METHODS OF EXTRACTION OF PLANT GROWTH HORMONES IN COCONUT WATER: I. UV CHARACTERIZATION

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ABSTRACT

Methods of extracting plant growth hormones, auxins, gibberellins, and cytokinins from coconut water (CW) were developed. The methods by-passed the pre-concentration stage of evaporation *in vacuo* and utilized CW as is. The methods are: (1) standard dialysis and the dialyzate adsorbed on an activated charcoal column and on resin (Amberlite XAD-4) column from which the adsorbate is eluted and fractionated (2) reverse dialysis and further adsorption and fractionation (3) direct solvent extraction with solvent recycling and concentration via a one-step process.

The extracted growth hormones were identified by UV analysis in the 180-350 nm region.

Introduction

Our country is in serious economic difficulties due to huge foreign debts and shortage of dollar reserves. A very important step to hasten economic recovery is to develop our agro-industrial technologies. One means of doing this is to utilize our rich and abundant agro-resources, one of which is coconut. Considering the tremendous volume of coconut water disposed by our coconut-processing industries, its use as a source of complex biochemicals is indeed challenging.

This paper will deal on coconut water, (CW)/milk, the liquid endosperm of the fruit of *Cocos nucifera* L. as a source of plant growth hormones, and the methods by which these substances can be extracted economically and in large amounts for application to agriculture and tissue culture. A number of studies have reported the presence of auxin-like (Dix and Van Staden, 1982) gibberellin-like (Radley and Dean, 1958) and cytokinin-like (Mauney *et. al.* 1952, Zwar *et al.*, 1963; Van Staden and Drewer, 1974; Letham, 1974) substances in coconut water. The methods of extraction require a pretreatment whereby the filtered coconut water is first concentrated under vacuum at 35°C.

Materials and Methods

Coconut water was obtained from 9-10 month-old coconuts and filtered. The filtered coconut water was sterilized at 121°C at 15 psi for 10 minutes for easier handling. Fresh coconut water can be used provided extraction is within 10 hours. Beyond this period, CW becomes contaminated with microbial growth.

Dialysis

One liter of sterile CW was dialyzed against distilled water with 2 changes of 500 ml each per 4-5 hours shaking as shown in Fig. 1. To handle larger volumes, re-

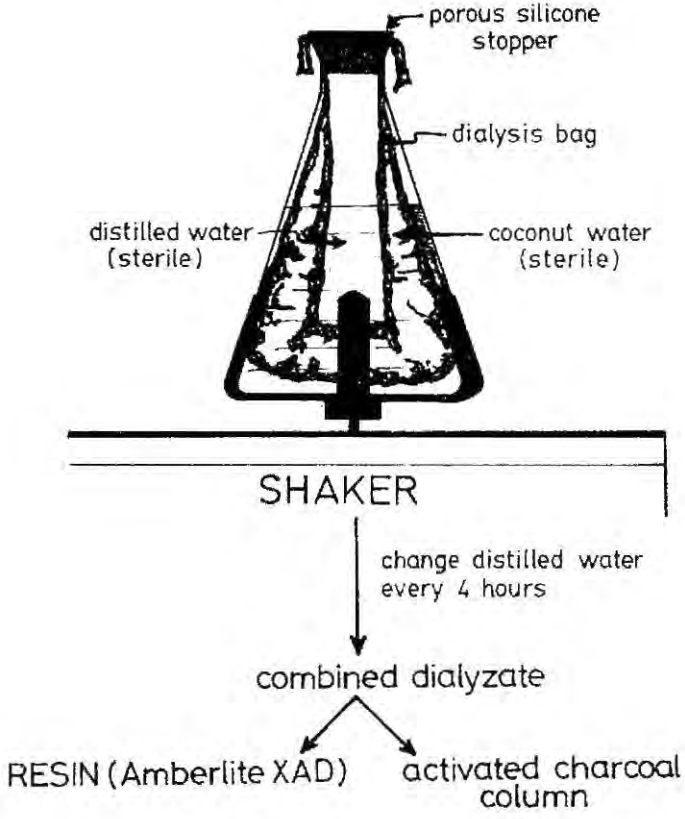


Fig. 1. Schematic diagram of Standard Dialysis Set-Up.

verse dialysis was done using 3 liters of sterile CW and 2 1/2 liters sterile distilled H_2O in the reservoir and the mixture was pumped into the dialysis bag containing 500 ml sterile water. The dialyzate was pumped out at the same rate (Fig. 2). At the end of the operation, (10-8 hours), the dialyzate in the bag was combined with the pumped dialyzates.

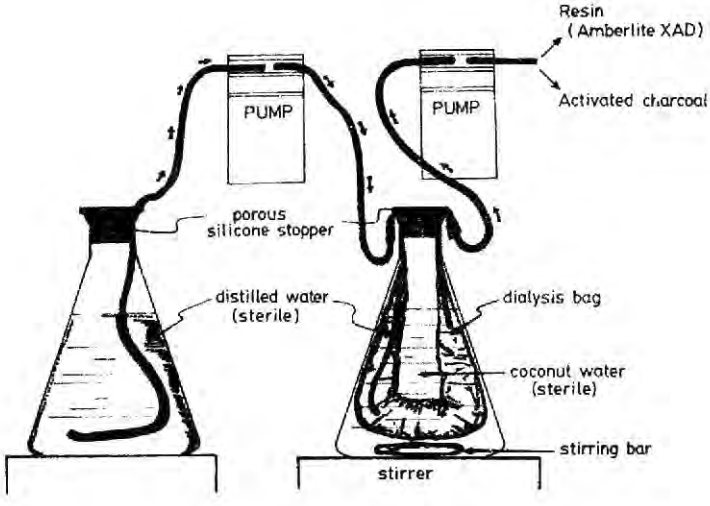


Fig. 2. Schematic diagram of Reverse Dialysis Set-up.

Adsorption on columns

Charcoal Column

Column I consists of 5 g of activated charcoal (Sigma No. C-5260, 250-350 mesh). The charcoal was poured as a slurry into glass column (15 x 1.5 cm) fitted inside with a fluted filter paper to prevent clogging and aid the flow of dialyzate through the column. The column was first washed with 25% (v/v) acetic acid in ethanol, followed by water, then 25% acetonitrile, water (10 bed volumes), and finally with 4N HCl until the column was free of sugars. The Molisch test was used to test for the presence of sugars.

Resin Column

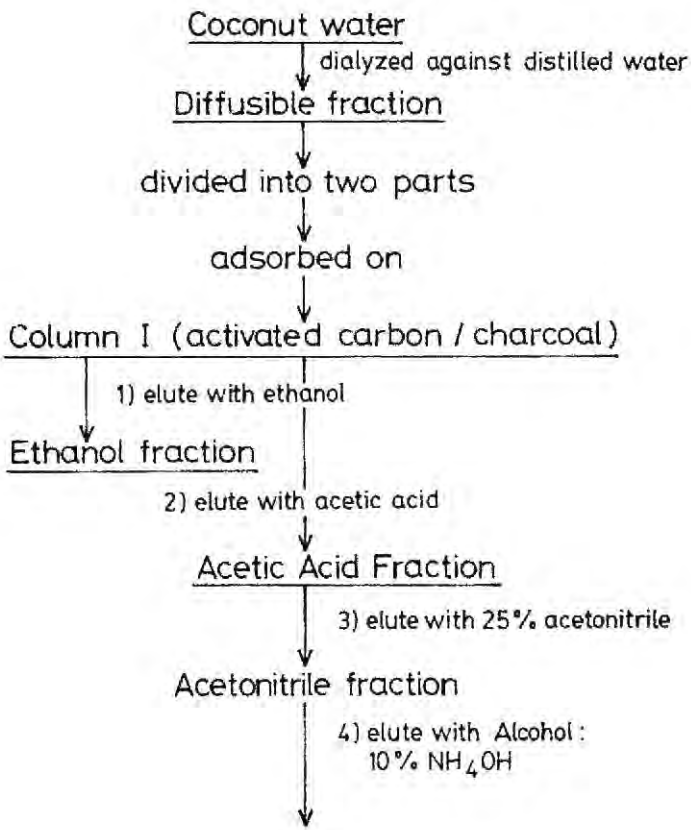
Column II consisted of Amberlite XAD-4 resin. The resin was purified first with warm running tap water followed by acetone, then with acetonitrile and lastly with ether. The column was finally washed with 10 bed volumes of distilled water. The other half of the dialyzate was then passed through the column.

Elution and fractionation

Charcoal Column

The adsorbate in the charcoal column was eluted stepwise as shown in Fig. 3. Two hundred ml of ethanol run through the column formed the alcohol fraction.

Fig. 3. The schematic procedure for the fractionation of the coconut water diffusible fraction.



Resin Column

The resin adsorbate was eluted out with 200 ml of methanol. The methanol eluate was further fractionated (Fig. 4) and concentrated at a reduced pressure at 40°C. The concentrate was diluted with water to about 100 ml (pH 4.8) and designated as the methanol eluate. The methanol eluate was extracted with ether (ether extract 1). The aqueous phase was acidified to pH 2 and then extracted with ether (ether extract 2). After the second ether extraction, the aqueous portion was adjusted to pH 11 and then extracted with ether (ether extract 3). The ether fractions and aqueous fractions were concentrated (40°C) and the concentrate diluted to 100 ml with water.

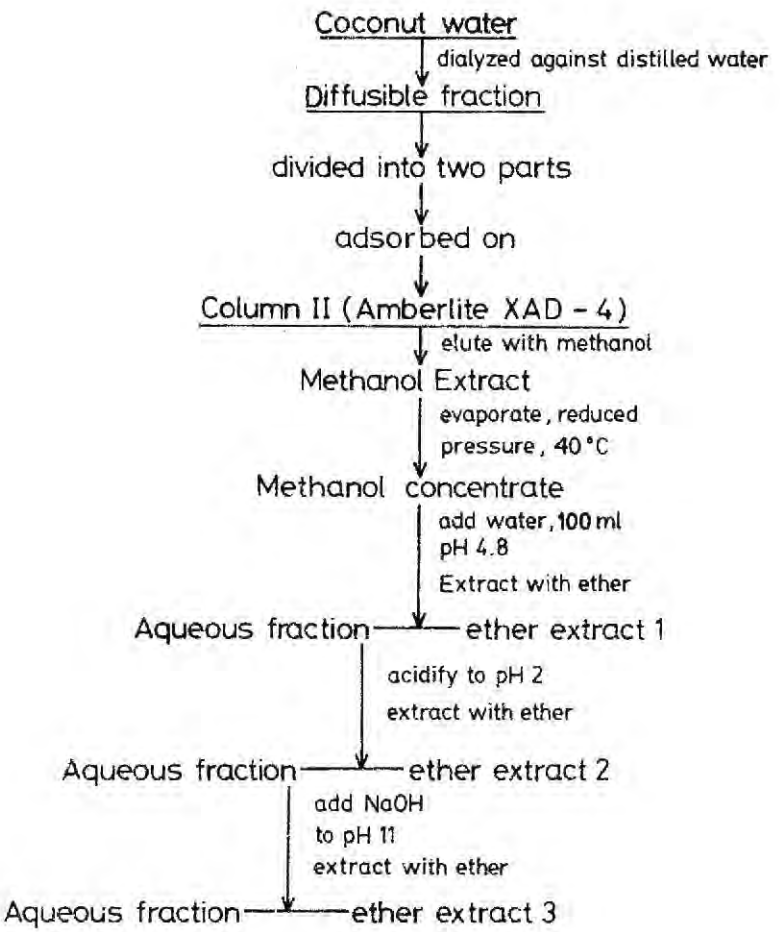


Fig. 4. The schematic procedure for the fractionation of the coconut water diffusible fraction.

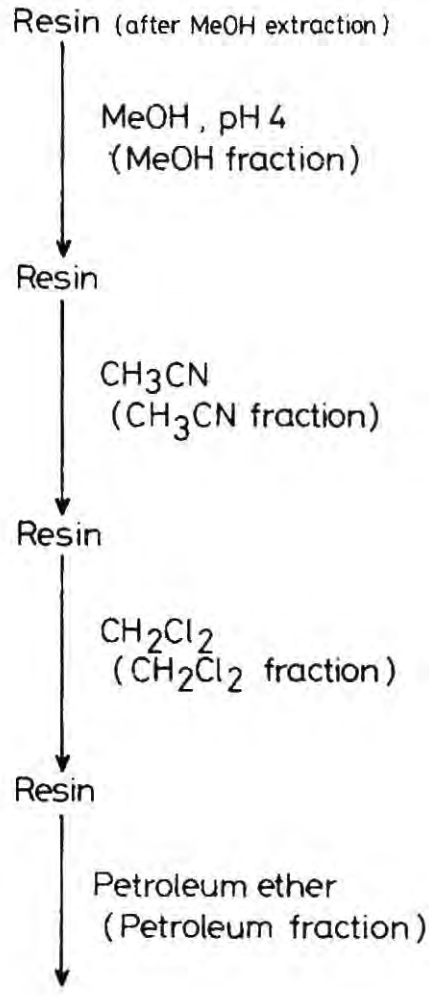


Fig. 5. Sequential Polarity of Solvents.

The resin adsorbate was further eluted out with solvents of decreasing polarity. The scheme of elution is shown in Fig. 5. Methanol, pH 4 (200 ml) was followed by acetonitrile (200 ml), then with methylene chloride (200 ml) and finally with petroleum ether (200 ml).

The solvents were recovered and the concentrates diluted to 100 ml with water.

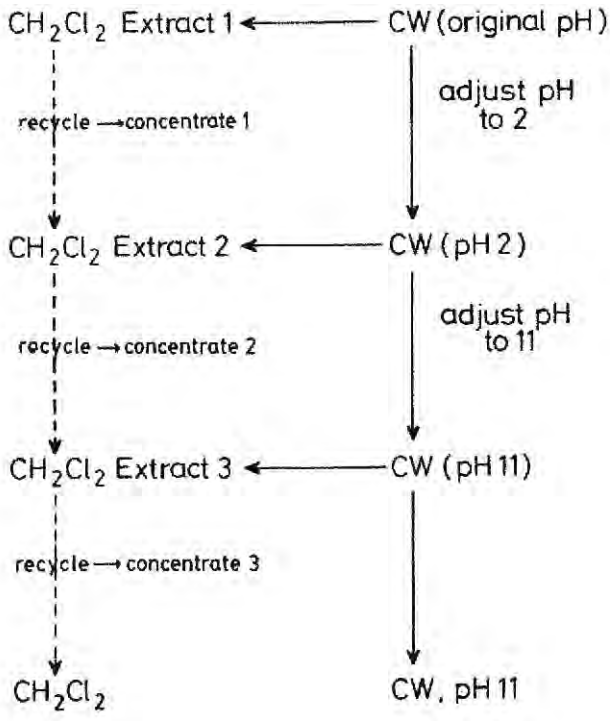


Fig. 6. Direct Solvent Extraction by Methylene Chloride.

Direct solvent extraction

Coconut water was extracted with methylene chloride (b.p. 40.1°C) at its original pH in the ratio of 1 of solvent to 10 of CW. The methylene layer was removed from the aqueous phase. The solvent was then recycled by distillation *in vacuo* and the concentrate was designated as CH_2Cl_2 extract 1. The aqueous portion was acidified to pH 2 and extracted with CH_2Cl_2, the solvent was recycled and the concentrate that was left designated as CH_2Cl_2 2. The aqueous phase after the second extraction was adjusted to pH 11 and again extracted with CH_2Cl_2. Again the solvent was recycled and the concentrated residue designated as CH_2Cl_2 3. The extraction, fractionation diagram is illustrated in Fig. 6.

The solvents were recovered and the concentrates diluted to 100 ml with water.

Characterization and identification of the extracts/fractions of CW

The UV absorption curves for the standard growth hormones and the various coconut water extracts/fractions were determined from 180 nm to 350 nm, using a Uvicon 810/820 recording spectrophotometer. The standard growth hormones were indoleacetic acid, gibberellic acid, kinetin, zeatin and abscissic acid. The UV spectral curves of the CW extracts/fractions were compared with spectral curves of the standards for the identification of the growth hormones present.

Results and Discussion

UV spectral curves (180-350 nm) for the plant growth hormones are shown in Fig. 7. Indoleacetic acid has 3 absorption peaks at 190, 221, and 280 nm (Fig. 7a), gibberellic acid at 190 and 265 (Fig. 7b), kinetin at 189, 210, 267 (Fig. 7c), zeatin at 190, 225, 269 (Fig. 7d), and abscissic acid at 245 and 190 (Fig. 7e).

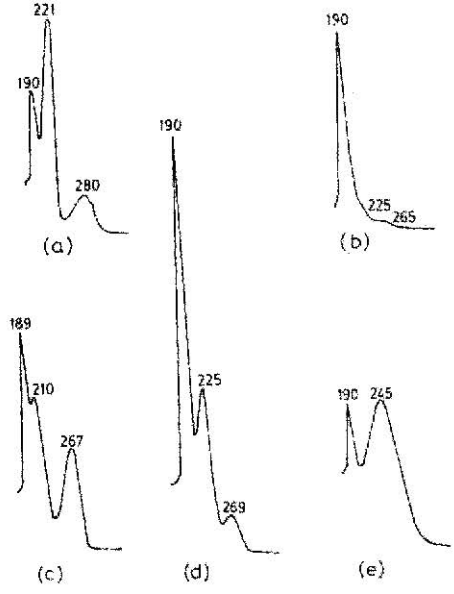


Fig. 7. UV spectral curves of plant growth hormones:
(180-350 nm)
(a) indoleacetic acid, 50 ug/ml
(b) gibberellic acid, 4.7 ug/ml
(c) kinetin, 3.5 ug/ml
(d) zeatin, 10 ug/ml
(e) abscissic acid, 4.5 ug/ml

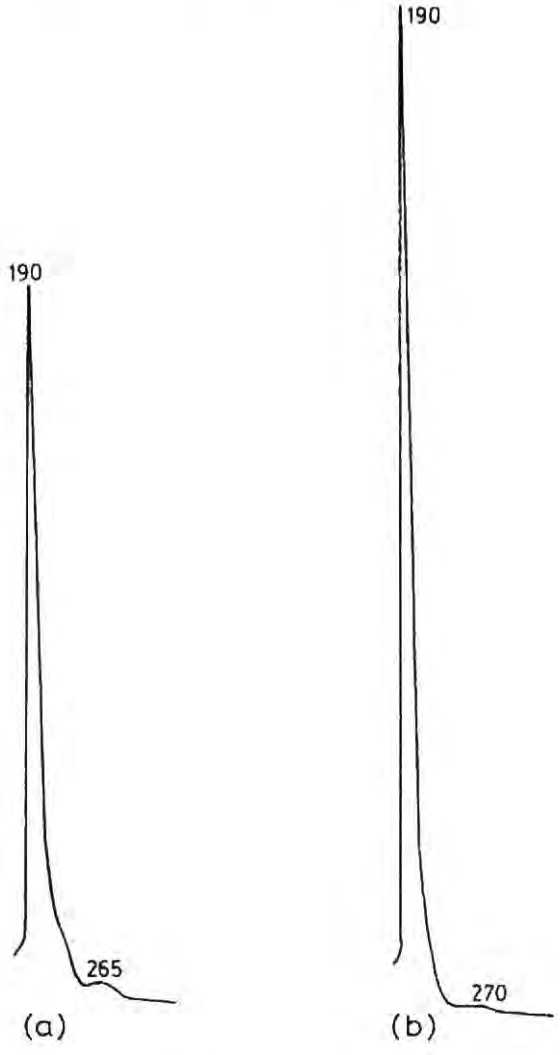


Fig. 8. UV spectral curves (180-235 nm) of:
(a) fresh coconut water (cw), (diluted 100x)
(b) CW dialyzate, (diluted 20x)

UV spectral curves of fresh coconut water (Fig. 8a), diluted 100x show an approximate concentration of about 1.2 g per liter of substances absorbing at 190 nm. These substances may consist of adenine-like organics, plant growth hormones, soluble nucleic acids, proteins, aromatic amino acids, etc. Fig. 8b shows about 0.4

g per liter of these absorbing substances at 190 nm. The high molecular weight substances have been separated from the lower molecular weight compounds by dialysis.

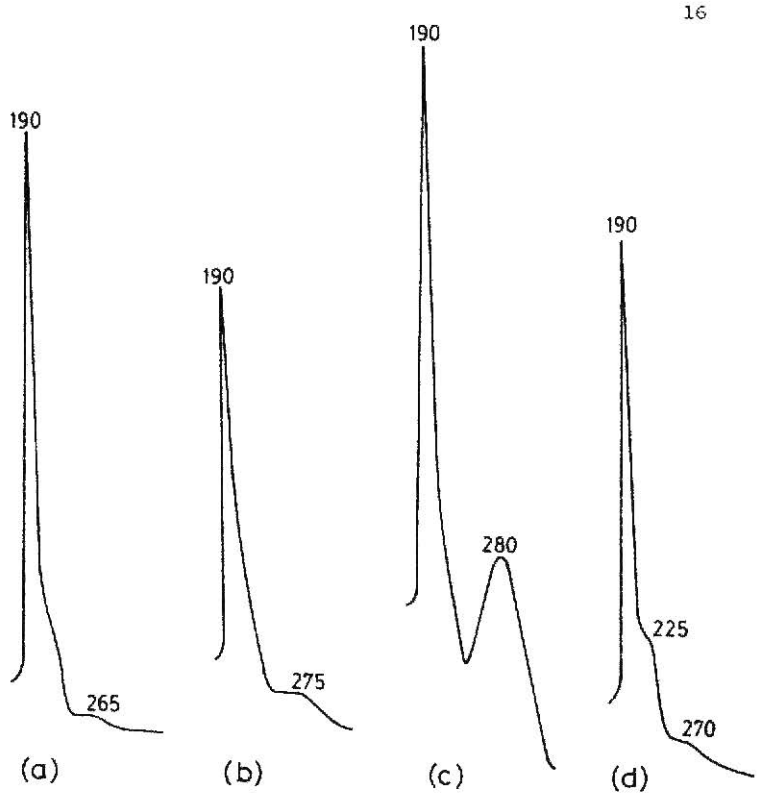


Fig. 9. UV spectral curves (180-350 nm) of CW fractions eluted from activated charcoal column by:
(a) ethanol, diluted 10x
(b) glacial acetic acid, diluted 20x
(c) acetinitrile
(d) ethanol: 10% NH_4OH, diluted 5x

The charcoal fractions (Fig. 9) indicate the presence of auxin/kinetin-like substances, 113.3 μg/ml in the ethanol fractions (Fig. 9a) and gibberellins, 115.6 μg/ml in the glacial acid fractions (Fig. 9b). An unidentified growth hormone (11.5 μg/ml) is present in the acetonitrile fraction (Fig. 9c) with an absorption peak at 190 and 280 nm. This plant growth hormone needs to be further studied. Zeatin-like subtances are present in the ethanol: 10% NH_4OH, fraction 4.8 μg/ml (Fig. 9d).

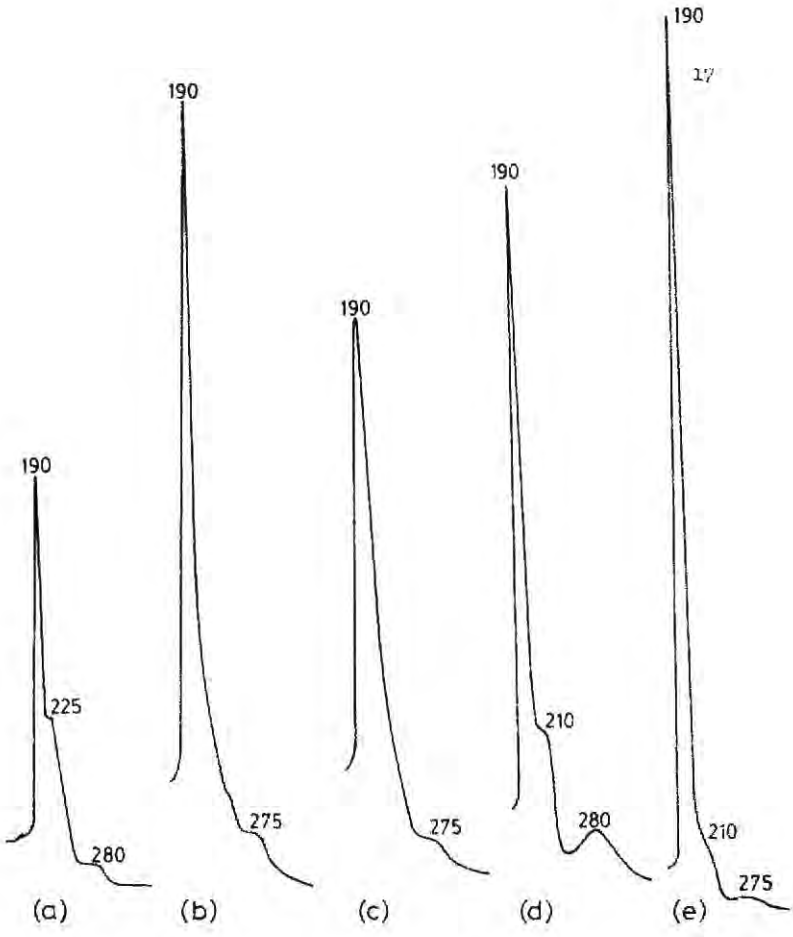


Fig. 10. UV spectral curves (180-350 nm) of methanol eluate from resin Amberlite XAD-4 and the ether fractionation of the methanol eluate

(a) methanol eluate, diluted 10x
(b) ether extract 1, pH 4.8
(c) ether extract 2, pH 2
(d) ether extract 3, pH 11
(e) aqueous layer, pH 11

The resin fraction (Fig. 10) indicates a mixture of the growth hormones in the methanol eluate (Fig. 10a). Ether extract 1 (Fig. 10b) contains about 15.11 μg/ml of gibberellin-like substances. Ether extract 2, (Fig. 10c) contains about 9.78 μg/ml of gibberellin-like substances while ether extract 3 (Fig. 10d) has about 13.56 μg/ml kinetin/zeatin-like substances. The aqueous layer, pH 11 (Fig. 10e) has mostly zeatin-like substances, 36 μg/ml.

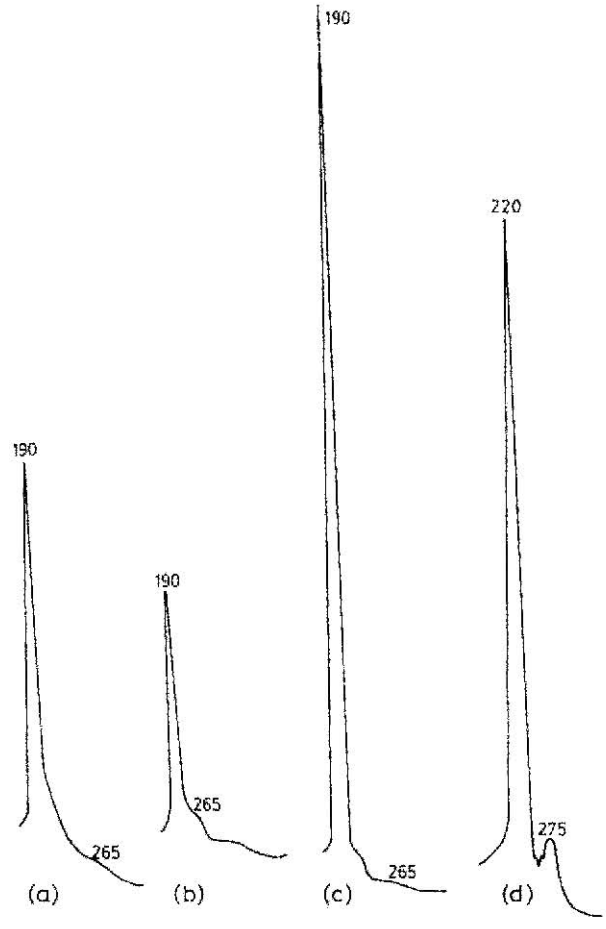


Fig. 11. UV spectral curves (180-350 nm) of resin eluates by sequential polarity of eluants:

(a) CH_3OH, pH 4
(b) CH_3CN
(c) CH_2Cl_2
(d) petroleum ether eluate

Further elution of the resin columns by solvents of decreasing polarity have UV spectral curves as shown in Fig. 11. Zeatin-like substances, 8.9 μg/ml, (Fig. 11a) are present in the methanol pH 4 eluate and also in the CH_3CN eluate, 27.3 μg/ml, (Fig. 11b). Gibberellins/zeatins may be present in the CH_2Cl_2 eluate, 24.4 μg/ml, (Fig. 11c). The 190 nm absorption peak is absent in the petroleum ether eluate which means that substances absorbing in this region have already been stripped from the resin column.

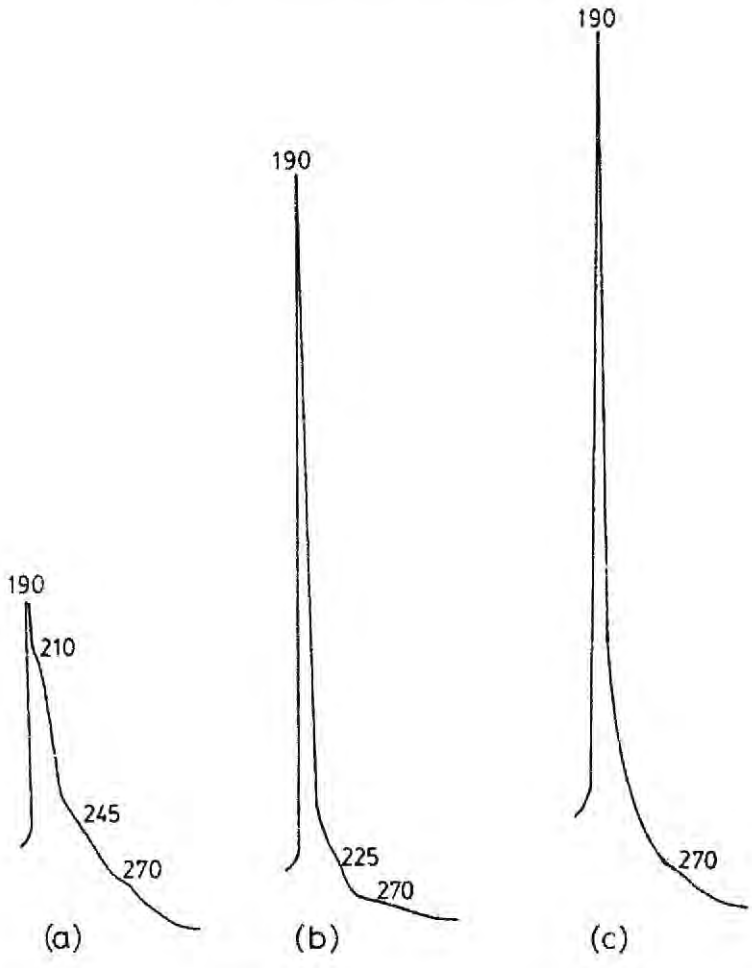


Fig. 12. UV spectral curves (180-350 nm) of direct methylene chloride extracts of CW:
(a) CH_2Cl_2, orig. CW, pH 5.5, diluted 10x
(b) CH_2Cl_2, pH_2 diluted 20x
(c) CH_2Cl_2 3, pH 11

Fig. 12 shows the UV absorption spectra of the methylene chloride fractions from the direct solvent extraction method. Fig. 12a shows a mixture of kinetins, auxins, and a slight hump in the 245 nm region and contains around 44.4 μg/ml of these hormones. Zeatin-like substances 253.40 μg/ml, (Fig. 12b) are present in the CH_2Cl_2 extract, pH 2. Gibberellins, 14.22 μg/ml (Fig. 12c) are present in the CH_2Cl_2 extract 3, pH 11.

Summary and Conclusion

The results show that gibberellins are present in the glacial acetic acid fraction of the charcoal adsorbate, ether extract 1, pH 4.8 (resin), ether extract 2, pH 2 (resin), CH_2Cl_2 eluate and the CH_2Cl_2 extract 3, pH 11, of the direct solvent extraction. Kinetins are present in ethanol fraction (charcoal), ethanol eluate (resin), ether extract 3, pH 11 and CH_2Cl_2 extract 1. Zeatins are present in methanol: 10% NH_4OH eluate (charcoal), methanol eluate (resin), ether extract 3 and aqueous extract 3, pH 11 (resin), CH_3OH, pH 4, CH_3CN, CH_2Cl_2 eluates and CH_2Cl_2 extract 2, pH 2. Auxins are easily oxidized but their presence can be detected in ethanol extract (charcoal), and CH_3OH, pH 4.8 eluate (resin) and CH_2Cl_2 1 extract, pH 5.5.

An interesting plant growth hormone is present in the acetonitrile fraction (charcoal).

Plant growth hormones can be extracted from coconut water by a direct process of dialysis, column (charcoal or resin) adsorption, and elution. Direct solvent extraction of the plant growth hormones with solvent recycling and concentration via a one-step process is faster, simpler and more economical. The reverse dialysis method can be adapted to a continuous and large scale operation.

UV analysis of coconut water extracts show that CW is rich in gibberellins and cytokinins, particularly kinetins and zeatins.

This research study shows that the technology of extracting plant growth hormones from coconut water can be developed. With such a technology, the importation of these high-cost biochemicals will be minimized, and dollar reserves will be saved. Moreover, the coco-chemical industries will be boosted by the additional use of coconut water not only for the manufacture of vinegar and nata de coco, etc. but also as a rich source of complex biochemicals, such as plant growth hormones, needed in agriculture and high-technology research in tissue culture.

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