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*Article*

# Analyses of Phytohormones in Coconut (*Cocos Nucifera* L.) Water Using Capillary Electrophoresis-Tandem Mass Spectrometry

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**Abstract:** Capillary electrophoresis (CE) coupled with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) is reported as an alternative and potentially useful method for the simultaneous analysis of various classes of phytohormones with diversified structures, including indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), abscisic acid (ABA), gibberellic acid (GA), zeatin (Z), $N^6$-benzyladenine (BA), α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). The key to the CE-MS/MS analysis was based on electroosmotic flow reversal using a cationic polymer-coated capillary. Under optimum conditions, a baseline separation of eight phytohormones was accomplished within 30 min using 60 mM ammonium formate/formic acid buffer of pH 3.8 with −20 kV as the separation voltage. The accessibility of MS/MS together with the characterization by migration properties obtained by CE allows for the development of CE-MS/MS as an emerging potential method for the analysis of different classes of phytohormones in a single run. The utility of the CE-MS/MS method was demonstrated by the comprehensive screening of phytohormones in coconut (*Cocos nucifera* L.) water after pre-concentration and purification through solid-phase extraction (SPE) cartridge. IAA, ABA, GA and Z were detected and quantified in the purified coconut water extract sample.

**Keywords:** capillary electrophoresis; tandem mass spectrometry; phytohormones; cationic polymer-coated capillary; solid-phase extraction

## 1. Introduction

Phytohormones are a group of naturally occurring organic compounds that play crucial roles in mediating plant growth through a whole range of developmental processes. These structurally diverse compounds mainly include auxins, cytokinins, abscisic acid, gibberellins and ethylene [1–3]. In addition to the plant-related roles, some phytohormones show significant human health benefits and could potentially be used for biomedical applications [4–6]. Thus, the rapid analyses of phytohormones are of great importance to both plant physiologists, in understanding various physiological processes regulated by phytohormones, and scientists from various disciplines (e.g., clinicians and molecular biologists), especially in view of their potential role in medical applications.

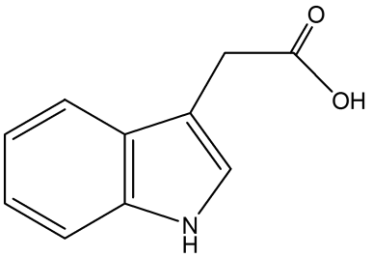
Although gas and liquid chromatography combined with mass spectrometry (GC-MS, LC-MS) [7–10] as well as enzyme-linked immunosorbent assay or radioimmunoassays (ELISA, RIA) [11,12] of the LC fractionated samples are the most commonly utilized techniques in the field of phytohormone analysis, capillary electrophoresis (CE) is steadily being used as an analytical technique particularly in the analysis and separation of solutes within a biological matrix [13–15]. Compared with LC and GC, CE is a separation technique with the following characteristics, namely simplicity in the method development, high separation efficiency, and low consumption of sample and reagents that would result in a low running cost. However, the complex nature of real plant extracts presents the main challenge for CE.

Mass spectrometry (MS) is a desirable mode of detection for CE, since it represents a higher sensitivity and universal specificity than any other available CE detectors and it has the advantage of positive analyte identification. Following the first investigation of Thomson (1912), the technologies associated with MS have progressed extremely rapidly [16]. More recently, CE-MS has emerged as a powerful tool for the characterization of charged species, and this technology has been successfully applied for the analyses of phytohormones, such as cytokinins [17,18], and gibberellins [19,20]. However, to our knowledge, so far there are very limited reported works carried out using the CE-MS approach to simultaneously separate or determine different classes of phytohormones in a single experiment [21].

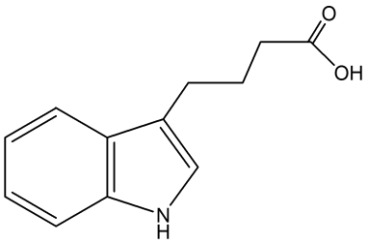
Previously, there were a few papers reported for the determination of phytohormones by CE with UV detection [13–15]. Unfortunately, the CE methods could not be converted to CE-MS, due to the fact that MS sensitivity may deteriorate as the bulk flow of non-volatile buffer (e.g., phosphate ions) and/or surfactant enters the MS source region [22]. Chemically, most auxins (e.g., indole-3-acetic acid, indole-3-butyric acid, α-naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid), gibberellins (e.g., gibberellic acid) and abscisic acid contain a carboxylic group (Figure 1), which exist as anions at a relatively high pH level, while cytokinins are amphoteric compounds (Figure 1), which are able to ionize under different pH values. Analysis of anions by CE is usually performed in the negative mode, in which polycationic polymers were added to the buffer solution to reverse the direction of electroosmotic flow (EOF) [14]. However, it is quite difficult to transfer this methodology to CE-MS

experiments due to the experimental incompatibility of such a buffer modifier with MS. In the present study, this problem was overcome by employing cationic polymer-coated capillary to reverse the EOF [19]. By using these coated capillaries with positively charged surface, it was possible to create an EOF constantly towards the anode (MS side) without adding any buffer modifiers.

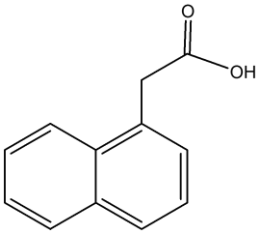
**Figure 1.** Chemical structures of phytohormones.



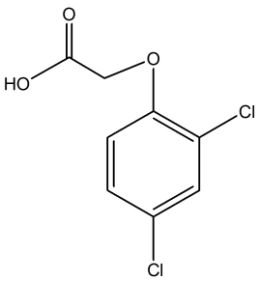

indole-3-acetic acid (IAA)



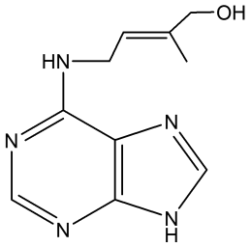

indole-3-butyric acid (IBA)



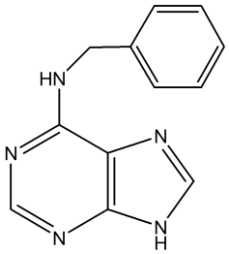

*α*-naphthaleneacetic acid (NAA)



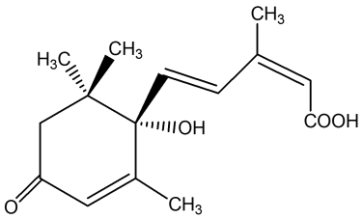

2,4-dichlorophenoxyacetic acid (2,4-D)



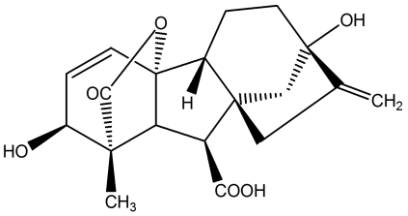

zeatin (Z)




$N^6$-benzyladenine (BA)




abscisic acid (ABA)




gibberellic acid (GA)

Since free phytohormones present in plants are at very low concentrations (typically nanogram per gram fresh weight or lower), the various problems associated with the isolation and identification of phytohormones in plant extracts are challenging [23]. Our original methodology employed $C_{18}$ solid-phase extraction (SPE) cartridges mainly for the pre-concentration of phytohormones [8]. However, mixed-phase SPE cartridges that combine reversed-phase and anion-exchange chromatography separation were obviously the ideal pre-concentration and sample clean-up method for phytohormones [24]. Therefore, in our current work, Mixed-Mode Anion-eXchange (MAX) SPE was employed as an efficient approach to pre-concentrate and purify phytohormones within a biological matrix.

As a further extension of our earlier works involving the analyses of cytokinins and gibberellins using CE-MS [17–20], we would like to report on a simple CE-MS/MS methodology combined with SPE for the simultaneous analysis of the major classes of phytohormones. The methodology could provide a

suitable approach for the analysis of the various classes of phytohormones, including indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), $\alpha$-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), zeatin (Z), $N^6$-benzyladenine (BA), gibberellic acid (GA), and abscisic acid (ABA) in a single analysis. The effects exerted by pH and concentration of buffer, applied voltage and sheath liquid on the resolution of CE separation were systematically investigated and optimized. With the optimized CE-MS/MS approach, selective identification and also structural elucidation of phytohormones were achieved. The developed methodology was then used to screen for the presence of endogenous phytohormones in coconut water after pre-concentration and sample clean-up using SPE. Four phytohormones (IAA, ABA, GA and Z) in young coconut (*Cocos nucifera* L.) water were successfully identified and quantified.

## 2. Experimental Section

### *2.1. Reagents and Materials*

The phytohormone standards: IAA (PhytoTechnology Laboratories, Shawnee Mission, KS, USA), IBA (Sigma-Aldrich, St. Louis, MO, USA), NAA (PhytoTechnology Laboratories), 2,4-D (PhytoTechnology Laboratories), Z (Sigma-Aldrich), BA (Sigma-Aldrich), GA (Sigma-Aldrich) and ABA (Sigma-Aldrich) were used in the analysis. Unless otherwise noted, all analytes were dissolved in ultra-pure water. Methanol (HPLC grade) was from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were of analytical reagent grade. Ammonium formate and ammonium acetate was purchased from Hayashi Pure Chemical Ind. Ltd (Osaka, Japan); formic acid and acetic acid were purchased from Fisher Scientific (Hanover Park, IL, USA); ammonia solution (28%) was purchased from APS Finechem (Seven Hills, Australia); ethanol was purchased from Merck (Darmstadt, Germany). Ultra-pure water (MilliQ, Waters, Milford, MA, USA) was used throughout the study. The pH value of the running buffer solutions was adjusted by mixing an equal concentration of two solutions in different ratios, which was monitored using a pH meter (CORNING 440, Corning Glass Works, Corning, NY, USA).

### *2.2. Instrumentation*

All CE-MS experiments were performed with an Agilent capillary electrophoresis system in conjunction with an Agilent Trap XCT mass spectrometer equipped with an Agilent CE-ESI-MS sprayer kit (G1607A) and an Agilent CE-ESI-MS adapter kit (G1603A) (Agilent Technologies, Waldbronn, Germany). The CE-ESI-MS adapter kit includes a capillary cassette, which facilitates thermostating the capillary, and the CE-ESI-MS sprayer kit, which simplifies coupling the CE system with MS system equipped with an electrospray source. The design of the sprayer consists of a triaxial flow arrangement, in which the CE eluent is mixed with a sheath liquid at the sprayer tip, and then nebulized with nitrogen gas. Sheath liquid with the flow rate of 4 μL $min^{-1}$ was delivered using a Hewlett Packard 1100 series isocratic pump equipped with a 1:100 splitter. All system control, data acquisition and data analysis were performed with the Agilent CE ChemStation software (Agilent Technologies, 2004) and Agilent LC/MSD Trap software (Agilent Technologies, 2004).

### *2.3. CE-ESI-MS Conditions*

Separations were carried out on commercially available SMILE(+), a cationic capillary coated with successive multiple ionic polymer layers [25], which was purchased from Nacalai Tesque (Kyoto, Japan). The capillary dimensions were 50 μm i.d. × 100 cm total length. The capillary temperature was thermostated to 20 °C. Prior to first use, a new capillary was pretreated with 50 mM acetic acid (pH 3.4) for 20 min. Before each injection, the capillary was preconditioned for 6 min by flushing with the running electrolyte. For sample injections, a small ultra-pure water plug was hydrodynamically injected into the capillary with an injection time of 2 s at 50 mbar; the sample previously dissolved in ultra-pure water, was then injected at a pressure of 50 mbar for 5 s; and finally the running electrolyte was injected at 50 mbar for 2 s.

ESI-MS analysis was performed in the negative mode, and the ion trap was scanned at *m/z* 50–400 in full scan mode. The maximum accumulation time for the ion trap was set at 200 ms and the target count was set at 100,000. The actual accumulation time was controlled by ion charge control (ICC), which was used to prevent ion saturation in the ion trap. The capillary voltage was set at 3500 V. The nebulizer gas pressure, drying gas flow rate and drying gas temperature for the ESI source were set at 15 psi, 5 L $min^{-1}$ and 350 °C, respectively. Other instrument parameters were optimized for generating the highest signal intensities. For the CE-MS/MS experiments, helium was adopted as the collision gas, and product ion scan was performed using the deprotonated molecular ion as the precursor ion. Negative product ion mass spectra of all the phytohormones were shown in Figure 2. The multiple reaction monitoring (MRM) mode was used to monitor the transitions from the precursor ions to the most abundant product ions.

**Figure 2.** The negative product spectra of (**A**) indole-3-acetic acid (IAA); (**B**) α-naphthaleneacetic acid (NAA); (**C**) indole-3-butyric acid (IBA); (**D**) zeatin (Z); (**E**) 2,4-dichlorophenoxyacetic acid (2,4-D); (**F**) $N^6$-benzyladenine (BA); (**G**) abscisic acid (ABA); and (**H**) gibberellic acid (GA) *, each with the deprotonated molecule ($[M-H]^-$) as a precursor ion. The chemical structures of the compounds are displayed and their cleavage sites are indicated with arrows pointing to the most abundant product ions and their *m/z*. Concentration of each analyte: 100 μM. For the CE-ESI-MS conditions, please refer to the Experimental Section.* During the MS fragmentation, there were five fragmentation sites for GA.

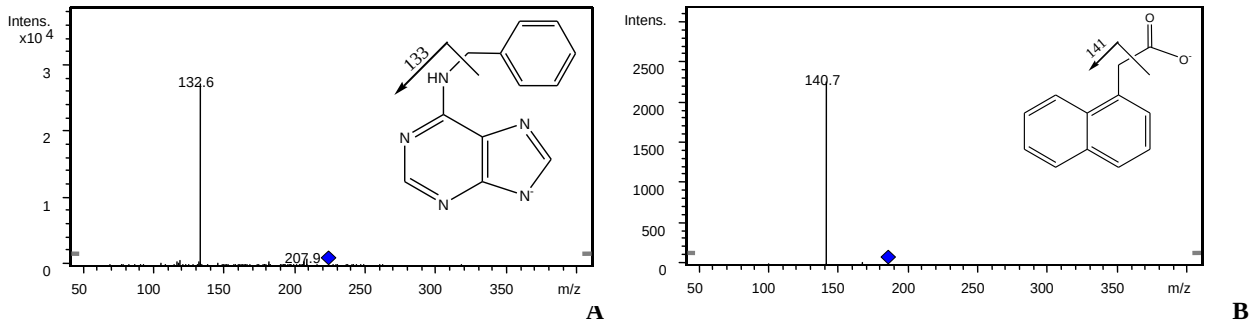
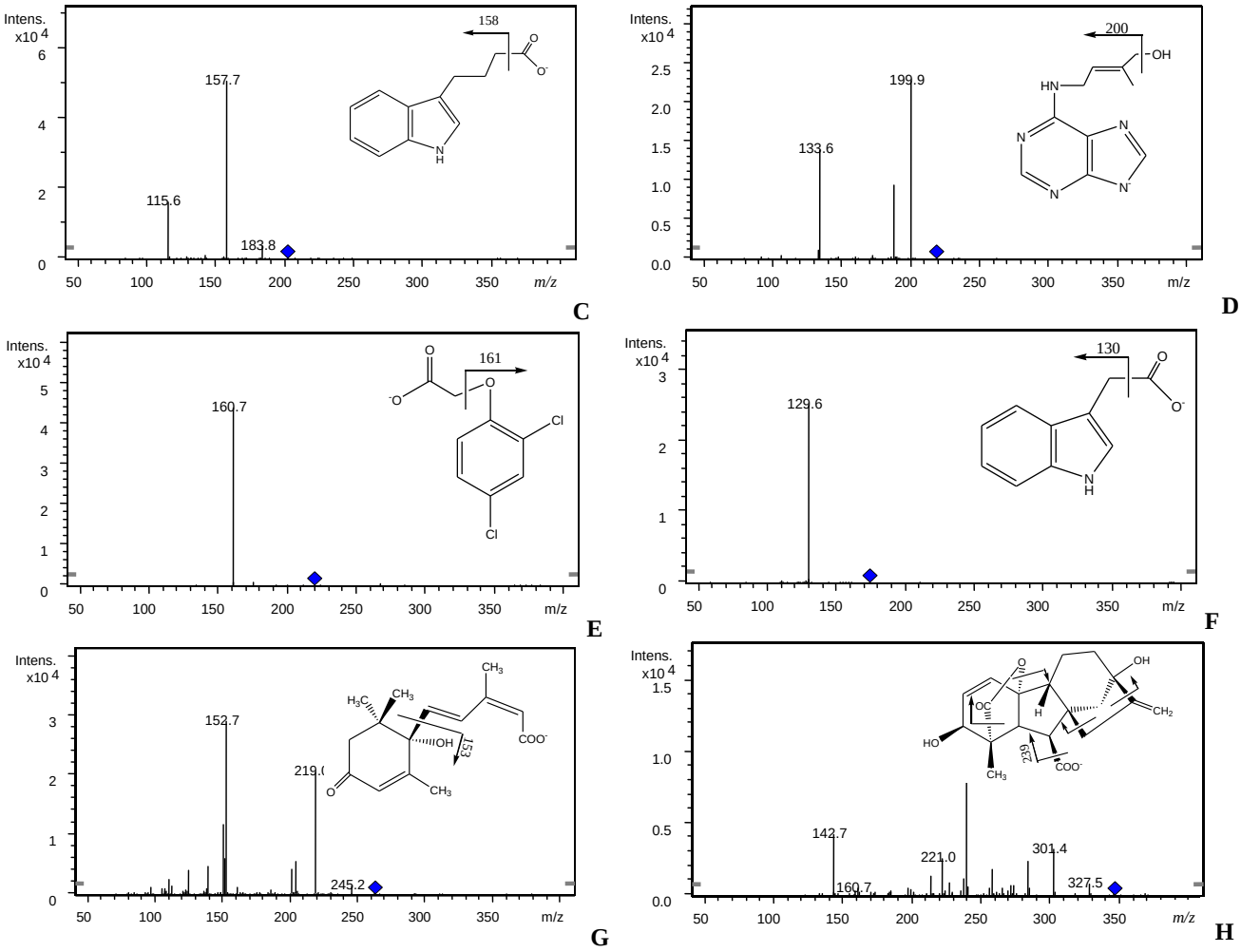


**Figure 2.** *Cont.*




*2.4. Sample Preparation*

Coconut water was obtained from fresh young coconut fruits of green variety (Emanate Agricultural Industries Ltd., Selangor, Malaysia), and the analysis was performed within two weeks after harvest. Before further pre-concentration and purification, the coconut water was spiked with 2% (v/v) formic acid and gone through filter paper (Whatman, 12.5 cm, No. 542) to remove suspended matters. The pre-concentration and purification of endogenous phytohormones using the SPE was performed according to Dobrev *et al.* [24] with some further modification. As the reported SPE protocol [24] was developed for extraction and purification of IAA and ABA, we further optimized and validated the protocol for the eight phytohormones.

The pre-conditioning of the Oasis MAX extraction cartridge (3 mL, 60 mg, Waters) was as follows: it was first washed with 5 mL of methanol and followed by 5 mL water. After 50 mL acidified eight phytohormone standard solution or coconut water were applied, cartridges were washed with 5 mL 1 M formic acid solution, then washed with 5 mL 1 M ammonia solution, and finally 5 mL 0.2 M ammonium hydroxide in 80% methanol. The putative phytohormones were eluted with 5 mL of 1 M formic acid in 80% methanol.

The recoveries of eight phytohormone standards ranged from 86.2% to 96.7%. The RSDs ($n$ = 6) of the recoveries for eight phytohormone standards were in the range of 3.4%–7.8%. The RSDs showed that this protocol is accurate for pre-concentration and purification of the eight phytohormones.

For coconut water samples, the eluates collected from twenty SPE cartridges were evaporated at room temperature and dissolved in 0.1 mL ultra-pure water for further CE-MS/MS analysis. Alternatively, the sample extracts were further diluted to the calibration range, and analyzed by CE-MS/MS for quantification of phytohormones with high concentrations (*i.e.*, IAA, ABA, and GA).

## 3. Results and Discussion

### *3.1. Optimization of CE Separation Conditions*

During the preliminary investigations, three buffer systems, including 50 mM ammonium acetate/ammonium hydroxide buffer of pH 10.0, 50 mM ammonium acetate/ammonium hydroxide buffer of pH 6.5, and 50 mM ammonium formate/formic acid buffer of pH 4.0, were studied to achieve the best resolution and shorten the overall analytical time for the eight phytohormones.

Since the cationic polymer-coated capillary was used in the current study, the reverse EOF was highly suppressed in the alkaline medium. Therefore, the analytes migrated to anode mainly by electrophoretic mobility at high pH level. At pH 10.0, IAA, IBA, NAA, 2,4-D, ABA and GA were fully negatively charged. As cytokinin bases have a basic pKa of 4.2 associated with the amino group at position 6 and an acidic pKa *ca.* 10 associated with the imidazole hydrogen at $N^9$ [26], Z and BA were only partially negatively charged. As a result, the separation time was *ca.* 120 min, when the buffer of pH 10.0 was used, which was considered too long to be an ideal separation time (Figure 3A).

When 50 mM ammonium acetate/ammonium hydroxide buffer of pH 6.5 was applied, the total separation was completed within 10 min (Figure 3B). However, total overlapping of the Z and BA peaks, and total or partial overlapping of IAA, NAA and 2,4-D peaks were observed. At this pH of 6.5, it was unlikely to resolve the eight phytohormones completely. Therefore, this buffer system was considered to be unsuitable as a potential separation buffer.

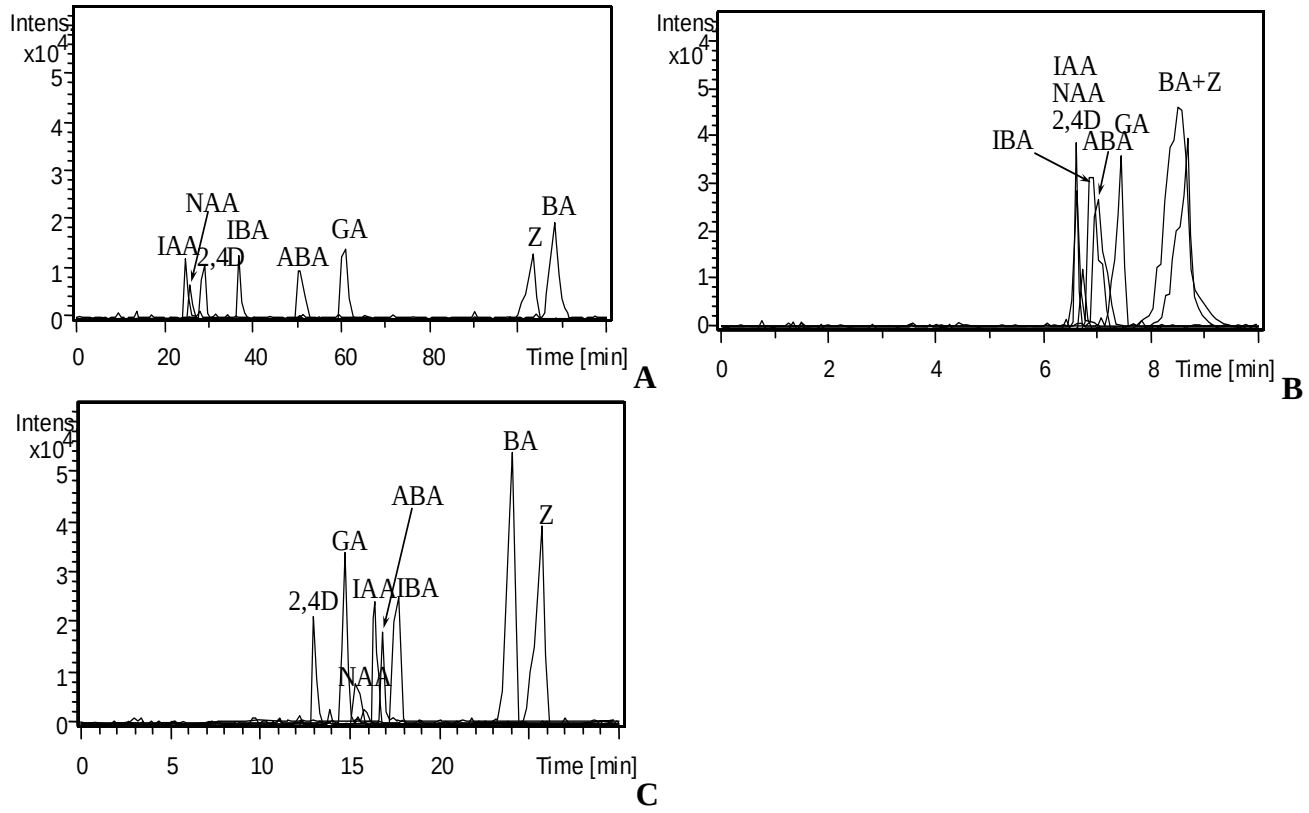
As indicated in Figure 3C, the best separations were achieved with an acidic buffer medium containing 50 mM ammonium formate/formic acid buffer (pH 4.0). At pH 4.0, IAA, IBA, NAA, 2,4-D, ABA and GA were partially negatively charged, while Z and BA were partially positively charged due to acidic pKa *ca.* 4. Owing to the high EOF by the low pH, the separation could be achieved in a reasonable time. Therefore, the acidic buffer would be applied for further optimization.

To simplify the study, the resolution of two neighboring peaks was employed to evaluate the optimum separation conditions in the present work. Typically, the resolution ($R_{A,B}$) of two neighboring peaks A and B was calculated with the following equation [27]:

$$R_{A,B} = \frac{2(t_B - t_A)}{W_B + W_A} \quad (1)$$

where $t_A$, and $t_B$ represent the migration time and $W_A$, and $W_B$ represent the width of the peaks A and B measured by extrapolating the relatively straight sides to the baseline, respectively.

**Figure 3.** Multiple reaction monitoring (MRM) mass electrophorograms of eight phytohormones using the buffer containing (**A**) 50 mM ammonium acetate/ammonium hydroxide buffer of pH 10.0; (**B**) 50 mM ammonium acetate/ammonium hydroxide buffer of pH 6.5; and (**C**) 50 mM ammonium formate/formic acid buffer of pH 4.0. Applied voltage: −20 kV; temperature: 20 °C; sheath liquid: 10 mM ammonium hydroxide in water–methanol (50:50, v/v).




The concentration of the running buffer and its pH are important parameters for obtaining efficient separations. The pH of the running buffer is a key parameter since it determines the extent of ionization of each solute, while the ionic strength of the buffer has a significant effect on the solute mobility and separation efficiency. Therefore, an attempt was made to optimize these conditions using a response surface methodology (RSM) to provide a map of responses in the form of a three-dimensional (3D) graph. RSM provides better results compared to classical optimization performed using one variable at a time [28,29].

The RSM was obtained by 30 independent experiments, with each central point repeated three times. The experiments indicated that there was no change in the migration order for the eight analytes throughout the whole range of buffer pH and concentrations examined. The migration times for the analytes increased in the order 2,4-D > GA > NAA > IAA > ABA > IBA > BA > Z. The effects of buffer pH and concentration on resolutions of neighboring peaks were plotted in the response surface map in Figure 4A–G. Although the resolution increased as ionic strength increase, it is at the cost of increased migration time. As a result, the 60 mM formate buffer of pH 3.8 was found to be a suitable compromise in resolving all the tested compounds.

**Figure 4.** Simultaneous effects of pH and buffer concentration (mM) on the resolutions of (**A**) 2,4-dichlorophenoxyacetic acid (2,4-D) and gibberellic acid (GA); (**B**) gibberellic acid (GA) and $\alpha$-naphthaleneacetic acid (NAA); (**C**) $\alpha$-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA); (**D**) indole-3-acetic acid (IAA) and abscisic acid (ABA); (**E**) abscisic acid (ABA) and indole-3-butyric acid (IBA); (**F**) indole-3-butyric acid (IBA) and $N^6$-benzyladenine (BA); and (**G**) $N^6$-benzyladenine (BA) and zeatin (Z) at the applied voltage −20 kV. For other experimental conditions, please refer to Figure 3C.

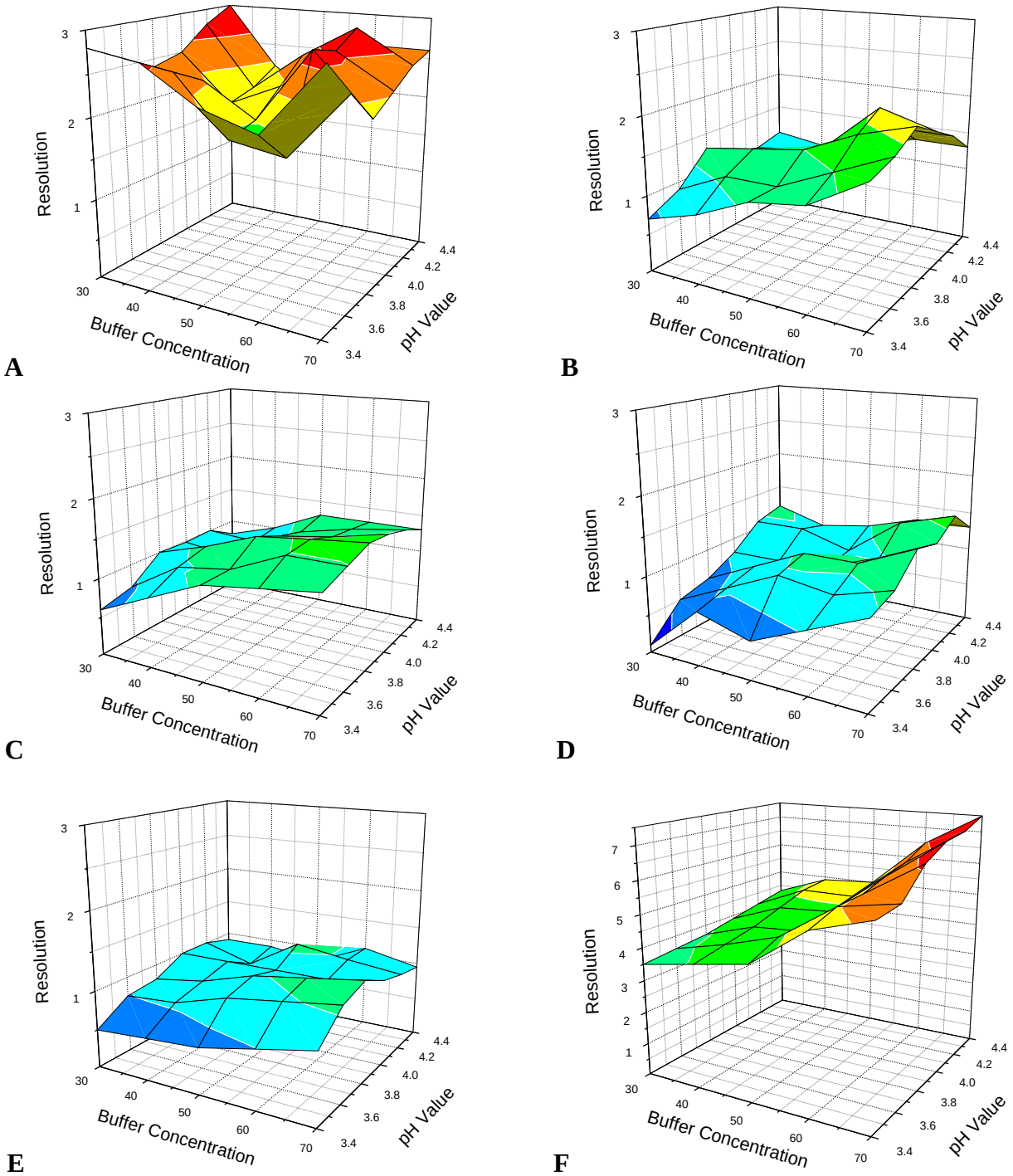
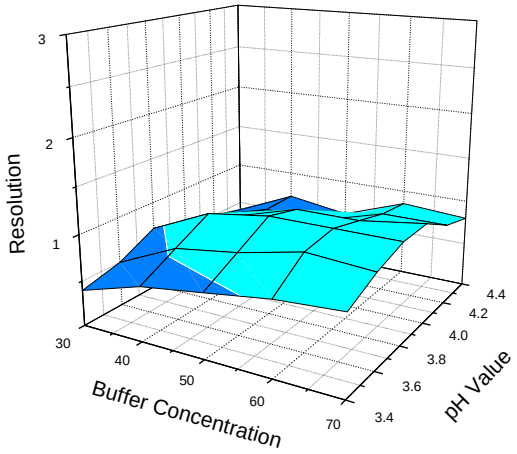


**Figure 4.** *Cont.*




**G**

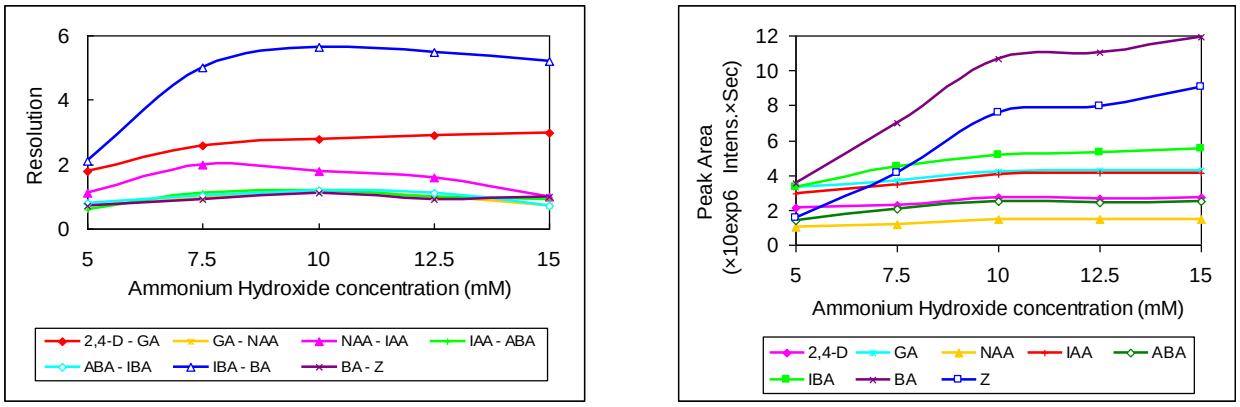
The effects of the voltage applied from 10 to 30 kV with reverse polarity were investigated using a running buffer consisting of 60 mM ammonium formate/formic acid (pH 3.8). Generally, with the increase of separation voltage, the migration time decreased and the peak shapes became sharper accordingly. However, higher voltages also exhibited higher currents and increased Joule heating. A voltage of −20 kV was considered to be the suitable voltage as the eight phytohormones were separated within a reasonable analysis time and provided satisfactory resolution. The separation of eight phytohormones under optimum conditions of 60 mM ammonium formate/formic acid buffer (pH 3.8) with a separation voltage of −20 kV was achieved within 30 min.

### *3.2. Selection of Sheath Liquid*

It is well known that the choice of sheath liquid has significant effects on the sensitivity for CE-MS analysis [30]. Different concentrations (5–15 mM) of ammonium hydroxide were dissolved in methanol–water (50:50, v/v), respectively, and the effect of each sheath liquid composition was investigated. We discovered that the sheath liquid did not only influence the sensitivity of analyte detection but also on the resolution of the separation [17]. A possible explanation for this phenomenon is the formation of moving ionic boundary during separation [31]. As shown in Figure 5A, the best resolutions for most neighboring peaks were obtained using 10 mM ammonium hydroxide in methanol–water (50:50, v/v).

The effects of sheath liquid on the peak response sensitivity of the eight phytohormones were also investigated. The peak intensity of analyte was calculated based on the peak area of each phytohormone on the MRM mass electropherogram. As shown in Figure 5B, the maximum values for most peak intensities were achieved with 10 mM or higher concentration of ammonium hydroxide in water–methanol (50:50, v/v) solution used as the sheath liquid. Based on the results, the optimum sheath liquid condition was 10 mM ammonium hydroxide in methanol–water (50:50, v/v), which resulted in optimum resolutions and higher intensities of MS signals.

**Figure 5.** Effects of ammonium hydroxide concentration in sheath liquid on (**A**) resolution; and (**B**) the sensitivity of phytohormones. The peak intensity of the analytes was calculated based on the peak area of each phytohormone on the multiple reaction monitoring (MRM) mass electropherograms. Experimental conditions: running buffer: 60 mM ammonium formate/formic acid buffer of pH 3.8; applied voltage: −20 kV; normal sample injection; flow rate of the sheath liquid: 4 μL $min^{-1}$. For CE-ESI-MS conditions, please refer to the Experimental Section.



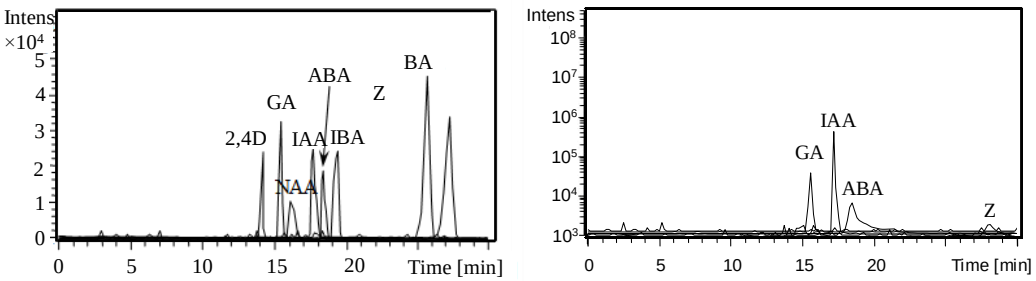

A B

As the ammonium hydroxide concentrations in sheath liquid affected both resolution and sensitivity of the eight phytohormones, the effects of 9 mM, 10 mM and 11 mM ammonium hydroxide (triplicate of each experiment) on resolutions for neighboring peaks and peak response sensitivity of eight phytohormones were studied for robustness testing. The results indicated that the small change in the conditions did not significantly ($p > 0.05$) affect the determination of phytohormones.

### *3.3. Validation of CE-MS/MS Method*

Figure 6A shows the MRM mass electropherograms of the eight phytohormone standard mixture obtained by CE-MS/MS under optimal conditions: 60 mM ammonium formate/formic acid buffer (pH 3.8).

**Figure 6.** Multiple reaction monitoring (MRM) mass electropherograms of (**A**) the eight phytohormone standard mixtures; and (**B**) coconut water obtained by capillary electrophoresis-tandem mass spectrometry (CE-MS/MS) under optimized conditions: a running buffer consisting of 60 mM ammonium formate/formic acid (pH 3.8); applied voltage: −20 kV; temperature: 20 °C; sheath liquid: 10 mM ammonium hydroxide in water–methanol (50:50, v/v).



The reproducibility of the migration time of the eight phytohormones under optimum CE-MS/MS conditions was investigated by doing repeated injections ($n$ = 6) of a mixture of standards at a concentration of 30 μM. The RSDs of the migration times for all analytes were in the range of 0.6%–1.1% (Table 1). The high reproducibility in migration time indicated that this method was accurate, robust and suitable for screening of putative phytohormones in biological samples. A linear correlation was found between the concentration and the peak area for all the phytohormones in the range of 5–50 μM; typically $R^2$ values were in the range of 0.988–0.994. Three independent injections were carried out for each calibration point. The quantitative data obtained from the analysis of phytohormones by CE-MS/MS are summarized in Table 1. The reproducibility of peak areas obtained for all the phytohormones with RSD values ($n$ = 6) was in the range of 3.4%~6.5% which are considered relatively low, due to the inherent limitation of the ion trap MS instruments for quantification work. Based on the S/N = 3, the LODs of CE-MS/MS method were in the range of 0.2–1.6 μM, which were comparable or even lower than HPLC-UV method [8]. Based on the S/N = 10, the LOQs of CE-MS/MS ranged from 0.7 to 5.2 μM depending on the analytes.

**Table 1.** Response characteristics of phytohormones using capillary electrophoresis-tandem mass spectrometry (CE-MS/MS) under optimized conditions *.

| Phytohormones | Migration Time (min) [a] | | MRM Transition | Peak Area (Cps × s) [a] | | Calibration Equation [b] | $R^2$ | LOQ (μM) | LOD (μM) |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | RSD (%) | (*m/z*) | Mean | RSD (%) | | | | |
| 2,4-D | 14.2 | 0.8 | 219→161 | 396,042 | 3.4 | y = 13148x + 1573 | 0.993 | 2.1 | 0.6 |
| GA | 15.4 | 0.7 | 345→239 | 466,253 | 4.2 | y = 15410x + 3961 | 0.994 | 1.6 | 0.5 |
| NAA | 16.2 | 0.8 | 185→141 | 308,019 | 3.8 | y = 10097x + 5109 | 0.991 | 5.2 | 1.6 |
| IAA | 17.8 | 0.9 | 174→130 | 432,170 | 4.1 | y = 14192x + 6395 | 0.992 | 2.3 | 0.7 |
| ABA | 18.4 | 0.7 | 263→153 | 389,975 | 4.7 | y = 24176x + 7372 | 0.993 | 3.9 | 1.2 |
| IBA | 19.5 | 0.6 | 202→158 | 494,861 | 3.9 | y = 15929x + 16923 | 0.994 | 2.0 | 0.6 |
| BA | 25.6 | 0.9 | 224→133 | 1,584,351 | 6.5 | y = 53396x − 17542 | 0.99 | 0.7 | 0.2 |
| Z | 27.1 | 1.1 | 218→200 | 1,273,235 | 5.9 | y = 42769x − 9838 | 0.988 | 0.9 | 0.3 |

* Optimum experimental conditions: running buffer: 60 mM ammonium formate/formic acid buffer of pH 3.8; applied voltage: −20 kV; temperature: 20 °C, sheath liquid: 10 mM ammonium hydroxide in water–methanol (50:50, v/v). For the other CE-ESI-MS conditions, please refer to the Experimental Section; [a] The data were measured with repeated injections under ($n$ = 6) of a mixture of the phytohormone standards at a concentration of 30 μM each; [b] In the calibration equations, x represents concentration of the analyte (μM) and y represents the peak area (Cps × s).

### *3.4. Analysis of Phytohormones in Coconut Water*

As the CE-MS/MS method provided high selectivity and sensitivity as well as good accuracy, precision, and robustness during the validation procedure, the major challenge was to apply this method to screen for naturally occurring phytohormones (different classes) in coconut water.

Under the optimum CE-MS/MS conditions (60 mM ammonium formate/formic acid buffer of pH 3.8), the presence of IAA, ABA, GA and Z in the purified coconut water extract was successfully screened based on ion transition used for the MRM detection as well as the migration time. Figure 6B shows the MRM mass electropherograms of coconut water extract sample, obtained by the CE-MS/MS

method. Despite the fact that coconut water is a complex biological matrix, which contains numerous endogenous organic compounds, the well-defined MRM mass electropherogram was obtained without significant interference from the other naturally present compounds in the matrix. Additionally, in this screening exercise, GA and Z were also identified, which demonstrated that the current method is more sensitive and selective than the previous developed HPLC-UV methods [8]. The estimated original concentration of naturally occurring phytohormones in the coconut water, disregarding the loss during the purification steps, was listed in Table 2.

**Table 2.** The levels of phytohormones in coconut water as determined by capillary electrophoresis-tandem mass spectrometry (CE-MS/MS) with multiple reaction monitoring (MRM) experiments.

| **Phytohormones** | **IAA** | **ABA** | **GA** | **Z** |
|---|---|---|---|---|
| Estimated Concentration ($\times 10^{-3}$ μM) | 198.2 | 15.3 | 49.6 | 0.12 |

In essence, this developed CE-MS/MS method allowed for the positive identification and quantification of phytohormones present in pmol $mL^{-1}$ range in coconut water. It is noted that the LODs of the current optimum CE-MS/MS method could not match the LODs of some reported LC-MS methods [9,10]. This limitation arises from the inherent low injection volume of analyte sample in the CE experiments. Nevertheless, compared with other analytical techniques, the CE-MS/MS method has several advantages: (i) various classes of phytohormones with diversified structures could be separated and analyzed without derivatization in a short analytical time; (ii) relatively high sensitivity could be achieved; (iii) phytohormones could be selectively determined without serious matrix interference, and (iv) running cost of CE-MS experiment is relatively low compared to GC-MS or LC-MS experiment. Furthermore, the present methodology provides high resolving power, excellent reproducibility and low sample consumption. Thus, the current CE-MS/MS method is still useful as a possible alternative analytical approach for phytohormone analyses. The higher LODs of the current CE-MS/MS method compared to LC-MS work will be compensated by further developments of new CE technology or by novel on-line pre-concentration method for future works. Meanwhile, it is anticipated that the sensitivity of the CE-MS method could be further improved by using the next generation of mass spectrometer coupled to CE.

## 4. Conclusions

This work demonstrated that CE-MS or CE-MS/MS can be used to separate different classes of phytohormones (including auxins, cytokinins, abscisic acid and gibberellin) with a special cationic polymer-coated capillary. Compared with the HPLC-UV technique for the analysis of phytohormones [8], the present CE-MS/MS technique offers improvement to LOD, unequivocal identification and quantification, and elimination of the adverse effects from matrix interference associated with the more conventionally applied UV detection method. The method was linear over eight phytohormone standard concentrations of 5–50 μM.

The effectiveness of this novel CE-MS/MS method was evaluated by the unambiguous identification and quantification of phytohormones in coconut water with the combination of reversed-phase anion-exchange SPE. Four phytohormones in coconut water, including IAA, ABA, GA and Z, were identified in a single analysis. The detection of various classes of phytohormones by our various work,

namely auxins, gibberellin, abscisic acid and cytokinins in coconut water is important to plant scientists and laboratories using coconut water routinely as a growth supplement in plant tissue culture, as it may in part provide an adequate biochemical explanation to the effectiveness of coconut water as a supplement in regulating plant growth and development. It is also plausible that there may be more endogenous phytohormones (known and novel ones) present in the coconut water that we were unable to detect due to the current LOD of our CE-MS/MS method.

Nevertheless, more work is still needed to further improve on the LOD of the present CE-MS approach in measuring phytohormones present in biological samples, as we are well aware of the current sensitivity of the various GC-MS and LC-MS approaches [7–10]. Also, we would like to evaluate our CE-MS method on a wider range of biological samples including leaves, roots, *etc*.

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## Author Contributions

Swee Ngin Tan: experimental setup, data acquisition, and manuscript preparation. Jean Wan Hong Yong: concept approval, data review and manuscript revision. Liya Ge: experimental design, data analysis and interpretation, and manuscript improvement.

## Conflicts of Interest

The authors declare no conflict of interest.

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