Analytical Methods

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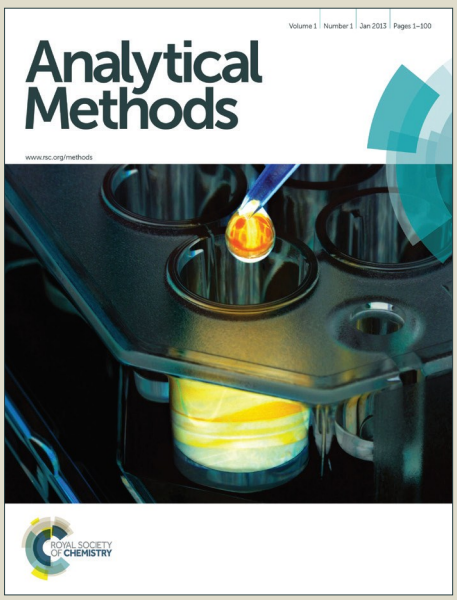

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Paper

Extraction of plant growth regulators present in *Kappaphycus alvarezii* sap by imidazolium based ionic liquids: Detection and quantification by HPLC–DAD technique

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Three different imidazolium based ionic liquids namely 1-butyl-3-methylimidazolium hexafluorophosphate [Bmim][PF_6], 1-methyl-3-octylimidazolium tetrafluoroborate [Omim][BF_4] and 1-butyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide [Bmim][NTf_2] were used to extract plant growth regulators (PGRs) present in the sap (*K*-sap) obtained by the mechanical expulsion from the fresh *Kappaphycus alvarezii* seaweed. [Bmim][PF_6] was able to extract up to 65% of the total *trans*-zeatin and about 18% of the total indole-3-acetic acid (IAA) present in the sap as estimated using HPLC-DAD (diode array detector). However [Bmim][NTf_2] was not able to extract any of the PGRs. Furthermore, addition of [Omim][BF4] to the sap resulted formation of instant white precipitate with *trans*-zeatin preferentially bound on it. From the overall studies it was concluded that, [Bmim][PF_6] can be effectively used to extract PGRs from seaweeds or plant extracts.

Introduction

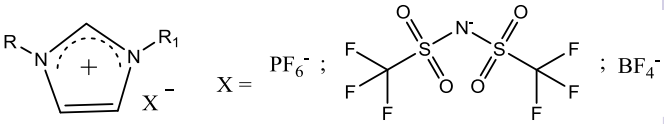
Kappaphycus alvarezii is one of the commercially important red seaweeds growing in tropical waters. This seaweed is the major source of κ-carrageenan, an important polysaccharide used extensively in food and beverage industries as food stabilizer and texture provider. [1] It has been disclosed that the liquid obtained by the mechanical expulsion of the fresh seaweed (K-sap) is a potent plant stimulant with proven ability to enhance growth and quality of various crops when applied as foliar sprays. [2,3] The juice is found to be rich of various micro and macronutrients and plant growth regulators (PGRs). [3,4]

Room temperature ionic liquids (RTILs) are semiorganic salts and remain in liquid state below 100 ^{o}C and are considered as a novel class of benign media alternative to the conventionally used organic solvents for various applications. [5-7] Apart from the applications of these liquids in other fields, researchers are inclined towards using these solvents for liquid–liquid extractions of metal ions and organic compounds. [8,9] ILs can be hydrophilic or hydrophobic in nature depending on the structures of the cations or anions. [6,7] Hydrophobic ionic liquids are the preferential choice as extraction media due to their ability to form biphasic layers with aqueous solutions. [10]

We have recently demonstrated identification and quantification of few PGRs in the sap expelled out from fresh *Kappaphycus alvarezii* by mechanical crushing. [4] Further we were able to separate selectively Gibberellic acid-3 (GA_3) from the sap and used this as a new formulation of foliar spray, which substantially increased the biomass of corn stover. [11] However, the biggest disadvantage of extraction of PGRs by conventional method is the large scale usage of environmentally deleterious volatile solvents and involvement of tedious multi-step extraction procedures. The unique extraction ability of ionic liquids as environment friendly solvents urged us to develop a facile method for the extraction of PGRs from the seaweed juice.

Although ILs are used for the extraction of natural products such as alkaloids, drug molecules etc., [12,13] but to the best of our knowledge other than a single report on extraction of indole-3-butyric acid from pea plants using imidazolium based hydrophobic ionic liquids containing PF_6 and BF_4 anions, [14] there are no reports available on the application of the ILs in the extraction of PGRs. However, in one of our recent research endeavour, we were able to bind selectively *trans*-zeatin present in the *K*-sap with [Bmim][BF_4]. [15]

Herewith, we report outcome of our studies pertaining to the ability of few imidazolium based ionic liquids for the extraction of PGRs present in *K-sap* and their quantification employing HPLC-DAD technique. The structure of the ILs used for the present study is depicted in Figure 1.



R = -CH_3 ; -C_8H_{17} R_1= -C_4H_9 ; -CH_3

Fig. 1: Structure of ionic liquids used to extract plant growth regulators present in *Kappaphycus alvarezii* sap.

Materials & Methods

Materials

Kappaphycus alvarezii was collected from the cultivation sites of south east coast of India, Mandapam (9.28°N 79.12°E)

during April, 2013. A specimen of the sample was deposited to the Institute herbarium for future references. Standard indole-3-acetic acid (IAA) and Gibberellic acid (GA_3) were purchased from S.D. Fine Chemicals, Mumbai and *trans*-zeatin, 1-butyl-3-methylimidazolium hexafluorophosphate [Bmim] [PF_6], 1-methyl-3-octylimidazolium tetrafluoroborate [Omim][BF_4], 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [Bmim][NTf_2] were procured from Merck, Germany. All chemicals were used as received without further purification. Milli Q water and HPLC grade methanol was used for all the experiments. Standard PGR samples were prepared as 25 mgL^{-1} IAA, GA_3 in methanol and 25 mgL^{-1} *trans*-zeatin in *n*-butanol.

Preparation of *Kappaphycus alvarezii* sap:

Fresh *Kappaphycus alvarezii* was mechanically crushed to obtain the sap (*K*-sap) as shown in ESI Figure S1. [2]

HPLC-UV-DAD (diode array detector) analysis

Shimadzu prominence HPLC system was used for the analysis of PGR rich ionic liquid phase after dilution with methanol. The HPLC solvent system was comprised of A: water/0.1% HCOOH and B: Methanol/0.1%HCOOH. HPLC elution was carried out using Enable C18H 5µm 150 mm x 4.6 mm column following method reported by Pan *et al*., (2010) with minor modifications. [16] HPLC gradient programme used in the analysis was as follows (Table 1).

Table 1: Gradient table for the HPLC analysis of PGR rich ionic liquid phase

Time (min)	Flow (mL/min)	Volume % (A)	Volume % (B)
0	0.8	70	30
2	0.8	70	30
20	0.8	0	100
22	0.8	0	100
25	0.8	70	30

Injection volume for all samples and standards was maintained as 25µl. Column heater temperature was set at 40 ^oC and UV detection was performed using UV-DAD detector. HPLC chromatograms were monitored at 205, 254 and 280 nm depending on the nature of the sample. Data processing was done using LC Solution ™ software provided by the HPLC manufacturer. All samples were analyzed in triplicate and the average of peak areas was taken for calculations. Concentration of plant growth regulators in IL phase were calculated from peak area in comparison with calibration curves.

Optimization of sap and IL volume ratio:

The effective volume ratio of *K*-sap and IL on the extraction efficiency was studied. Different ratios of IL to *K*-sap ranging from 1: 2 to 1: 10 were used for extraction experiments and the samples were analyzed on HPLC. No significant effect on the extractability of the PGRs in the IL was observed above the ratio 1:4. Hence, IL to K-sap ratio was optimized as 1: 4 for all the experiments.

Optimization of extraction time:

Samples at different extraction time of 5, 10, 15, 30, 45, 60 and 120 minutes stirring were analysed in the HPLC for comparative study. It was found that there was no change in the peak area of detected PGRs in samples after 30 minutes of extraction. So extraction time of 35 min was fixed for all subsequent extraction experiments.

Extraction procedure:

Extraction experiments were carried out at room temperature (25 ^OC) in tightly stoppered glass bottles containing optimized quantities of *K*-sap and respective ILs. The mixture was stirred for 35 minutes at 450 rpm. IL phase was carefully separated from the aqueous seaweed extract phase after centrifuging at 8000 rpm for 20 minutes. The separated IL layer was diluted 15 times with methanol and subjected to HPLC analyses.

Effect of temperature on extraction efficiency:

In order to study the influence of temperature on the extraction efficiency of PGRs by the ILs, the extraction was also carried out at 40 and 50 ^OC. In a typical experiment, [Bmim][PF_6] was added to *K-sap* solution at 1: 4 ratio (i.e., 250 µL IL was added to 1 ml of sap) and stirred at 40 and 50 ^OC for 35 min. The IL phase thus obtained was subjected to analysis on HPLC-DAD as described above.

Results and discussions:

The main criterion for choosing IL solvent systems for extraction from the seaweed sap is their ability to form biphasic layers with the *K-Sap* due to the hydrophobic nature of these room temperature molten salts. Hydrophobicity of ILs depends mainly on the nature of the anion and the carbon chain length attached to the cation of the ILs. Accordingly [Bmim][PF_6], [Omim][BF_4] and [Bmim][NTf_2] were selected as suitable ILs due to their hydrophobic nature and ability to form biphasic systems with the seaweed juice.

Extractability of plant growth regulators present in seaweed extract:

Extraction experiment was performed by taking 1.0 ml of *K*-sap against 250 µL of the respective ILs followed by vigorous stirring as described in the experimental section at three at 25 ^oC. Addition of [Omim][BF_4] to seaweed extract resulted formation of white precipitate similar to the precipitate formed upon addition of 1-butyl-3-methyl imidazolium tetrafluoroborate in the sap. [15] The ionic liquids [Bmim][PF_6] and [Bmim][NTf_2] formed distinct biphasic systems with *K*-sap. In both the cases IL phase was separated by centrifugation.

Detection of plant growth regulators in the ionic liquid phase:

The IL phases obtained with [Bmim] [PF_6] and [Bmim][NTf_2] were diluted fifteen times with HPLC grade methanol and subjected to HPLC analysis along with individual standard IAA, Kinetin, Zeatin and GA_3 (25 mgL^{-1} each) solutions, which were reported to be present in the seaweed sap. [4] The chromatogram of [Bmim][NTf_2] phase didn't show peaks matching with chromatograms of any of the standard PGRs *viz*. IAA, zeatin, kinetin and GA_3. However, in [Bmim] [PF_6] phase, IAA and zeatin were detected by matching RT with the respective standard samples as shown in Figure 2. The respective chromatogram of the standard PGRs are shown in ESI Figure S2 and S3.

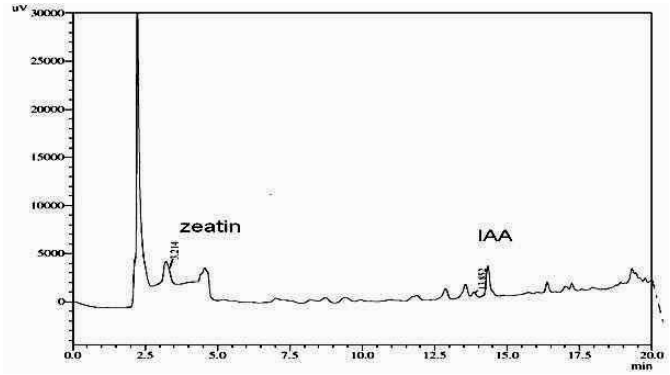


Fig. 2 : HPLC chromatogram of diluted [Bmim][PF_6] phase

The identities of the peaks were further confirmed by spiking [Bmim] [PF_6] phase samples with standard IAA and trans-zeatin as shown in Figure 3. Enhancement of peak areas of the peaks at 3.2 and 13.8 was observed, which indicated the presence of trans-zeatin and IAA in the IL phase.

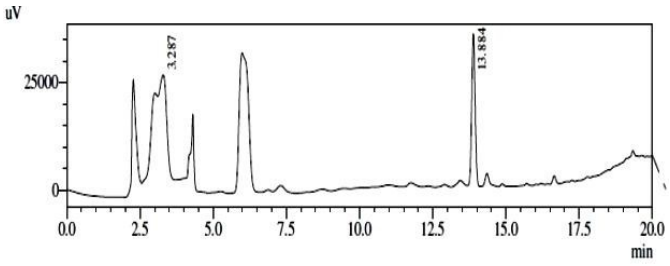


Fig. 3 : HPLC chromatogram of diluted [Bmim] [PF_6] phase after spiking with IAA and *trans*-zeatin

The possible reason behind the extraction of IAA and trans-zeatin in [Bmim][PF_6] can be attributed to the interaction of the anionic entity of the IL with the OH group of the PGR molecule *via* hydrogen bonding. The $\pi-\pi$ interaction between the cationic entity of ILs and the aromatic groups of PGRs may be also responsible for their selective extraction. In addition to this, polarity of solvent, $n-\pi$ interaction among solvent and solute also play a role in most of the natural product –IL extraction processes. [17] PF_6^- is known to interact with IAA in plant extract as reported by Absalan *et al.*,(2008) in an interfering extraction study. [14] The interaction between the anion PF_6^- and hydroxyl group of PGRs is the main driving force for the solvation of the PGRs in the IL phase. [18] Although both the [Bmim][PF_6] and Bmim][NTf_2] are promising ionic liquids for extraction of bioactive molecules, in this case, the later is not effective which may be attributed to the specific solvent properties such as low polarity, bulkier anion and inferior ionic nature of this IL in comparison to the former. The ionic nature of ILs characterised by effective ionic concentration C_{eff} for the two ionic liquids [Bmim][PF_6] and [Bmim][NTf_2] are reported to be 3.3 and 2.1 respectively. [18,19]

Quantification of plant growth regulators in ionic liquid phase:

Quantification of IAA and trans-zeatin was carried out using HPLC as described above. Samples collected from IL phase were injected to the HPLC after dilution with methanol. Calibration plot of IAA and *trans*-zeatin were prepared using five different concentrations of 3, 5, 8, 13 and 25 mgL^{-1} using reagent grade standards by diluting with above sample solution so as to compensate the variation in instrument response due to heavy matrix of ionic liquid and seaweed extract as shown in Figure 4 and 5. The details of calibration plot are shown in Table S1 (ESI). Concentration of IAA and *trans*-zeatin present in the sample was calculated from HPLC peak area according to the calibration plot. The concentration of IAA and zeatin in the original IL phase was estimated to be 3.87 mgL^{-1} and 12.4 mgL^{-1} respectively with respect to K-sap. Thus approximately partitioning up to 18.40 % of the total IAA [estimated to be 21.03 mg L^{-1} by conventional method] [4] and 65.36 % of total trans-zeatin [estimated to be 18.97 mg L^{-1} by conventional method] [4] was achieved by [Bmim][PF_6].

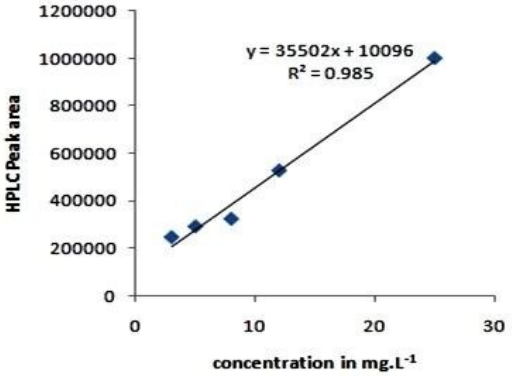


Figure 4 : Calibration plot for IAA

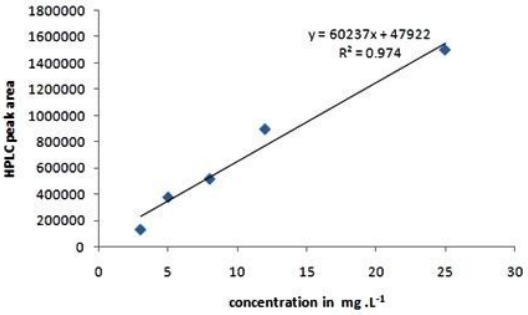


Figure 5. Calibration plot for *trans*-zeatin

The extraction carried out with [Bmim][PF_6] at 40 ^{O}C resulted extraction of 11.75 mgL^{-1} of zeatin (ESI, Figure. S4), which was increased to 11.75 mgL-1, when extraction was carried out at 50 ^{O}C (ESI, Figure. S5). However, in both the cases, no extraction of IAA took place as confirmed by recording HPLC chromatogram of the phase spiked with standard trans-zeatin and IAA (ESI, Figure. S6). The results indicated marginal increase in the extraction efficiency of the IL at 50 ^{O}C.

In summary, herein the extraction ability of three different imidazolium based hydrophobic ionic liquids for plant growth regulators present in *Kappaphycus alvarezii* sap was studied. The extraction protocol used is facile and based on simple stirring followed by centrifugation to separate PGR rich ionic liquid phase. Among the ILs, [Bmim][PF_6] was found to extract up to 65% of the total trans-zeatin and about 18% of the total indole-3-acetic acid (IAA) present in the sap into the ionic liquid phase. The extraction efficiency of the ionic liquid was found to marginally enhance at 50 ^{O}C for the extraction of trans-zeatin. [Bmim][NTf_2] was not able to extract any of the PGRs**.** Furthermore, addition of [Omim][BF_4] to

the sap resulted in formation of white precipitate with trans-zeatin preferentially bound on it. From the overall studies it was concluded that, [Bmim][PF_6] can be a good choice to extract PGRs from seaweed extracts. Although, no attempt was made to recover ionic liquid from the IL phase in the present study but recovery of [Bmim][PF_6] is possible [20] there by making ILs as reusable solvent systems for such extraction techniques.

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Notes and references

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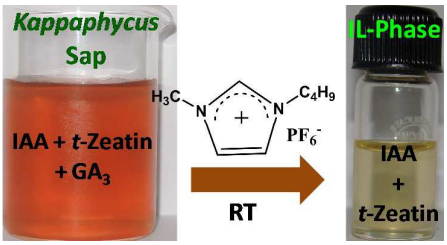
Extraction of plant growth regulators present in *Kappaphycus alvarezii* sap by imidazolium based ionic liquids: Detection and quantification by HPLC–DAD technique

Arun Kumar Das & Kamalesh Prasad *



Among three different hydrophobic ionic liquids, [Bmim][PF_6] was found to extract selectively *trans*-zeatin and indole-3-acetic acid from *Kappaphycus alvarezii* Sap.