Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1048 (2004) 119–126
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Analysis of some cytokinins in coconut (*Cocos nucifera* L.) water by micellar electrokinetic capillary chromatography after solid-phase extraction

Liya Ge[a], Jean Wan Hong Yong[a], Swee Ngin Tan[a,*], Xin Hao Yang[b], Eng Shi Ong[c]

[a] *Natural Sciences Academic Group, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Singapore*
[b] *Singapore Epson Industrial Pte Ltd., Engineering Department, Plating Division, 86 Second Lok Yang Road, Singapore 628162, Singapore*
[c] *Applied Science School, Temasek Polytechnic, 21 Tampines Avenue 1, Singapore 529757, Singapore*

Received 14 January 2004; received in revised form 1 July 2004; accepted 14 July 2004

Abstract

Micellar electrokinetic capillary chromatography (MECC) was developed for the separation of cytokinins including *trans*-zeatin, *trans*-zeatin-*O*-glucoside, dihydrozeatin, dihydrozeatin-*O*-glucoside, *meta*-topolin riboside, N^6-isopentenyladenine and N^6-benzylaminopurine. Under the optimum conditions, i.e. a combination of 10 mM phosphate and 10 mM borate as the running buffer containing 50 mM sodium dodecyl sulphate at pH 10.4, the separation of seven cytokinin standards was accomplished within 11 min. The C_{18} solid-phase extraction (SPE) method was used to pre-concentrate the putative cytokinins present in the coconut water. Following which, the eluate was further purified using mixed mode Oasis MCX SPE columns and this additional step helps to reduce matrix interference during MECC. After the two solid-phase extraction steps, the optimized MECC method was able to screen for certain cytokinins (zeatin-*O*-glucoside and dihydrozeatin-*O*-glucoside) present in coconut water. After this screening, the presence of zeatin-*O*-glucoside and dihydrozeatin-*O*-glucoside in coconut water was further confirmed by independent high-performance liquid chromatography and liquid chromatography–mass spectrometry experiments.

Keywords: Solid-phase extraction; Cytokinins; Coconut water

1. Introduction

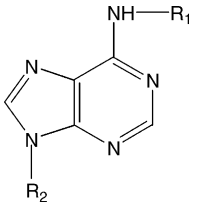
The analysis of cytokinins, which represent one major group of phytohormones, is an important area of research in plant science. Cytokinins play various important functions in cell division, chlorophyll formation, stimulating differentiation of plant tissues, seed germination, bud formation, release of buds from apical dominance, leaf expansion, and reproductive development [1–8]. In addition to the above plant-related roles, some derivatives of cytokinins could potentially be useful to reduce the growth of some types of mammalian tumors [9–13]. The activity of cytokinins is essentially dependent on their chemical structures. All native cytokinins are derivatives of adenine with at least one substituent at the N^6 position (see Table 1). Thus, the rapid analyses of cytokinins are of great importance to both plant physiologists, in understanding various physiological processes, and scientists from various disciplines (e.g. clinicians and molecular biologists) especially in view of its potential role in suppressing mammalian tumor growth.

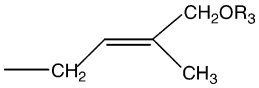
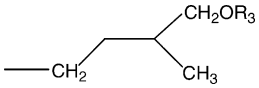
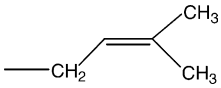
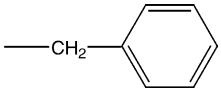
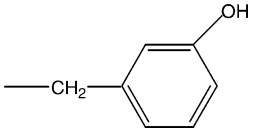
Capillary electrophoresis (CE) is an analytical separation technique that brings speed, quantification, reproducibility, and automation to the highly resolving but labour-intensive traditional flat-bed electrophoresis. CE is playing an increasingly important role in the fast growing field of biochemical analysis of complex mixtures due to the many advantages offered by this method [14,15]. So far, relatively few papers have reported on the analysis of cytokinins by CE [16–20]. In comparison with immunoassays such as enzyme-linked

* Corresponding author. Tel.: +65 7903810; fax: +65 68969432.
E-mail address: sntan@nie.edu.sg (S.N. Tan).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.07.031

Table 1
Structures, names and abbreviations of the cytokinins used in this study



R_1	R_2	R_3	Compound	Abbreviation
	H	H	*trans*-Zeatin	Z
	H	G	*trans*-Zeatin-*O*-glucoside	ZOG
	H	H	Dihydrozeatin	DZ
	H	G	Dihydrozeatin-*O*-glucoside	DZOG
	H	–	N^6-Isopentenyladenine	iP
	H	–	N^6-Benzylaminopurine	BA
	R	–	*meta*-Topolin riboside	mTR

H: hydrogen; R: β-D-ribofuranosyl; G: β-D-glucopyranosyl.

immunosorbent assays (ELISA), radioimmunoassay (RIA) [21,22], or chromatography such as high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS) [23–27], CE generally offers shorter analysis time and potentially lower costs as the buffer salts and solvents required cost much less than the radioactive or heavy isotope-labelled internal standards.

Cytokinins are present in plants in very low concentrations (usually at levels below 30 pmol g^{-1} of fresh weight [28]). They often occur together with the other structurally similar substances, and are therefore below the detection limits in CE. To overcome this limitation, some pre-concentration and purification approaches have been developed for cytokinin analyses, including large-volume stacking [20], liquid–liquid solvent extraction [26,27,29], and solid-phase extraction (SPE) [30]. In SPE method, a high extraction recovery can usually be obtained for many compounds with a suitable sorbent and operating procedure. Fast and efficient separation of cytokinins can also be achieved by using mixed-mode SPE [30]. In [30], the quantification of cytokinins after SPE was via either HPLC–ELISA (or RIA) or HPLC–MS.

Coconut water, which contains a large spectrum of biochemicals that can act as growth factors individually or synergistically [31], is commonly used at 2–15% (v/v) as a medium for plant tissue culture [3–5,32]. A number of attempts to identify the ingredients in coconut water have been reported. One component isolated from 700 gallons of coconut liquid endosperm was 1,3-diphenylurea, which shows cytokinin-like activity [33]. Later two adenine-type cytokinins—zeatin-riboside (ZR) [34,35] and zeatin (Z) [35] were shown to be present in this endosperm. A new cytokinin, determined to be ZR with a large sugar conjugate at the hydroxyl group on the side chain, was reported to be present in coconut water in 1995 [36,37]. In this paper, we first demonstrate the separation of cytokinin standards by micellar electrokinetic capillary chromatography (MECC). Following which, we used the method to screen for putative cytokinins in coconut water by MECC. The presence of the two cytokinins in coconut water, namely zeatin-*O*-glucoside (ZOG) and dihydrozeatin-*O*-glucoside (DZOG), was further confirmed by independent HPLC and LC–MS experiments.

2. Experimental

2.1. Reagents and materials

The cytokinin standards: *trans*-zeatin (Z), dihydrozeatin (DZ), *trans*-zeatin-*O*-glucoside (ZOG), dihydrozeatin-*O*-glucoside (DZOG), *m*-topolin riboside (mTR), isopentenyladenine (iP) and benzylaminopurine (BA) were either obtained from OlChemIm, Czech Republic or from Prof. Stuart Letham at the Australian National University (Table 1). All the standards were dissolved in ethanol–water (50:50, v/v). Methanol (HPLC grade) was from J.T. Baker (Phillipsburg, NJ, USA); triethylamine (HPLC grade) was from BDH

(England, UK). All other chemicals were analytical reagent grade. Boric acid, disodium hydrogenphosphate and ethanol were purchased from Merck (Darmstadt, Germany); sodium dodecyl sulfate (SDS) and sodium hydroxide were purchased from Fluka (Steinheim, Switzerland); acetic acid and formic acid were purchased from Fisher Scientific (Hanover Park, IL, USA); ammonia solution (28%) was purchased from APS Finechem (NSW, Australia). Purified water (MilliQ, Waters, Milford, MA, USA) was used throughout the study. The pH of the buffer solutions was adjusted by adding sodium hydroxide solution and was monitored using a pH meter (Corning 440, Corning Glass Works, NY, USA).

2.2. Apparatus and related procedures

The separation of cytokinins was carried out using a capillary electrophoresis system (P/ACE MDQ; Beckman Coulter, CA, USA) equipped with a photodiode array detection (DAD) system. Instrumentation, system control and data analyses were carried out using a PC with the accompanying software (P/ACE System MDQ Software, version 2.3). The electrophoretic separations were carried out in an uncoated fused-silica capillary (76 μm i.d. × 363 μm o.d.; Polymicro Technologies, Phoenix, AZ, USA) using a suitable running buffer. The optimum MECC running buffer consisted of 10 mM phosphate, 10 mM boric acid and 50 mM SDS at pH 10.4, unless stated otherwise. The wavelength for detection was set at 254 nm. The capillary had an effective length of 47 cm (total length: 57 cm) and was operated at an applied voltage of 15 kV. Sample introduction was accomplished by vacuum injection for 5 s under a pressure of 0.5 psi (psi = 6894.76 Pa). Typically a mixture of seven cytokinin standards was injected into the CE capillary. All samples were filtered through a 0.45-μm Whatman glass microfiber filter before injecting into the CE instrument. At the beginning of each working day, the capillary was rinsed sequentially with sodium hydroxide (0.1 M), water and the separation buffer. Each rinsing step was about 5 min and between each sample injection, the above rinsing procedure was adopted except the time taken for each rinsing step was 3 min. These standard conditions were used for all experiments.

HPLC experiments were carried out using a Waters 2695 Separations Module (Waters) linked simultaneously to a DAD system (PDA 2996 detector, Waters). Data were processed by the accompanying system software (Millennium^{32} Software, Data Handling System for Windows, version 4.0). Samples were dissolved in mobile phase (initial conditions), filtered through a 0.45-μm Whatman glass microfiber filter and injected into a C_{18} column (Platinum 100 Å, 5 μm, 250 mm length, 4.6 mm diameter, Alltech, Deerfield, IL, USA). The column thermostat was set at 25 °C. Solvent A consisted of 40 mM acetic acid, pH adjusted to the range of 3.78–3.80 with triethylamine [39] and solvent B was methanol. The flow-rate was 1 mL min^{-1} throughout the whole separation. Prior to each analysis the column was washed with mobile phase (methanol–acetic acid buffer, 95:5, v/v). The absorbance of the column eluent was monitored at 269 nm with the programmable DAD system.

A LC–MS system (Model 1100 Series, Agilent Technologies, Palo Alto, CA, USA) coupled with an electrospray ionization (ESI) interface was used in the scan mode for standards and selected ion monitoring (SIM) at *m*/*z* 381, 383, 384 and 385 for real samples. $[M + H]^+$ and $[M + 2]^+$ were selected as the SIM ion for quantification. Conditions for the MS were as follows: ESI temperature was 380 °C; detector voltage was 1.5 kV; nebulizing gas (nitrogen) flow was 4.5 L min^{-1}. The data were processed by the accompanying system software (LC/MSD Chemstation). The chromatographic separation conditions were the same as described earlier for the HPLC.

2.3. Cytokinins isolation and sample pretreatment involving SPE procedure

Coconut water was obtained from fresh coconut fruits (Emanate Agricultural Industries, Selangor, Malaysia) purchased from a supermarket. It is noteworthy that coconut water contains other substances including sugars [38].

The pH of coconut water was adjusted to 3 by adding acetic acid and filtered through filter paper (Whatman, 12.5 cm, No. 542) to remove suspended matters. Then, 100 mL filtrate were transferred to C_{18} SPE columns (J.T. Baker, Phillipsburg, NJ, USA; 500 mg, 3 mL), which had been previously washed sequentially with methanol–acetic acid (100:1, v/v), methanol–water–acetic acid (50:50:1, v/v/v), methanol–water–acetic acid (30:70:1, v/v/v), and then finally with water [39]. After washing the column with 4 mL water adjusted to pH 3 with acetic acid, the putative cytokinins were eluted with 6 mL ethanol–water–acetic acid (80:20:1, v/v/v) and then the eluate was evaporated at room temperature under vacuum and finally dissolved in 0.5 mL water–ethanol (50:50, v/v) for MECC analysis. Alternatively the eluate collected from six C_{18} SPE columns was combined, evaporated and further purified using the mixed-mode SPE columns (Oasis MCX, Waters 3 mL) [30].

The procedure for further purification of cytokinins collected from the eluate of C_{18} SPE columns is shown in Fig. 1. The pre-conditioning of the mixed mode SPE columns was as follows: washing with 3 mL of methanol followed by 3 mL formic acid (1 M). The extracts from the C_{18} SPE column were re-dissolved in 3 mL formic acid (1 M) and applied to the mixed mode SPE column. The mixed mode SPE column was then washed and eluted with the order of solutions as shown in Fig. 1. The interaction of cytokinins with C_{18} and MCX columns has been discussed in this work [30]. The majority of cytokinin bases, ribosides and glucosides were eluted in the second eluate. Cytokinin nucleotides, if present in eluate one of the mixed mode columns, have been kept and these will be further analyzed in a later study. The eluate was dried in vacuum at room temperature and dissolved in 0.5 mL water–ethanol (50:50, v/v) for further analyses.

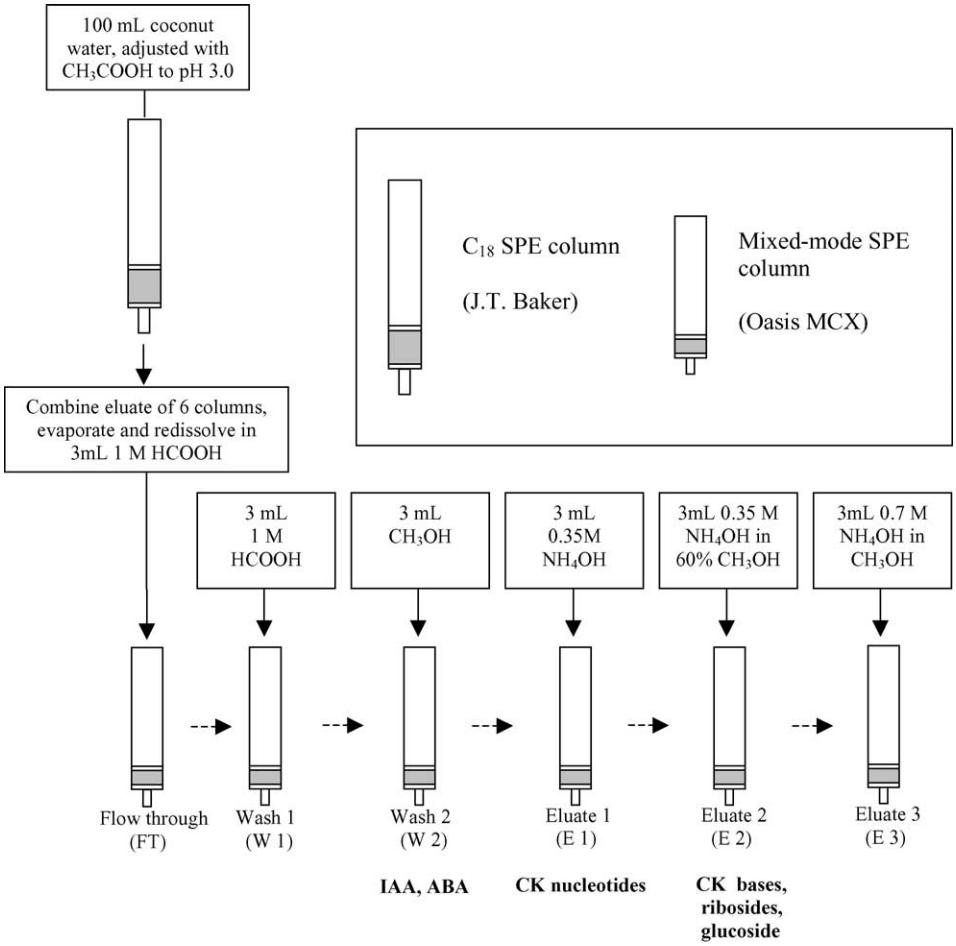


Fig. 1. Schematic diagram illustrating the pre-concentration and purification procedures for the putative cytokinins in coconut water using two types of solid-phase extraction columns [30].

3. Results and discussion

3.1. Development of MECC separation

The chemical composition and concentration of running buffer could have significant effects on MECC separation. During the initial phase of the investigation, no satisfactory resolution could be achieved for all standards of cytokinins when pure 20 mM phosphate or boric acid buffer containing 50 mM SDS at pH 10.4 was employed as running buffer for the separation. Pure borate buffer containing 50 mM SDS gave a better resolution for Z, DZ and mTR compared to pure phosphate buffer containing 50 mM SDS; however, pure phosphate buffer containing 50 mM SDS provided better resolution for the following pairs of peaks: BA and iP, and DZOG and Z. Fig. 2 shows the well-resolved electropherogram for the seven cytokinins in a mixture of 10 mM phosphate and 10 mM borate buffer (pH 10.4) containing 50 mM SDS.

The selectivity in CE separation is greatly affected by pH of buffer solutions. In MECC, the pH of the buffer solution affects the mobility, solubility and the partitioning of analytes into the micellar phase in the separation model. Typically, increasing buffer pH leads to the shortening of separation time due to the enhancement of EOF (Fig. 3). At pH 10, the following pairs of peaks: Z and DZOG, BA and iP cannot be resolved. At pH 11, the peaks for Z and DZ cannot be

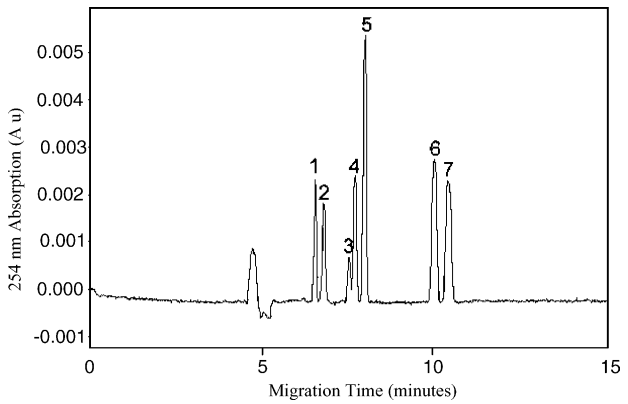


Fig. 2. Electropherogram of cytokinin standards using a combination of 10 mM phosphate and 10 mM borate buffer (pH 10.4) containing 50 mM SDS. CE conditions: fused-silica capillary, 75 μm i.d., 57 cm length; separation voltage, 15 kV; pressure injection, 5 s (at 0.5 psi). Peak identity: (1) ZOG; (2) DZOG; (3) Z; (4) DZ; (5) mTR; (6) BA; (7) iP.

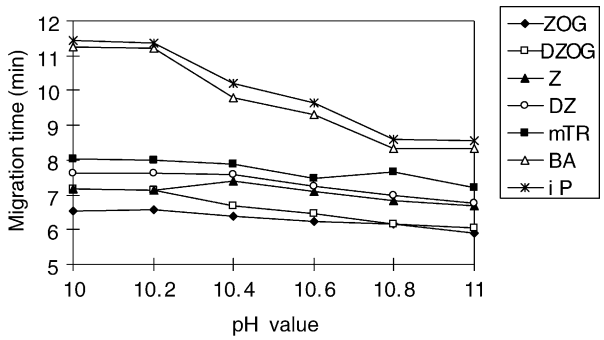


Fig. 3. Separation of cytokinin standards in buffers with different pH. Buffer solution contained a combination of 10 mM phosphate, 10 mM borate buffer and 50 mM SDS. Other experimental conditions were as described in Fig. 2.

resolved. The best resolution was obtained later when a pH of 10.4 (10 mM borate and 10 mM phosphate in the presence of 50 mM SDS) was used (Fig. 3). Cytokinin bases have a basic pK_a of ca. 4 and acidic pK_a of ca. 10. [40] Thus, under the present optimum experimental conditions at pH 10.4, the predominant form of cytokinins is the anionic species.

In MECC, separation can be achieved based on the differences in the distribution of the solutes between the hydrophobic and the charged micellar phase [41]. Typically, the migration time for all the cytokinins increase with increasing SDS concentration, in a mixture of 10 mM phosphate and 10 mM borate buffer of pH 10.4. The migration order of the cytokinins changed slightly when the concentration of SDS was changed from 10 to 100 mM. Couples of ZOG–DZOG, BA–iP and Z–DZ tend to co-migrate at lower SDS concentration. The unresolved pairs were later separated when the concentration of SDS was increased to 50 mM and beyond. Therefore, the best resolution was obtained when SDS concentration was set at 50 mM in a buffer solution containing 10 mM phosphate and 10 mM borate (pH 10.4).

In addition to the running buffer conditions, the effect of separation voltage on the resolution was examined over the range of 10–30 kV in a buffer solution consisting of 10 mM phosphate and 10 mM borate with 50 mM SDS (pH 10.4). Generally, an increase of EOF at high applied field resulted in a shorter analysis time but also caused a decrease in the resolution for the separation of cytokinins. Thus, a separation voltage was set at 15 kV to achieve a good resolution and an analysis time of less than 15 min.

From the above optimization, a combination of 10 mM phosphate and 10 mM borate buffer (pH 10.4) containing 50 mM SDS as well as a separation voltage of 15 kV was chosen as the optimum separation conditions to resolve (with some compromise) the seven cytokinins for investigation. Under these optimum MECC conditions, the separation of the seven cytokinins was achieved within 11 min. If rinsing time was included in a MECC experiment, the time needed for the separation of these cytokinins was about half an hour.

The reproducibility of the migration time of the cytokinins under optimum MECC conditions was investigated by doing repeated injections (n = 6) of a mixture of the cytokinin standards at a concentration of 50 μM. The relative standard deviations (R.S.D.) for all the cytokinins were in the range of 0.7–1.2% (Table 2). The high reproducibility in migration time indicated that this method was probably reliable for analyzing cytokinins in plant samples. A linear correlation was found between the concentration and the peak area ratios for all cytokinins in the following range: Z, 25–200 μM; DZ, 20–200 μM; ZOG, 25–200 μM; DZOG, 30–200 μM; mTR, 12.5–200 μM; BA, 20–200 μM; iP, 25–200 μM; typically r values were in the range of 0.991–0.999. Three independent injections were carried out for every calibration point. Since all seven cytokinins have slightly different UV spectra for maximum absorption, a compromise wavelength was set at 254 nm in this work. Based on the signal/noise ratio = 3, the limits of detection under optimum MECC conditions were estimated to be in the range of 2–18 μM. The quantitative data obtained from the analysis of cytokinins by MECC are summarized in Table 2.

3.2. Analyses of putative cytokinins in coconut water using MECC

We used the MECC (operating under the optimum conditions described earlier) to screen for putative cytokinins in coconut water. Fig. 4A shows the MECC electropherograms of extracts purified using C_{18} SPE column, and Fig. 4B shows the MECC electropherograms of extracts purified using both C_{18} and mixed mode SPE columns. Under optimum MECC conditions of 10 mM phosphate and 10 mM borate

Table 2
Response characteristics of cytokinins using micellar electrokinetic capillary chromatography under optimized conditions

Cytokinin	Migration time (min)		Equation of calibration curve[a]	R^2	Linear range (μM)	Detection limit (μM)
	Mean	R.S.D. (%)				
ZOG	6.4	0.7	$y = 146.33x + 889.71$	0.992	25–200	2.2
DZOG	6.7	0.7	$y = 255.62x - 2895.2$	0.994	30–200	14.0
Z	7.4	0.9	$y = 221.67x - 303.81$	0.997	25–200	4.9
DZ	7.6	0.7	$y = 257.58x + 1719.6$	0.999	20–200	9.8
mTR	7.9	1.2	$y = 307.39x + 1824.3$	0.991	12.5–200	3.5
BA	9.8	1.1	$y = 431.00x - 1848.9$	0.996	20–200	7.0
iP	10.2	0.9	$y = 454.28x - 6887.1$	0.994	25–200	18.0

[a] In the calibration equations, x represents concentration of the analyte (μM) and y represents the peak area (μAu s).

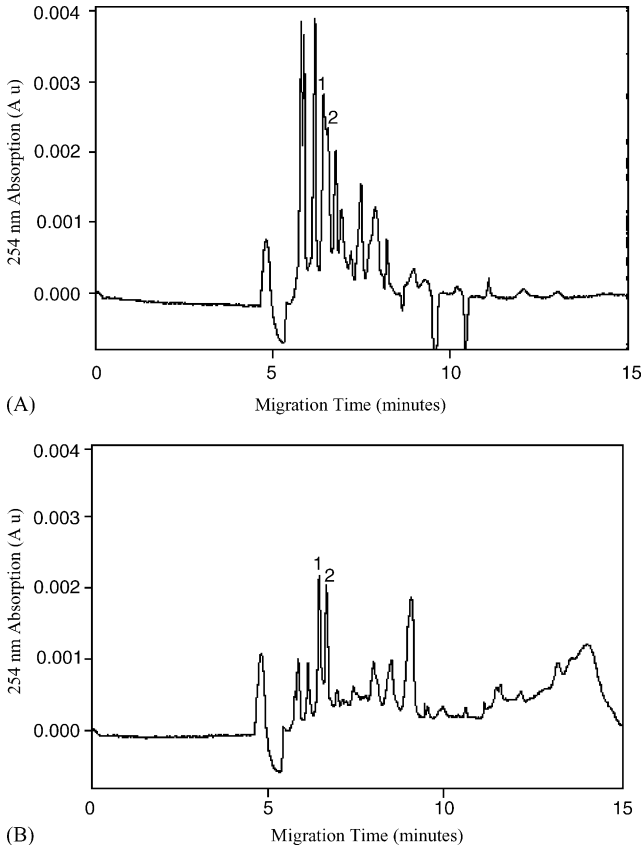


Fig. 4. Electropherograms of putative cytokinins in coconut water using MECC under optimized conditions. (A) Extracts purified using C_{18} SPE columns only. (B) Extracts purified using both C_{18} SPE and MCX SPE columns. Buffer solution contained a combination of 10 mM phosphate and 10 mM borate buffer (pH 10.4) with 50 mM SDS (pH 10.4). Other experimental conditions were as described in Fig. 2.

buffer of pH 10.4 containing 50 mM SDS, the presence of ZOG and DZOG in the purified coconut water extracts was successfully screened based on their migration times as well as by using standard addition method. The estimated concentrations of ZOG and DZOG in the coconut water were 97.9 pmol mL^{-1} (R.S.D. = 1.4%, n = 3) and 71.9 pmol mL^{-1} (R.S.D. = 2.2%, n = 3), respectively, disregarding the loss during the purification steps. Thus, the absolute levels of these *O*-glucosides in coconut water will certainly be higher if we had used ^{3}H-labelled cytokinins as recovery markers. The further identification and quantification of the other cytokinins (cytokinin nucleotides, bases and ribosides) in coconut water is now under further investigation, such as the possible occurrence of cytokinin nucleotides in eluate one.

Our data indicating the presence of cytokinin *O*-glucosides in coconut water will definitely be useful to researchers using coconut water as a growth supplement in tissue culture medium. So far, the application of CE for the identification of cytokinins in plant samples have been reported by Pacáková et al. [19], whereas Liu et al. [20], have reported on the CE detection of abscisic acid in tobacco flowers. However, no attempts were made to estimate the levels of cytokinins in these two CE approaches.

3.3. HPLC experiments

The identities of the putative ZOG and DZOG in the coconut water were further evaluated using HPLC. Both isocratic elution and gradient elution were tested to find the optimum separation conditions for the two putative *O*-glucosides. With respect to separation efficiency and shorter run time, the gradient elution approach was eventually used as the preferred method (the column was eluted isocratically with methanol–acetic acid buffer (5:95, v/v) for 3 min, then a linear gradient to methanol–acetic acid buffer (25:75, v/v) in 30 min, and finally isocratically at methanol–acetic acid buffer (25:75, v/v) for 17 min). In gradient elution, different compounds are eluted by increasing the strength of the organic solvent. The sample was injected while a weaker mobile phase was being applied to the system. The strength of the

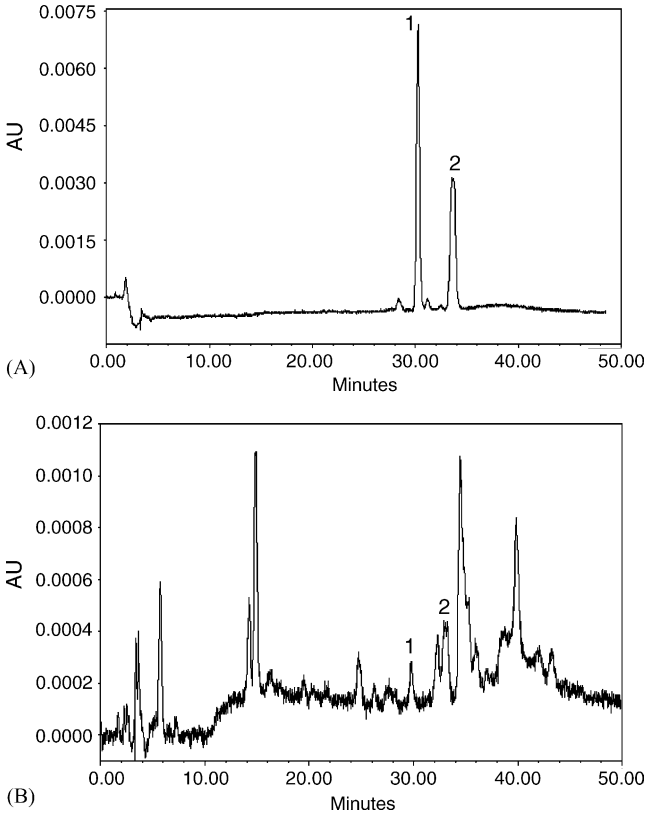


Fig. 5. UV profiles of standard cytokinin *O*-glucosides and putative cytokinin *O*-glucosides in purified coconut water extracts using HPLC. (A) UV profile of ZOG and DZOG standards at 269 nm. (B) UV profile of putative ZOG and DZOG in purified coconut water extracts at 269 nm. *Note*: Injection volume was 10 μL for cytokinin standards and 30 μL for the purified coconut water extracts. Coconut water extracts were purified earlier using both C_{18} SPE and MCX SPE columns. HPLC running conditions: the column was eluted isocratically with methanol–acetic acid buffer (5:95, v/v) for 3 min, then a linear gradient to methanol–acetic acid buffer (25:75, v/v) in 30 min, and finally isocratically at methanol–acetic acid buffer (25:75, v/v) for 17 min. Peak identities: (1) ZOG; (2) DZOG.

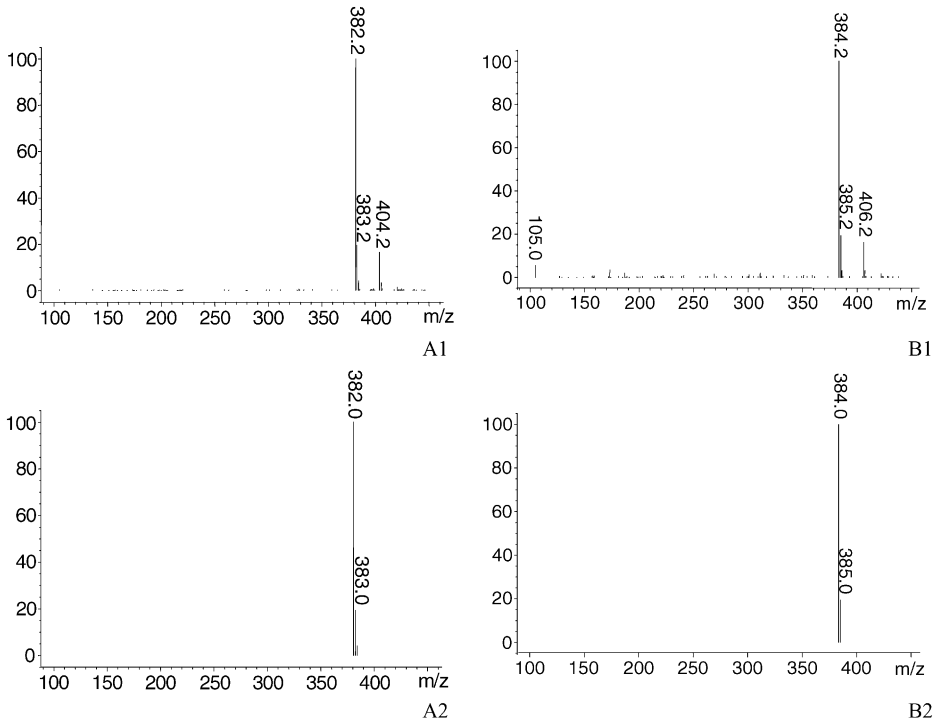


Fig. 6. LC–ESI-MS electrospray spectra of standard cytokinin *O*-glucosides and putative cytokinin *O*-glucosides in purified coconut water extracts in SIM mode. (A1) Electrospray spectrum of ZOG standard. (B1) Electrospray spectrum of DZOG standard. (A2) Electrospray spectrum of putative ZOG in purified coconut water extracts. (B2) Electrospray spectrum of putative DZOG in purified coconut water extracts. *Note*: LC–ESI-MS electrospray was carried out in positive ion and scan mode between *m*/*z* 50 and 450 per second. Coconut water extracts were purified earlier using both C_{18} SPE and MCX SPE columns. LC conditions were as described in Fig. 5.

mobile phase was later increased in increments by raising the organic solvent fraction, which subsequently resulted in elution of the retained components. This was done in a stepwise or linear fashion. Gradient elution achieved the following improvements over isocratic elution: better resolution, and equal bandwidths (Fig. 5) [42]. Fig. 5A shows the representative HPLC chromatograms of a mixture of ZOG and DZOG standards at 269 nm wavelength. The retention times of ZOG and DZOG were 30.1 min (R.S.D. = 0.7%, n = 3) and 34.3 (R.S.D. = 0.5%, n = 3), respectively. On the basis of retention time as well as the results obtained from using the standard addition method (data not shown), the presence of ZOG and DZOG in the coconut water was highly probable (Fig. 5B). Despite the many efforts taken to shorten the time taken for HPLC separation, the total run time typically exceeded an hour due to the need for column washing (methanol–acetic acid buffer (95:5, v/v) for 5 min) and column equilibration (15 min).

3.4. LC–ESI-MS measurements

The aim of this MS investigation was to confirm the identity of the two putative cytokinin O-glucosides found in coconut water. For the interface, ESI mode was selected on the basis that it was suitable for ionizing polar and non-polar compounds. ESI is a mild ionization method that produces some related molecular ions and these ions are typically protonated molecular ions $[M + H]^+$. Information on the molecular weight of these compounds can be easily obtained [43]. The electrospray spectra of ZOG and DZOG are shown in Fig. 6A1 and B1. For the purified coconut water extracts, ZOG was eluted at 30.8 min and was accompanied by a *m*/*z* of 382.0 as the $[M + H]^+$ ion and a *m*/*z* of 383.0 as the $[M + 2]^+$ ion; DZOG was eluted at 34.1 min and was accompanied by a *m*/*z* of 384.0 as the $[M + H]^+$ ion and a *m*/*z* of 385.0 as the $[M + 2]^+$ ion (Fig. 6A2 and B2). The LC retention times and the electrospray spectra of these two putative compounds in the purified samples, matched those observed in the earlier HPLC results and the electrospray spectra of authentic standards, respectively.

4. Conclusions

This work demonstrated that MECC is one possible method for screening for some putative cytokinins in coconut water after appropriate sample pre-concentration and purification. C_{18} SPE columns have been successfully used as a pre-concentration tool while further sample purification was carried out using mixed mode SPE columns. A buffer solution with 10 mM phosphate and 10 mM borate buffer (pH 10.4) containing 50 mM SDS, provided favourable conditions to separate the seven cytokinins. The method displayed good reproducibility and was linear over standard concentrations

of 30–200 μM. One major advantage of the present CE technique is that the separation was completed within a shorter time when compared to the HPLC approach [39]. Z and DZ were resolved easily under the present conditions. The identity of the two major cytokinin *O*-glucosides detected in coconut water was further confirmed by independent HPLC and LC–MS experiments. For future work, it will be interesting to couple the CE approach to MS for the analyses of cytokinins in biological samples.

Acknowledgements

The authors are grateful to Nanyang Technological University (NTU, Singapore) for financial support. L.G. acknowledges the award of NTU Postgraduate Research Scholarship. We are grateful to Agilent Technologies Singapore Pte Ltd for the technical assistance in performing the LC–MS experiments. The gifts of cytokinin standards from Stuart Letham, and discussions with Charles Hocart and Hua Lin are much appreciated.

References

[1] C.B. Taylor, Plant Cell 9 (1997) 981.
[2] K. Zhang, D.S. Letham, P.C.L. John, Planta 200 (1996) 2.
[3] N.T. Thanh-Tuyen, E.V. De Guzman, Plant Sci. Lett. 29 (1983) 81.
[4] S. Kintzios, J.B. Drossopoulos, E. Shortsianitis, D. Peppes, Scientia Hort. 85 (2000) 137.
[5] T. Tiainen, Plant Sci. 88 (1993) 83.
[6] C. Riou-Khamlichi, R. Huntley, A. Jacqmard, J.A.H. Murray, Science 283 (1999) 1541.
[7] K. Furuhashi, Plant Sci. 79 (1991) 241.
[8] D.W.S. Mok, M.C. Mok (Eds.), Cytokinins: Chemistry, Activity and Function, CRC Press, Boca Raton, FL, 1994, p. 1 (Chapter 1).
[9] K. Vermeulen, M. Strnad, V. Kryštof, L. Havlíček, A. Van der Aa, M. Lenjou, G. Nijs, I. Rodrigus, B. Stockman, H. van Onckelen, D.R. Van Bockstaele, Z.N. Berneman, Leukemia 16 (2002) 299.
[10] M. Hajdúch, L. Havlíček, J. Veselý, R. Novotný, V. Mihál, M. Strnad, Adv. Exp. Med. Biol. 457 (1999) 341.
[11] S.I.S. Rattan, B.F.C. Clark, Biochem. Biophys. Res. Commun. 201 (1994) 665.
[12] R.C. Gallo, J. Whang-Peng, S. Perry, Science 165 (1969) 400.
[13] H. Meisel, S. Gunter, D. Martin, E. Schlimme, FEBS Lett. 433 (1998) 265.
[14] P. Thibault, Dovichi N.J., in: P. Camilleri (Ed.), Capillary Electrophoresis—Theory and Practice, CRC Press, London, 1998, p. 23 (Chapter 2).
[15] S.N. Tan, L. Hua, Curr. Top. Anal. Chem. 3 (2002) 83.
[16] P. Barták, P. Bednář, Z. Stránský, P. Boček, R. Vespalec, J. Chromatogr. A 878 (2000) 249.
[17] P. Barták, D. Pěchova, P. Tarkowski, P. Bednář, M. Kotouček, Z. Stránský, R. Vespalec, Anal. Chim. Acta 421 (2000) 221.
[18] P. Barták, J. Ševčík, T. Adam, D. Friedecký, K. Lemr, Z. Stránský, J. Chromatogr. A 818 (1998) 231.
[19] V. Pacáková, K. Štulík, V. Vlasáková, A. Březinová, J. Chromatogr. A 764 (1997) 331.
[20] B.F. Liu, X.H. Zhong, Y.T. Lu, J. Chromatogr. A 945 (2002) 257.
[21] A. Székács, G. Hegedüs, I. Tóbiás, M. Pogány, B. Barna, Anal. Chim. Acta 421 (2000) 135.
[22] G.M. Banowetz, in: D.W.S. Mok, M.C. Mok (Eds.), Cytokinins: Chemistry, Activity and Function, CRC Press, Boca Raton, FL, 1994, p. 305 (Chapter 23).
[23] C.H. Hocart, J. Wang, D.S. Letham, J. Chromatogr. A 811 (1998) 246.
[24] J. Barcizewski, M. Mielcarek, M. Stobiecki, Biochem. Biophys. Res. Commun. 279 (2000) 69.
[25] V. Vlasáková, A. Březinová, J. Holík, J. Pharm. Biomed. Anal. 17 (1998) 39.
[26] C.C. Chou, W.S. Chen, K.L. Huang, H.C. Yu, L.J. Liao, Plant Physiol. Biochem. 38 (2000) 309.
[27] C. Chen, in: H.F. Linskens, J.F. Jackson (Eds.), High Performance Liquid Chromatography in Plant Sciences, Springer, Berlin, 1987, p. 23.
[28] P. Redig, O. Shaul, D. Inzé, M.V. Montagu, H. van Onckelen, FEBS Lett. 391 (1996) 175.
[29] N.M. Brito, S. Navickiene, L. Polese, E.F.G. Jardim, P.B. Abakerli, M.L. Ribeiro, J. Chromatogr. A 957 (2002) 201.
[30] P.I. Dobrev, M. Kamínek, J. Chromatogr. A 950 (2002) 21.
[31] E.M. Shantz, F.C. Steward, J. Am. Chem. Soc. 74 (1952) 6133.
[32] E.F. George, Plant Propagation by Tissue Culture, Part 1, The Technology, 2nd ed., Exegetics, Edington, 1993, p. 319.
[33] E.M. Shantz, F.C. Steward, J. Am. Chem. Soc. 77 (1955) 6351.
[34] D.S. Letham, in: F. Wightman, G. Setterfield (Eds.), Biochemistry and Physiology of Plant Growth Substances, Runge Press, Ottawa, 1968, p. 19.
[35] J. Van Staden, S.E. Drewes, Physiol. Plant 34 (1975) 106.
[36] H. Kobayashi, N. Morisaki, Y. Tago, Y. Hashimoto, S. Iwasaki, E. Kawachi, R. Nagata, K. Shudo, Experientia 51 (1995) 1081.
[37] H. Kobayashi, N. Morisaki, Y. Tago, Y. Hashimoto, S. Iwasaki, E. Kawachi, R. Nagata, K. Shudo, Chem. Pharm. Bull. (Tokyo) 45 (1997) 260.
[38] J. Arditti, R. Ernst, Micropropagation of Orchids, Wiley, 1992, p. 47 (Chapter 2).
[39] J.W.H. Yong, S.C. Wong, D.S. Letham, C.H. Hocart, G.D. Farquhar, Plant Physiol. 124 (2000) 767.
[40] R. Horgan, I.M. Scott, in: L. Rivier, A. Crozier (Eds.), Principles and Practice of Plant Hormone Analysis, Academic Press, London, 1987, p. 303 (Chapter 5).
[41] S. Terabe, K. Otsuka, K. Ichikawa, A.A. Tsicjoua, T. Ando, Anal. Chem. 56 (1984) 111.
[42] L.R. Synder, M.A. Stadalius, M.A. Quarry, Anal. Chem. 55 (1983) 1412.
[43] R.E. Ardrey, Liquid Chromatography–Mass Spectrometry: An Introduction, Wiley, Chichester, 2003, p. 75 (Chapter 4).