFBBr reacts at both the amino and carboxylic acid group of the ACC molecule, resulting in ACC-bis-PFB with M_r of 461.

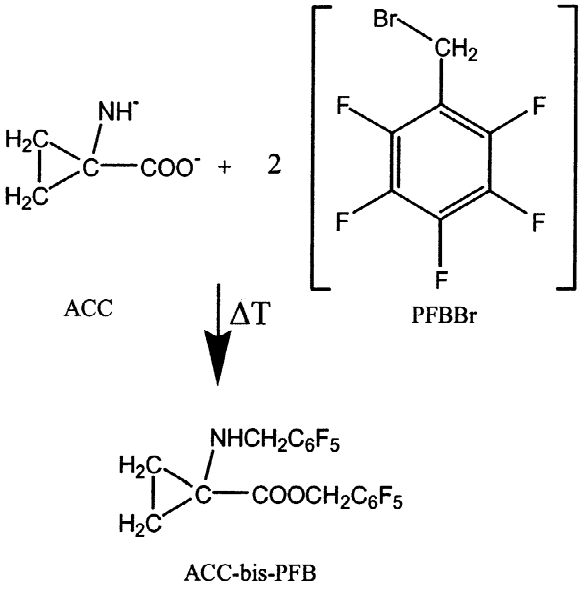

Fig. 1. Derivatisation reaction under strong alkaline conditions of 1-aminocyclopropane-1-carboxylic acid (ACC) by pentafluorobenzyl bromide (PFBBr), resulting in the formation of ACC-bis-PFB. The reaction took place for 15 min at 60 °C.

ACC-bis-PFB was analysed in the negative chemical ionisation mode because of the compound's high electron-capture characteristics. Under the ionisation conditions used, dissociative electron capture occurs after which a PFB group splits off, resulting in a negatively charged ion with m/z 280 which can be sensitively detected. Fig. 2 shows the total ion current (TIC) chromatogram, together with the mass chromatogram for m/z 280. Although several components are detected in TIC, only one signal with a retention time of 10.85 min is found at the diagnostic ion used for ACC quantification whereas in a blank sample no signal at 10.85 min was found (data not shown). The latter was derivatised as described above but without addition of ACC. The mass spectrum of 5 pmol ACC-bis-PFB and [$^2H_4$]ACC-bis-PFB is depicted in Fig. 3. The de-protonated [ACC-PFB]$^-$ and [$^2H_4$ACC-PFB]$^-$ molecules show a single, specific and intense peak at, respectively, m/z 280 and 284. The absence of a m/z 280 signal in the blank allows us to select this fragment as diagnostic ion for quantitative analysis.

3.2. Quantitative analysis of ACC-bis-PFB by GC–CI^--MS

For quantification purposes, 1 pmol [$^2H_4$]ACC was spiked with different amounts of genuine ACC ranging between 100 fmol and 100 pmol. Each concentration ratio was prepared and analysed three to seven times. These mixtures were derivatised as described above and analysed by GC–CI^--MS. As m/z 280 and 284 were the most abundant fragments of, respectively, ACC-bis-PFB and [$^2H_4$]ACC-bis-PFB, these traces were selected as diagnostic ions used for quantification. The ratio between the concentrations was plotted against the ratio of the concomitant areas of the unlabelled and heavy labelled ACC. After logarithmic transformation, the area-to-area ratio (m/z 280/m/z 284) was plotted against the molar ratio. The resulting calibration graph demonstrated a linear fit in the tested range from 100 fmol ACC/1 pmol [$^2H_4$]ACC to 100 pmol ACC/1 pmol [$^2H_4$]ACC. Although 10 fmol could still be detected, it slightly shifted from the linear range. The linear regression equation for the calibration graph was $y=(1.009\pm0.034)x+(0.002\pm0.091)$ ($n=41$, $R^2=0.993$). From these data,

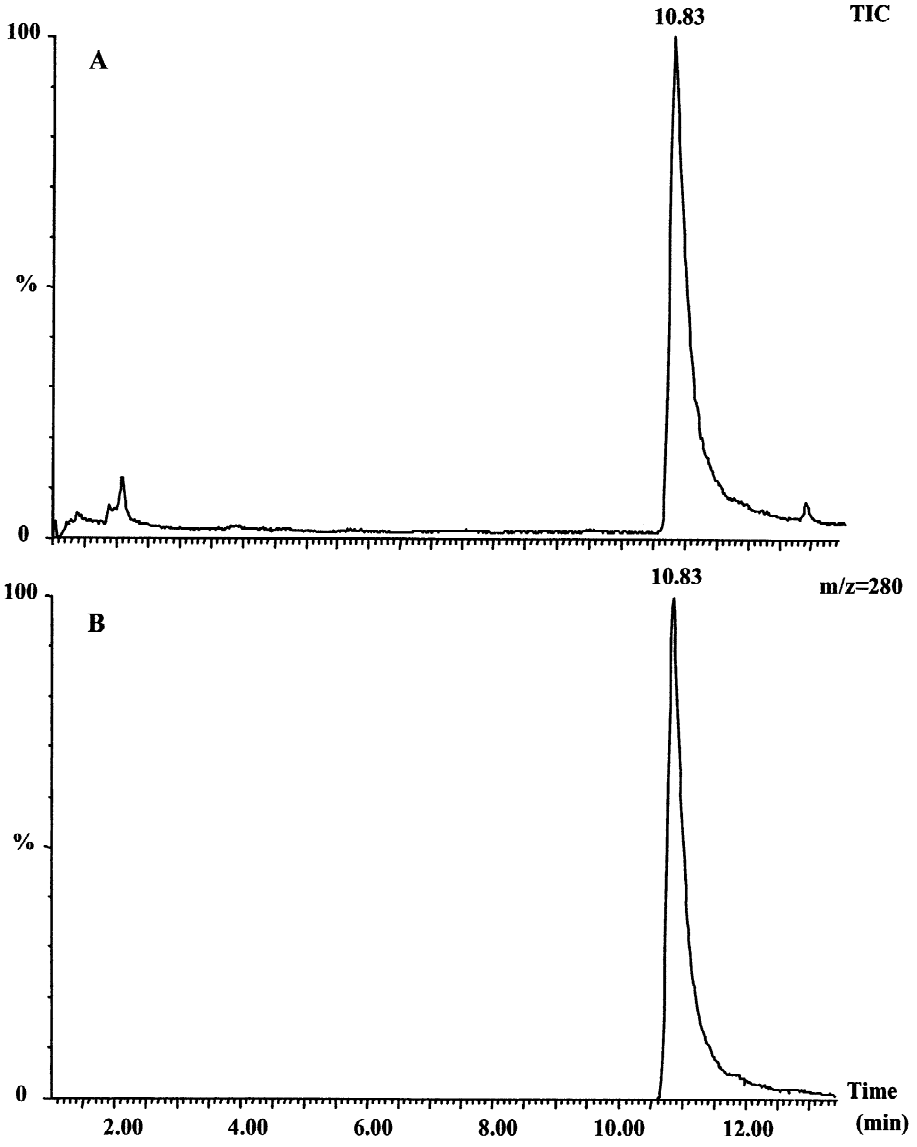

Fig. 2. Chromatograms (total scanning) of 5 pmol of ACC-bis-PFB standard. Column, Varian (CP-SIL 5CB, Low Bleed/MS, 15 m, 0.25 mm I.D., film thickness 0.25 μm); carrier gas, He (Air Liquide); flow rate, 1.5 ml/min; oven gradient, 11 min at 150 °C, linear gradient to 300 °C in 7.5 min, 1 min at 300 °C. (A) TIC chromatogram (m/z 95–495 in 1 s); (B) mass chromatogram for m/z 280, corresponding to the de-protonated [ACC-PFB]$^-$ molecule.

we can conclude that ACC levels in biological samples can be determined by quantitative GC–CI$^-$-MS analysis of the unlabelled and $^2H_4$-labelled compounds within a range of 100 fmol to 100 pmol, without the necessity of an external calibration curve.

3.3. Quantitative GC–CI$^-$-MS analysis of ACC-bis-PFB in plant samples

A. thaliana Col.0 wild type plants and *B. napus* L. pods and their dehiscence zones were extracted and purified by means of RP-C_{18} and strong cation-

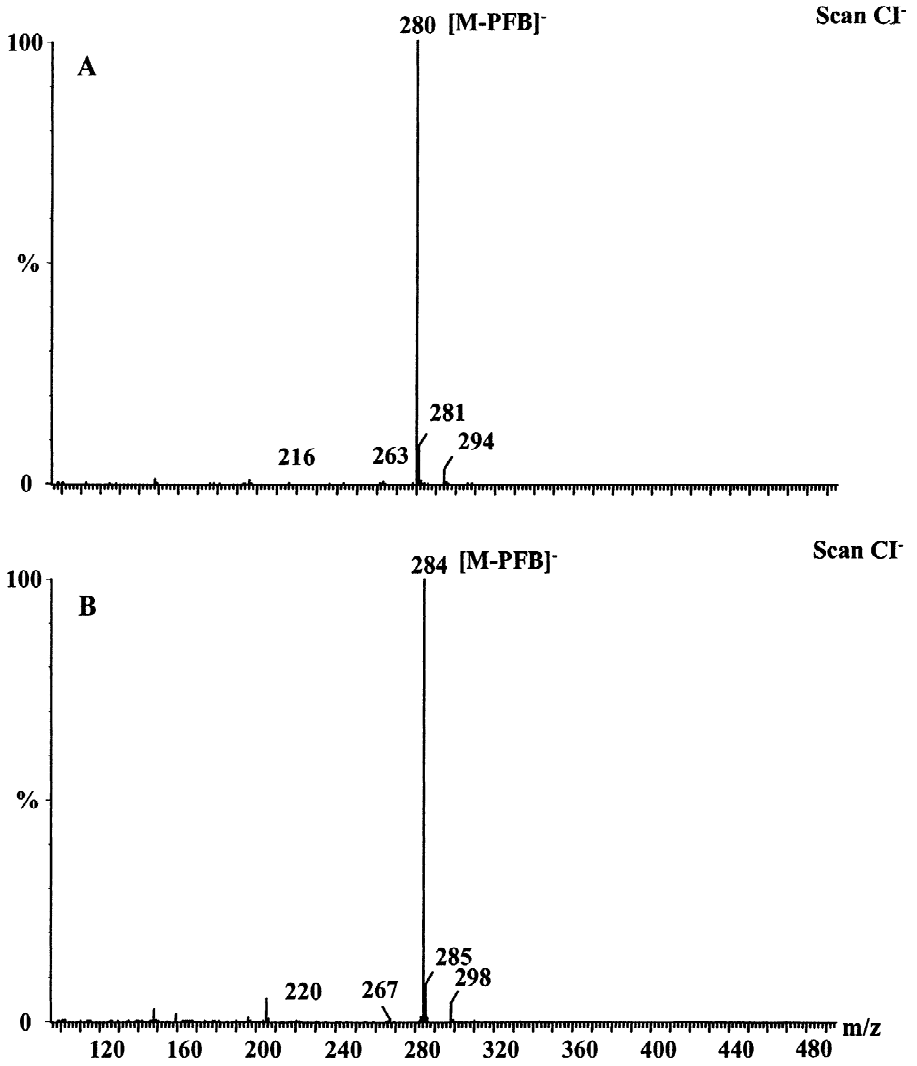

Fig. 3. (A) CI^- mass spectrum of ACC-bis-PFB corresponding to the peak signal observed at $t_R = 10.85$ min in Fig. 2; (B) mass spectrum of a $[^2H_4]$ACC-bis-PFB peak, obtained by the same procedure as for ACC-bis-PFB. Both signals correspond to 5 pmol of compound.

exchange cartridges. To check the presence of putative contaminating compounds at the trace m/z 284, no heavy-labelled ACC was added to the extract. The isolated ACC was derivatised and determined on GC–CI^--MS as described above. At the m/z 284, selected for detection of the heavy labelled standard, no signal at the ACC specific retention time was observed in any sample. At m/z 280 some other compounds were detected. In order to obtain a decent differentiation between the latter and the ACC-bis-PFB, an isocratic GC run for routine analyses was carried out (Fig. 4).

By recording the samples in scan mode (not shown), it became clear that m/z 280 was the sole signal detected at a retention time of 10.85 min in the *Arabidopsis* and *Brassica* samples. These data demonstrate that the signals selected for quantification of ACC and $[^2H_4]$ACC are representative for these two compounds. Hence, the extraction, derivatisation and detection procedure presented here is

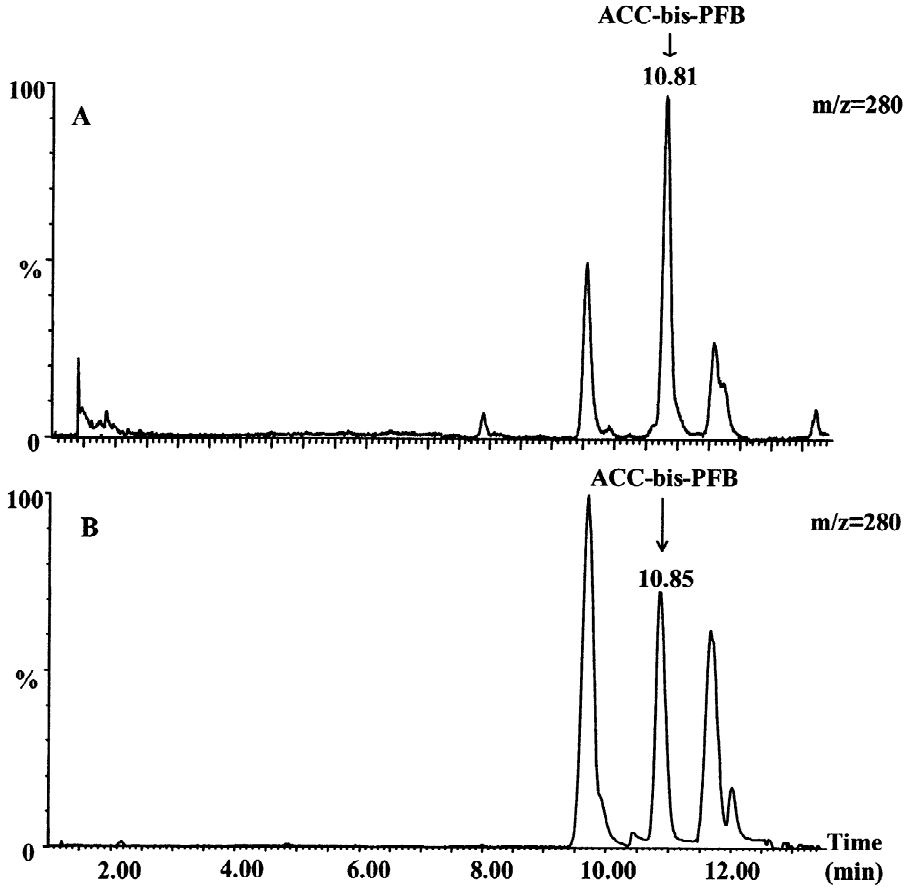

Fig. 4. Chromatograms of plant samples (selected ion monitoring) for m/z 280 (ACC-PFB). Conditions are the same as for the reference sample. (A) A 7-day-old *A. thaliana* plant sample and (B) pod wall dehiscence zone of *B. napus* 6 weeks after anthesis.

shown to be specific enough for the determination of ACC contents in *Arabidopsis* and *Brassica* samples.

Based on these results, we compared this method with the LC–MS/MS technique published by Chauvaux et al. [11]. Therefore, samples were extracted and purified as described in Section 2 and split in two equal aliquots. One aliquot was derivatised by PFBBr and measured on GC–CI^--MS, while the other aliquot was derivatised by PITC and quantified on LC–ES^+-MS/MS. Three samples were taken from *Brassica* pods and complete *Arabidopsis* seedlings, giving fresh masses of ~500 and 150 mg, respectively. As shown in Table 1, for *Brassica* samples comparable results of ~1400 pmol/g fresh mass were obtained by GC–CI^--MS and LC–ES^+-MS/MS. Half of the sample was injected for LC–ES^+-MS/MS determination, whereas 1/100 was used for GC–CI^--MS detection demonstrating once more the superior sensitivity of the latter method. The ACC content in *A. thaliana* samples analysed by GC–CI^--MS is 118±11 pmol/g fresh mass. The results obtained by LC–ES^+-MS/MS are expressed as less than the sample specific detection limit. This sample specific detection limit covers the detection limit of the LC–ES^+-MS/MS including the sample specific recovery losses after the extraction and derivatisation losses. The detection limit of 226±16 pmol/g fresh mass indicates that the *A. thaliana* data obtained by GC–CI^--MS (118±11 pmol/g fresh mass) might be realistic. The mean extraction recovery obtained by the extraction procedure here described is ~70%, whereas the extraction protocol

Table 1
Comparison of GC–CI^--MS with LC–ES^+-MS/MS quantification of ACC in three samples of *B. napus* pods 6 weeks after anthesis and three samples of 7-day-old *A. thaliana* Col.0

Sample No.	Endogenous ACC individual values (pmol/g fresh mass)	
	GC–MS	LC–MS/MS
B. napus		
1	1523	1644
2	1545	1431
3	1166	1264
A. thaliana		
1	124	<244[a]
2	125	<214[a]
3	105	<220[a]

After extraction, 200 pmol [$^2H_4$]ACC was added and samples were purified over RP-C_{18} and strong cation-exchanger cartridges. The eluted samples were split in two equal parts, dried and derivatised and measured with, respectively, PITC and LC–ES^+-MS/MS on one the hand and with PFBBr and GC–CI^--MS on the other.

[a] Internal ACC level below detection limit of LC–MS/MS.

described by Chauvaux et al. [11] resulted in a mean recovery of 10%.

4. General conclusion

Netting and Duffield [18] showed PFBBr derivatisation to be a highly effective method for the derivatisation and detection of amino acids. Application of this method to ACC results in the formation of a molecule with M_r of 461. As ACC is a small molecule, detection on MS results in a high background signal, inherent to low-molecular-mass ions. By derivatisation with PFBBr, the molecule can easily be detected in negative chemical ionisation with m/z 280 being the most abundant ion. As no interference with other compounds was observed, this trace was selected for quantification purposes. Determination of this component resulted in a detection limit of 10 fmol. Furthermore, a linear fit in the 100 fmol–100 pmol range was found, allowing the method to be used for the analysis of ACC since endogenous ACC levels in biological samples are mostly within this range. Singh et al. [19] demonstrated that LC–negative atmospheric pressure ionisation ($APCI^-$)-MS/MS yielded detection through dissociative electron capture at attomolar level for the PFB derivative of the amino acid phenyl alanine. Therefore, we tested the ionisation properties of ACC-bis-PFB in the $APCI^-$ mode which produced a detection limit of 250 fmol (data not shown). Chauvaux et al. [11] showed the phenylthiohydanthion derivative of ACC to have a detection limit of 100 fmol using LC–ES^+-MS/MS. However, a drawback of this technique was the large amount of preparative work and the consequently low extraction and derivatisation recovery (<10%). Based on these results, we preferred GC–MS rather than LC–MS/MS despite the selectivity of the latter. Taking the complete extraction protocol, recovery values up to 70% were reached. The combination of a rapid, high yield purification method with a stable derivatisation procedure and a sensitive detection method allowed the detection of ACC in samples with fresh masses of ~100 mg. Although ACC usually is present in nanomolar concentrations in whole plants, this method allows ACC determination at an organ level or in seedlings with low fresh masses. ACC quantification at organ level at specific developmental stages can then be used to unravel relations between plant development and ACC or ethylene levels. As shown before, ethylene plays an important role in seedling development, for example the triple response [20]. Therefore, this method will also enable the study of the role of ethylene biosynthesis in the development of, for example, *Arabidopsis* seedlings at specific phenotypic stages.

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References

[1] D.O. Adams, S.F. Yang, Proc. Natl. Acad. Sci. USA 76 (1979) 170.
[2] K.J. Bradford, S.F. Yang, Plant Physiol. 65 (1980) 322.
[3] S.F. Yang, N.E. Hoffman, Annu. Rev. Plant Physiol. 26 (1984) 1579.
[4] C.C. Lizada, S.F. Yang, Anal. Biochem. 100 (1979) 140.
[5] L.W. Coleman, C.F. Hodges, J. Plant Physiol. 138 (1991) 7.
[6] R.G. Savidge, G.M.C. Mutumba, J.K. Heald, P.F. Wareing, Plant Physiol. 71 (1983) 434.
[7] B.A. McGaw, R. Horgan, J.K. Heald, Anal. Biochem. 149 (1985) 130.
[8] R. Miller, J. La Grone, P. Skolnick, K.M. Boje, J. Chromatogr. 578 (1992) 103.
[9] D. Lanneluc-Sanson, C.T. Phan, R.L. Granger, Anal. Biochem. 155 (1986) 322.
[10] N. Chauvaux, W. Van Dongen, E.L. Esmans, H.A. Van Onckelen, J. Chromatogr. A 657 (1993) 337.
[11] N. Chauvaux, W. Van Dongen, E.L. Esmans, H.A. Van Onckelen, J. Chromatogr. A 775 (1997) 143.
[12] E. Prinsen, W. Van Dongen, E.L. Esmans, H.A. Van Onckelen, J. Chromatogr. A 826 (1998) 25.
[13] E. Prinsen, unpublished results.
[14] M.A. Else, M.B. Jackson, Aust. J. Plant Physiol. 25 (1998) 453.
[15] K. Petritis, V. Dourtoglou, C. Elfakir, M. Dreux, J. Chromatogr. A 896 (2000) 335.
[16] D.M. Penrose, B.A. Moffat, B.R. Glick, Can. J. Microbiol. 47 (2001) 77.
[17] J. Persson, T. Näsholm, Physiol. Plant 113 (2001) 352.
[18] A.G. Netting, A.M. Duffield, Biomed. Mass Spectrom. 12 (1985) 668.
[19] G. Singh, A. Gutierrez, K. Xu, I.A. Blair, Anal. Chem. 72 (2000) 3007.
[20] J. Kieber, Annu. Rev. Plant Physiol. Plant Mol. Biol. 48 (1997) 277.