ELSEVIER

Journal of Chromatography A 826 (1998) 25–37

JOURNAL OF CHROMATOGRAPHY A

Micro and capillary liquid chromatography–tandem mass spectrometry: a new dimension in phytohormone research

Els Prinsen[a,*], Walter Van Dongen[b], Eddy L. Esmans[b], Henri A. Van Onckelen[a]

[a]*University of Antwerp (UIA), Biology Department, Universiteitsplein 1, B-2610 Antwerpen, Belgium*

[b]*University of Antwerp, (RUCA–UIA), Chemistry Department, Nucleoside Research and Mass Spectrometry Unit, Groenenborgerlaan 171, B-2020 Antwerpen, Belgium*

Received 10 March 1998; received in revised form 17 September 1998; accepted 21 September 1998

Abstract

Quantification of phytohormones in small amounts of material requires sensitive analytical techniques. The analysis of indoles and cytokinins using electrospray tandem mass spectrometry (MS–MS) was described earlier. Gradient elution resulted in an improved detection limit. Micro liquid chromatography (LC) and capillary LC, in combination with large volume injections, resulted in a 200 to 1200 times net increase in sensitivity in comparison with conventional isocratic LC–MS–MS. We obtained a linearity range between 1 fmol and 5 pmol for capillary LC–MS–MS and between 5 fmol and 1 nmol for micro LC–MS–MS.

Keywords: Liquid chromatography–mass spectrometry; Column switching; Plant hormones; Indoles; Auxins; Cytokinins

1. Introduction

During the last two decades, the use of physicochemical methods based on mass spectrometric detection in phytohormone research gained importance. By the time Hedden wrote his review on modern methods for the quantitative analysis of plant hormones in 1993 [1], liquid chromatography–mass spectrometry (LC–MS)-based methods were still in their infancy and detection limits were inferior to those obtained using gas spectrometry–mass spectrometry (GC–MS) [1]. Due to its intrinsic requirement for intensive purification, UV-based detection [2,3] has been abandoned. Nowadays, cytokinins are still analysed by electron impact (EI^+) GC–MS after trimethylsilylation, permethylation or acetylation [4–7], whereas good detection limits for derivatised indole-3-acetic acid (IAA) are obtained in EI^+ [8,9] or negative ion chemical ionisation (NICI) GC–MS [10,11]. The necessary derivatisation of cytokinins is however obstructive and, depending on the conditions, multiple derivatives occur [12,13], whereas derivatisation of IAA is still time-consuming and can result in severe recovery losses. The first reports on the analysis of cytokinins [14] and IAA conjugates and catabolites [15,16] used LC–frit fast atomic bombardment (FAB)-MS. Yang et al. [17] demonstrated the first LC–MS interface, atmospheric pressure chemical ionisation (APCI), which allowed LC–MS analysis of cytokinins in biologically relevant concentration ranges. Under LC–APCI-MS in the selected ion monitoring (SIM) mode, considerable fragmentation of the protonated molecules was ob-

*Corresponding author. Tel.: +32-3-8202266; fax: +32-3-8202271; e-mail: prinsen@uia.ua.ac.be

0021-9673/98/$ – see front matter
PII: S0021-9673(98)00763-8

served. Consequently, despite its excellent detection limit, a baseline separation of the different metabolites of each cytokinin type was still essential because of a lack of selectivity. The electrospray ionisation process by itself is extremely gentle, optimising the probability of obtaining molecular mass information [18,19], and is therefore very suitable for the analysis of phytohormones. We described the use of electrospray LC–MS–MS for the analysis of cytokinins [20] and indole compounds [21]. In combination with conventional LC, detection limits of 1 pmol cytokinin or 0.1 pmol IAA injected on-column were achieved under single reaction monitoring conditions. In spite of the structural analogy between the different compounds analysed simultaneously, the unique diagnostic transitions for each individual compound used for single reaction monitoring (SRM) enabled fast analysis under less chromatographic resolution [20,21]. Bartók et al. [22] showed the use of in-source collision-induced dissociation MS for the analysis of cytokinins without precursor ion selection. In contrast to tandem mass spectrometry (MS–MS), where the first quadrupole functions as a separate unit for precursor ions, single quadrupole MS is not suitable for the analysis of complex mixtures with good resolution, without preliminary chromatographic separation.

Improvement of the mass sensitivity is a must. This resulted in a general trend towards miniaturisation of instrumentation as well as in sample size [23]. Miniaturisation implicates (1) a mobile flow-rate characteristic of the different column diameters and (2) a decrease in injection volume under isocratic conditions parallel to the decrease in column diameter, the latter resulting in a decreased loading capacity and a decreased dynamic range [23]. For a concentration-sensitive device, the detector signal depends on the concentration of the analyte in the carrier flow [24]. The down-scaling factor being C_{max} ratio$=(d_{c1}/d_{c2})^2$ [24]. Electrospray ionisation (ESI) MS, being concentration-sensitive, is compatible with miniaturised chromatographic conditions for improving mass sensitivity. Indeed, coupling of capillary LC to ESI improved mass sensitivity for the analysis of DNA adducts [25,26].

Plant hormones are present and active in minor concentrations in plant tissue. To allow quantification in a restricted amount of material, i.e. protoplasts, chloroplasts, seedlings, seeds, buds or apical root- and stem regions, a sensitive analytical technique is a prerequisite in today's phytohormone research. Moreover, analysing a physiological process requires the analysis of a decent number of biological samples. In this paper, we compare the performance and robustness of micro (1 mm column) and capillary (300 μm column) LC–(+)ESI-MS–MS for the routine analysis of cytokinins and auxins. To allow large or extra-large volume injections in combination with the ideal peak-eluting volume, on-column focussing and column switch configurations were performed, respectively, in combination with gradient elution. Criteria such as sensitivity, linearity range and robustness are discussed in this paper.

2. Experimental

2.1. Products

The authentic and deuterium-labelled cytokinins used for standard calibration curves and internal tracers, respectively, were purchased from Apex Organics (Honiton, UK). Authentic indole compounds were from Sigma (Bornem, Belgium). [^{13}C]IAA and [^{15}N]Trp were from Cambridge Isotopes (Andover, MA, USA). Other heavy labelled indole tracers were synthesised starting from the above-mentioned tracers, as described by Prinsen et al. [21]. Diazomethane, which was used for the methylation of carboxyl–indole compounds, was synthesised as described by Schlenk and Gellerman [27]. All of the stable isotopes used are shown in Table 1.

2.2. Plant material and sample preparation

Nicotiana tabacum L cv. Petit Havana SR1 plants were cultivated in vitro under sterile conditions at 25°C, 16 h light, 8 h darkness on Murashige and Skoog medium [28] with 3% sucrose, 200 mg/l *myo*-inositol, 10 mg/l thiamine dichloride, 1 mg/l pyridoxine hydrochloride, 1 mg/l nicotinic acid and 0.8% agar. Plant leaf material (50–100 mg fresh mass) was extracted in Bieleski solvent [29]. Extracts were obtained after immunoaffinity purification using a broad-spectrum monoclonal anti-cytokinin anti-

Table 1
Stable isotopes for the analysis of cytokinins and indole compounds

Compound	Abbreviation	Source
Dihydrozeatin	[$^{2}H_5$]DHZ	Apex Organics
Dihydrozeatin-N_9-riboside	[$^{2}H_5$][9R]DHZ	Apex Organics
Dihydrozeatin-N_9-glucoside	[$^{2}H_5$](9G)DHZ	Apex Organics
Dihydrozeatin-N_7-glucoside	[$^{2}H_5$](7G)DHZ	Apex Organics
Dihydrozeatin-*O*-glucoside	[$^{2}H_5$](OG)DHZ	Apex Organics
Dihydrozeatin-*O*-glucoside-N_9-riboside	[$^{2}H_5$](OG)[9R]DHZ	Apex Organics
Isopentenyl adenine	[$^{2}H_6$]iP	Apex Organics
Isopentenyl adenosine	[$^{2}H_6$][9R]iP	Apex Organics
Isopentenyl adenine-N_9-glucoside	[$^{2}H_6$](9G)iP	Apex Organics
Isopentenyl adenine-N_7-glucoside	[$^{2}H_6$](7G)iP	Apex Organics
Dihydrozeatin-N_9-riboside-monophosphate	[$^{2}H_5$][9R-MP]Z	Apex Organics
Isopentenyl adenosine-monophosphate	[$^{2}H_6$][9R-MP]iP	Apex Organics
Indole-3-acetic acid	Phenyl-[$^{13}C_6$]IAA	Cambridge Isotope Labs.
Indole-3-acetamide	Phenyl-[$^{13}C_6$]IAM	Synthesis from [$^{13}C_6$]-IAA [40]
Indole-3-ethanol	Indole-[$^{15}N_1$]IEt	Synthesis from indole-[$^{15}N_1$]-Trp (Cambridge Isotope Labs.) [41]
Indole-3-acetonitrile	[$^{13}C_1$]IAN	Gift from N. Ilic, USA [42]

Abbreviations following Crouch et al. [43].

body, in combination with solid-phase extraction as described by Redig et al. [30]. We added [$^{13}C_6$]IAA, ^{13}C-labelled indole-3-acetamide ([$^{13}C_6$]IAM), ^{15}N-labelled indole-3-ethanol ([$^{15}N_1$]IEt), ^{13}C-labelled indole-3-acetonitrile ([$^{13}C_1$]IAN), [$^{2}H_5$]DHZ, [$^{2}H_5$][9R]DHZ, [$^{2}H_5$](9G)DHZ, [$^{2}H_5$](7G)DHZ, [$^{2}H_5$](OG)DHZ, [$^{2}H_5$](OG)[9R]DHZ, ^{2}H-labelled isopentenyl adenine ([$^{2}H_6$]iP), 2H-labelled isopentenyl adenosine ([$^{2}H_6$][9R]iP), [$^{2}H_6$](9G)iP, ^{2}H-labelled isopentenyl adenine-N_9-glucoside ([$^{2}H_6$](7G)iP) (20–50 pmol each), [$^{2}H_5$][9R-MP]Z and [$^{2}H_6$][9R-MP]iP (50–100 pmol each) as internal tracers. The [$^{2}H_5$]DHZ, [$^{2}H_5$][9R]DHZ, [$^{2}H_5$](9G)DHZ, [$^{2}H_5$](7G)DHZ, [$^{2}H_5$](OG)DHZ, [$^{2}H_5$](OG)[9R]DHZ) and [$^{2}H_5$][9R-MP]DHZ were also used as internal tracers for the quantification of the corresponding zeatin type derivatives. The N_7 and *O*-glucoside derivatives were not retained on the immunoaffinity column and were therefore isolated from all other cytokinins before analysis. Analysis of this *O*- and N_7 glucoside fraction was performed on a LC–MS run separate from other cytokinin derivatives. Cytokinin ribotides were treated with alkaline phosphatase after fractionation from the free bases, ribosides and glucosides and prior to further purification because excessive degradation of ribotides occurs during manipulation. This implies that each individual sample results in three different cytokinin fractions. IAA was purified further as described in Prinsen et al. [3]. IAA was methylated prior to LC–MS–MS [21].

For on-column focussing or column switch applications, samples were dissolved in 10 μl of 20% methanol, which were then diluted with 30 μl of Milli-Q water, to obtain a final methanol (MeOH) concentration of 5%.

2.3. LC and LC–LC conditions

2.3.1. Isocratic conventional HPLC

Conventional LC was performed using a Kontron 325 pump (Kontron Instruments, Milan, Italy). Samples (10–100 μl) were injected (Kontron 465 injector using a 25-μl or a 100-μl sample loop) on a C_8 reversed-phase column (LiChrosphere 60 RP Select B; 5 μm; 125×4.6 mm I.D.; Merck, Darmstadt, Germany) and eluted with methanol–0.01 *M* NH_4OAc, pH 7 (50:50, v/v, for auxins; 90:10, v/v, for cytokinin bases and ribosides, and 35:65, v/v, for cytokinin glucosides) at a flow-rate of 0.8 ml/min [20,21]. Using a post-column split of 1/20 (Accurate, LC-Packings, Amsterdam, Netherlands), the effluent was introduced into the MS source at a flow-rate of 40 μl/min.

2.3.2. Conventional HPLC with gradient elution

The gradient elution used in combination with conventional LC was as follows: sample loading at MeOH–0.01 *M* NH_4OAc, pH 7 (5:95, v/v) for 1 min followed by a linear gradient from 5:95 (v/v) MeOH–0.01 *M* NH_4OAc, pH 7 to 90:10 (v/v) MeOH–0.01 *M* NH_4OAc, pH 7, over 4 min. Using a post-column split of 1/20 (Accurate, LC-Packings), the effluent was introduced into the MS source at a flow-rate of 40 μl/min.

2.3.3. Micro LC with on-column focussing

Micro LC was performed on a Hypersil 5 μm C_8 BDS 150×1 mm I.D. column (Alltech, Deerfield, IL, USA). Micro LC was also evaluated on an Alltech 100×1 mm column and on Prodigy 5 μm OD83 100 Å, 50×1 mm and 30×1 mm columns (Phenomenex, Torrance, CA, USA). The total sample volume injected ranged from 10 to 20 μl. The mobile phase consisted of the following gradient systems (Fig. 1).

2.3.3.1. For the analysis of cytokinin free bases, ribosides and N_9-glucosides (Fig. 1a)

Sample loading for 1 min in 5:95 (v/v) MeOH–0.01 *M* NH_4OAc, pH 7, followed by a linear gradient of MeOH–0.01 *M* NH_4OAc, pH 7, from 5:95 (v/v) to 90:10 (v/v) over 2.5 min. The 90:10 (v/v) MeOH–0.01 *M* NH_4OAc, pH 7, conditions were retained during the last 0.5 min of the run.

2.3.3.2. For the analysis of cytokinin O-glucosides, O-glucoside-ribosides and N_7 glucosides (Fig. 1b)

Sample loading for 1 min in 5:95 (v/v) MeOH–0.01 *M* NH_4OAc, pH 7, followed by a discontinuous linear gradient of MeOH–0.01 *M* NH_4OAc, pH 7, from 5:95 (v/v) to 35:65 (v/v) over 3 min, followed by a linear gradient of MeOH–0.01 *M* NH_4OAc, pH 7, from 35:65 (v/v) to 90:10 (v/v) over 2 min. Isocratic conditions of 90:10 (v/v) MeOH–0.01 *M* NH_4OAc, pH 7, were retained during the last 0.5 min of the run.

2.3.3.3. For the analysis of indole compounds (Fig. 1c)

Sample loading for 1 min in 5:95 (v/v) MeOH–0.01 *M* NH_4OAc, pH 7, followed by a linear gradient from 5:95 to 90:10 (v/v) MeOH–0.01 *M* NH_4OAc, pH 7, over 5 min. The isocratic 90:10

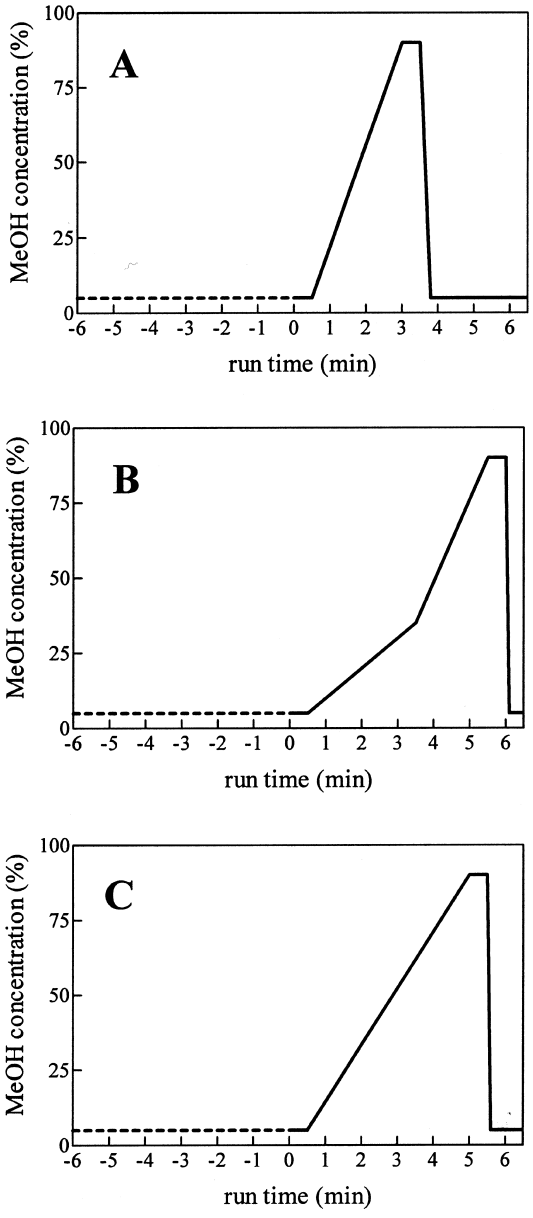

Fig. 1. Solvent gradient used for micro and capillary LC expressed as MeOH concentration (%). The remaining solvent percentage consisted of 0.01 *M* NH_4OAc, pH 7. The gradient shown in (a) is used for the analysis of cytokinin free bases, ribosides and N_9-glucosides. The gradient shown in (b) is used for the analysis of cytokinin *O*-glucosides, *O*-glucoside-ribosides and N_7-glucosides. The gradient shown in (c) is used for the analysis of all indole compounds. A constant flow-rate of 100 or 10 μl/min was used for micro and capillary LC, respectively. The dashed line corresponds to the additional sample loading performed under column switch conditions.

(v/v) MeOH–0.01 M NH_4OAc, pH 7, conditions were retained during the last 0.5 min of the run.

A constant flow rate of 100 μl/min was achieved by using two Kontron 422 pumps equipped with 0.01–2 ml/min pump heads in a Master–Slave configuration using a Biocompatible polyether ether ketone (PEEK) mixing tee (JOUR Research, Onsala, Sweden) as a low volume solvent-mixing device. The effluent was directly introduced into the MS source at a flow-rate of 100 μl/min. A Kontron 332 UV detector equipped with a 1 μl capillary U-shaped flow cell (LC-Packings) was used at λ=268 nm.

2.3.4. Micro LC with column switch configuration (micro LC–LC)

Tandem micro LC was obtained by using a column switch configuration set-up as outlined in Fig. 2a. Automatic column-switching was performed using an injector-driven ten-port multifunctional valve with a micro-electric two position valve actuator (Valco Instruments, Houston, TX, USA). The time-based settings for the injector-driven valves are schematically drawn in Fig. 2c. To obtain additional sample filtration, we used a through hole 0.5 mm stainless-steel pre-column filter unit (JOUR Research) in the inlet position of the ten-port valve (position 1 in valve B, Fig. 2a–b). For the analysis of all cytokinin fractions and indole compounds, sample loading (10–100 μl) was performed on a Prodigy 5 μm, OD83 100 Å, 30×1 mm (Phenomenex) cartridge. This precolumn length is essential for the retention of indole-3-acetamide. For all other applications, an analytical precolumn (5×1 mm I.D.) cartridge filled with C_8 select B packing material (LC-Packings) can be used. Sample loading was performed by a Kontron 325 pump (flow-rate, 50 μl/min; 0.01 *M* NH_4OAc, pH 7) over 6.5 min, i.e. the time necessary for backflushing the preceding sample from the parallel precolumn. After 6.5 min of sample loading, precolumn 1 was backflushed, using the appropriate solvent gradient delivery system, as outlined in Fig. 1. The Kontron 422 pump settings are outlined in Fig. 2c. The flow-rate was 100 μl/min. During backflush, the analyte concentrated on the precolumn was introduced to the Hypersil 5 μm C_8 BDS, 150×1 mm I.D. (Alltech) analytical column. The effluent of the analytical column was directly introduced into the MS source at a flow-rate of 100 μl/min. Depending on the sample volume injected, the Kontron 465 injector was equipped with a 10-, 25- or 100-μl sample loop. While sample 1 was introduced to the analytical column, a second sample was loaded on a parallel precolumn (precolumn 2 in Fig. 2).

2.3.5. Capillary LC with on-column focussing

Capillary LC was performed on a C_8 BDS 150× 0.3 mm I.D. column (FUS-15-03-C_8, LC-Packings). The total sample volume injected ranged from 1 to 10 μl. The mobile phase consisted of the appropriate gradient systems as described for micro LC. A flow-rate of 10 μl/min was achieved by using a 1/70 pre-column splitter (Accurate, LC-Packings). The effluent from the analytical column was introduced into the MS source at a flow-rate of 10 μl/min.

2.3.6. Capillary LC with column switch (capillary LC–LC)

Tandem capillary LC was obtained by using a column switch configuration setup as outlined in Fig. 2b. Automatic column-switching was performed using an injector-driven ten-port multifunctional valve with a micro-electric two position valve actuator (Valco Instruments) (Fig. 2c). Sample loading (1–20 μl) was performed on an analytical precolumn (5×0.3 mm I.D.) cartridge filled with C_8 Select B packing material (LC-Packings) driven by a Kontron 422 pump (flow-rate, 10 μl/min; 0.01 *M* NH_4OAc, pH 7) over 6.5 min, i.e. the time necessary for backflushing the preceding sample from the parallel precolumn. After sample loading, the precolumn was backflushed, using the above-mentioned compound-specific solvent gradients and a Kontron 325 pump (flow-rate, 0.7 ml/min) in combination with an 1/70 accurate pre-column splitter to obtain a 10 μl/min flow-rate on the Hypersil 5 μm C_8 BDS, 150×0.3 mm I.D. (Alltech), analytical column. The effluent was directly introduced into the MS source at a flow-rate of 10 μl/min. Depending on the sample volume injected, the Kontron 465 injector was equipped with a 10- or a 25-μl sample loop.

2.4. (+)ESI-MS–MS conditions

The LC system was linked to a Quattro II mass

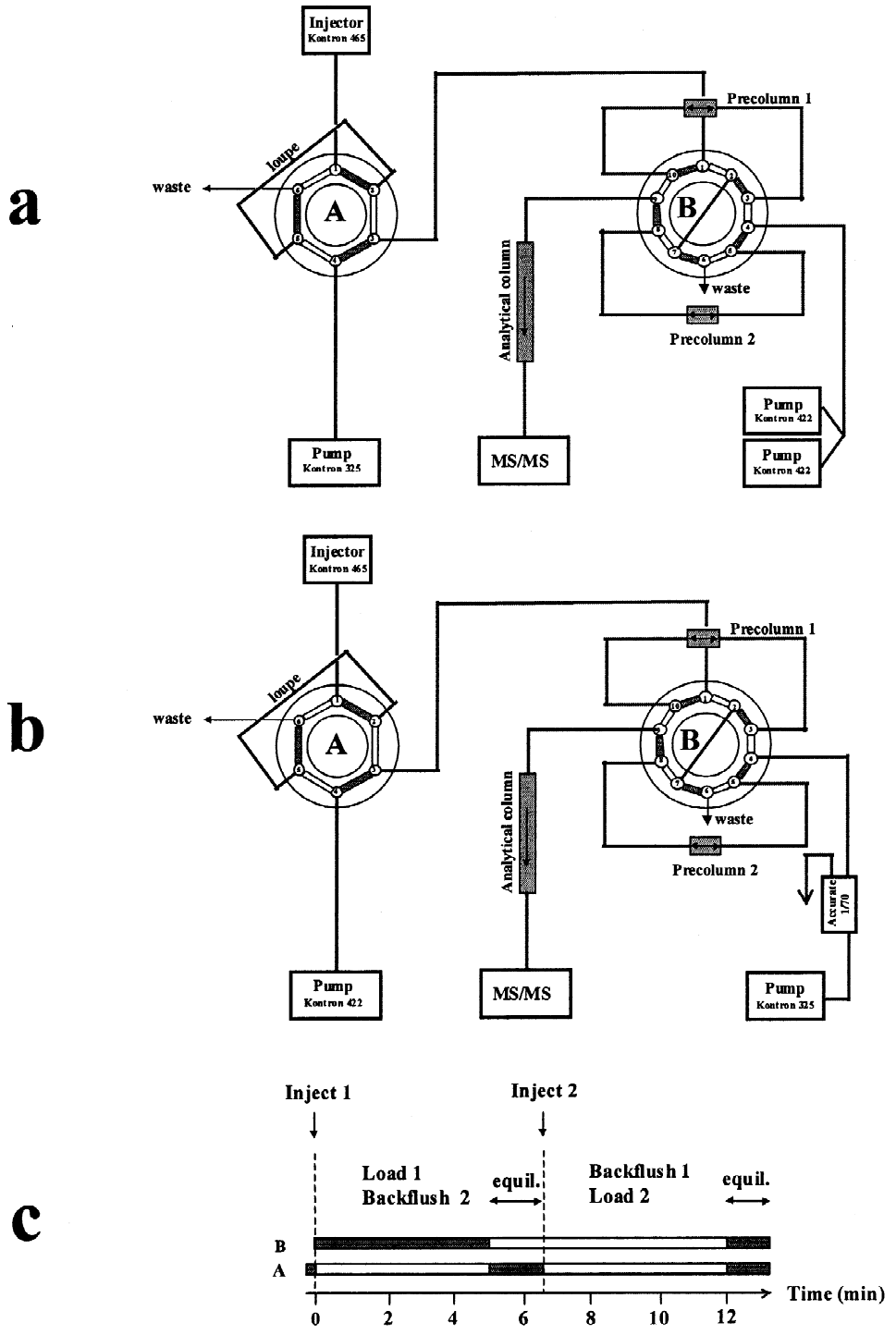

Fig. 2. Schematic diagrams of the column switch injection methods used for micro (a) and capillary (b) tandem LC. The time-based settings for the injector-driven valves are summarised in (c). Valve A=injector-driven six-port valve containing sample loop. Valve B=injector-driven ten-port multifunctional valve with a micro-electric two-position valve actuator.

spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface and a megaflow probe (Micromass UK). The source temperature was 80°C, the nebulising gas flow-rate was 20 l/h, the drying gas flow-rate was 400 l/h and the capillary voltage was +3.5 kV. The cone voltage depended on the compound under investigation [20,21] and is summarised in Table 2. Collision-

Table 2
Diagnostic transitions, specific cone voltage and collision energy used for the analysis of different cytokinins and indole compounds (the latter after methylation) by LC–(+)ESI-MS–MS ($P_{AR}=4\cdot10^{-3}$ mbar)

Compound	Precursor ion	Diagnostic transition	Cone (V)	Collision energy (eV)
IAA-Me	190	190→130	25	17
[$^{13}C_6$]IAA-Me	196	196→136	25	17
IAN	157	157→130	20	20
[$^{13}C_1$]IAN	158	158→130	20	20
IEt	162	162→144	20	12
$^{15}N_1$]IEt	163	163→145	20	12
IAM	175	175→130	20	20
$^{13}C_6$]IAM	181	181→136	20	20
Z	220	220→136	20	20
DHZ	222	222→136	20	20
$^{2}H_5$]DHZ	225	225→136	20	20
iP	204	204→136	20	20
[$^{2}H_6$]iP	210	210→137	20	20
[9R]Z	352	352→220	20	20
[9R]DHZ	354	354→222	20	20
[$^{2}H_5$][9R]DHZ	357	357→225	20	20
[9R]iP	336	336→136	20	20
[$^{2}H_6$][9R]iP	342	342→210	20	20
(9G)Z	382	382→220	20	20
(9G)DHZ	384	384→222	20	20
[$^{2}H_5$](9G)DHZ	387	387→225	20	20
(9G)iP	366	366→204	20	20
[$^{2}H_6$](7G)iP	372	372→210	20	20
(7G)Z	382	382→220	20	20
(7G)DHZ	384	384→222	20	20
[$^{2}H_5$](7G)DHZ	387	387→225	20	20
(OG)Z	382	382→220	20	20
(OG)DHZ	384	384→222	20	20
[$^{2}H_5$](OG)DHZ	387	387→225	20	20
(OG)[9G]Z	514	514→382	20	20
(OG)[9G]DHZ	516	516→384	20	20
[$^{2}H_5$](OG)[9R]DHZ	519	519→387	20	20

IAA-Me=Indole-3-acetic acid methyl ester; [$^{13}C_6$]IAA-Me=^{13}C-labelled indole-3-acetic acid methyl ester; IAN=indole-3-acetonitrile; $^{13}C_1$-IAN=^{13}C-labelled indole-3-acetonitrile; IEt=indole-3-ethanol; [$^{15}N_1$]IEt=^{15}N-labelled indole-3-ethanol; IAM=indole-3-acetamide; [$^{13}C_6$]IAM=^{13}C-labelled indole-3-acetamide; Z=zeatin; DHZ=dihydrozeatin; [$^{2}H_5$]DHZ=^{2}H-labelled dihydrozeatin; iP=isopentenyl adenine; [$^{2}H_6$]iP=^{2}H-labelled isopentenyl adenine; [9R]Z=zeatin-N_9-riboside; [9R]DHZ=dihydrozeatin-N_9-riboside; [$^{2}H_5$][9R]DHZ=^{2}H-labelled dihydrozeatin-N_9-riboside; [9R]iP=isopentenyl adenosine; [$^{2}H_6$][9R]iP=^{2}H-labelled isopentenyl adenosine; (9G)Z=zeatin-N_9-glucoside; (9G)DHZ=dihydrozeatin-N_9-glucoside; [$^{2}H_5$](9G)DHZ=^{2}H-labelled dihydrozeatin-N_9-glucoside; (9G)iP=isopentenyl adenine-N_9-glucoside, [$^{2}H_6$](7G)iP=^{2}H-labelled isopentenyladenine-N_9-glucoside; (7G)Z=zeatin-N_7-glucoside; (7G)DHZ=dehydrozeatin-N_7-glucoside; [$^{2}H_5$]-(7G)DHZ=^{2}H-labelled dehydrozeatin-N_7-glucoside; (OG)Z=zeatin-*O*-glucoside; (OG)DHZ=dihydrozeatin-*O*-glucoside; [$^{2}H_5$](OG)DHZ=^{2}H-labelled dihydrozeatin-*O*-glucoside; (OG)[9G]Z=zeatin-*O*-glucoside-N_9-riboside; (OG)[9G]DHZ=dihydrozeatin-*O*-glucoside-N_9-riboside; $^{2}H_5$-(OG)[9R]DHZ=^{2}H-labelled=dihydrozeatin-*O*-glucoside-N_9-riboside.
Summarised from Prinsen et al. [20] and Bartók et al. [21].
Abbreviations following Crouch et al. [43].

activated dissociation (CAD) of the protonated molecular ion ($[MH]^+$) was obtained using argon as a collision gas at the appropriate compound-specific collision energy, ranging between 10–20 eV (Table. 2) and a P_{AR} (argon pressure) of $4 \cdot 10^{-3}$ mbar. A cross-flow counter-electrode (Micromass) was used to avoid excessive contamination of the source for routine analysis of biological samples.

Quantification was done by SRM of the $[MH]^+$ ion (dwell time, 0.05 s; inter channel delay, 0.01 s; span, 0 u) and the appropriate product ion (see Table 2). The mass spectrometer was tuned using a 10^{-4} *M* zeatin–riboside solution dissolved in 100% methanol. The signal-to-noise (*S*/*N*) ratios of the response were calculated on the unsmoothed mass spectra, with reference to the base peak in the mass spectrum. All mass spectra were background subtracted and smoothed once. All data were processed using Masslynx software (VG Micromass).

3. Results and discussion

3.1. Improving concentration sensitivity by gradient elution and improving mass sensitivity by downscaling the chromatographic dimensions

Calculating the downscaling factor, C_{max} ratio= $(d_{c1}/d_{c2})^2$ [24], theoretical net gains of 21 and 230 could be obtained using 1 mm or 300 μm columns, respectively, compared to a conventional 4.6-mm column. In combination with concentration-sensitive detection, such as electrospray mass spectrometry, the response is proportional to the analyte concentration in the chromatographic peak. To obtain an optimal peak-eluting volume, we improved the chromatographic conditions by using gradient elution. A 15-s peak time window at the appropriate flow-rate resulted in a maximal eluting volume of 25 μl for micro LC and 4 μl for capillary LC. The 25 μl peak-eluting volume obtained for micro LC corresponded to a total of 15 000 numerical plates (data not shown). The chromatography of a 10-fmol isopentenyl adenine reference solution by micro and capillary LC is shown in Fig. 3a–b, respectively. Reducing the peak elution time window has consequences for the time span available for measuring each individual SRM transition when multiple SRM transitions are analysed simultaneously. A minimum of ten measuring points for each SRM transition is necessary. For this reason, a dwell time of 0.05 s was programmed for each SRM transition in combination with a 0.01-s inter-channel delay.

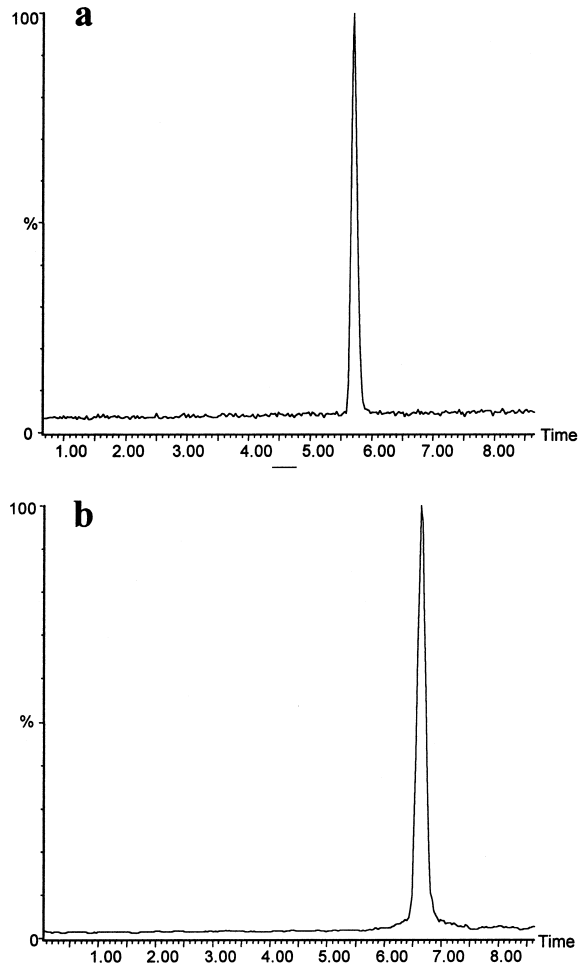

Fig. 3. Chromatography of 10 fmol of isopentenyl adenine (diagnostic transition: 204→136) by micro (a) and capillary (b) LC–(+)ESI-SRM-MS–MS. Time is given in min.

Fig. 4 shows the UV trace of the precolumn during sample loading (a), the UV trace of the analytical column (b) and the chromatography of cytokinins present in tobacco leaves.

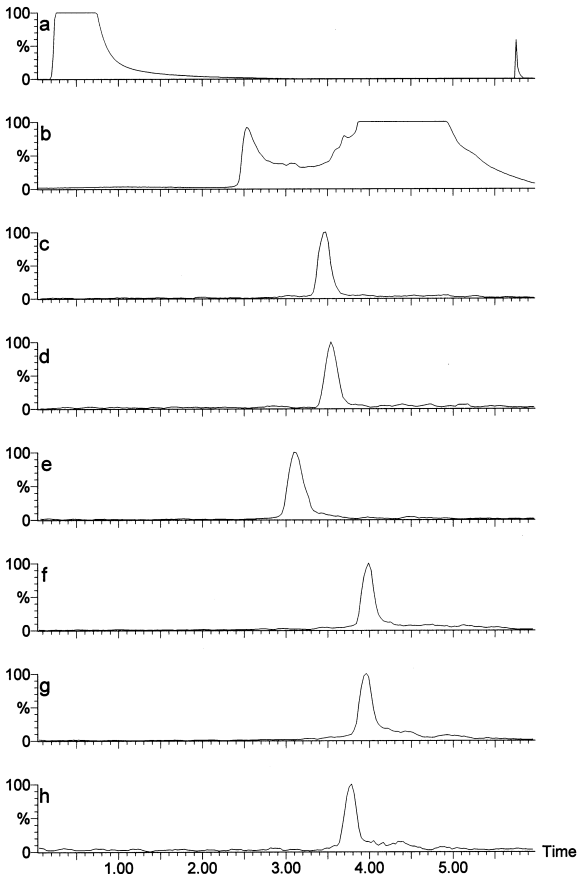

Fig. 4. UV (268 nm) trace of the precolumn (a), UV (268 nm) trace of the analytical column (b) and chromatography of zeatine (diagnostic transition: 220→136) (c), zeatin-N_9-riboside (diagnostic transition: 352→220) (d), zeatin-N_9-glucoside (diagnostic transition: 382→220) (e), isopentenyl adenine (diagnostic transition: 204→136) (f), isopentenyl adenosine (diagnostic transition: 336→204) (g) and isopentenyl adenine-N_9-glucoside (diagnostic transition: 366→204) (h) in a biological sample analysed by micro LC–LC–(+)ESI–SRM–MS–MS. Time is given in min.

3.2. Large and extra-large volume injections, on-column focussing versus column switch

Miniaturisation is inevitably related to a downscaling of the maximum injection volume [31]. This results in a net gain when the total volume of a sample is limited or when samples can be concentrated off-line in an unlimited manner. However, when handling biological samples, downscaling the sample volume is laborious and often results in poor recovery. Essentially, this means that only when in combination with large or extra-large volumes will column miniaturisation result in a net gain in concentration and, consequently, a net gain in mass sensitivity. On-column focussing and column switch configurations in combination with gradient elution allowed larger injection volumes in combination with an optimal peak-eluting volume. Large and extra-large injection volumes can be obtained by (1) on-column focussing followed by gradient elution or (2) focussing on a precolumn followed by backflush loading of the analyte on the analytical column.

Through on-column focussing, we could obtain large volume injections (maximum, 10 μl). With this option, up to 10 or 2 μl could be injected on a 1 mm or 300 μm column, respectively. Sample loading was performed under non-eluting conditions corresponding to 5–10% MeOH. This had some implications for sample preparation as the cytokinins isopentenyl adenine and isopentenyl adenosine, in particular, are not easily dissolved in low concentrations of methanol. Dissolving samples directly in 5% methanol in water resulted in excessive recovery losses. Consequently, we dissolved samples in 10 μl of 20% methanol in water, which we finally diluted with 30 μl of Milli-Q water to obtain a MeOH concentration of 5%.

Extra-large volume injections (max. 100 μl for a 1 mm or 25 μl for a 0.3-mm column) were obtained with a column switch configuration, as shown in Fig. 1. This configuration has the additional advantage of providing an extra on-line purification step for the sample. Moreover, dissolving biological samples in a total volume of 100 μl has some advantages in terms of improved recovery. By the sequential loading of two precolumns, we were able to load a sample while the first precolumn was being backflushed. This sequential loading reduced the time needed for sample loading and resulted in substantial speeding up of sample analysis. It is important to note that, when an identical stationary phase is used for both the precolumn and the analytical column in a column switch configuration, optimal performance is only obtained in combination with a fast linear gradient for backflushing. Isocratic backflushing of the pre-

column at high methanol concentrations (90% MeOH) will elute the analytical compounds under solvent conditions that will not retain these compounds on an analytical column with an identical stationary phase. Minor retention on an analytical column is however still advised [32].

3.3. Detection limits

In Table 3, we compared the theoretical downscaling factor ($f_{\text{theor.}}$), S/N, detection limits and the linearity range for conventional, micro and capillary LC in the presence or absence of gradient elution. These data show that the S/N obtained for capillary LC equals the S/N obtained for micro LC, despite the theoretical downscaling factor.

It is also worth mentioning that restriction of the total number of scans per SRM transition, as a consequence of peak shape (peak-eluting volume), should always be considered. When multiple SRM transition channels were analysed simultaneously, a 0.01 s interchannel delay and a minimal 0.05 s dwell time per SRM transition is essential to obtain at least ten scan points per individual SRM transition.

The sensitivity of this method was optimised at the level of mass spectrometric detection. As a high electrolyte concentration may interfere with the mass spectrometric response of the analyte [33], we investigated the effect of electrolyte concentration on the mass spectrometric response (data not shown). Ammonium acetate concentrations ranging between 10 m*M* and 100 n*M* were tested. The highest sensitivity was obtained with 10 μ*M* ammonium acetate, in the case where the analyte was directly introduced into the MS source without prior LC separation. However, optimal LC conditions and, consequently, an optimal signal/noise ratio was obtained using 10 m*M* ammonium acetate and a solvent pH of 6.6 (data not shown). It is important to note that optimal sensitivity was obtained when a limited amount of SRM transitions were analysed simultaneously.

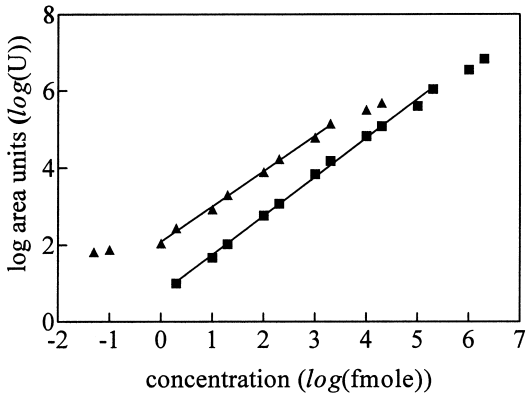

Fig. 5. Detection limit and linearity range for isopentenyl adenine for micro LC–(+)ESI-SRM-MS–MS (■) and capillary LC–(+)ESI-SRM-MS–MS (▲). Data are the mean±standard deviation (n_y replicates, 2). The error bars are not displayed (smaller than the symbols).

3.4. Conventional versus micro- and capillary LC coupled to electrospray tandem mass spectrometry

Fig. 5 shows the linearity range for both the 1 and 0.3 mm columns. Although we were able to analyse as little as 0.1 fmol of isopentenyl adenine by capillary LC, the linearity ranged from 1 fmol to 5 pmol (Fig. 5) (slope, 0.9146±0.01151; *y*-intercept, 2.089±0.02301; *x*-intercept, −2.284; 1/slope, 1.093; goodness of fit, r^2 0.9978, *sy.x*, 0.05195; *P*-value

Table 3
Comparison of conventional, micro and capillary LC–(+)ESI-MS–MS for 1 pmol of iP injected

LC nomenclature	Column dimension (mm)	$f_{\text{theor.}}$	Gradient elution (+/−)	S/N limit (fmol)	Detection	Linearity range
Conventional	4.6	1	−	5	600	1–10 nmol
Conventional	4.6	1	+	14	200	0.2–1000 pmol
Micro	2	5	+	115	40	n.a.
Micro	1	21	+	1056	5	5 fmol–1 nmol
Capillary	0.3	235	+	1256	0.1	1 fmol–5 pmol

S/N: signal-to-noise ratio; theoretical downscaling factor, $f_{\text{theor}}=C_{\max}$ ratio$=(d_{c1}/d_{c2})^2$; n.a. not analysed.

<0.0001; n_x=8, n_y=2). For micro LC, linearity was observed between 5 fmol and 1 nmol (slope, 1.009±0.01168; *y*-intercept, 0.7411±0.03820; *x*-intercept, −0.7345; 1/slope, 0.9911; goodness of fit, r^2 0.9976, *sy.x* 0.07752; *P*-value <0.0001; n_x=11, n_y=2). The upper limit of the linearity range is the result of the loadability of both the precolumn and the analytical column. The total length of the 1 mm precolumn used (30×1 mm I.D., Phenomenex) was six-times larger than the capillary precolumns available (5×0.3 mm I.D., LC-Packings). This results in a higher loadability of the 1 mm precolumns and, therefore, a larger linearity range.

3.5. Robustness and repeatability

Capillary dimensions, using a 300 μm column, require the use of a microflow processor for micro flow (1–10 μl) delivery. These flow-rates are obtained using dedicated low volume pumps [34] or splitters based on flow resistance. All splitters that are available to obtain microflow rates are resistance-sensitive. The resistance sensitivity of a splitter can be partially corrected by using a balanced column flow splitter [35]. The balance column emulates viscosity changes during the gradient and, therefore, the split ratio is maintained during the gradient. Permanent observation of the flow-rate during processing is however essential. For routine analysis, automation is preferred. The use of pre-column splitters or balanced column splitters, implicates that a minor obstruction at the level of the analytical column, due to impurities or viscosity fluxes in biological samples, will always result in fluctuations in the splitting factor or even a total off-splitting of the analytical column, whereas the pressure displayed remains constant. Fluctuations in the retention time, as shown in Fig. 6, or even a final drop in the through-column flow will be the result. On the other hand, using a direct flow delivery, any obstruction will result in an overpressure error feedback signal and a resetting of the injector. Consequently, further automatic injection will be cancelled.

The retention time for isopentenyl adenine was evaluated for both 1 and 0.3 mm columns by continuously analysing biological samples for 24 h. The retention time of 150 consecutive injections is shown in Fig. 6. The retention time was stable during

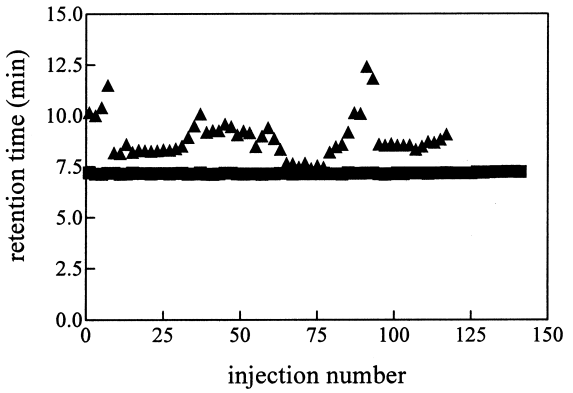

Fig. 6. Repeatability of the retention time of isopentenyl adenine during 145 consecutive injections of biological samples using micro LC–(+)ESI-SRM-MS–MS (■) and capillary LC–(+)ESI-SRM-MS–MS (▲). Each data point displayed is an individual value.

the entire period for the 1 mm column. The fluctuations in retention time shown for the 0.3 mm column are the result of flow fluctuations due to fluctuations in the split ratio.

To evaluate the repeatability of micro LC–MS–MS, the response for 1 pmol isopentenyl adenine was measured at an interval of every 20 injections of biological samples and this was done over a time span of 24 h. The response during 250 consecutive injections is shown in Fig. 7. A 20% fluctuation in

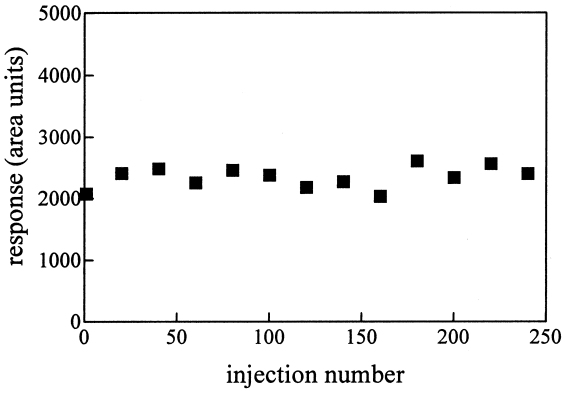

Fig. 7. Repeatability of the response of 1 pmol of isopentenyl adenine during 250 consecutive injections of biological samples using micro LC–(+)ESI-SRM-MS–MS. At intervals of 20 injections, a 1-pmol reference solution was inserted between the consecutive samples for diagnostic purposes. Each data point displayed is an individual value.

response was observed. As internal tracers are used routinely, these fluctuations do not alter the quantification efficiency. The repeatability was enhanced using a cross-flow counter-electrode.

The robustness of micro LC–MS–MS has been evaluated for routine analysis over a time span of one year, resulting in the analysis of over 3000 samples on one individual analytical column. In comparison to conventional LC–MS–MS, no specific extra precautions in sample preparation besides sample filtration were necessary.

4. Conclusion

The analysis of phytohormones using LC–ESI-MS–MS was described earlier [20,21] and proved to be very useful for routine analysis [30,36–38]. In this paper, we aimed to improve the sensitivity of LC–(ESI)-MS–MS by miniaturisation of the LC dimensions in combination with large and extra-large volume injections in order to allow the analysis of phytohormones in a limited amount of starting material. The data presented here show the performance of micro (1 mm column) and capillary (300 μm column) LC for routine analysis of cytokinins and auxins.

4.1. Detection limits and the linearity range

Capillary and nano LC [25,26,39] allow excellent detection in the femtomole and subfemtomole range. We analysed the detection limits and linearity range for both micro and capillary LC for the analysis of cytokinins and indole compounds. For capillary LC, we showed a detection limit of 0.1 fmol and linearity between 1 fmol and 5 pmol. For micro LC, a detection limit of 5 fmol and linearity between 5 fmol and 1 nmol could be observed. We advise the use of capillary LC when the amount of starting material is limited and concentrations above 5 pmol can be excluded. Especially in combination with capillary LC, the amount of tracer used should be low enough to work within the linearity range.

4.2. Optimising the total sample analysis time

Automatic column-switching was performed using an injector-driven ten-port multifunctional valve with a micro-electric two-position valve actuator in combination with a conventional automated injector. This ten-port valve in combination with a conventional automated injector equals the performance of the fully automated micro autosampler (FAMOS, LC-Packings). With the ten-port valve, we were able to load two precolumns in parallel, reducing the analysis time to 6.5 min per sample. The final sample analysis time can be further reduced by reducing the length of the analytical column. Separation of the two isomers zeatin-*O*-glucoside and zeatin-N_7-glucoside in the sample mixture is a prerequisite for column length.

In conclusion, robustness is expressed by the following attributes: (1) a large and biologically relevant linearity range, (2) direct flow delivery, (3) reproducible retention times, (4) short sample loading time, (5) reasonable tubing diameter, (6) constant flow through the analytical column, (7) the ability to use guard columns and to replace pre-column filters, (8) easy evaluation of the flow-rate, (9) easy detection of potential leakage and (10) a high flow-rate through the MS capillary, requiring less elaborate positioning. We therefore suggest the use of micro LC for the routine analysis of large numbers of biological samples. The robustness of micro LC–LC–MS–MS for the analysis of phytohormones in a minute amount of starting material has been evaluated over a time span of nearly one year, resulting in the analysis of over 3000 samples on one individual analytical column. However, we emphasize the power of further miniaturisation through capillary and nano LC when the amount of starting material available requires further miniaturisation, if dedicated low volume pumps are available.

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.L.E. and H.A.V.O. E.P. and H.A.V.O. are respectively Post Doctoral Fellow and Research Director of the Fund for Scientific Research Flanders (F.W.O.). Dr. N. Ilic (University of Maryland, USA) synthesised the [$^{13}N_1$]-IAN tracer.

References

[1] P. Hedden, Annu. Rev. Plant Physiol. Plant Mol. Biol. 44 (1993) 107.
[2] M. Strnad, T. Vanek, P. Binarová, M. Kamínek, J. Hanus, in M. Kutácek, M.C. Eliot, I. Machácková (Editors), Molecular Aspects of Hormonal Regulation of Plant Development, SPB Academic Publishers, The Hague, 1990, p. 41.
[3] E. Prinsen, P. Redig, M. Strnad, I. Galís, W. Van Dongen, H. Van Onckelen, in K. Gartland, M. Davey (Editors), Methods in Molecular Biology, Vol. 44, *Agrobacterium* Protocols, Humana Press, Totowa, NJ, 1995, p. 245.
[4] H. Soejima, T. Suqiyama, K. Ishihara, Plant Physiol. 100 (1992) 1724.
[5] B.A. McGaw, R. Horgan, J.K. Heald, G.J. Wullems, R.A. Schilperoort, Planta 176 (1988) 230.
[6] I.M. Scott, R. Horgan, Planta 161 (1984) 345.
[7] P.-O. Björkman, E. Tillberg, Phytochem. Anal. 7 (1996) 57.
[8] A. Edlund, S. Eklöf, B. Sundberg, T. Moritz, G. Sandberg, Plant Physiol. 108 (1995) 1043.
[9] D.M. Ribnicky, T.J. Cooke, J.D. Cohen, Planta 204 (1998) 1.
[10] E. Epstein, J.D. Cohen, J. Chromatogr. 209 (1981) 413.
[11] E.A. Scheider, C.W. Kazakoff, F. Wightman, Planta 165 (1985) 232.
[12] L.M. Palni, R.E. Summons, D.S. Letham, Plant Physiol. 72 (1983) 858.
[13] N. Watanabe, T. Yokota, N. Takahashi, Plant Cell Physiol. 23 (1982) 479.
[14] N. Imbault, T. Moritz, O. Nilsson, H.-J. Chen, M. Bollmark, G. Sandberg, Biol. Mass Spectrom. 22 (1993) 201.
[15] A. Östin, T. Moritz, G. Sandberg, Biol. Mass Spectrom. 21 (1992) 292.
[16] A. Östin, C. Catalá, J. Chamarro, G. Sandberg, J. Mass Spectrom. 30 (1995) 1007.
[17] Y.-Y. Yang, I. Yamaguchy, Y. Kato, E.W. Weiler, N. Murofushi, N. Takahashi, J. Plant Growth Regul. 12 (1993) 21.
[18] A.L. Burlingame, R.K. Boyde, S.J. Gaskell, Anal. Chem. 66 (1994) 634R.
[19] W.M.A. Niessen, A.P. Tinke, J. Chromatogr. A 703 (1995) 37.
[20] E. Prinsen, P. Redig, W. Van Dongen, E.L. Esmans, H. Van Onckelen, Rapid Commun. Mass Spectrom. 9 (1995) 948.
[21] E. Prinsen, W. Van Dongen, E. Esmans, H. Van Onckelen, J. Mass Spectrom. 32 (1997) 12.
[22] T. Bartók, G. Börcsök, R. Komoróczy, F. Sági, J. Mass Spectrom. 31 (1996) 756.
[23] K.B. Tomer, M.A. Moseley, L.J. Mass Spectrom. Rev. 13 (1994) 431.
[24] G. Hopfgartner, K. Bean, J. Henion, R. Henry, J. Chromatogr. 647 (1993) 51.
[25] K. Vanhoutte, P. Joos, F. Lemière, W. Van Dongen, E.L. Esmans, J. Mass Spectrom. Rapid Commun. Mass Spectrom., (1995) S143.
[26] K. Vanhoutte, W. Van Dongen, I. Hoes, F. Lemière, E.L. Esmans, H. Van Onckelen, E. Van den Eeckhout, R.E.J. van Soest, A.J. Hudson, Anal. Chem. 69 (1997) 3161.
[27] H. Schlenk, J.L. Gellerman, Anal. Chem. 32 (1960) 1412.
[28] T. Murashige, F. Skoog, Physiol. Plant. 15 (1964) 473.
[29] R.L. Bieleski, Anal. Biochem. 9 (1964) 431.
[30] P. Redig, T. Schmülling, H. Van Onckelen, Plant Physiol. 112 (1996) 141.
[31] D. Hawkes, Lab. Equipment Dig. 30 (1992) 10.
[32] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[33] C. Enke, Anal. Chem. 69 (1997) 4885.
[34] C. Ericson, S. Hjertén, Anal. Chem. 70 (1998) 366.
[35] C. Yan, R. Dadoo, R.N. Zare, D.J. Rakestraw, D.S. Anex, Anal. Chem. 68 (1996) 2726.
[36] P. Redig, O. Shaul, D. Inzé, M. Van Montagu, H. Van Onckelen, FEBS Lett. 391 (1996) 175.
[37] E. Miklashevichs, I. Czaja, A. Cordeiro, E. Prinsen, J. Schell, R. Walden, Plant J. 12 (1997) 489.
[38] J.A. Dieleman, F.W.A. Verstappen, B. Nicander, D. Kuiper, E. Tillberg, J. Tromp, Physiol. Plant. 99 (1997) 456.
[39] J.P. Chervet, M. Ursem, J.P. Salzmann, Anal. Chem. 68 (1996) 1507.
[40] H.A. Van Onckelen, P. Rüdelsheim, D. Inzé, A. Follin, E. Messens, S. Horemans, J. Schell, M. Van Montagu, J. de Greef, FEBS Lett. 181 (1985) 357.
[41] F. Hofmann, T. Rausch, W. Hilgenberg, J. Label. Compd. Radiopharm. XVIII (1981) 1491.
[42] B.B. Stowe, Anal. Biochem. 5 (1963) 107.
[43] N.R. Crouch, M.T. Smith, J. Van Staden, Plant Growth Regul. 13 (1993) 13.