JOURNAL OF MASS SPECTROMETRY, VOL. 32, 12–22 (1997)

HPLC Linked Electrospray Tandem Mass Spectrometry: A Rapid and Reliable Method to Analyse Indole-3-Acetic Acid Metabolism in Bacteria

Els Prinsen,[1]* Walter Van Dongen,[1,2] Eddy L. Esmans[2] and Henri A. Van Onckelen[1]
[1] University of Antwerp (UIA), Dept. of Biology, Universiteitsplein 1, B-2610 Antwerpen, Belgium.
[2] University of Antwerp (RUCA, UIA), Dept. of Chemistry, Nucleoside Research and Mass Spectrometry Unit, Groenenborgerlaan 171, B-2020 Antwerpen, Belgium.

Electrospray tandem mass spectrometry in combination with high-performance liquid chromatography can be used for the simultaneous detection and quantification of indole-3-acetic acid, indole-3-methanol, tryptophan, indole-3-acetamide, indole-3-ethanol, tryptamine, indole-3-acetaldehyde, indole-3-acetonitrile, indole-3-aldehyde, indole-3-lactic acid, indole-3-pyruvate and anthranilate on an individual basis. Although methylation is not an absolute prerequisite for analysis of most carboxyl-type indole compounds, samples were methylated before analysis and this improved the detection limits for tryptophan, indole-3-acetic acid, anthranilate and indole-3-lactate up to 1000-fold. Moreover, owing to this methylation no ion suppression conditions, necessary for chromatography of organic acids, have to be used and stability of indole-3-pyruvate was achieved. A detection limit of 100 fmol indole-3-acetic acid methyl ester injected on-column was obtained under multiple reaction monitoring conditions. In view of the analysis of a large number of samples, the chromatographic conditions selected are a compromise between speed of analysis and resolution.

J. Mass Spectrom. **32**, 12–22 (1997)

No. of Figures: 8 No. of Tables: 4 No. of Refs: 22

KEYWORDS: anthranilate; electrospray tandem mass spectrometry; high-performance liquid chromatography; indole-3-acetic acid; tryptophan

INTRODUCTION

Most current physico-chemical assay systems for plant hormones incorporate a high-resolution chromatographic step, usually high-performance liquid chromatography (HPLC) or capillary gas chromatography (GC) using fluorescence or mass spectrometry (MS) as a highly selective detection system (for a review, see Ref. 1). Until now, the most sensitive method described for the analysis of indole-3-acetic acid (IAA) is GC/MS analysis of the pentafluorobenzyl ester of indole-3-acetic acid. This derivatization results in a higher sensitivity under negative ion chemical ionization conditions using ammonia as reagent gas. With such an analysis, a detection limit of 5 pg of IAA as its pentafluorobenzyl ester was achieved.[2]

Tryptophan (Trp) has been analysed by capillary GC/MS as its N-acylalkyl ester using the quinolinium ion (m/z 130) and the molecular ion (m/z 260) as diagnostic ions for GC/MS selected-ion monitoring (GC/SIM-MS).[3] Endogenous indole-3-pyruvate (IPyA) has been identified by full-scan GC/MS.[4] In order to allow the purification of this labile IPyA, the compound was converted into its pentafluorobenzyl oxime derivative in the crude extract. This derivative also allowed the sensitive detection and measurement of IPyA in the picogram range using GC and negative chemical ionization. Proof of the existence of endogenous IPyA and measurement of the endogenous levels has been lacking owing to the highly labile nature of this compound. The first report of the application of liquid chromatography/tandem mass spectrometry (LC/MS/MS) for the identification of IAA metabolites was the identification of hydroxylated derivatives and amino acid ester-linked conjugates of IAA using fast atom bombardment (FAB) in combination with capillary reversed-phase HPLC.[5,6]

Bacteria of the nitrogen-fixing genera *Azospirillum* and *Rhizobium* live in association and symbiosis, respectively, with roots of many plants. Like most rhizosphere bacteria, members of these bacterial genera produce phytohormones. This excretion of phytohormones by associated bacteria may promote plant growth and improve crop yield (for recent reviews, see Refs 7 and 8). The study of the biosynthetic pathways of the plant hormone IAA in bacteria requires the screening of all IAA intermediates and their breakdown products (see Fig. 1) in a large number of samples.

Badenoch-Jones *et al.*[9] for the first time unequivocally identified and semi-quantitatively determined

* Correspondence to: E. Prinsen.

CCC 1076–5174/97/010012–11

Received 16 July 1996
Accepted 9 September 1996

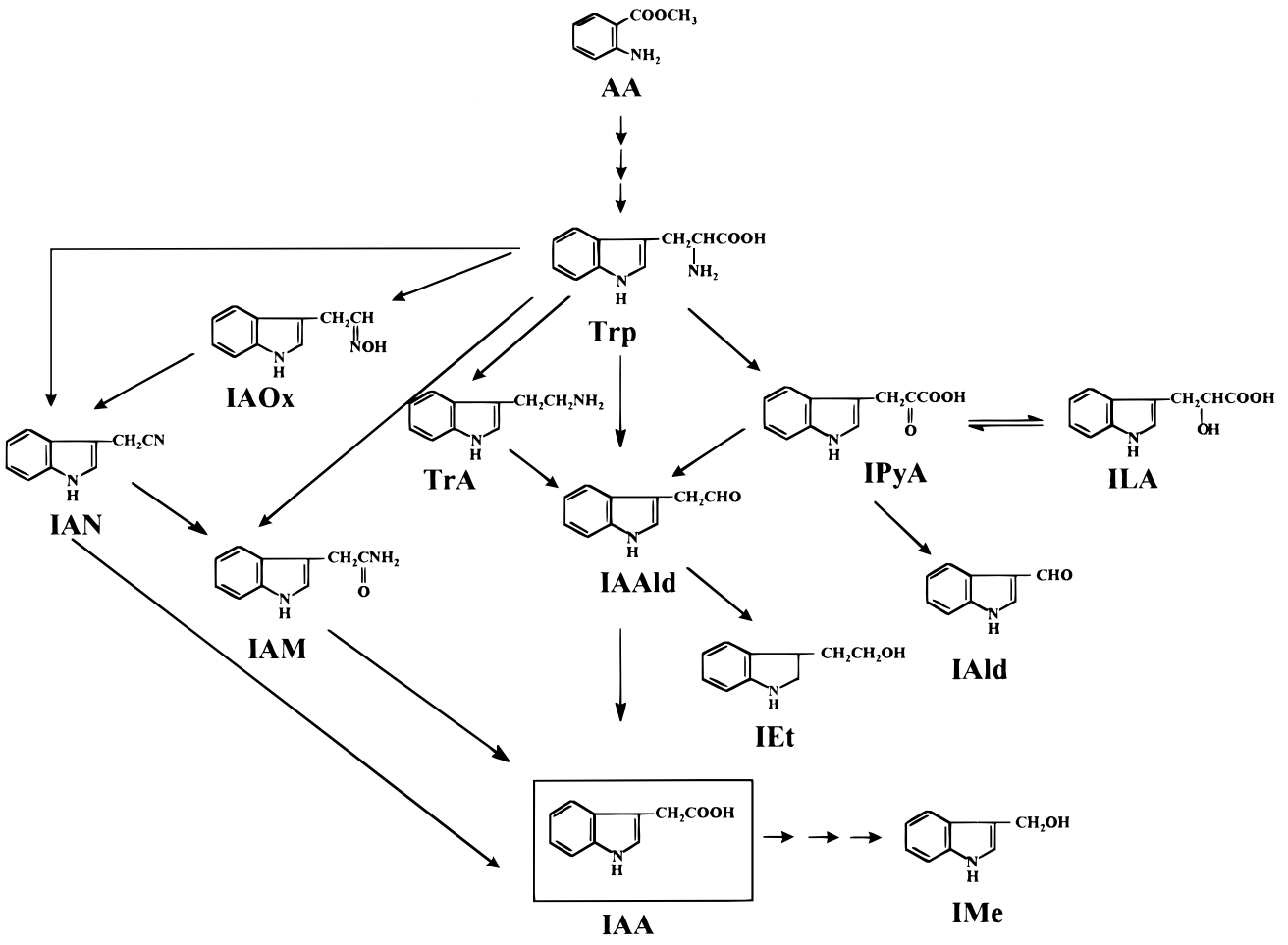

Figure 1. Biosynthetic pathways described for IAA. AA = anthranilate; IAA = indole-3-acetic acid; IAAld = indole-3-acetaldehyde; IAld = indole-3-aldehyde; IAM = indole-3-acetamide; IAN = indole-3-acetonitrile; IAOx = indole-3-acetaloxime; IEt = indole-3-ethanol; ILA = indole-3-lactic acid; IMe = indole-3-methanol; IPyA = indole-3-pyruvic acid; TrA = tryptamine; Trp = tryptophan.

several indole compounds in culture supernatants of *Rhizobium* strains using GC/MS. Lebuhn and Hartmann[10] optimized a method for the determination of substances occurring in auxin metabolism and Trp catabolism. The latter method was based on solid-phase extraction in combination with two isocratic reversed-phase HPLC separations under different liquid-phase conditions and simultaneous detection with fluorescence and UV absorbance at different wavelengths. By measuring various chromatographic and spectral parameters simultaneously, the determination reliability was improved. This method is time consuming, however, and therefore not appropriate for a kinetic screening of various bacterial strains. Although GC/MS is widely used for the analysis of IAA in plant tissue, for convenience HPLC with on-line UV detection or thin-layer chromatography (TLC) in combination with the Salkowski colour reaction is still often used for the analysis of IAA metabolism by bacteria, despite its poor selectivity and despite the fact that the accuracy of this method was already questioned in 1988.[11]

To devise a rapid, reliable and sensitive screening assay, we designed a single-step purification procedure in combination with electrospray tandem mass spectrometry (ES-MS/MS) which allows the concentration and quantification of all IAA intermediates present in the bacterial cells or secreted by the bacteria into the growth medium.

EXPERIMENTAL

Products

The unlabelled indole components were purchased from Sigma. The labelled tracers used are summarized in Table 1.

Bacterial growth conditions

Azospirillum brasilense strain SP248[16] was grown at 28 °C in MMAB[17] minimal medium on a G25 rotatory incubation shaker (New Brunswick Scientific) at 199 rpm. Upon harvest, the OD_{600} was measured (Spectronic 1001, Bausch & Lomb) and the antioxidant butylated hydroxytoluene was added. At an optical density of 0.9, bacterial cells were separated from 5 ml

Table 1 Stable isotopes for the analysis of different indole compounds

Compound	Abbrev.	Stable isotope	Source
Anthanilic acid	AA	$^{15}N_1$	Cambridge Isotope Labs.
Tryptophan	Trp	Indole-$^{15}N_1$	Cambridge Isotope Labs.
Indole-3-acetic acid	IAA	Phenyl-$^{13}C_6$	Cambridge Isotope Labs.
Indole-3-acetamide	IAM	Phenyl-$^{13}C_6$	Synthesized from phenyl-$^{13}C_6$-IAA[12]
Indole-3-ethanol	IEt	Indole-$^{15}N_1$	Synthesized from indole-$^{15}N_1$-Trp[13]
Indole-3-acetaldehyde	IAAld	Indole-$^{15}N_1$	Synthesized from indole-$^{15}N_1$-Trp[13]
Indole-3-acetonitrile	IAN	Side-chain α-carbon-$^{13}C_1$	Gift from N. Ilic, Beltsville, MD, USA[14]
Indole-3-pyruvic acid	IPyA	Indole-$^{15}N_1$	Synthetized from indole-$^{15}N_1$-Trp[15]

of culture medium by centrifugation (Medifuge, Heraeus, 10 min, 2000 *g*, room temperature). The bacterial cells and the culture media were immediately frozen upon harvest and stored at −20 °C until purification. The prepurification procedure is based on the method described earlier for IAA.[18]

Analysis of indole compounds in bacterial cells

Bacterial cells were resuspended in 200 µl of 100% ethanol to which 100 ng of $^{15}N_1$-Trp[(indole-^{15}N)-L-tryptophan, 98%+, Cambridge Isotope Laboratories], $^{13}C_6$-IAA [(phenyl-$^{13}C_6$]-indole-3-acetic acid, 99%, CIL), indole-$^{15}N_1$-IPyA (synthesized from indole-$^{15}N_1$-Trp[15]) and phenyl-$^{13}C_6$-IAM (synthesized from $^{13}C_6$-IAA[12]) and 500 ng of $^{15}N_1$-AA (^{15}N-anthranilic acid, 98%+, CIL), indole-$^{15}N_1$-IAAld (synthesized from indole-$^{15}N_1$-Trp[13]), $^{13}C_1$-IAN (kindly provided by N. Ilic, University of Maryland, USA) and indole-$^{15}N_1$-IEt (synthesized from indole-^{15}N-Trp[13]) were added as internal tracers. Bacterial cells were sonicated for 3 × 0.5 min at 300 W (Braun) and 0 °C. Cell debris was removed by centrifugation (MSE Microcentaur, 5 min., 13000 rpm, room temperature). These bacterial extracts were diluted to 5 ml with 0.05 M HCl and concentrated on an RP-C_{18} cartridge (Bond-Elut, Varian). After rinsing with an additional 10 ml of 0.05 M HCl, all indole compounds were eluted from this cartridge with 2 × 2 ml of acetonitrile. The acetonitrile was evaporated in a Speed Vac (Heto VR-1). Before analysis, the samples were dissolved in 100 µl of acidified methanol and methylated with ethereal diazomethane,[19] dried under a stream of nitrogen and dissolved in 100 µl of methanol–0.01 M ammonium acetate (50:50, v/v).

Analysis of indole compounds in bacterial growth media

To 2 ml of bacterial growth medium were added 2 ml of 0.1 M HCl and 100 ng of $^{15}N_1$-Trp, $^{13}C_6$-IAA, $^{15}N_1$-IPyA and $^{13}C_6$-IAM and 500 ng of $^{15}N_1$-AA, $^{15}N_1$-IAAld, $^{13}C_1$-IAN and $^{15}N_1$-IEt. This extract was concentrated on an RP-C_{18} cartridge as described above for the bacterial pellets.

LC/(+)ES-MS/MS conditions for the analysis of indole compounds

Samples containing indole compounds were analysed using an HPLC system linked to a Quatro II mass spectrometer equipped with an electrospray interface (LC/(+)ES-MS/MS) (Micromass). Samples (10 µl) were injected on an RP C-8 reversed-phase column (Merck; LiChrospher 60 RP Select B; 5 µm; 125 × 4 mm i.d.) and eluted with methanol–0.01 M ammonium acetate (50:50, v/v) at 0.8 ml min^{-1}. Using a post-column split of 1:20, the effluent was introduced into the electrospray source (source temperature 80 °C, capillary voltage +3.5 kV, cone voltage 20 V). Under these conditions, full-scan spectra of the different indole compounds were recorded (scan range 50–600 11 Da, scan speed 300 Da s^{-1}). Collision-activated dissociation (CAD) spectra of the protonated molecular ion ($[MH]^+$) were obtained at a collision energy of 20 eV and a P_{AR} of 4×10^{-3} mbar. Quantification was done

Table 2 Parent ions, diagonstic transitions or diagnostic ion used in multiple reaction monitoring (MRM) or selected-ion monitoring (SIM), specific cone voltage and collison energy used for the analysis of different indole compounds by LC (+)ES-MS/MS after methylation ($P_{AR} = 4 \times 10^{-3}$)

Compound	Parent ion	Diagonostic transition MRM Diagnostic ion SIM*	Cone voltage (V)	Collision energy (eV)	Detection limit (pmol)
IAA-Me	190	190 → 130	25	17	0.1
TRP-Me	219	219 → 160	20	20	1
IAN	157	157 → 130	20	20	10
IEt	162	162 → 144	20	12	0.1
IMe	165	165 → 130	10	10	1
IMe-Me	179	179 → 130	10	10	1
IAld	146	146 → 118	20	15	1
ILA-Me	220	220 → 160	20	15	0.1
IPyA-Me	218	SIM 218*	25	—	0.01
IAM	175	175 → 130	20	20	0.1
IAAld	160	160 → 118	20	16	10
TrA	161	161 → 144	12	12	0.1
AA-Me	152	152 → 120	15	15	10

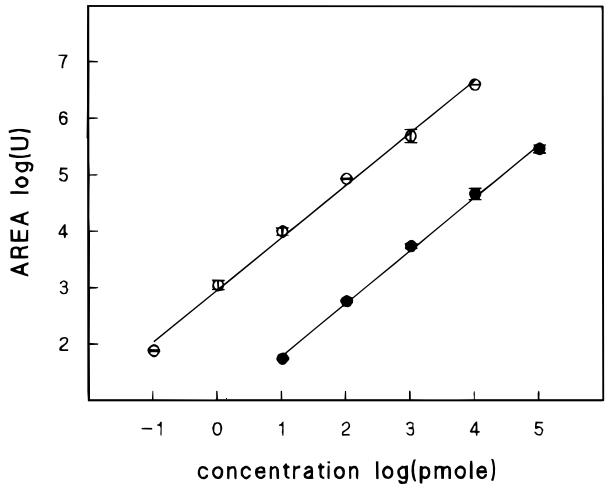

Figure 2. Linearity plot of IAA (solid symbols) and IAA-Me ester (open symbols) obtained by combined LC/(+)ES-MS/MS in multiple reaction monitoring (MRM) using a P_{AR} of 4×10^{-3} mbar, a cone voltage of 25 V and a collision energy of 17 eV. U = arbitrary peak area units. The data plotted are means ± SE ($n = 4$).

by multiple reaction monitoring (MRM) of the $[MH]^+$ ion (dwell time 0.1 s, span 0) and the appropriate product ion (see Table 2). All data were processed by Masslynx software.

RESULTS AND DISCUSSION

Optimization and diagnostic ions

Under LC/atmospheric pressure chemical ionization (APCI)-MS conditions, considerable fragmentation of the protonated molecule $[MH]^+$ was observed, giving rise to the quinolinium ion, present for nearly all indole compounds analysed. Therefore, despite the excellent sensitivity of APCI, we opted for electrospray ionization. Because of the presence of a carboxyl group, a very sensitive electrospray full-scan spectrum using negative ionization could be obtained for IAA, Trp, indole-3-lactic acid (ILA), IPyA and the Trp precursor anthranilic acid (AA) (data not shown). However, under the conditions tested, no product ions could be observed. Using positive-ion electrospray conditions, specific product ions were observed for most indole compounds analysed. In contrast to the electrospray full-scan spectra using the negative-ion mode, no sensitive full-scan spectra could be shown for the positive-ion mode. Because the negative charge characteristic of the free carboxyl group at neutral pH could interfere with analysis using the positive-ion mode, we opted to methylate the carboxyl group. This methylation, in combination with analysis by (+)ES-MS, allowed us to improve the response for IAA up to 200-fold (Fig. 2),

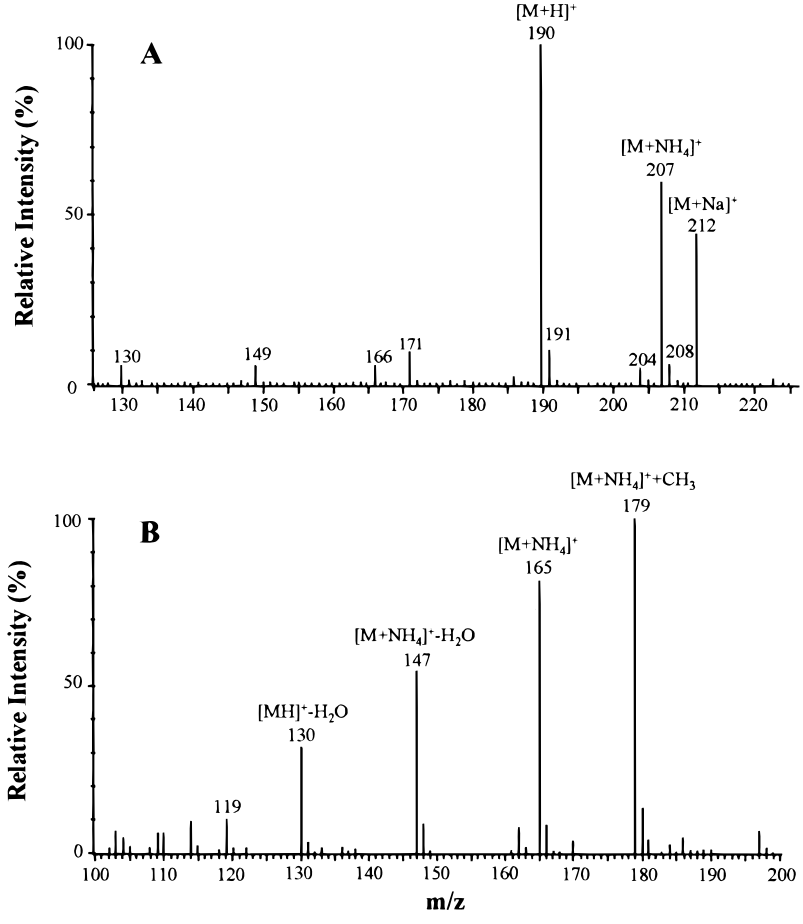

Figure 3. (A) Full-scan spectrum of indole-3-acetic acid methyl ester obtained by LC/MS with FIA using a capillary voltage of +3.5 kV, a cone voltage of 25 V and a source temperature of 80 °C. (B) Full-scan spectrum of indole-3-methanol obtained by LC/MS with FIA using a capillary voltage of +3.5 kV, a cone voltage of 10 V and a source temperature of 80 °C.

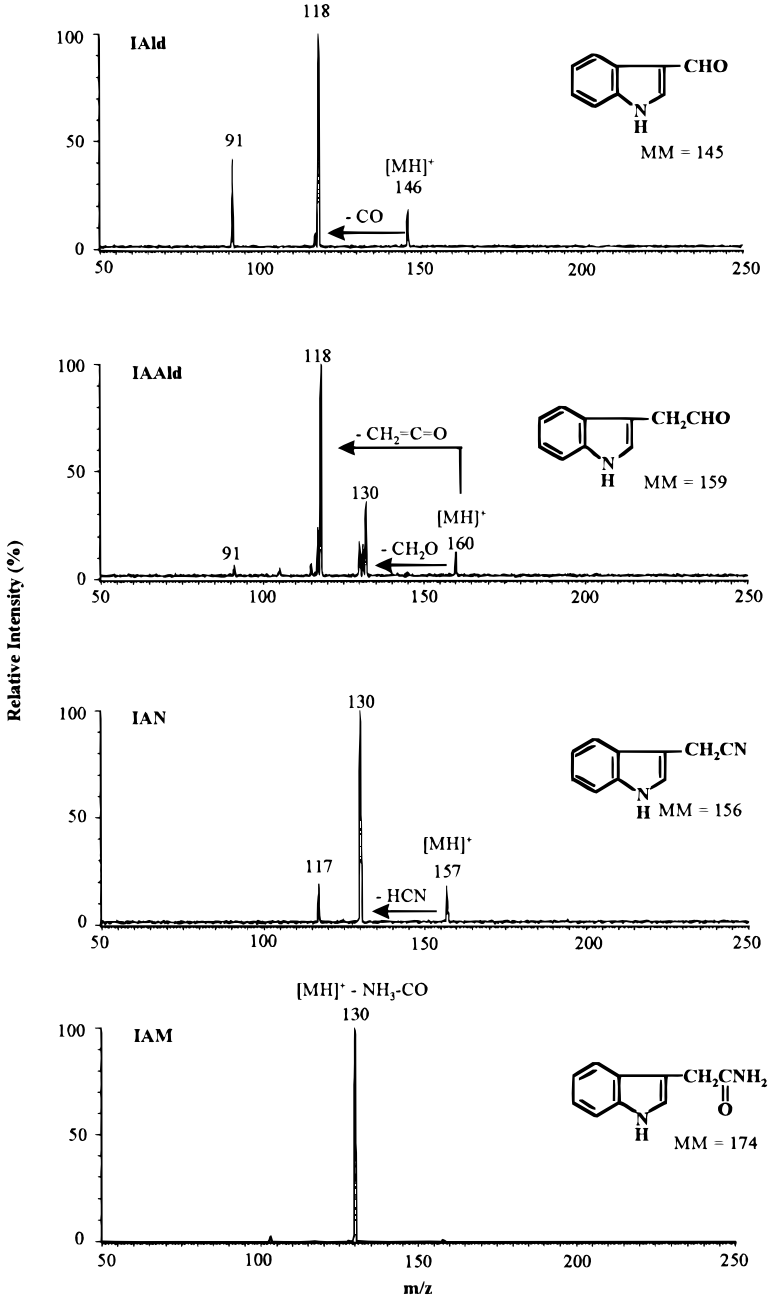

Figure 4. CAD spectra of $[MH]^+$ for indole-3-aldehyde (IAld), indole-3-acetaldehyde (IAAld), indole-3-acetonitrile (IAN) and indole-3-acetamide (IAM) obtained by combined LC/(+)ES-MS/MS using a P_{AR} of 4×10^{-3} mbar in combination with the appropriate cone voltage and collision energy for each individual compound as listed in Table 2. CAD spectra were obtained by FIA in MCA using 100 µl of 10^{-4} M stock solutions. MM = molecular mass.

whereas the detection limit of Trp was improved 10-fold. For 10 pmol of Trp injected, we obtained a signal-to-noise ratio of 3. A signal-to-noise ratio of 30 was obtained for 10 pmol of the methylated compound. For the analysis of ILA, IPyA and AA, this methylation is a prerequisite for measurement in a biologically relevant concentration range and resulted in an 1000-fold improvement of the detection limit. As methylation, using ethereal diazomethane, does not damage the other indole compounds in the extract, derivatization can be carried out on the combined extract.

To determine these indole compounds using MRM, we investigated the full-scan spectra and the product ion spectra for each individual compound. Under the mass spectral conditions described above, the (+)ES full-scan mass spectra of all indole compounds analysed by flow injection analysis (FIA) showed the protonated molecule $[MH]^+$ as the most abundant ion at a cone voltage ranging, depending on the compound analysed, between 10 and 25 V (see Table 2). The (+)ES full-scan mass spectrum of IAA-Me is given as an example [Fig. 3(A)]. In the case of IAA-Me, IAM and IAN, also $[M + Na]^+$ and $[M + NH_4]^+$ adducts were detected but they were not selected as parent ions for diagnostic purposes.

Even at a cone voltage as low as 10 V, fragmentation of indole-3-methanol (IMe) occurred [Fig. 3(B)]. The (+)ES full-scan mass spectrum of IMe contained ions at

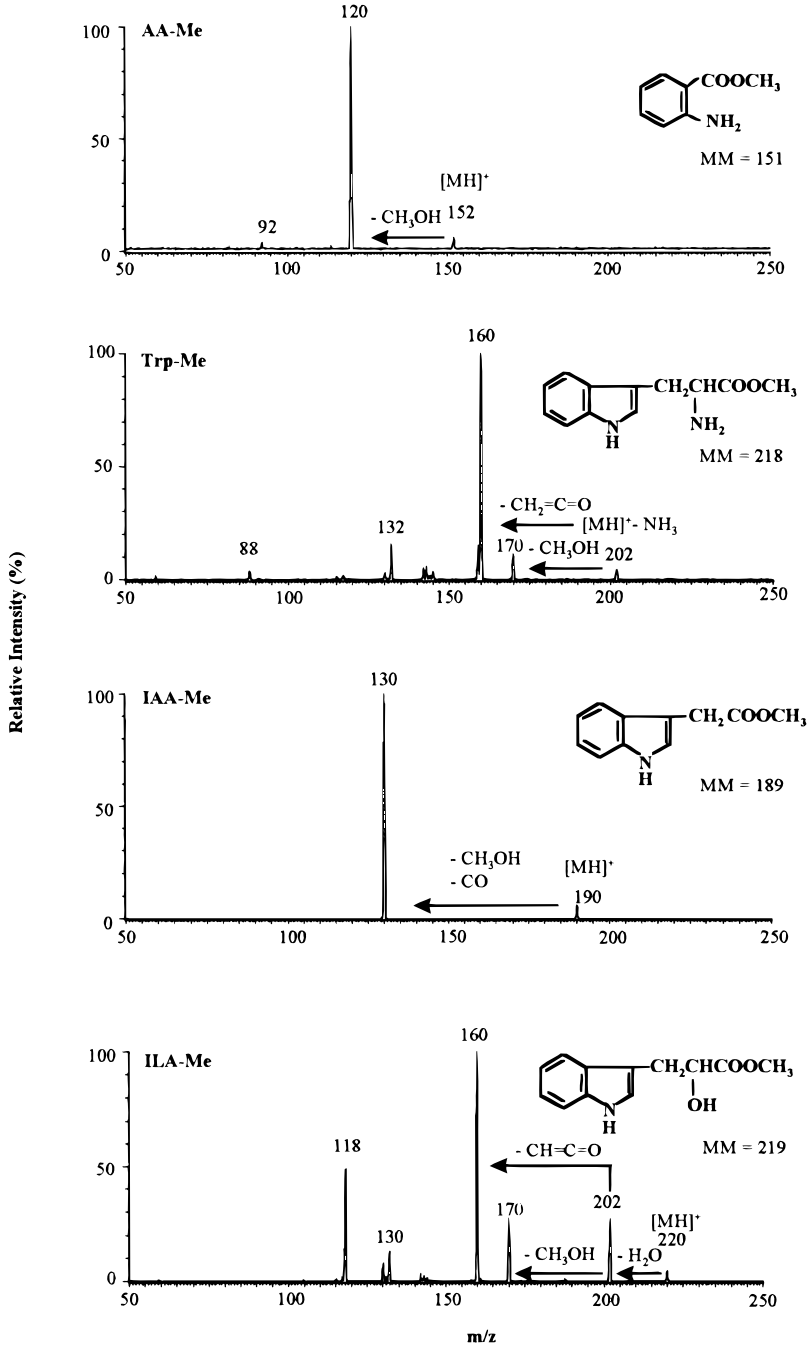

Figure 5. CAD spectra of the methylated compounds anthranilic acid methyl ester (AA-Me), tryptophan methyl ester (Trp-Me), indole-3-acetic acid methyl ester (IAA-Me) and indole-3-lactic acid methyl ester (ILA-Me) obtained by combined LC/(+)ES-MS/MS using a P_{AR} of 4×10^{-3} mbar in combination with the appropriate cone voltage and collision energy for each individual compound as listed in Table 2. CAD spectra were obtained by FIA in MCA using 100 µl of 10^{-4} M stock solutions. MM = molecular mass.

m/z 179 and 165. These ions can be assigned to $[M + NH_4]^+ + CH_3$ and $[M + NH_4]^+$, respectively. Fragment ions are observed at *m/z* 147 and 130. These ions can be explained by the loss of H_2O from $[M + NH_4]^+$ and $[MH]^+$, respectively. Both ions at *m/z* 179 and 165, being representative of the methylated and non-methylated compounds, respectively, were selected as parent ions for diagnostic purposes.

The parent ions selected for the analysis of each individual indole compound are summarized in Table 2. A high yield of positively charged product ions was obtained at a P_{AR} of 4×10^{-3} mbar and a collision energy in the collision cell ranging between 10 and 20 eV, depending on the individual compound analysed (see Table 2). The diagnostic transition of each individual compound selected for multiple reaction monitoring is given in Table 2.

Spectra of authentic indole compounds

As an example, Fig. 4 shows the CAD spectra of the $[MH]^+$ of indole-3-aldehyde (IAld), indole-3-acetaldehyde (IAAld), indole-3-acetonitrile (IAN) and indole-

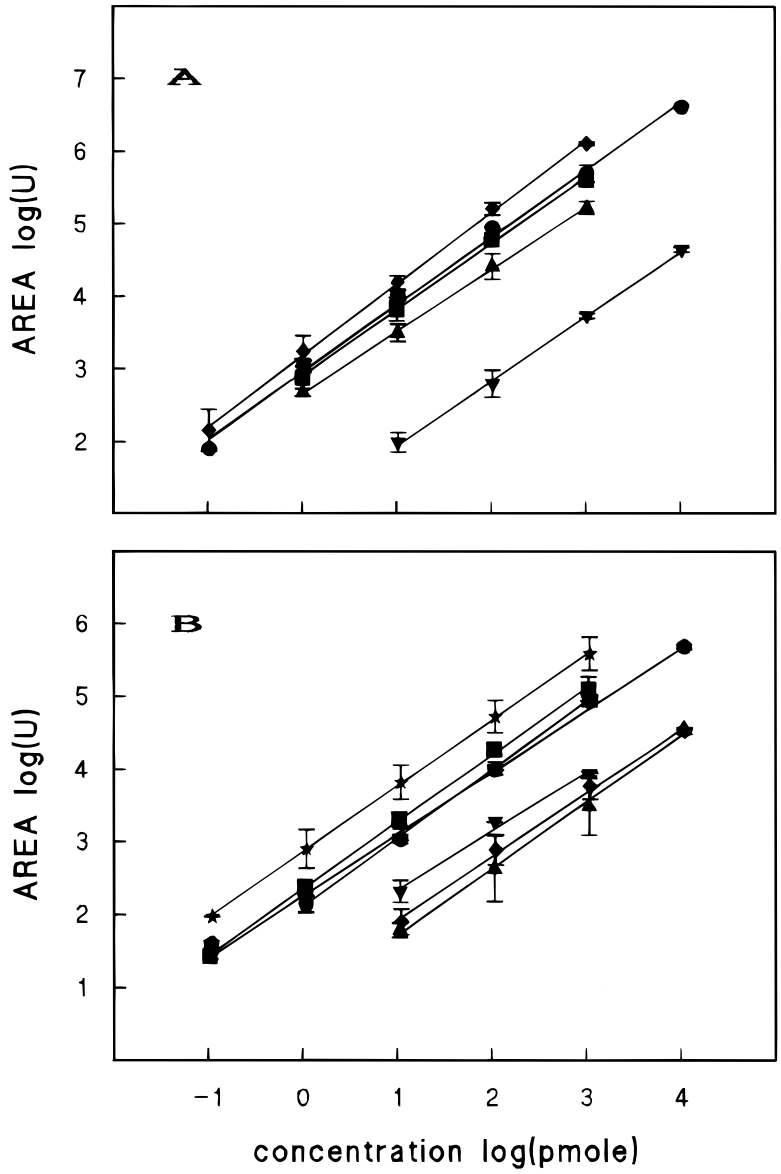

Figure 6. (A) Linearity plot for indole-3-pyruvic acid methyl ester (◆), anthranilic acid methyl ester (▼), tryptophan methyl ester (■), indole-3-lactic acid methyl ester (▲) and indole-3-acetic acid methyl ester (○). (B) Linearity plot for indole-3-acetamide (★),indole-3-ethanol (■), tryptamine (●), indole-3-acetaldehyde (▲), indole-3-acetonitrile (▼), indole-3-aldehyde (◑) and indole-3-methanol (◆) using the diagnostic ions listed in Table 2 for multiple reaction monitoring. Data were obtained using 10 µl of mixed stock solutions ranging between 10^{-2} and 10^{-8} M. U = arbitrary peak area units. The data plotted are means ± SE (n = 4).

3-acetamide (IAM), obtained by combined LC/(+)ES-MS/MS using a P_{AR} of 4×10^{-3} mbar in combination with the appropriate cone voltage and collision energy for each individual compound as listed in Table 2. Figure 5 shows the CAD spectra of the methylated compounds AA-Me, Trp-Me, IAA-Me and ILA-Me. All CAD spectra were obtained by FIA in multiple channel analysis (MCA) using 100 µl of a 10^{-4} M stock solution.

Owing to the unstable nature of IPyA, special precautions were required, particularly because degradation of IPyA into IAA occurred.[20] Butylated hydroxytoluene was added to the culture medium upon harvest and during purification only ethanol or acidified solvents were used. Cooney and Nonhebel[4] showed stable derivatization of IPyA as HFB-Me-IPyA prior to analysis by GC. This derivatization also seemed appropriate prior to analysis by LC/(+)ES-MS/MS (data not shown). A detection limit of 100 fmol injected could be obtained for HFB-Me-IPyA in MRM using 413 → 130 as a specific diagnostic transition (data not shown). However, several other indole compounds in the combined extract, i.e. IAAld, IAld and IAA, seemed to be degraded during this derivatization and for this reason we abandoned this possibility. Under the conditions tested, no sensitive product ion transition could be found for the analysis of Me-IPyA in MRM. Therefore, despite its inferior selectivity in comparison with MRM, (+)ES SIM was the only alternative which seemed to be highly sensitive. Using (+)ES SIM a detection limit of 10 fmol injected was obtained. No interfering compounds were found at the specific retention time of IPyA-Me. Measuring in the SIM mode implied that, owing to the similar molecular masses of Me-Trp and $^{15}N_1$-Me-IPyA, the chromatographic conditions should guarantee a baseline separation of these two com-

pounds. Methylation was performed immediately after purification. After this derivatization IPyA remained stable.

Linearity and detection limits

The linearity using the diagnostic transitions listed in Table 2 for MRM was analysed for different indole compounds. The data shown in Fig. 6(A) and (B) were obtained using 10 µl of different stock solutions ranging between 10^{-2} and 10^{-8} M. After logarithmic transformation, a linear regression function adequately described the relationship between concentration and integrated area units of the corresponding peak signals as shown by a 'lack-of-fit test'[21] (IAA-Me, $p = 0.11$, $n = 12$; Trp-Me, $p = 0.70$, $n = 16$; IPyA-Me, $p = 0.82$, $n = 15$; ILA-Me, $p = 0.72$, $n = 16$; AA-Me, $p = 0.55$, $n = 16$; IAM, $p = 0.99$, $n = 17$; IAN, $p = 0.17$, $n = 6$; IEt, $p = 0.24$, $n = 20$; IMe, $p = 0.15$, $n = 18$; TrA, $p = 0.54$, $n = 13$; IAld, $p = 0.63$, $n = 16$; IAAld, $p = 0.54$, $n = 13$), within the concentration ranges given for the individual compounds in Fig. 6(A) and (B). It should be mentioned that the individual response for each compound was reduced to 20% of the maximum response when 20 different MRM pairs were analysed simultaneously. This is due to the limited scan time available for each individual MRM pair. The linearity range and detection limit for each individual compound analysed, measured simultaneously for all compounds from a mixed reference stock solution, are summarized in Table 2.

Quantification using stable isotopes

The diagnostic ions used for the detection of the different stable isotopes are listed in Table 3. In Fig. 7, the ratio between the concentrations is plotted against the ratio of the concomitant areas of the unlabelled ($Area_x$) and labelled ($Area_{ix}$) compounds. To different concentrations of unlabelled compound (10^{-3}–10^{-8} M) called $concentration_x$, we added a constant amount of the stable isotope (10^{-5} M), called $concentration_{ix}$. The amount of stable isotope was comparable to the amount of tracer added to the samples. As an example, the calibration graphs after logarithmic transformation

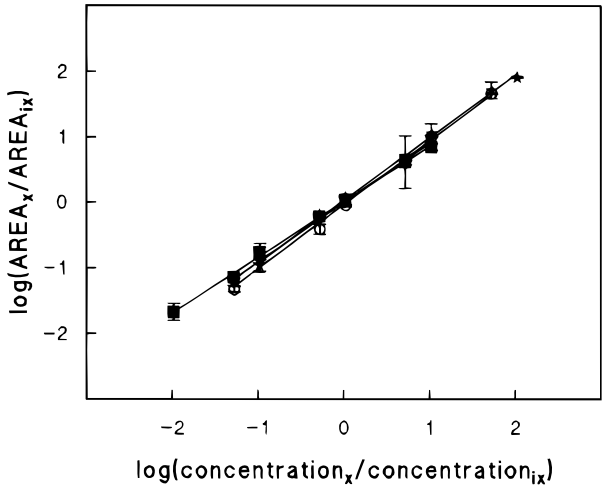

Figure 7. Calibration graphs for indole-3-acetic acid methyl ester (○), tryptophan methyl ester (■), anthranilic acid methyl ester (▼) and indole-3-acetamide (★). To different concentrations of unlabelled compounds (10^{-3}–10^{-8} M), called $concentration_x$, we added 10^{-5} M of the stable isotope, called $concentration_{ix}$. $Area_x$ and $Area_{ix}$ are the concomitant areas of the unlabelled and labelled compounds, respectively. The data plotted are means ± SE (n = 6).

for IAA-Me, Trp-Me, AA-Me and IAM are given in Fig. 7. The calibration graphs for the compounds analysed showed a significant linear fit with a slope of 1 and an intercept of 0 [$x = (0.98 \pm 0.024)y + (0.016 \pm 0.014)$]. A quantification using internal stable tracers, where calculation is based on the ratio between the areas of the diagnostic ions corresponding to the unlabelled and labelled compound, is therefore acceptable. For the quantification of the indole compounds for which no tracers were available, we used the relative response towards IAA and Trp.

LC/ES-MS/MS

Because of the selectivity of MRM, we can distinguish the different IAA intermediates, despite their structural analogy, without the necessity for a complete chromatographic baseline separation. As stated earlier, the chromatographic conditions should only guarantee a baseline separation of Trp and IPyA. Several mobile phases were tested for a maximum response (peak area)

Table 3 Parent ions and diagnostic transitions (or diagnostic ion for IPyA-Me*) used in multiple reactant monitoring (MRM) (or selected-ion monitoring (SIM) for IPyA-Me*) for the different stable isotopes analysed by LC/(+)ES-MS/MS (LC(+)ES-MS for IPyA-Me*) after methylation ($P_{AR} = 4 \times 10^{-3}$ mbar)[a]

Isotope	Parent ion	Diagnostic transitions MRM Diagnostic ions SIM*
Phenyl-$^{13}C_6$-IAA-Me	196	196 → 136
Indole-$^{15}N_1$-TRP-Me	220	220 → 161
Side-chain α-carbon-$^{13}C_1$-IAN	158	158 → 130
Indole-$^{15}N_1$-IEt	163	163 → 145
Indole-$^{15}N_1$-IPyA-Me	219	SIM 219*
Phenyl-$^{13}C_6$-IAM	181	181 → 136
Indole-$^{15}N_1$-IAAld	161	161 → 119
$^{15}N_1$-AA-Me	153	153 → 121

[a] The specific cone voltage and collison energy for each individual compound is given in Table 2.

in combination with minimum baseline noise. Previously we described the combination of optimum spray conditions obtained by a 1:20 split through the mass spectrometer and a chromatographic flow rate of 0.8 ml min^{-1} for the analysis of different cytokinins by LC/ES-MS/MS.[22] At a constant flow rate of 0.8 ml min^{-1} and a post-columns split of 1:20, we tested several solvent compositions. MeOH–H_2O–formic acid (70:29.5:0.5 or 50:49.5:0.5) gave very high background noise and a poor response for IAA.

Ammonium acetate at a concentration of 0.01 M was the best electrolyte concentration without reducing the sensitivity.[22] Indeed, using MeOH–NH_4OAc–HOAc (70:29.5:0.5), the response of IAA was improved tenfold, although the high background noise remained. Since chromatography of methylated compounds does not require ion suppression conditions, MeOH–NH_4OAc (50:50) seemed to give the best separation and the lowest background noise (data not shown). Figure 8 shows the separation of IAld, IAAld, indole-3-ethanol (IEt), IMe, IAN, tryptamine (TrA), IAM, IAA-Me, Trp-Me, AA-Me, ILA-Me and IPyA-Me by LC/(+)ES-MS/MS. All indole compounds, except IPyA-Me, were observed in MRM. For IPyA-Me, LC/MS analysis was performed under SIM conditions. Table 4 shows the quantification by LC/(+)ES-MRM-MS/MS of the endogenous pool of IAA

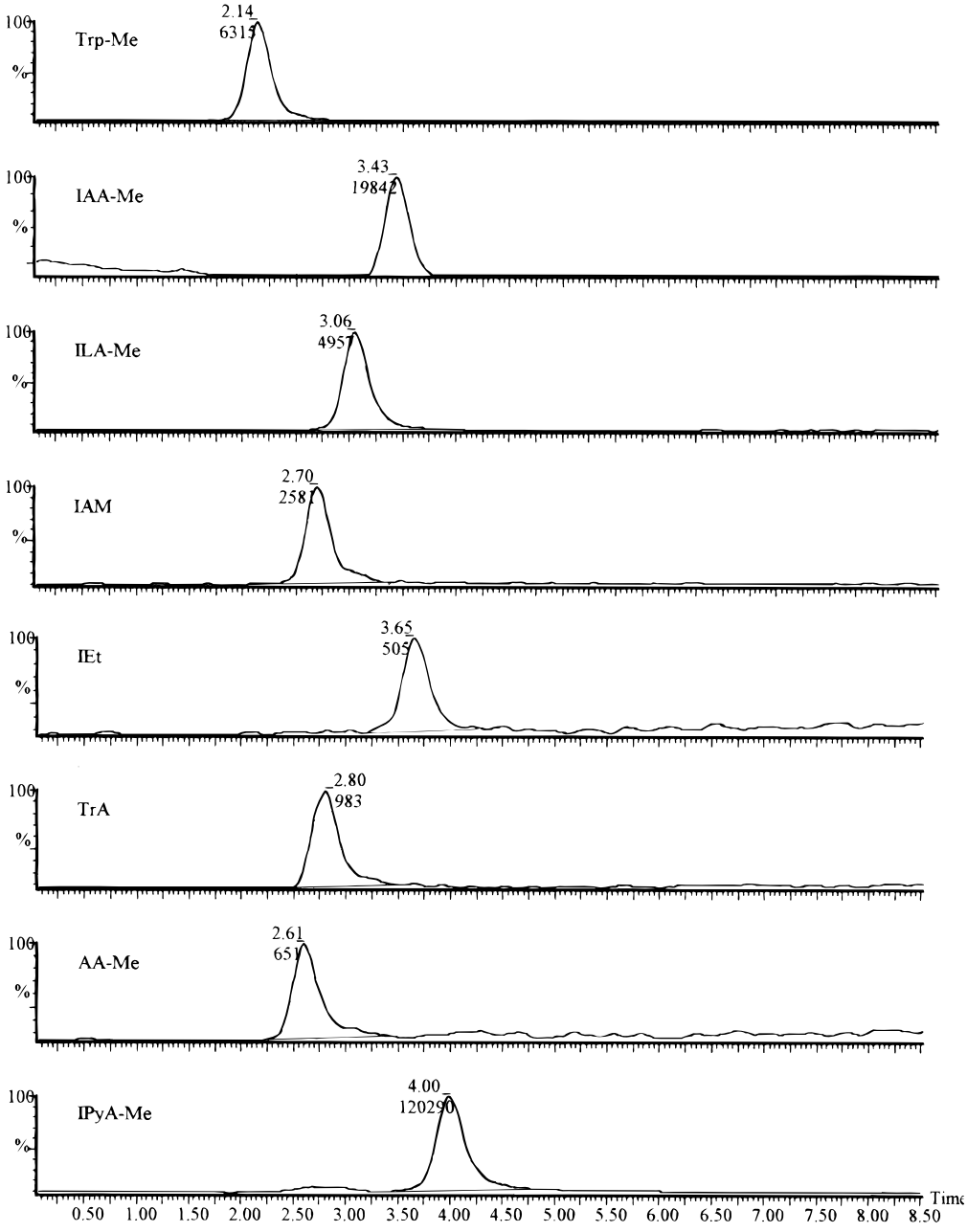

Figure 8. LC/(+)ES-MRM-MS/MS of tryptophan methyl ester (Trp-Me, 219 → 160), indole-3-acetic acid methyl ester (IAA-Me, 190 → 130), indole-3-lactatic acid methyl ester (ILA-Me, 220 → 160), indole-3-acetamide (IAM, 175 → 130), indole-3-ethanol (IEt 147 → 130), tryptamine (TrA, 161 → 144), anthranilic acid methyl ester (AA-Me, 152 → 120) and indole-3-pyruvic acid methyl ester (IPyA-Me, SIM 218) under the experimental conditions described in the Experimental section

Table 4 Endogeneous concentrations of different IAA metabolites present in an *Azosprillum brasilense* culture medium during the exponential growth stage analysed by LC/(+)ES-MRM-MS/MS

Indole compound	Concentration (pmol ml^{-1})	Indole compound	Concentration (pmol ml^{-1})
IAA	270	IAAld	10
Trp	78	TrA	5.0
ILA	10	IEt	5.0
IAld	130	IAM	17
AA	9100	IPyA	121000
IAN	22		

intermediates in an *Azospirillum brasilense* Sp245 culture medium at the exponential growth stage.

CONCLUSION

Parallel to the method described for the analysis of cytokinins,[22] we have shown here that LC/ES-MS/MS) in combination with a one-step solid-phase extraction is a very sensitive and reliable method for screening the IAA metabolism in bacteria. Depending on the compound analysed, a detection limit of 10 fmol–10 pmol injected was obtained, and a linear relationship was observed within a concentration range of 0.1 pmol–1 nmol injected. A collision energy ranging between 10 and 20 eV and a P_{AR} of 4×10^{-3} mbar is appropriate for the efficient fragmentation of most indole compounds. In view of the analysis of a considerable number of samples, the chromatographic conditions selected here were a compromise between speed of analysis and resolution. Notwithstanding the structural analogy between the different indole compounds analysed here, the unique diagnostic transition for each individual indole compound observed during MRM allowed fast analysis with less ideal chromatographic resolution. This reduces the total analysis time per sample from 60 to 6 min.

Although methylation is not an absolute prerequisite for the analysis of most carboxyl-type indole compounds, samples were methylated before analysis to improve the detection limits for Trp, IAA, AA and ILA up to 1000-fold. Moreover, this methylation allowed us to omit the ion suppression conditions necessary for the chromatography of organic acids.

Special attention was paid to control the stability of IPyA. Butylated hydroxytoluene was added upon harvest and, during manipulation of the extract, ethanol or acidic conditions were used.[10] After purification, methylation avoided further degradation. We have shown that the derivatization into the HFB oxime of the methylated IPyA[4] is very useful for the analysis of IPyA by ES-MS/MS. Nevertheless, this derivatization was disastrous for several other indole compounds (Trp, IAA, ILA and TrA). As a compromise, if the analysis of other indole compounds is desired, analysis of IPyA using LC/SIM-MS after methylation is preferred.

Although SIM is know to be less selective than MRM, we observed no interference with other compounds at the specific retention time of IPyA-Me. Owing to the similar molecular masses of Trp and the stable isotope of IPyA, chromatographic conditions were chosen in relation to the baseline separation of these two compounds. Although IPyA and IAA can be analysed very sensitively using GC/negative chemical ionization MS after appropriate derivatization,[2,4] we opted for designing a method which allows the rapid, reliable and sensitive screening of all possible IAA intermediates in a bacterial sample. The method described here allows a kinetic screening of all IAA precursors, necessary for the analysis of the different IAA biosynthetic pathways in bacteria, starting from less than 1 ml of bacterial medium. Moreover, this method can easily be extended for the screening of the heavily labelled reaction products after feeding with ^{13}C-or ^{15}N-labelled IAA precursors.

Acknowledgements

This work was supported by a Belgian programme on 'Interuniversity Poles of Attraction' initiated by the Belgian State, Prime Minister's Office, Science Policy Programming to E.E and H.V.O. E.P. and H.V.O. are Research Associate and Research Director, respectively, of the Belgian National Fund for Scientific Research (NFWO). The authors wish to thank Dr F. De Ridder for the statistical analysis and S. Oden for her skilful technical assistance. Dr N. Ilic (University of Maryland, USA) synthesized the $^{13}N_1$-IAN tracer. Dr R. Walden (Max Planck Institute, Cologne, Germany) is acknowledged for critical reading of the manuscript.

REFERENCES

1. P. Hedden, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 107 (1993).
2. E. Epstein and J. D. Cohen, *J. Chromatogr.* **209**, 413 (1981).
3. L. Michalczuk, K. Bialek and J. D. Cohen , *J. Chromatogr.* **596**, 294 (1992).
4. T. P. Cooney and H. M. Nonhebel, *Biochem. Biophys. Res. Commun.* **162**, 761 (1989).
5. A. Ostin, T. Moritz and G. Sandberg, *Biol. Mass Spectrom.* **21**, 292 (1992).
6. A. Ostin, C. Catalá, J. Chamarro and G. Sandberg, *J. Mass Spectrom.* **30**, 1007 (1995).
7. W. T. Frankenberger and M. Arshad, *Phytohormones in Soils. Microbial Production and Function*. Marcel Dekker, New York (1995).
8. C. L. Patten and B. R. Glick, *Can. J. Microbiol.* **42**, 207 (1996).
9. J. Badenoch-Jones, R. E. Summons, B. Entsch, B. G. Rolfe, C. W. Parker and D. S. Letham, *Biomed. Mass Spectrom.* **9**, 429 (1982).
10. M. Lebuhn and A. Hartmann, *J. Chromatogr.* **629**, 255 (1993).
11. A. Crozier, P. Arruda, J. M. Jasmin, A. M. Monteiro and G. Sandberg, *Appl. Environ. Microbiol.* **54**, 2833 (1988).
12. H. A. Van Onckelen, P. Rüdelsheim, D. Inzé, A. Follin, E.

Messen, S. Horemans, J. Schell, M. Van Montagu and J. De Greef, *FEBS Lett*. **181**, 357 (1985).
13. F. Hofmann, T. Rausch and W. Hilgenberg, *J. Labelled Compd. Radiopharm*. **18**, 1491 (1981).
14. B. B. Stowe, *Anal. Biochem*. **5**, 107 (1963).
15. J. Badenoch-Jones, R. E. Summons and D. S. Letham, *J. Labelled Compd. Radiopharm*. **20**, 1325 (1983).
16. V. L. D. Baldani, J. I. Baldani and J. Dobereiner, Biol. *Fertil. Soils* **4**, 37 (1987).
17. M. Vanstockem, K. Michiels, J. Vanderleyden and A. Van Gool, *Appl. Environ. Microbiol*. **53**, 1387 (1987).
18. E. Prinsen, A. Costacurta, K. Michiels, J. Vanderleyden and H. Van Onckelen, *Mol. Plant Microbe Interact*. **6**, 609 (1993).
19. H. Schlenk and J. L. Gellerman, *Anal. Chem*. **32**, 1412 (1960).
20. A. Ernstsen, G. Sandberg and A. Crozier, *Physiol. Plant*. **68**, 519 (1986).
21. J. Neter, M. Wasserman and H. Kutner, *Applied Linear Statistical Models*. Irwin, Boston (1990).
22. E. Prinsen, P. Redig, W. Van Dongen, E. L. Esmans and H. A. Van Onckelen, *Rapid Commun. Mass Spectrom*. **9**, 948 (1995).