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Research Article

GC-MS, HPLC AND AAS ANALYSIS OF FATTY ACIDS, AMINO ACIDS AND MINERALS IN RED ALGAE *AMPHIROA ANCEPS*

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ABSTRACT

Fatty acid, amino acid, minerals, and trace metals of Amphiroa anceps were determined. Fatty acids are determined by gas chromatography-mass spectrometry (GC-MS), using flame ionization detector. In the case of fatty acids total 12 components were find out in which the major fatty acids encountered are 16:0, 20:4, 18:1n9, 18:2n6, and 14:0, etc. Amino acids are determined using high performance liquid chromatography (HPLC) and total 18 amino acids were found on the dry weight basis. Tryptophan was estimated colorimetrically following digestion under alkaline condition and estimated with thioglycolic acid reagent. Minerals identified by using Flame Photometry are sodium, potassium and calcium whereas iron, copper, Manganese, magnesium, etc using Atomic Absorption Spectroscopy.

Keywords: Seaweeds, Red algae, Amphiroa anceps, Fatty acids, Amino acids, Minerals.

INTRODUCTION

Life originated in the sea and during evolution, marine organism have developed into very sophisticated physiological and biochemical system. Ancient time onwards Asian and European countries utilized marine algae as a source of polysaccharides for food and pharmaceutical uses. Now marine environment have proven to be a very rich source of potent compound that has demonstrated significant activities against cancer, inflammation, pain, allergy and human pathogens.

The investigation of fatty acids in the field of lipid chemistry between marine species has been started out since 1950s [1]. The fatty acids are essential for human nutrition and are of interest in biotechnology, food chain studies and cosmetics. The fatty acid distribution between different taxonomic classes of algae's observed large variations according to temperature fluctuations, intensity of light, and amount of nitrogen content and level of mineral content present in the algal strain [2]. Methyl esters of fatty acids (FAME) from animal and vegetable origin having 8-24 carbon atoms are separated and detected using GC-MS.

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer. The amino acid sequence helps to determine the properties of protein or peptide molecules. So, the amino acid analysis can be used to quantify proteins and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect the concentration of amino acid that might be present in their sample.

Seaweeds are incomparable source of minerals, macro elements and trace metals. Mineral content in seaweeds compared to land and animal products is generally higher concentration and the essential minerals and trace elements needed for human nutrition are present in seaweeds. A fraction of dry weight of seaweed account for minerals and the amount is slightly greater than that of land and animal products. From the literatures it was observed that seaweeds are valuable sources of protein, vitamins, and minerals which are essential for human nutrition [3].

MATERIALS AND METHODS

Collection and processing of marine algae

Red algae are collected from the Gulf of Mannar region of Mandapam coast, Tamilnadu, South-east coast of India. Study area is blessed with a diverse variety of algae. To avoid the species contamination, the algae which infested exclusively on the intertidal rocky region and other substratum were collected. Immediately after collection, they are washed in fresh sea water to remove epiphytes and other extraneous matter. Sample was kept under shade for 7 days. After drying the sample, make into powder form and then used for primary estimation of fatty acids, amino acids and minerals.

GC-MS analysis of FAME

Fatty Acid Methyl Esters (FAME) were analyzed and separated using Perkin Elmer Auto system XLGC and Perkin Elmer Turbo Mass Gold Mass spectrometer (Norwalk, CTO6859, USA). The column used was Perkin Elmer Elite-225 capillary column measuring 30m ×0.25mm with a film thickness of 0.25mm. Helium was used as carrier gas at a flow rate of 0.5ml/min. The inlet temperature was maintained at 265˚C. The oven temperature was initially held at 110˚C for 4min. and was programmed to increase to 240˚C at a rate of 2.7˚C/min., held at 240˚C for 3min. And then programmed to increase to 280˚C at a rate of 20˚C and held for 5min. The total run time was 62.15min. The MS transfer line was maintained at a temperature of 200˚C. The source temperature was maintained at 180˚C. GC-MS was carried out using EI and data were evaluated using total ion count (TIC) for compound identification and quantification. An external standard consists of 37 FAMEs including purchased from SUPELCO (SIGMA-ALDRICH, UK), was used for calibration and quantification of individual fatty acids. Detector used was Flame ionization detector [4].

A known weight of lipid, extracted by Folch method [5] was taken into a round bottom flask. Evaporated off the chloroform and added methanolic sodium hydroxide. Attached condenser, and refluxed under nitrogen until fat globules disappear. Added BF_3 solution and continued boiling for 5min. Removed from heat, and added saturated sodium chloride solution. Stopper the flask and mixed vigorously for 15sec while solution was still warm. Transfer it into a separating funnel. Washed the Round bottom flak with distilled water and transferred it into a separating funnel. Added petroleum ether to this separating funnel and shake the contents and left it for 5min under nitrogen for the separation. Transferred the lower aqueous layer to a round bottom flask and upper petroleum ether layer to another separating funnel. Lower aqueous layer in the round bottom flask are extracted twice with petroleum ether, and the upper petroleum ether layer was pooled with the above one in the separating funnel. Washed thrice the combined petroleum ether extracts with water, collected the upper petroleum ether layer, filtered it through anhydrous sodium sulphate, and evaporated off solvent under vacuum. Made up the contents with petroleum ether and transferred it into small vials. Methyl esters of the fatty acids thus obtained were separated by Gas chromatography-Mass Spectroscopy.

Determination of total amino acid profile

Acid hydrolysis

The amount of each amino acid present within a given protein does not vary from molecule to molecule and can provide useful information about the nature of the protein molecule. For the determination of amino acid the sample was weighed into a test tube. 6N HCl was added and the tubes were heat sealed after filling pure nitrogen gas. Hydrolysis was carried out in a hot air oven at 110^{o}C for 24hours. After the hydrolysis, the content were removed quantitatively and filtered. The content of the flask were flash evaporated to remove trace of HCl. The residue was made up to a definite volume with 0.05N HCl. The sample thus prepared was filtered again through a membrane filter of 0.45micron size [6].

The sample was injected to Shimadzu HPLC-LC-10AS consisting of column packed with a strong acidic cation exchange resin of styrene divinyl copolymer with sulphonic group. The column used is sodium type i.e. ISC-07/S1504 Na having a length of 19cm and diameter 5mm. The oven temperature was maintained at 60^{0}C. The amino acid analysis was done with non-switching flow method and fluorescence detection after post column derivatization with O-phthalaldehyde. Amino acid standard was also run to calculate the concentration of the amino acid depending on the standard chromatogram.

Alkaline Hydrolysis

Determination of tryptophan

The amino acid Tryptophan is not stable to acid digestion in the presence of even trace amount of oxygen and is estimated separately by alkali digestion using colorimeter.

Procedure

About 200-250mg of sample was hydrolyzed with 10 ml of 5% NaOH at 110°C for 24 hours in a sealed tube filled with pure nitrogen. The hydrolysate was neutralized to pH 7.0 with 6 N HCl using phenolphthalein indicators [7]. The volume was made up to 100ml with distilled water. The solution was then filtered through whatman filter paper No.1 and filtrate was used for estimation.

To a test tube containing 4ml of 50% H_2SO_4, 0.1 ml of 2.5% sucrose and 0.1 ml of 0.6% thioglycolic acid were added. These tubes were kept for 5min in water bath at 45-50°C and cooled. The sample was then added to the test tubes. A set of (0.1 to 0.8) standard Tryptophan (10µg/ml) was treated similarly. The volume was made up to 5 ml with 0.1N HCl and allowed to stand for 5minutes. The absorbance was measured using Hitachi-UV/Vis U-2910 spectrophotometer at 500 nm. The concentration was obtained by drawing standard graph.

Determination of Minerals

Procedure for Sample preparation

Heated a porcelain crucible to 600°C in a muffle furnace, cooled in a desiccator and weighed (W1). Weighed accurately about 2g of the dried sample into a porcelain crucible (W2). The crucible was placed on a fume hood and heated at a low flame until the material was charred. The charred material was kept inside the previously set muffle furnace and heated at 600°C for 6hrs to get white or grayish white ash. The crucible was cooled in a desiccator and weighed (W3)[8]. For the analysis of minerals collected the ash and dissolved in concentrated hydrochloric acid and made up to 50ml in a standard flask.

Identification of minerals

Estimation of various minerals such as copper, zinc, sodium, potassium, magnesium etc has been carried out as per the procedure outlined in standard manual.

Flame photometric analysis of minerals

The instrument used for analysis was BWB XP flame photometer (U.K) and the working standards in the range of 10, 20 and 40ppm were made from stock standard of 1000ppm for each mineral (Na, K, Ca). Standardized the instrument with the above series of working standards. Samples were aspirated into the flame and the corresponding readings were recorded. Calculations were carried out to find the mineral concentration of the sample. The characteristic color radiations are produced when salts such as Na, K, Ca, etc are introduced into a flame. Emission of such characteristics radiation by each element and the correlation of the emission intensity with the concentration of that element is the basis of flame photometry.

Atomic absorption spectroscopy for the analysis of trace metals

Instrument used was Varian spectra-220AA atomic absorption spectrophotometer. The working standards of appropriate range were made from stock standard of 1000ppm for each trace metal. Standardized the instrument with the above series of working standards. Samples were aspirated into the flame (combination of air and acetylene) and the corresponding absorption of characteristic radiation by the atomic vapor of the element was recorded. The minerals were analyzed by dissolving the ash (obtained in ash determination) in dil.Hydrochloric acid and estimated using Atomic absorption spectrophotometer (Spectra AA220, AAS VARIAN), with acetylene and air supplied in constant ratio for flame and hollow cathode lamp.

RESULTS AND DISCUSSION

Fatty Acids

Table 1 shows the profile of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids. From Table 1 it can be observed that there are total 12 fatty acid components are available in the *Amphiroa anceps*. The available saturated fatty acids (SFA) include Palmitic acid (57.57%), Myristic acid (4.25%), Stearic acid (2.70%), Lignoceric acid (0.34%),etc. monounsaturated fatty acids (MUFA) are Palmitoleic acid (2.48%), oleic acid (5.77%), Erucic acid (0.11%), and polyunsaturated fatty acids (PUFA) are Lignoceric acid (4.51%), Arachidonic acid (18.67%), Eicosapentaenoic acid (2.46%), Docosahexaenoic acid (0.96%). From the analysis of fatty acids it was observed that the most abundant fatty acids present in the Amphiroa anceps was Palmitic acid and the next abundant fatty acid is Myristic acid. From the literatures it was observed that seaweeds are an important source of long chain Polyunsaturated Fatty Acids i.e. from n-2 to n-6, which is fundamental for the development of cell membranes. In addition, these PUFA are precursors of eicosanoids, which influence inflammation processes and immune reactions. Anti-cancer properties can have been attributed to Palmitic acid and also some studies reveal that it hinder the fatty acids are useful for the treatment of cardiovascular diseases, hypertension, autoimmune diseases, diabetics, etc.

Table 1: Fatty acid analysis of *A. anceps* using GC-MS

Carbon Number	Fatty acid Name	Amphiroa anceps (%)
C14	Myristic acid	4.25
C16	Palmitic acid	57.57
C18	Stearic acid	2.70
C24	Lignoceric acid	0.34
	Total SFA	**64.86**
C16:1	Palmitoleic acid	2.48
C18:1n9	Oleic acid	5.77
C22:1	Erucic acid	0.11
	Total MUFA	**8.36**
C18:2n6	Linoleic acid	4.51
C20:4	Arachidonic acid	18.67
C20:5n3	Eicosapentaenoic acid	2.46
C22:6n3	Docosahexaenoic acid	0.96
	Total PUFA	**26.6**

Amino Acids

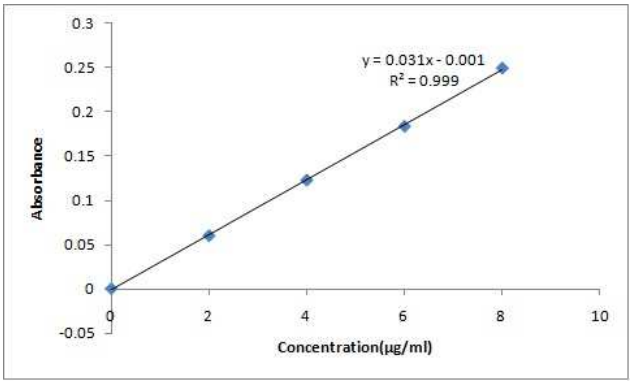
The sequence of amino acids in a protein or peptide determines the properties of the molecule. Amino acids present in amphiroa anceps was detected using HPLC and the red algae species featured

uniquely high concentration of Arginine, Aspartic acid, Glutamic acid, Valine, Isoleucine, Serine, Leucine, Threonine, Proline, Tyrosine, etc respectively. The various components available in the Amphiroa anceps amino acid are given in Table 2 shows the graph of amino acids which is present in the Amphiroa anceps. Table 2 also presents the quantitative and qualitative information on the available amino acids and Graph 1 shows the standard graph of tryptophan which is determined by digestion under alkaline conditions and estimated with thioglycolic acid reagent.

The dried sample of red algae Amphiroa anceps was found to contain 18 amino acids, namely, aspartic acid, glutamic acid, alanine, valine, glycine, arginine, serine, methionine, threonine, phenylalanine, tyrosine, isoleucine, leucine, histidine, cysteine, tryptophan and proline. It can be observed from Table 2 that Arginine is the major component and the quantity being 22.29g/16gN. The other components, namely, Aspartic acid (5.21g), Glutamic acid (4.44g), Valine (4.04g), Isoleucine (3.29g), Serine (2.92g), Leucine (2.88g), Threonine (2.64g), Proline (2.27g), Tyrosine (2.11g), are followed by Arginine. Further, it can be noted that Alanine is much less (0.45g) quantity compared to all other components available in Amphiroa anceps.

Table 2: Determination of total amino acid profile of *A. anceps* using HPLC

Amino acids	g/16gN
Aspartic acid	5.21
Serine	2.92
Threonine	2.64
Glutamic acid	4.44
Proline	2.27
Glycine	0.74
Alanine	0.45
Cysteine	0.49
Valine	4.04
Methionine	0.59
Isoleucine	3.29
Leucine	2.88
Tyrosine	2.11
Phenylalanine	1.16
Histidine	1.55
Lysine	1.46
Arginine	22.29
Tryptophan	1.33
Total	59.87



Graph 1: Colorimetric Estimation of Tryptophan

Minerals

The following minerals are identified in Amphiroa anceps (Table 3 and Table 4). It was observed that (Table 3) the Na (5.95mg) is the major constituent of algae and formed the bulk of total minerals. The next major constituent is potassium and magnesium. Copper is the least mineral product of these algae. From the above, it can be observed that Na/K ratio is below1.0 which reduces the risk of hypertension. Further, it can be observed that the mineral content available in *A.anceps* for human consumption is well within the limits (1.5-10mg). In general, algal product would supplement the daily intake of some trace elements for adults : Fe, 10-18mg; Zn, 15 mg; Mn, 2.5-5mg and Cu is 2-3mg determined mineral content in several red edible marine seed vegetables. Seaweeds contained high proportions of ash (21.1- 39.3%). In general red algae, ash content is 20.6 – 21.1%. Atomic absorption spectrophotometry of the ashes indicated that marine seaweeds contained high amount of both macro minerals (13.7mg, Na, K, Ca) and trace metals (1.35mg, Fe, Zn, Cu, Mg, Mn), than those reported for edible land plants. In the present study, it was identified that Na is the major mineral. Fayaz *et al*. used the ash of the sample for the estimation of mineral elements by AOAC procedure. The concentrations of the elements in *Amphiroa anceps* were determined with flame and atomic absorption spectrophotometer. Duplicate determinations for each element were carried out on dry weight basis. The concentration of the elements was determined from calibration. It was observed that Amphiroa anceps contains calcium 2.21mg, iron 0.39 mg and zinc 0.32mg, copper 0.01mg, manganese 0.14mg, magnesium 0.48mg, sodium 5.95mg, potassium 5.64mg of the sample. The presence of significant amounts of calcium and iron in *Amphiroa anceps* may be due to its metabolic system in which it is capable of directly absorbing elements from the sea water. Ca was the major constituent of these algae and formed the bulk of total minerals. Similar observation was made in the present investigation.

Table 3: Flame Photometric Analysis of Minerals

Minerals	Absorbance	Mineral content(mg)
Na	4.8	5.95
K	4.55	5.64
Ca	44.75	2.21

Table 4: Atomic Absorption Spectroscopic Analysis of trace metals

Minerals	Absorbance	Mineral content(mg)
Fe	0.7905	0.39
Cu	0.022	0.10
Zn	0.6514	0.32
Mn	0.298	0.14
Mg	0.97315	0.48

CONCLUSION

Fatty acids, amino acids and mineral contents were estimated in *Amphiroa anceps* on dry weight basis. The samples were collected from Gulf of Mannar region of South east coast of India, Tamilnadu. Fatty acids are detected using GC-MS, amino acids are by HPLC, and tryptophan estimated using colorimetry and minerals using flame atomic absorption spectroscopy. In the case of fatty acids total 12 components were identified and in which Palmitic acid (C16) and Arachidonic acid (C20:4) are the major components. Total Saturated Fatty Acids present 64.86%, Monounsaturated Fatty Acids 8.36%, and Polyunsaturated Fatty Acids 26.6%. Total 18 amino acids were found out using HPLC while in case of tryptophan which is estimated after alkaline hydrolysis. Among all the amino acids Arginine is the major constituents is the major constituent and followed by Aspartic acid, Glutamic acid and Isoleucine etc. The minerals such as Na, K, and Ca were identified using flame photometry and Mg, Mn, Zn, Cu, etc. The studies showed that the Amphiroa anceps could be used as a food supplement to meet the recommended daily intake of some essential minerals. The results obtained are of interest in the field of biochemistry and chemotaxonomy and also provide useful information to the pharmaceutical industry.

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