DOI:10.1021/jf904500e

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Detection and Quantification of Some Plant Growth Regulators in a Seaweed-Based Foliar Spray Employing a Mass Spectrometric Technique sans Chromatographic Separation

Kamalesh Prasad,* Arun Kumar Das, Mihir Deepak Oza, Harshad Brahmbhatt, Arup Kumar Siddhanta,* Ramavatar Meena, Karuppanan Eswaran, Mahesh Rameshchandra Rajyaguru, and Pushpito Kumar Ghosh*

Central Salt and Marine Chemicals Research Institute (Council of Scientific and Industrial Research), G. B. Marg, Bhavnagar 364 002, Gujarat, India

The sap expelled from the fresh harvest of *Kappaphycus alvarezii*, a red seaweed growing in tropical waters, has been reported to be a potent foliar spray. Tandem mass spectrometry of various organic extracts of the sap confirmed the presence of the plant growth regulators (PGRs) indole 3-acetic acid, gibberellin GA_3, kinetin, and zeatin. These PGRs were quantified in fresh state and after 1 year of storage by ESI-MS without recourse to chromatographic separation. Quantification was validated against HPLC data. The results may be useful in correlating with the efficacy of the sap. The methodology was extended to two other seaweeds. The method developed is convenient and precise and may find application in other agricultural formulations containing these growth hormones.

KEYWORDS: Mass spectrometric quantification; plant growth regulators; growth hormones; liquid seaweed fertilizer

INTRODUCTION

Seaweed extracts are utilized as foliar sprays for improved plant growth (*1*). Their efficacy is ascribed to the presence of auxins, cytokinins, and gibberellins besides other nutrients (*2*−*10*). The red seaweed *Kappaphycus alvarezii* (Doty) Doty, which grows in tropical waters, is cultivated extensively as a source of κ-carrageenan (*11*). The sap from the fresh weed can be mechanically expelled and is a promising foliar spray (*12*). Yields of many crops registered a pronounced increase upon application of the sap at 2.5−5.0% (v/v, dilution with water) level (Supporting Information, Table S1). The results achieved with soybean under rain-fed conditions have been reported recently, where 46% increase in yield was observed upon application of the sap at 12.5% concentration (*13*). Sugar cane and several other crops have also responded well to the sap (Supporting Information, Table S1) (*14*), and sugar cane trials are underway presently in the state of Uttar Pradesh over 30000 acres. A plant has been commissioned recently for commercial production of sap and κ-carrageenan from fresh seaweed. Elucidation of the specific PGR constituents present in the sap, and their absolute concentrations, is therefore of considerable importance.

There is extensive literature on the study of plant growth regulators (PGRs) in seaweeds through conventional bioassay and colorimetric tests (*15*−*19*). These assays provide useful indicators of the classes of PGRs present along with their gross estimates. However, individual constituents are hard to identify. With the introduction of new analytical techniques, studies were conducted by various research groups to characterize PGRs in various formulations in greater detail. Reports are available on the identification of iso-pentenadenosine (cytokinin), abscisic acid (ABA), adenine, indole acetic acid, *trans*-zeatin, *trans*-zeatin-riboside, dihydro-*trans*-zeatin, isophenyladenine, and isopentenyl adenosine from different seaweed extracts using GLC and GC-MS techniques (*20*−*22*). One difficulty is the somewhat tedious sample preparation involved. LC-MS is also employed widely by various researchers (*23*−*26*). It is a convenient technique but, as found in the course of the present work, resolution of certain PGRs may pose difficulties. We report herein analyses of the *K. alvarezii* sap for PGRs employing a mass spectrometric technique sans chromatography, which has been described previously for the study of various organic compounds in complex systems (*27*, *28*), although not for the study of PGRs. The technique involves MS/MS for identification followed by ESI-MS for quantification. With the help of this technique, four PGRs in the sap were identified and quantified. Quantification was validated against HPLC. The technique was further employed to elucidate the PGRs in two additional seaweed sap samples.

MATERIALS AND METHODS

Chemicals and Standards. Standard indole acetic acid (IAA) ($C_{10}H_9NO_2$, MW 175.18), kinetin ($C_{10}H_9N_5O$, MW 215.08), gibberellin GA_3 ($C_{19}H_{22}O_6$, MW 346.38), indole propionic acid (IPA) ($C_{11}H_{11}NO_2$, MW 189.21), indole butyric acid (IBA) ($C_{12}H_{13}NO_2$, MW 204.21), 6-benzyl amino purine (BAP) ($C_{12}H_{11}N_5$, MW 225.25), and 9-phenanthrene methanol (PM) ($C_{15}H_{12}O$, MW 208.26) were purchased from SD Fine Chemicals, Mumbai, India, whereas *trans*-zeatin ($C_{10}H_{13}N_5O$, MW 219.11), propyl paraben, methyl paraben, and potassium benzoate were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO. Diethyl

*Authors to whom correspondence should be addressed [e-mail (K.P.) kamlesh@csmcri.org, (A.K.S.) aks@csmcri.org, or (P.K.G.) pkghosh@csmcri.org].

 Published on Web 03/31/2010 © 2010 American Chemical Society

ether (DEE), *n*-butanol, dichloromethane (DCM), ethyl acetate, and ammonium hydroxide were purchased from Ranbaxy Chemicals, Mumbai, India. All chemicals were of analytical grade and were used as received without further purification. HPLC grade solvents were used for HPLC and MS experiments.

Preparation of PGR Samples. Standards were prepared as follows: 4.4 mg of IAA in 2 mL of MeOH, 1 mg of kinetin in 10 mL of MeOH/H_2O (9:1 v/v), 1 mg of *trans*-zeatin in 10 mL of MeOH/H_2O (9:1 v/v), and 1.7 mg of GA_3 in 2 mL of MeOH. PGR extracts of sap were prepared from fresh *Kappaphycus* sap sample obtained by mechanically expelling sap from the fresh seaweed as per the patented procedure (*12*), filtering the mass through a nylon cloth having mesh size approximately 20−50 μm and preserving the sap using a mixture of 0.02% propyl paraben, 0.2% methyl paraben, and 0.1% potassium benzoate. A specimen of the *K. alvarezii* used in the present work has been deposited in our institute herbarium for further referencing (AL-II-120-11). Auxins, gibberellins, and cytokinins were thereafter extracted from sap using DEE, ethyl acetate, and *n*-butanol, respectively, following literature procedures (Supporting Information, sections 2 and 3) (*18*). Note that for extraction with the above solvents, fresh sap was used each time. The extraction of the PGRs for the 1-year-old samples was carried out following the same protocol. The sap from *Gracilaria edulis* (AL-II-43-12) and *Sargassum tenerrimum* (AL-II-148-08) was also analyzed in a similar manner.

Colorimetric Assay. Standards were put on the TLC plates (Merck, Kieselgel 60 F_{254} precoated plates of dimension 20 × 20 cm with layer thickness of 0.25 mm) along with the corresponding organic extract of sap, that is, DEE extract for auxins, *n*-butanol extract for cytokinins, and ethyl acetate extract for gibberellins. The TLC plates were run with the following solvent compositions: isopropanol/ammonium hydroxide/water (8:1:1 v/v) for auxins; chloroform/water (4:1 v/v, the lower layer was used) for cytokinins; isopropanol/water (4:1 v/v) for gibberellins. The spots were visualized directly under UV (254 nm) in the case of cytokinins, whereas gibberellins and auxins were viewed after the spots had been sprayed with ethanolic sulfuric acid (120 °C) and Ehrlich's reagent, respectively. The corresponding spots were scooped out and eluted in methanol for gibberellin and auxin and in *n*-butanol for cytokinins and used for colorimetric estimations as described in the Supporting Information.

ESI-MS Measurements. For mass spectrometry, a Waters Q-Tof Micromass instrument equipped with an electrospray ionization interface, MCP detector, and Waters MassLynx software (version 4.0) was used. Samples were introduced by direct injection with a syringe pump. Standards of IAA, IPA, and IBA at concentrations of 140, 280, 560, and 1120 ppm and of GA_3 at concentrations of 25, 50, 100, 200, 400, and 800 ppm were prepared in methanol, whereas kinetin at concentrations of 10, 20, 62.5, 125, 250, 500, and 1000 ppm and *trans*-zeatin at concentrations of 19.75, 36, 75, and 150 ppm were prepared in *n*-butanol. Fifty microliters of acetic acid was added into each 10 mL aliquot of sample to facilitate ionization. The mass spectrometer was run employing direct flow injection technique. Mass fragmentation patterns of IAA and GA_3 were recorded in ESI negative mode (ESI^-), whereas the ESI positive mode (ESI^+) was employed for zeatin and kinetin. The mass spectrometric parameters were optimized for each PGR. Of these, desolvation temperature (150 °C), source temperature (90 °C), syringe rate (10 μL/min), ion energy (2.0 V), and collision energy (7.0 V) were maintained constant for all of the ESI-MS measurements, whereas capillary voltage and sample cone voltage were optimized for each sample. For MS/MS studies, the parameters were once again optimized to obtain proper mass fragmentation. Details of ESI-MS and MS/MS of standards are provided in the Supporting Information. Crude organic extracts of the sap were passed through a silica gel column (100−200 mesh, the dimensions of the column were 12.5 cm (height) × 2.5 cm (i.d.) and the total volume eluted was 25 mL) followed by filtration through a 0.45 μm Whatman glass microfiber filter prior to injection into the mass spectrometer.

Quantification of PGRs using mass spectroscopy was done according to the procedure of Hoffmann and Stroobant (*29*). The technique is based on comparison of the intensity of the signal corresponding to the product that has to be quantified with that of an internal standard (IS). IPA (590 ppm) and BAP (1000 ppm) were chosen as IS for IAA and cytokinins, respectively, as these yielded similar fragmented nuclei. PM (1400 ppm), which is soluble in MeOH, was used as internal standard for gibberellins. Calibration plots were made by varying the concentrations of standard PGRs while the concentration of IS remained fixed. Five measurements were made at each concentration. Organic extracts of sap were similarly analyzed after addition of the respective IS. Five measurements were made for each sample. Details are provided in the Supporting Information (section 6).

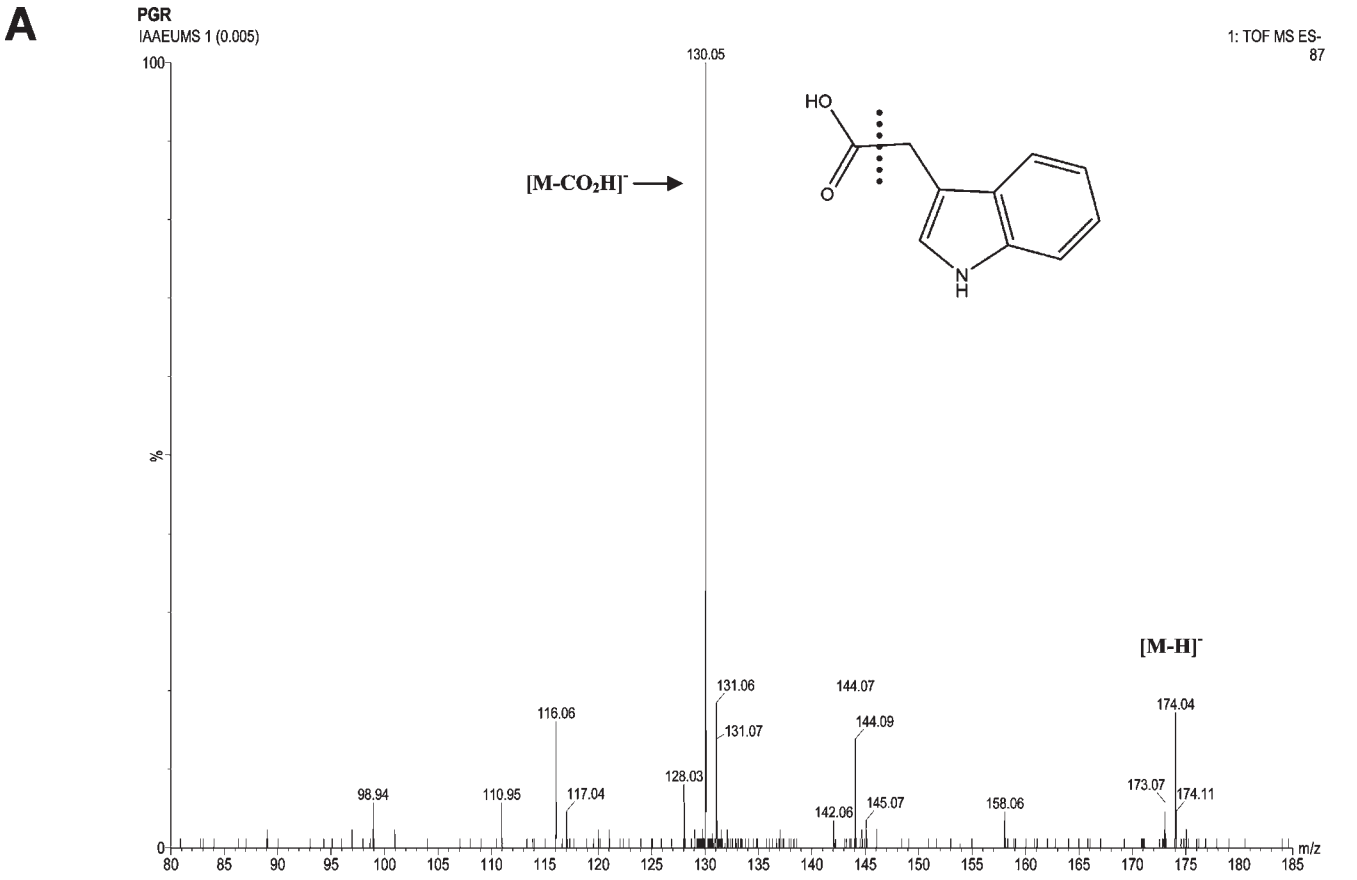
HPLC Analysis. The HPLC used was a Shimadzu instrument (Shimadzu Corp., Kyoto, Japan) comprising a Rheodyne injector, a 250 mm × 4.6 mm (i.d.) Nucleosil C18 stainless steel column (5 μm particle size, 300 Å pore size) (Sigma-Aldrich, Inc., St. Louis, MO), 6AD pumps, an SPD-10A UV−vis detector, and an LC-10 chromatography manager. Standards and organic extracts of sap were injected with a 20 μL syringe (Hamilton, Reno, NV). Isocratic elution was performed at 40 °C with MeOH/H_2O (3:7, v/v) mobile phase containing 0.1% acetic acid at a constant flow rate of 1.3 mL min^{-1}. UV detection was carried out at 254 nm except in the case of gibberellin, for which the detection wavelength was 205 nm (*17*). Besides retention time (t_R), LC-MS was used to ascertain the identities of the peaks. After their identification, concentrations of the different PGRs were estimated on the basis of HPLC peak areas measured against standards.

RESULTS AND DISCUSSION

The sap obtained from *K. alvarezii* has been analyzed previously for macro- and micronutrients, the most prominent of which is potassium (Supporting Information, Table S2). To our knowledge, the composition of PGRs in the sap has not been reported thus far, and therefore we undertook this study. To ascertain the different classes of PGRs in the sap, we carried out at first the conventional colorimetric analyses. The detection wavelengths used were 530 nm for auxins and 254 nm for cytokinins and gibberellins. The estimated concentrations in sap were 56.04 ± 8.68, 77.20 ± 17.38, and 128.42 ± 9.98 ppm for total auxins, total cytokinins, and total gibberellins, respectively (Supporting Information, section 4).

Identification of PGRs by ESI-MS and MS/MS. Colorimetric studies provided positive indication of the presence of auxin, cytokinin, and gibberellin constituents in the sap. To probe the constituents more precisely, MS spectra were recorded. The *m*/*z* peak at 174 for DEE extract (**Figure 1A**), the *m*/*z* peaks at 216 (**Figure 2A**) and 220 (**Figure 3A**) for *n*-butanol extract, and the *m*/*z* peak at 345 (**Figure 4A**) for ethyl acetate extract matched well with those of standard IAA, kinetin, *trans*-zeatin, and GA_3, respectively. The MS/MS fragmentation patterns also matched with those of the standard: 174 → 130 (IAA) (**Figure 1B**); 216 → 148 → 136 (kinetin) (**Figure 2B**); 220 → 148 → 136 (*trans*-zeatin) (**Figure 3B**); 345 → 239 (GA_3) (**Figure 4B**). Fragmentation patterns for the standards are provided in the Supporting Information, Figures S4, S5, S6, and S7. These results are in good agreement with reported data (*30*).

It can be observed from the above data that kinetin was detected in the sap in small amounts, although it is usually not considered to be a natural substance. However, Bentley-Mowat and Reid have previously reported the presence of kinetin like substances in algae (*31*). Furthermore, the presence of natural kinetin in DNA and cell extracts was reported by Barciszewski et al. (*32*) and, more recently, Liya Ge and co-workers have reported the presence of kinetin and kinetin riboside in coconut water by LC−tandem mass spectrometry (*33*). In our studies the mass fragmentation was found to be identical to that of an authentic sample of kinetin and also tallied with the mass spectra available in the database (www.massbank.jp). A second sample of *Kappaphycus* sap was also subjected to analysis, and the presence of kinetin was once again observed. As kinetin is responsible for cell division, it may contribute toward the overall efficacy of the sap. Kinetin was, however, absent in the sap obtained from the brown seaweed *Sargassum tenerrimum* and the red seaweed *Gracilaria edulis*. However, we were able to detect IAA (13.2 ppm), zeatin (11.40 ppm), and GA_9 in the former, whereas the latter contained IPA (9.11 ppm), zeatin (14.24 ppm), and GA_3 (23.14 ppm) (Supporting Information, section 8). The main limitation of the



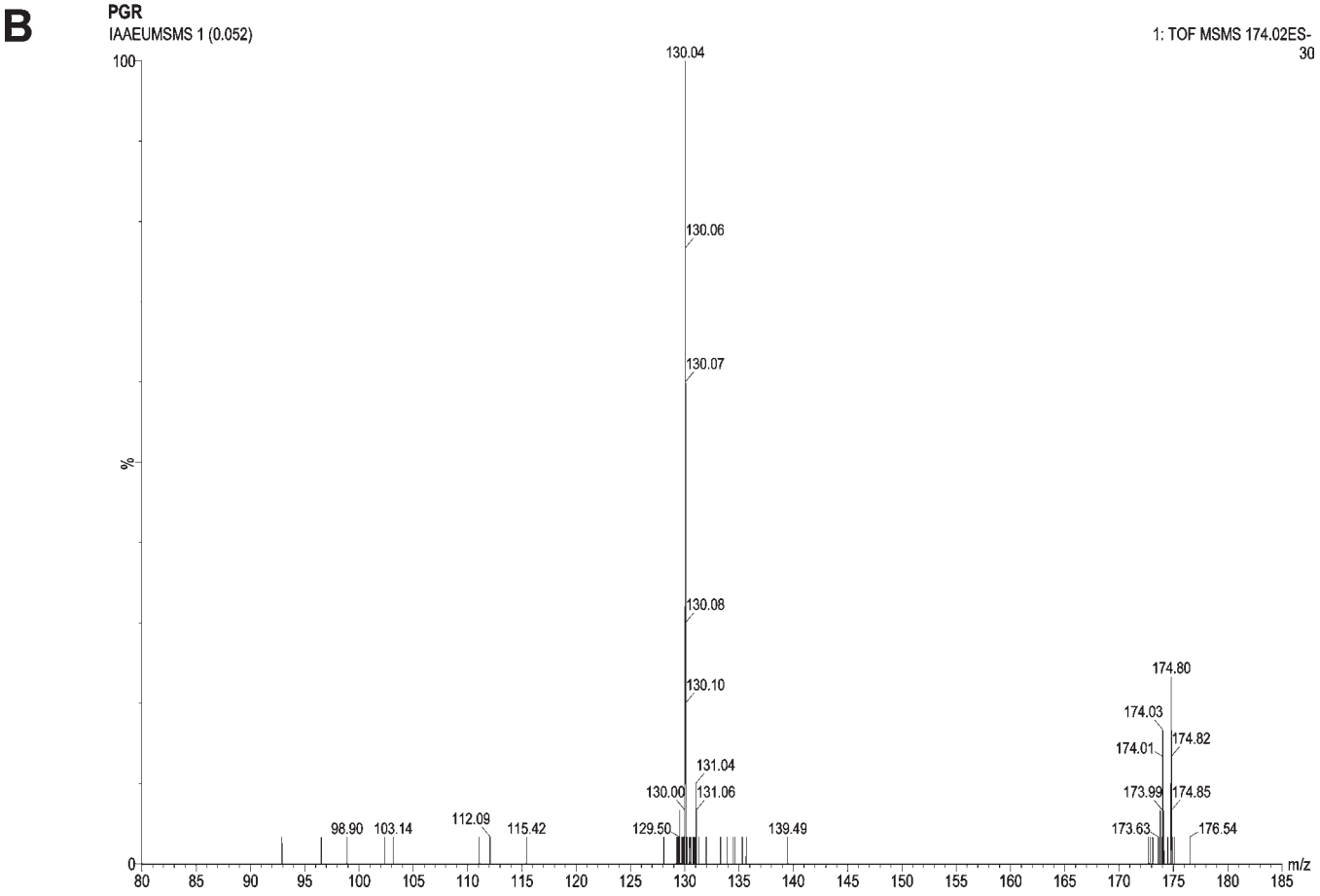


Figure 1. (**A**) Mass spectrum of IAA fraction extracted from sap. (**B**) MS/MS spectrum of precursor ion $(M - H)^-$ (*m*/*z* 174.04) in **A**.

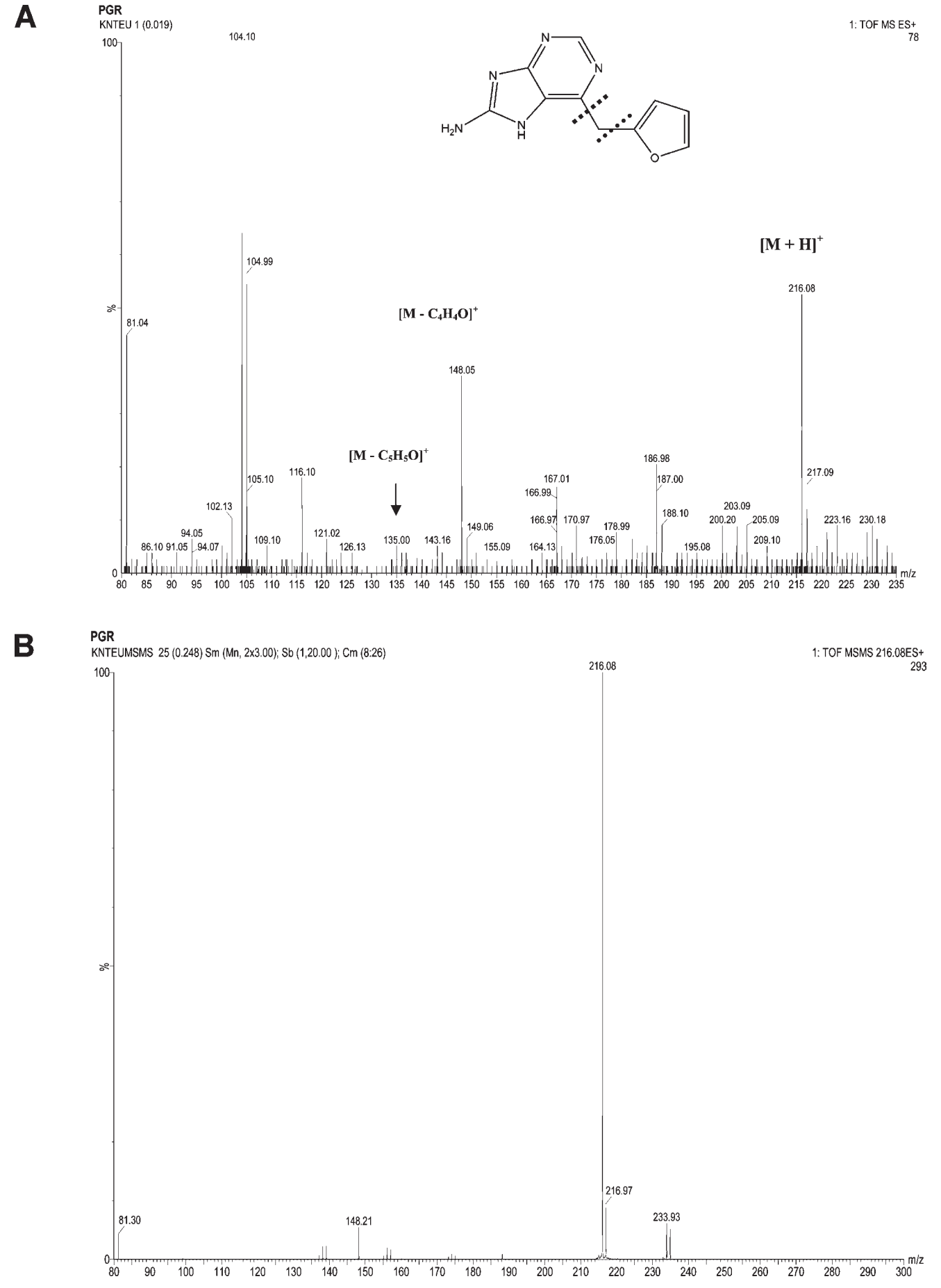
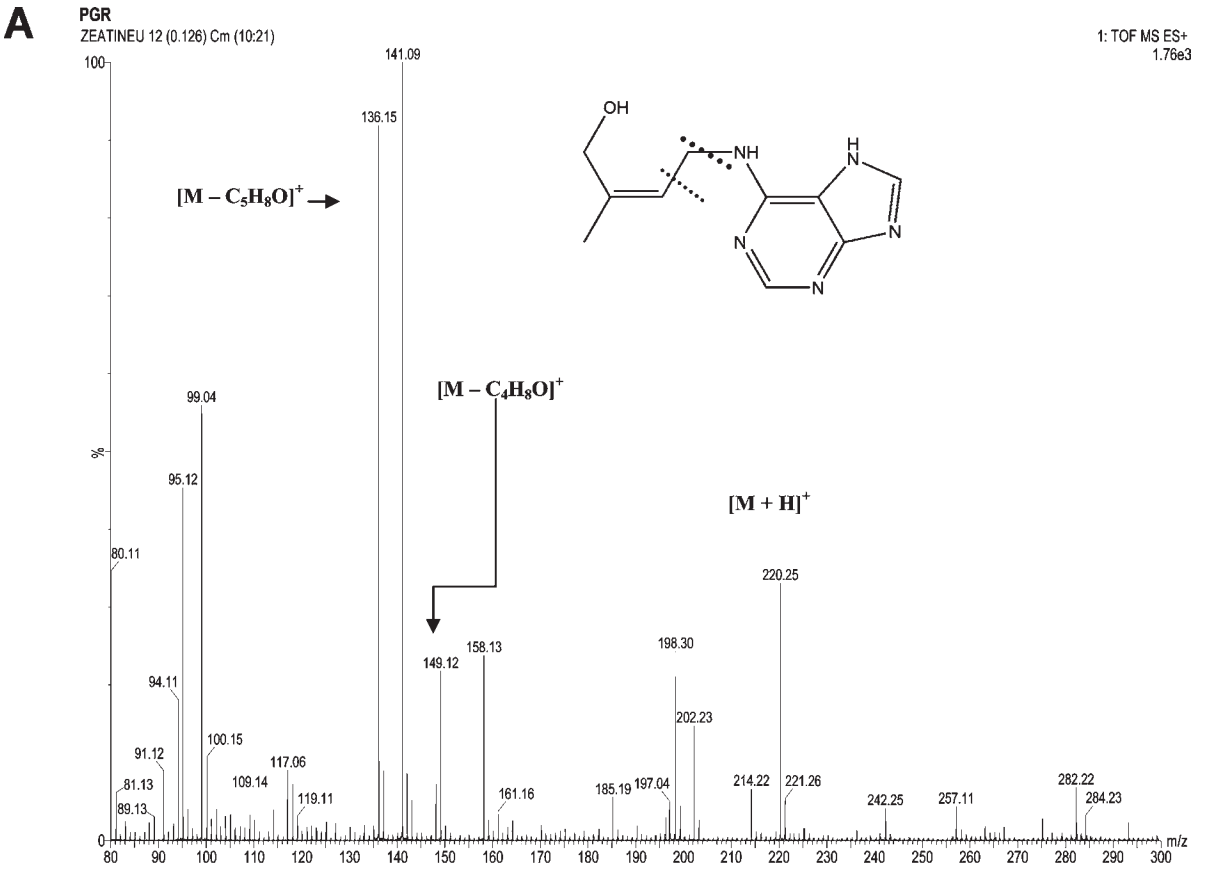


Figure 2. (**A**) Mass spectrum of kinetin fraction extracted from sap . (**B**) MS/MS spectrum of precursor ion $(M + H)^+$ (*m*/*z* 216.08) in **A**.



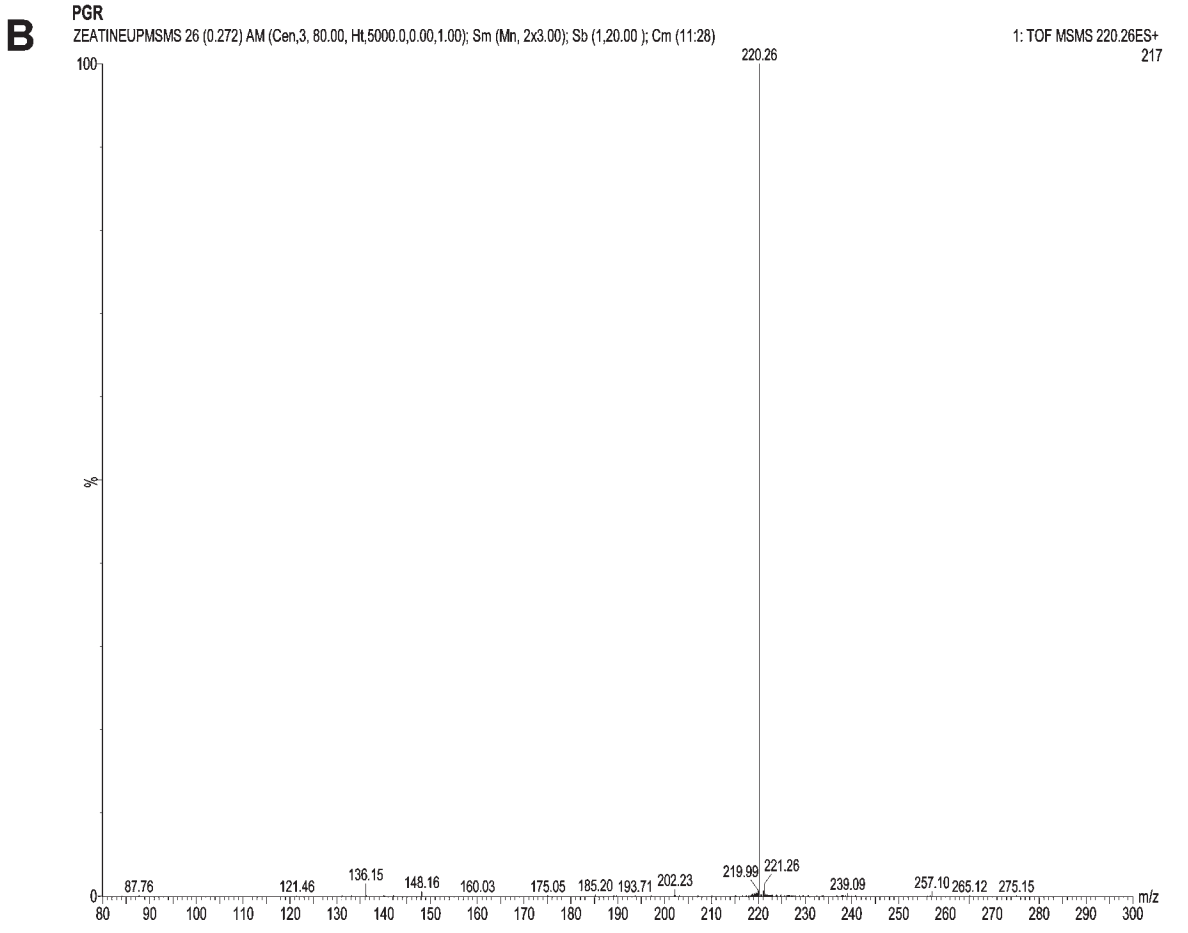


Figure 3. (**A**) Mass spectrum of zeatin fraction extracted from sap. (**B**) MS/MS spectrum of precursor ion $(M + H)^+$ (*m*/*z* 220.25) in **A**.

Figure 4. (**A**) Mass spectrum of GA_3 fraction extracted from sap. (**B**) MS/MS spectrum of precursor ion $(M - H)^-$ (*m*/*z* 345.08) in **A**.

method is that an elaborate database of mass spectra of PGRs must be accessible to the analyst for proper identification and, furthermore, the availability of standards for quantification. For example, whereas we could identify GA_9 in *S. tenerrimum*, we were unable to quantify for want of a standard.

Quantification of PGRs Using ESI-MS. The detailed procedures including the generation of standard plot for each PGR are provided in the Supporting Information, section 6). It is reported in the literature that, to minimize ion suppression, addition of a control matrix similar to the sample matrix is necessary to carry out the quantification (*29*, *34*). Hence, internal standards having matrices similar to those of the PGRs detected in the sap were used in the quantification. ES^- mode was suitable for ionization of IAA, IPA, IBA, and GA_3, whereas ES^+ mode was suitable for ionization of kinetin and *trans*-zeatin. For the quantification of IAA, the intensity ratio of IAA to IPA (I_{IAA}/I_{IPA}) was plotted against the corresponding quantity ratio (M_{IAA}/M_{IPA}). The fit was good, with $R^2 = 0.99$. Thereafter, the IAA in the DEE extract of the sap layer was estimated and found to be 23.36 ± 0.15 ppm (average of five measurements). In a similar manner, calibration plots of I_{KNT}/I_{BAP} versus M_{KNT}/M_{BAP} and I_{ZTN}/I_{BAP} versus M_{ZTN}/M_{BAP} gave R^2 values of 0.95 and 0.90, respectively. The concentrations of kinetin and zeatin in the sap were estimated to be 7.94 ± 0.30 and 23.97 ± 0.47 ppm, respectively, on the basis of analyses of the *n*-butanol extract of sap. For quantification of GA_3, phenanthrene has been used as IS in GC studies of gibberellins (*35*). However, in view of its insolubility in methanol, PM was used as IS instead. To quantify GA_3, a calibration plot was first made of I_{GA3}/I_{PM} versus M_{GA3}/M_{PM}, with $R^2 = 0.96$. The concentration of GA_3 in the sap, on the basis of the analysis of the ethyl acetate extract, was estimated to be 27.87 ± 0.14 ppm.

The PGRs in the sap were once again analyzed after 1 year of storage with preservatives, maintaining the same extraction protocol. The respective concentrations remained almost unchanged (IAA, 23.36 ppm; kinetin, 7.94 ppm; zeatin, 23.97 ppm ; GA_3, 27.87 ppm), confirming the efficacy of the preservative used (Supporting Information, section 6).

The concentrations of the PGRs in the sap are substantially higher than in terrestrial plants. However, relatively higher concentrations of PGRs in certain seaweeds have been reported by other researchers also (*36*). This is the likely reason behind the popularity of seaweed extracts as biofertilizers. Besides the intrinsically higher levels of the PGRs in the sap, the solvent extraction procedure, with pH adjustment, may have resulted in a more complete extraction from the aqueous to solvent phase (*36*). The above results were validated through studies employing HPLC with a UV−vis detector (vide infra).

Quantification through HPLC. HPLC analyses of the standard PGRs as well as the PGR fractions extracted from the *Kappaphycus* sap were carried out under identical conditions (Supporting Information, section 7). Under the HPLC conditions employed, the values of t_R were 7.6, 9.4, and 13.7 min for the three standard auxins, that is, IAA, IPA, and IBA, respectively. For the standard cytokinins, that is, kinetin, *trans*-zeatin, and benzyl aminopurine, the t_R values were 13.25, 12.89, 5.6 min, respectively. For GA_3, which was the sole gibberellin standard available to us, the t_R value was 9.62 min. When the standards kinetin and *trans*-zeatin were taken together, resolution of the two constituents was difficult in view of the closeness of the t_R values for the HPLC conditions adopted. As a result, these compounds were found to coelute (confirmed through MS studies), and hence their individual concentrations could not be determined and comparison with ESI-MS results was not possible. Reoptimization of the chromatographic technique, including optimization of column, solvent composition, flow rate, and temperature may help in achieving a better resolution. For the HPLC of the DEE extract of sap, the chromatogram was complex. However, the peak due to IAA could be identified and was well resolved. By this technique the concentration of IAA in sap was estimated to be 26.20 ppm versus 23.36 ppm by ESI-MS. In the case of the gibberellin extract of sap, the chromatogram was uncomplicated and the GA_3 peak was well resolved, yielding a concentration of 30.85 ppm in sap versus 27.87 by ESI-MS. The close agreement between the HPLC and ESI-MS data can be taken as a validation of the latter methodology.

The results from the above experiments carried out with the sap of *K. alvarezii* confirm the development of an easy and accurate method of quantification of a few PGRs (IAA, kinetin, zeatin, GA_3) using ESI-MS. Measurements were made on diethyl ether, *n*-butanol, and ethyl acetate extracts prepared from the sap. Other than the crude separation of the different classes of PGRs through solvent extraction, no further separation was required. The sap was found to contain 23.4 ppm of IAA and 27.9 ppm of GA_3 by the above method, in good agreement with the values of 26.3 and 30.5 ppm estimated by HPLC. The concentrations obtained by ESI-MS were lower in both cases, and this is ascribed to the low probability of interference from impurities given the uniqueness of the MS fingerprint. Thus, the values obtained by ESI-MS may be considered as truer estimates. Kinetin and zeatin were found to be present to the extent of 7.94 and 23.97 ppm, respectively, whereas estimation of these individual concentrations by HPLC proved intractable. The concentrations of the PGRs remained virtually unchanged after 1 year of storage with preservatives. The presence of other PGRs in the sap cannot be ruled out, and studies are in progress in this direction. Preliminary assessment was also made of the constituents of the sap of *G. edulis* and *S. tenerrimum*. These saps were devoid of kinetin, and the sap of *G. edulis* contained IPA instead of IAA, whereas that of *S. tenerrimum* contained GA_9 instead of GA_3. Precise information of the identities and concentrations of individual PGR constituents would help in fine-tuning formulations and application level of the sap. There may be other stimulants also, which we hope to explore in future studies.

ACKNOWLEDGMENT

We are grateful to the referees for their valuable suggestions, which have been incorporated in the revised manuscript. K.P. thanks Prof. R. Rengasamy and Dr. A. Muruganantham for providing training on the colorimetric method of PGR detection. The We also thank A. Bose of M/s Pepsico India Holdings Ltd. (licensee) and A. Seth of Aquagri Pvt. Ltd. (licensee) for providing impetus to this work, facilitating the training on colorimetric detection, and sharing information on field trials conducted with *Kappaphycus* sap (Table S1). We are grateful to National Institute of Nutrition, Hyderabad, India, for analyses of clarified sap (Table S2). We thank Dr. P. Paul for facilitating the MS and HPLC studies and the Council of Scientific & Industrial Research, New Delhi for supporting the initiative as an in-house laboratory project.

Supporting Information Available: Additional experimental details and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Chapman, V. J.; Chapman, D. J. Chapter 2. In *Seaweeds and Their Uses*; Chapman and Hall: London, U.K., 1980; pp 334−339.

(2) Metting, B.; Zimmerman, W. J.; Crouch, I. J.; van Staden, J. In *Introduction to Applied Phycology*; SPB Academic Publishing: The Hague, The Netherlands, 1990; pp 269−275.

(3) Sanderson, K. J.; Jameson, P. E. The cytokinins in a liquid seaweed extract: could they be active ingredients? *Acta. Hortic.* **1986**, *179*, 113–116.
(4) Tay, S. A. B.; Palni, L. M. S.; MacLeod, J. K. Identification of cytokinin glucosides in a seaweed extract. *J. Plant Growth Regul.* **1987**, *5*, 133–138.
(5) Sanderson, K. J.; Jameson, P. E.; Zabkiewicz, J. A. Auxin in a seaweed extract: identification and quantification of indole-3-acetic acid by gas chromatography−mass spectrometry. *J. Plant Physiol.* **1987**, *129*, 363–367.
(6) Crouch, I. J.; van Staden, J. Evidence for the presence of plant growth regulators in commercial seaweed products. *Plant Growth Regul.* **1993**, *13*, 21–29.
(7) Stirk, W. A.; van Staden, J. Isolation and identification of cytokinins in a new commercial seaweed product made from *Fucus serratus*. *J. Appl. Phycol.* **1997**, *9*, 327–330.
(8) Brain, K. P.; Chalopin, M. C.; Turner, T. D.; Blunden, G.; Wildgoose, P. B. Cytokinin activity of commercial aqueous seaweed extract. *Plant Sci. Lett.* **1973**, *1*, 241–245.
(9) Sivasankari, S.; Venkatesalu, V.; Anantharaj, M.; Chandrasekaran, M. Effect of seaweed extracts on the growth and biochemical constituents of *Vigna sinensis*. *Bioresour. Technol.* **2006**, *97*, 1745–1751.
(10) Ballester, A. Growth regulators from the seaweed *Chondrus crispus* L. (Lyn.). *J. Exp. Mar. Biol. Ecol.* **1975**, *20*, 179–182.
(11) Lewis, J. G.; Stanley, N. F.; Guist, G. In *Algae and Human Affairs*; Lembi, C. A., Waaland, J. R., Eds.; Cambridge University Press: Cambridge, U.K., 1990; pp 205−209.
(12) Eswaran, K.; Ghosh, P. K.; Siddhanta, A. K.; Patolia, J. S.; Periyasamy, C.; Mehta, A. S.; Mody, K. H.; Ramavat, B. K.; Prasad, K.; Rajyaguru, M. R.; Kulandaivel, S.; Reddy, C. R. K.; Pandya, J. B.; Tewari, A. U.S. Patent 6893479, May 17, 2005; U.S. Patent EP 1534757, May 10, 2006.
(13) Rathore, S. S.; Chaudhary, D. R.; Boricha, G. N.; Ghosh, A.; Bhatt, B. P.; Zodape, S. T.; Patolia, J. S. Effect of seaweed extract on the growth, yield and nutrient uptake of soyabean (*Glycine max*) under rainfed conditions. *S. Afr. J. Bot.* **2009**, *75*, 351–355.
(14) Desai, N. *Business India* **2009** (Sept 6), 106.
(15) Gordon, S. A.; Paleg, L. G. Observations on the quantitative determination of indoleacetic acid. *Physiol. Planta.* **1957**, *10*, 39–47.
(16) Mowat, J. A. A survey of results on the occurrence of auxins and gibberellins in algae. *Bot. Mar.* **1964**, *8*, 149–155.
(17) Harborne, J. B. Chapter 3. In *Phytochemical Methods − A Guide to Modern Techniques of Plant Analysis*, 2nd ed.; Chapman and Hall: London, U.K., 1984; pp 212.
(18) Mahadevan, A.; Sridhar, R. In *Methods in Physiological Plant Pathology*, 4th ed.; Sivakami Publications: Chennai, India, 1996; pp 76.
(19) Bernart, M.; Gerwick, W. H. 3-(Hydroxyacetyl)indole − a plant growth regulator from the oregon red alga *Prionitis lanceolata*. *Phytochemistry* **1990**, *29*, 3697–3698.
(20) Kingman, A. R.; Moore, J. Isolation, purification, and quantitation of several growth-regulating substances. *Bot. Mar.* **1982**, *25*, 149–153.
(21) Zhang, W.; Chapman, D. J.; Phinney, B. O.; Spray, C. R.; Yamane, H.; Takahashi, N. Identification of cytokinins in *Sargassum muticum* (Phaeophyta) and *Porphyra perforata* (Rhodophyta). *J. Phycol.* **1991**, *27*, 87–91.
(22) Stirk, W. A.; van Staden, J. Comparison of cytokinin and auxin like activity in some commercially used seaweed extracts. *J. Appl. Phycol.* **2005**, *18*, 503–508.
(23) Stirk, W. A.; Novak, O.; Strnad, M.; van Staden, J. Cytokinins in Macroalgae. *Plant Growth Regul.* **2003**, *41*, 13–24.
(24) Stirk, W. A.; Arthur, G. D.; Lourens, A. F.; Novak, O.; Strnad, M.; van Staden, J. Changes in cytokinin and auxin concentrations in seaweed concentrates when stored at an elevated temperature. *J. Appl. Phycol.* **2004**, *16*, 31–39.
(25) Pryde, A.; Schuler, A.; Vonder Mühll, F. P. A. Determination of an experimental plant growth regulator on wheat and cotton plants by reversed-phase ion-pair partition high-performance liquid chromatography. *Anal. Chim. Acta* **1979**, *111*, 193–199.
(26) Tsivou, M.; Kioukia-Fougia, N.; Lyris, E.; Aggelis, Y.; Fragkaki, A.; Kiousi, X.; Simitsek, Ph.; Dimopoulou, H.; Leontiou, I. P.; Stamou, M.; Spyridaki, M. H.; Georgakopoulos, C. An overview of the doping control analysis during the Olympic Games of 2004 in Athens, Greece. *Anal. Chim. Acta* **2006**, *555*, 1–13.
(27) Zhang, Y.; Conrad, A. H.; Conrad, G. W. Detection and quantification of 3,5,3′-triiodothyronine and 3,5,3′-triiodothyronine by electrospray ionization tandem mass spectrometry. *J. Am. Soc. Mass. Spectrom.* **2005**, *16*, 1781–1789.
(28) Liebisch, G.; Binder, M.; Schifferer, R.; Langmann, T.; Schulz, B.; Schmitz, G. High throughput quantification of cholesterol and cholesteryl ester by electrospray ionization tandem mass spectrometry (ESI-MS/MS). *Biochim. Biophys. Acta: Mol. Cell Biol. Lipids* **2006**, *1761*, 121–128.
(29) Hoffmann, E.; Stroobant, V. In *Mass Spectroscopy: Principles and Applications*; Wiley: New York, 2001; p479.
(30) Chiwocha, S. D. S.; Cutler, A. J.; Loewen, M. A.; Ross, R. S.; Kermode, A. R. A method of profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (*Lactuca sativa* L.) seeds. *Plant J.* **2003**, *35*, 405–417.
(31) Bentley-Mowat., J. A.; Reid, S. M. Investigation of the radish bioassay for kinetin and demonstration of kinetin like substances in algae. *Ann. Bot.* **1968**, *32*, 23–32.
(32) Barciszewski, J.; Siboska, G. E.; Pedersen, B. O.; Clark, B. F. C.; Rattan, S. I. S. Evidence for the presence of kinetin in DNA and cell extracts. *FEBS Lett.* **1996**, *393*, 197–200.
(33) Ge, L.; Yong, J. W. H.; Goh, N. H.; Chia, L. S.; Tan, S. N.; Ong, E. H. Identification of kinetin and kinetin riboside in coconut (*Cocos nucifera* L.) water using a combined approach of liquid chromatography−tandem mass spectrometry, high performance liquid chromatography and capillary electrophoresis. *J. Chromatogr., B* **2005**, *829*, 26–34.
(34) Villagrasa, M.; Guillamón, M.; Eljarrat, E.; Barceló, D. Matrix effect in liquid chromatography−electrospray ionization mass spectrometry analysis of benzoxazinoid derivatives in plant material. *J. Chromataogr., A* **2007**, *1157*, 108–114.
(35) Rachev, R.; Gancheva, V.; Bojkova, S.; Christov, C.; Zafirova, T. Gibberllin biosynthesis by *Fusarium moniliforme* in the presence of hydrophobic resin Amberlite XAD-2. *Bulg. J. Plant. Physiol.* **1997**, *23*, 24–32.
(36) Kingmann, A. R.; Moore, J. Isolation, purification and quantification of several growth regulating substances in *Ascopyllum nodosum*. *Bot. Mar.* **1982**, *25*, 149–159.

Received for review December 21, 2009. Revised manuscript received March 16, 2010. Accepted March 18, 2010.