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Research article

Simultaneous determination of different endogenetic plant growth regulators in common green seaweeds using dispersive liquid–liquid microextraction method

Vishal Gupta [a], Manoj Kumar [a], Harshad Brahmbhatt [b], C.R.K. Reddy [a,*], Abhiram Seth [c], Bhavanath Jha [a]

[a] Discipline of Marine Biotechnology and Ecology, Central Salt and Marine Chemicals Research Institute, Council of Scientific and Industrial Research (CSIR), Bhavnagar 364021, India
[b] Analytical Sciences, Central Salt and Marine Chemicals Research Institute, Council of Scientific and Industrial Research (CSIR), Bhavnagar 364021, India
[c] Aquagri Processing Pvt Ltd, 18, Anand Lok, New Delhi 110049, India

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ABSTRACT

A simple and rapid HPLC-based method was developed for simultaneous determination of major classes of plant growth regulators (PGRs) in *Monostroma* and different species of *Ulva*. The plant growth regulators determined included gibberellic acid (GA_3), indole-3-acetic acid (IAA), abscisic acid (ABA), indole-3-butyric acid (IBA), salicylic acid and kinetin riboside (KR) and their respective elution time was 2.75, 3.3, 3.91, 4.95, 5.39 and 6.59 min. The parameters optimized for distinct separation of PGRs were mobile phase (60:40 methanol and 0.6% acetic acid in water), column temperature (35 °C) and flow rate (1 ml/min). This method presented an excellent linearity (0.2–100 μg/ml) with limit of detection (LOD) as 0.2 μg/ml for ABA, 0.5 μg/ml for KR and salicylic acid, and 1 μg/ml for IAA, IBA and GA_3. The precision and accuracy of the method was evaluated after inter and intra day analysis in triplicates. The effect of plant matrix was compensated after spiking and the resultant recoveries estimated were in the range of 80–120%. Each PGR thereby detected were further characterized by ESI-MS analysis. The method optimized in this study determined IBA along with IAA for the first time in the seaweed species investigated except *Ulva linza* where the former was not detected. In all the species studied, ABA level was detected to be the highest while kinetin riboside was the lowest. In comparison to earlier methods of PGR analysis, sample preparation and analysis time were substantially reduced while allowing determination of more classes of PGRs simultaneously.

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1. Introduction

Plant growth regulators (PGRs) are structurally diverse group of naturally occurring substances that interacts with each other in a complex manner to regulate almost every aspect of plant life [1]. They are highly functional and even at trace quantities trigger a variety of basic physiological processes including cell division, enlargement and differentiation, organogenesis, seed dormancy and germination, leaf senescence and abscission as well as defence against abiotic and biotic stresses [2]. Endogenic cytokinins together with their riboside conjugate and auxins have been reported from seaweed species such as *Porphyra perforata*, *Sargassum muticum* [3–5], *Laminaria japonica* [6], *Dictyota humifusa*, *Ulva fasciata* [7], *Undaria pinnatifida* [8] and *Caulerpa paspaloides* [9]. Recently, Yokoya et al. [10] quantified cytokinin, auxin and abscisic acid in common red algae from Brazilian coast. The inherent growth regulating substances including both PGRs and minerals in seaweed were attributed to their application as fertilizer in agriculture [11]. The foliar spray of seaweed extract or its direct application to root has shown wide range of beneficial effects on terrestrial crops ranging from early seed germination, improved crop performance, increased resistance toward abiotic and biotic stress and enhanced post harvest shelf-life of perishable products [12,13]. As a result, the seaweed industry has emerged with a new sector of 'phycosupplement' with an estimated global market value of US$53 million [14]. Nowadays, the seaweed-based fertilizers are gaining importance over petrochemical based fertilizers because of the detrimental effects caused by petrochemical based fertilizers on soil productivity upon prolonged use. The commonly used seaweed-based fertilizers in the agriculture and horticulture are mainly prepared out of brown seaweeds namely *Ascophyllum nodosum*, *Ecklonia maxima*, *Macrocystis pyrifera* [12] owing to their round the year availability. However, the foliar spray of red seaweed *Kappaphycus alvarezii* sap on soyabean has resulted in an increase of crop yields as high as 46% over control [15]. The analysis of this sap for PGRs has revealed the presence of IAA, GA_3, kinetin and zeatin [16] in addition to several micro and macronutrients [17].

* Corresponding author. Tel.: +91 278 256 5801/256 3805x614; fax: +91 278 256 7562/256 6970.
E-mail address: crk@csmcri.org (C.R.K. Reddy).

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Further field trials of this sap on other agricultural crops has also shown encouraging results over control and showed increased yields of 30–40% in sugarcane, 26% in potato, 16% in barley and gram, and 13–15% in corn (Abhiram Seth personal communication).

The genus *Ulva* with its worldwide distribution, wider adaptability to diverse environmental conditions, higher growth rates and amenability for depolymerization makes it an attractive feedstock for developing fertilizer and bio-refinery products. The removal of moisture from the biomass indeed is the bottleneck toward the economy of energy industry therefore an integrated process involving extraction of liquid sap having agriculture applications could make overall process much competitive. Development of such innovative processes will also be advantageous in utilizing the massive biomass of *Ulva* produced during sudden outbreak as green tides or blooms recently reported in China [18], the Baltic Sea [19] and Chile [20].

The analytical techniques employed for PGR analysis include high pressure liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC-MS), capillary electrophoresis etc. Of these, HPLC with its inherent advantages such as wide application, short analysis time and high separation efficiency merits its choice as a better tool over others. However, intracellular traces of PGRs and coexistence of many interfering substances make the analysis difficult and necessitate for sample preparation and purification prior to analysis. The most commonly used pre-treatment techniques are liquid-liquid extraction (LLE), solid phase extraction (SPE), column chromatography and solid phase microextraction (SPME). Of these techniques, dispersive liquid–liquid microextraction (DLLME) method developed by Razaee et al. [21] is relatively new and has several advantageous over others such as 1) sample enrichment, 2) dispenses the need of purification through column, 3) low organic solvent consumption and 4) overall short operation time. The DLLME method together with HPLC coupled to ultraviolet detector (UV) has been employed for the analysis of polycyclic aromatic hydrocarbons [22] and psycotropic drugs [23]. Lu et al. [1] for the first time successfully demonstrated DLLME-HPLC-FLD as a powerful pre-concentration method sensitive for detecting auxins.

However, the requirement of rapid methodologies for sample derivatization has shifted the bottleneck for sample throughput from analysis to sample preparation. Therefore the objective of this investigation was to optimize a method for simultaneous determination of broad classes of plant growth regulators such as ABA, IAA, IBA, GA_3, KR along with salicylic acid in common macrophytic marine green algae *Monostroma* and *Ulva*.

2. Results and discussion

The interaction of different endogenetic hormone response pathways plays a crucial role in regulating various developmental and physiological processes. Therefore a method determining simultaneously different classes of endogenetic plant growth regulators (PGRs) will facilitate the understanding of various cellular, physiological and biochemical responses. In this study, a simple and rapid HPLC based method was optimized for simultaneous determination of major classes of PGRs such as auxins (IAA and IBA), ABA, GA_3 in common green seaweeds. The resolution of hydrophobic PGRs in HPLC analysis was found to significantly be affected by mobile phase composition and concentration. The distinct separation of these PGRs was attained in mobile phase constituted with methanol: 0.6% acetic acid (60:40). The modified mobile phase composition was distinct and simple compared to the binary mixtures used in majority of the studies in seaweed PGR analysis [7,10,24,25]. Also the modified composition was different from those reported for PGR analysis in terrestrial plants [26,27]. In addition to mobile phase, the column temperature and flow rate were the other determinants that offer better resolution of the analytes. The separation column temperature and pump flow rate was optimized as 35 °C and 1.0 ml/min respectively. The standards were detected at all the three wavelengths selected under the optimized conditions and quantification was performed at 208 nm for GA_3 and at 265 nm for other PGRs. The retention time of the standard analytes such as ABA, IAA, IBA, GA_3, salicylic acid and KR was 3.91, 3.3, 4.95, 2.75, 5.39 and 6.59 min respectively (Supplementary Fig. 1).

The linear range along with the limit of detection (LOD) for the target compounds are summarized in Table 1. The LOD was found to be lowest for ABA (0.2 μg/ml) followed by KR, salicylic acid (0.5 μg/ml), and IAA, IBA and GA_3 (1.0 μg/ml) (Table 1). The linear fitting of the repeated data was found significant at $p \leq 0.01$ and the evaluation results of the linear model by regression variance analysis are summarized in Table 2. The results revealed a good linear fit. The precision in the analysis was validated with inter/intra-day analysis in replicate and the relative deviation observed was in the range of 0.21–0.92%. The samples were found stable when stored at −20 °C even for a month and the observed relative reduction was 1.72% for all the targeted compounds except KR which was not recovered after storage (data not shown). The overall assay procedure has shown a short separation time of 7 min only. Therefore this optimized method reduced the sample preparation and required analysis time in addition to simultaneous profiling of more classes of PGRs. These merits make this method a choice for high throughput analysis over the other extraction and analysis strategies employed for seaweed PGR analysis [7,10].

The method precision and accuracy was found to be comparable with those of the methods previously developed for simultaneous analysis of PGRs from various plant matrices [1,2,28]. Recently, Lu et al. [29] developed a method employing SPE strategy followed by the analysis on pressurized capillary electrochromatography that showed LOD as lower as 0.2 μg/ml for endogenic (IAA) and ectogenic hormones (BA, IAA, IPA, NAA and KT). The other extraction method based on SPME also showed LODs in the range of 0.121–0.442 μg/ml for IAA, ABA and IBA [2]. The comparison of the method investigated in this study with other reported methods for PGR detection is illustrated in Table 3. The LODs for the sample analyzed herein were in the similar range as reported in some of the earlier reported methods thus demonstrating another advantage of dispensing the need of expensive cartridges. The earlier developed methods were evaluated at a few real samples however the method optimized in this study was evaluated on different green seaweed species thereby giving precision to the analysis. Matrix effects were also compensated in this method to attain accuracy in quantification. Signal recoveries were in the range of 80-92% for PGRs except GA_3 for which the signal recovery was 120% in *Ulva reticulata*. The LOD and signal recovery was inferior in this study compared to the report of Lu et al. [1] in which the same sample enrichment method was employed for analysis of auxins in *Chlorella vulgaris* with FLD detection system. The method exploited

Table 1
Analytical performance data for major endogenetic plant growth regulators.

Analyte	Linear range (μg/ml)	Regression equation	*R*	LOD (μg/ml)
ABA	0.2–100	$y = 26{,}547x - 10{,}400$	0.995	0.5
GA_3	0.2–100	$y = 53{,}554x + 11{,}074$	0.999	1
IAA	0.2–100	$y = 96{,}168x - 17{,}311$	0.995	1
IBA	0.2–100	$y = 74{,}274x - 72{,}997$	0.990	1
KR	0.2–100	$y = 15{,}770x - 46{,}378$	0.976	0.5
Salicylic acid	0.2–100	$y = 69{,}670x + 40{,}625$	0.971	0.5

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