

**Analytical and biochemical studies of selected
seaweeds obtained from the eastern coast of South
Africa`s Indian Ocean in KwaZulu-Natal**

By

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As the candidate's supervisor, I have approved this thesis for submission.

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DECLARATION 1: PLAGIARISM

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Publication 1

Title: Chemical composition and seasonal variation on elemental uptake of selected seaweeds from the Indian Ocean, KwaZulu-Natal, South Africa.

Authors: Judie Magura, Roshila Moodley and Sreekanth B. Jonnalagadda.

Journal: Manuscript submitted to Journal of Environmental Science and Health on the 8th of October 2015 (Currently under review).

Publication 2

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In preparation of the above manuscripts, I performed all the experiments and interpreted the data. The co-authors contributed in editing, and verifying the scientific content as well as editing the manuscript.

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ABSTRACT

Seaweeds are known to possess excellent sources of proteins, vitamins, dietary fibre, macro and micro-elements, as well as important bioactive compounds. Thus, they can contribute to the nutritional requirements of humans and may also be beneficial to human health. The use of seaweeds for human consumption and as medicine has strong roots in the Asian cultures and they have also gained importance in many other parts of the world due to their health benefits. In South Africa, despite the abundance of seaweeds, only a few seaweed species are exploited for the hydrocolloid industry and as feed for abalone, particularly in the Cape provinces. The coast of KwaZulu-Natal possesses diverse algal flora yet there is a lack of information regarding the nutritional and medicinal properties of these seaweeds.

The main aim of this study was to investigate the nutritional and medicinal properties of selected seaweeds namely *Halimeda cuneata*, *Spyridia hypnoides*, *Codium capitatum*, *Hypnea spicifera* and *Sargassum elegans* (the latter three are edible), found along the east coast of KwaZulu-Natal. The proximate composition of the three edible seaweeds and the effect of seasonal variation on elemental uptake in all five seaweeds was investigated. As brown seaweeds are generally known to accumulate high concentrations of toxic elements, the distribution of essential elements and the toxic element arsenic (total and inorganic) in *S. elegans* from eight different sites along the east coast of KwaZulu-Natal were also investigated. The brown seaweed, *S. elegans*, was also investigated for its secondary metabolites.

The edible seaweeds had a moisture level of 85.4 to 89.5%, protein of 6.1 to 11.8%, lipids of 7.5 to 13.1% and carbohydrates (which was obtained by difference) of 37.8 to 71.9 %. In general, elemental concentrations in the five seaweeds varied significantly with season ($p < 0.05$) and were found to be in decreasing order of $\text{Ca} > \text{Mg} > \text{Fe} > \text{Cu} > \text{Mn} > \text{As} > \text{Zn} > \text{Ni} > \text{Cr} > \text{Pb} >$

Co \approx Se. In *S. elegans*, elemental concentrations varied significantly with location ($p < 0.05$) and total As was found to be extremely high, ranging from 42.1 to 105.4 $\mu\text{g g}^{-1}$, of which, 21.4 to 53.0 $\mu\text{g g}^{-1}$ were in inorganic form. The phytochemical investigation of *S. elegans* revealed the presence of three bioactive compounds, β -sitosterol, fucosterol and phaeophytin **a**. This showed that *S. elegans* could be a potential and alternate source of these compounds. This study also shows that *C. capitatum* and *H. spicifera* could be potential sources of most essential nutrients and may contribute positively to the diet without posing the risk of adverse health effects due to low concentrations of toxic elements. However, consumption of *S. elegans* for nutritional or medicinal purposes could increase exposure to inorganic As which could cause adverse health effects therefore it should be avoided.

TABLE OF CONTENTS

DECLARATION 1: PLAGIARISM	II
DECLARATION 2: PUBLICATIONS	III
ACKNOWLEDGMENTS	IV
ABSTRACT	V
TABLE OF CONTENTS	VII
LIST OF FIGURES	X
LIST OF TABLES	XI
ABBREVIATIONS	XII
CHAPTER 1	1
1.1 INTRODUCTION	1
1.2 PROBLEM STATEMENT.....	3
1.3 AIMS.....	4
1.4 OBJECTIVES.....	4
REFERENCES	5
CHAPTER 2	8
LITERATURE REVIEW	8
2.1 SEaweEDS.....	8
2.2 THE SEaweED INDUSTRY IN SOUTH AFRICA.....	9
2.3 SEaweED USES IN AFRICA	11
2.4 HYDROCOLLOIDS.....	12
2.4.1 AGAR	12
2.4.2 ALGINATE	13
2.4.3 CARRAGEENAN.....	13
2.5 NUTRITION IN SEaweEDS	14
2.6 SECONDARY METABOLITES IN SEaweEDS.....	15
2.7 <i>SARGASSUM</i>	19
2.7.1 SCIENTIFIC CLASSIFICATION OF <i>SARGASSUM</i>	19
2.7.2 BIOACTIVE COMPOUNDS FOUND IN THE FAMILY SARGASSACEAE	21
2.7.3 <i>SARGASSUM</i> AS A FOOD SOURCE	22
2.7.4 <i>SARGASSUM ELEGANS</i> SUHR (1840)	23
2.8 SEaweED SPECIES IN THIS STUDY	24
2.8.1 <i>HALIMEDA CUNEATA</i> HERING IN KRAUSS (1846).....	24
2.8.2 <i>SPYRIDIA HYPNOIDES</i> (BORY DE SAINT-VINCENT) PAPENFUSS (1968)	24
2.8.3 <i>CODIUM CAPITATUM</i> P. SILVA (1959).....	25
2.8.4 <i>HYPNEA SPICIFERA</i> (SUHR) HARVEY (1847).....	26

2.9 ESSENTIAL ELEMENTS IN SEAWEEDS.....	27
2.10 ESSENTIAL NUTRIENTS IN HUMANS.....	28
2.10.1 MICRONUTRIENTS	28
2.10.2 MACRONUTRIENTS	31
2.11 THE TOXIC ELEMENT ARSENIC (As).....	33
2.12 SEASONAL VARIATION.....	34
2.13 ANALYTICAL AND PHYTOCHEMICAL TECHNIQUES	35
2.13.1 MICROWAVE DIGESTION	35
2.13.2 INDUCTIVELY COUPLED PLASMA-OPTICAL EMISSION SPECTROMETRY (ICP-OES)	37
2.13.2.1 ADVANTAGES AND DISADVANTAGES OF ICP-OES	38
2.13.2.2. ICP-OES INTERFERENCES	38
2.13.3 CHROMATOGRAPHY	39
2.13.3.1 THIN LAYER CHROMATOGRAPHY (TLC).....	40
2.13.3.2 COLUMN CHROMATOGRAPHY	40
2.13.4 SPECTROSCOPIC TECHNIQUES	41
2.13.4.1 NUCLEAR MAGNETIC RESONANCE (NMR).....	42
2.13.4.2 INFRARED SPECTROSCOPY (IR).....	43
2.13.4.3 MASS SPECTROMETRY	44
REFERENCES.....	45
CHAPTER 3.....	55
CHEMICAL COMPOSITION AND SEASONAL VARIATION ON ELEMENTAL UPTAKE OF SELECTED SEAWEEDS FROM THE INDIAN OCEAN, KWAZULU-NATAL, SOUTH AFRICA.....	55
ABSTRACT.....	55
3.1 INTRODUCTION.....	56
3.2 MATERIALS AND METHODS.....	57
3.2.1 SAMPLING	57
3.2.2 ANALYSIS OF PROXIMATE COMPOSITION	58
3.2.3 REAGENTS	58
3.2.4 DETERMINATION OF ELEMENTAL CONTENT	58
3.2.5 STATISTICAL ANALYSIS	59
3.3 RESULTS AND DISCUSSION	59
3.3.1 PROXIMATE COMPOSITION.....	59
3.3.2 ELEMENTAL ANALYSIS	61
3.3.3 TOXIC ELEMENTS.....	67
3.3.4 CONTRIBUTION TO THE DIET	69
3.4 CONCLUSION	73
REFERENCES.....	74
CHAPTER 4.....	79

BIOACTIVE COMPOUNDS, ELEMENTAL CONCENTRATIONS, TOTAL AND INORGANIC ARSENIC IN <i>SARGASSUM ELEGANS</i> SUHR (1840).	79
ABSTRACT	79
4.1 INTRODUCTION	80
4.2 MATERIALS AND METHODS	81
4.2.1 GENERAL EXPERIMENTAL PROCEDURE	81
4.2.2 SAMPLING	81
4.2.3 PREPARATION OF EXTRACTS	82
4.2.4 PHYTOCHEMICAL SCREENING	82
4.2.5 CHARACTERIZATION AND QUANTIFICATION METHODS	82
4.2.6 ISOLATION OF COMPOUNDS FROM <i>S. ELEGANS</i>	83
4.2.7 REAGENTS	85
4.2.8 DETERMINATION OF ELEMENTAL CONTENT	85
4.2.9 DETERMINATION OF INORGANIC ARSENIC	86
4.2.10 STATISTICAL ANALYSIS	86
4.3 RESULTS AND DISCUSSION	87
4.3.1 STRUCTURE ELUCIDATION OF COMPOUNDS FROM <i>S. ELEGANS</i>	87
4.3.2 ELEMENTAL ANALYSIS	90
4.4 CONCLUSION	94
REFERENCES.....	96
CHAPTER 5	101
5.1 SUMMARY	101
5.2 FINDINGS FROM THE CHEMICAL ANALYSIS OF THE EDIBLE SEaweEDS	101
5.3 FINDINGS FROM THE ELEMENTAL ANALYSIS	101
5.4 FINDINGS FROM THE PHYTOCHEMICAL STUDY OF <i>S. ELEGANS</i>	102
5.5 OVERALL CONCLUSION	102
5.6 RECOMMENDATIONS FOR FURTHER STUDY	103
APPENDIX	104

LIST OF FIGURES

FIGURE 1. BASIC STRUCTURE OF SEAWEEDS.	8
FIGURE 2. BEACH CAST.	10
FIGURE 3. A FARMER FEEDING FRESH KELP TO ABALONE (ROBERTSON-ANDERSON ET AL., 2006). 11	
FIGURE 4. CHEMICAL STRUCTURES OF (A) CHLOROPHYLL A AND (B) PHAEOPHYTIN A.	17
FIGURE 5. CHEMICAL STRUCTURES OF (A) CHOLESTEROL AND (B) FUCOSTEROL.	18
FIGURE 6. <i>SARGASSUM ELEGANS</i>	23
FIGURE 7. <i>HALIMEDA CUNEATA</i>	24
FIGURE 10. <i>SPYRIDIA HYPNOIDES</i>	25
FIGURE 8. <i>CODIUM CAPITATUM</i>	26
FIGURE 9. <i>HYPNEA SPICIFERA</i>	27
FIGURE 11. CEM MARS 6 MICROWAVE.	36
FIGURE 12. CEM MARS 6 (A) EASYPREP™ AND MARSXPRESS™ VESSELS.	36
FIGURE 13. ICP-OES OPTIMA 5300 DV AT THE SCHOOL OF CHEMISTRY AND PHYSICS (UKZN). 37	
FIGURE 14. A SCHEMATIC DIAGRAM OF THE TLC TECHNIQUE.	40
FIGURE 15. A COLUMN USED TO SEPARATE COMPOUNDS IN THIS STUDY.	41
FIGURE 16. INFRARED SPECTROMETER AT THE SCHOOL OF CHEMISTRY & PHYSICS (UKZN).	44
FIGURE 17. MEAN CONCENTRATIONS OF AS IN THE FIVE SEAWEED SPECIES STUDIED DURING THE FOUR DIFFERENT SEASONS, N = 3. DIFFERENT SUPERSCRIT LETTERS WITHIN COLUMNS INDICATE MEAN SEPARATIONS BY TUKEY`S OR GAMES-HOWELL POST-HOC TESTS AT THE 5% LEVEL.	67
FIGURE 18. MEAN CONCENTRATIONS OF Pb IN THE FIVE SEAWEED SPECIES STUDIED DURING THE FOUR DIFFERENT SEASONS, N = 3. DIFFERENT SUPERSCRIT LETTERS WITHIN COLUMNS INDICATE MEAN SEPARATIONS BY TUKEY`S OR GAMES-HOWELL POST-HOC TESTS AT THE 5% LEVEL.	68
FIGURE 19. STRUCTURE OF COMPOUND 1 (B-SITOSTEROL).	88
FIGURE 20. STRUCTURE OF COMPOUND 2 (FUCOSTEROL).	89
FIGURE 21. STRUCTURE OF COMPOUND 3 (PHAEOPHYTIN A).	90

LIST OF TABLES

TABLE 1. COMPOUNDS ISOLATED FROM DIFFERENT <i>SARGASSUM</i> SPECIES AND THEIR USES.....	21
TABLE 2. TRADITIONAL USES OF <i>SARGASSUM</i> AS A FOOD SOURCE.	22
TABLE 3. DIETARY REFERENCE INTAKES (DRIs) - RECOMMENDED INTAKES FOR INDIVIDUALS. ..	30
TABLE 4. TOLERABLE UPPER INTAKES LEVELS (UL).	30
TABLE 5. DIETARY REFERENCE INTAKES (DRI) - ACCEPTABLE MACRONUTRIENT DISTRIBUTION RANGES.	31
TABLE 6. PROXIMATE CHEMICAL COMPOSITION OF THE SEAWEED SAMPLES ANALYSED (MEAN \pm SD, N=3) AT THE 95% CONFIDENCE INTERVAL.	61
TABLE 7. ELEMENTAL CONCENTRATIONS (MEAN \pm SD, N=3) IN THE CERTIFIED REFERENCE MATERIAL, <i>WHITE CLOVER</i> BCR-402.	62
TABLE 8. CONCENTRATION (IN $\mu\text{G G}^{-1}$, DRY WEIGHT) OF ELEMENTS IN SEAWEEDS (MEAN \pm SD, N = 3) FOR THE FOUR DIFFERENT SEASONS.	65
TABLE 9. DIETARY REFERENCE INTAKE (RECOMMENDED DIETARY ALLOWANCE (RDA) AND TOLERABLE UPPER INTAKE LEVEL (UL)) FOR EACH ESSENTIAL ELEMENT AND ESTIMATED CONTRIBUTION OF SEAWEEDS (<i>C. CAPITATUM</i> , <i>H. SPICIFERA</i> AND <i>S. ELEGANS</i>) TOWARDS THE RDA FOR MOST INDIVIDUALS.	72
TABLE 10. PRELIMINARY PHYTOCHEMICAL SCREENING OF CRUDE EXTRACTS OF <i>S. ELEGANS</i>	87
TABLE 11. CONCENTRATIONS (IN $\mu\text{G G}^{-1}$, DRY WEIGHT) OF ELEMENTS IN <i>S. ELEGANS</i> (MEAN \pm SD, N = 3) AT THE EIGHT DIFFERENT SITES.	92
TABLE 12. CONCENTRATIONS (IN $\mu\text{G G}^{-1}$, DRY WEIGHT) OF TOTAL AND INORGANIC ARSENIC IN <i>S. ELEGANS</i> (MEAN \pm SD, N = 3).	93

ABBREVIATIONS

ALARP	As low as reasonably practicable
AMDR	Acceptable macronutrient distribution range
ANOVA	Analysis of variance
AOAC	Association of analytical communities
BMDL	Benchmark dose lower confidence limit
br s	Broad singlet
CFIA	Canadian Food Inspection Agency
¹³C-NMR	C-13 nuclear magnetic resonance spectroscopy
COSY	Correlated spectroscopy
CRM	Certified reference material
d	Doublet
dd	Double doublet
DEPT	Distortionless enhancement by polarization transfer
DRI	Dietary reference intake
DW	Dry weight
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nations

FSANZ	Food Standards Australia New Zealand
HMBC	Heteronuclear multiple bond coherence
¹H-NMR	Proton nuclear magnetic resonance spectroscopy
HR-ESI-MS	High-resolution electrospray ionisation mass spectrometry
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
ICP	Inductively coupled plasma
ICP-OES	Inductively coupled plasma-optical emission spectroscopy
IR	Infrared
JECFA	Joint Food and Agriculture Organization/ World Health Organization Expert Committee of Food Additives
m	Multiplet
MALDI	Matrix assisted laser desorption ionization
MS	Mass spectroscopy
NOESY	Nuclear overhauser effect spectroscopy
PFA	Perfluoroalkoxy
PTFE	Polytetrafluoroethylene
PTWI	Provisional Tolerable Weekly Intake
q	Quartet

RDA	Recommended dietary allowance
RF	Radio frequency
R_f	Retention factor
s	Singlet
t	Triplet
TLC	Thin layer chromatography
TOF	Time of flight
UL	Tolerable upper intake level
WHO	World Health Organisation

CHAPTER 1

1.1 Introduction

Seaweeds have been consumed traditionally in Asian countries since ancient times, but only a few coastal communities outside Asia have occasionally used seaweeds as components of their dishes (Ródenas de la Rocha et al., 2009; Bocanegra et al., 2009). However, due to increasing consumer interest in foods that do not only meet nutritional needs but also have health benefits and the influx of Asian cuisines, the use of seaweeds as foodstuffs and as components of functional foods has steadily gained importance in many parts of the world. Apart from their proven nutritional properties in Asian cuisines, seaweeds are exclusively used for the extraction of important food hydrocolloids such as agar, alginates and carrageenan in the rest of the world. Their availability, almost throughout the year, and relatively easy collection (seaweeds can be picked on foot during low tide) makes them an inexpensive natural food resource.

Bioactive compounds found in seaweeds have attracted the interest of health conscious societies and scientists as they are regarded as natural producers of active compounds and an alternative to synthetic substances (Rajapakse and Kim, 2011; Chojnacka and Kim, 2015). Seaweeds grow in harsh environments where they are exposed to a combination of light and high concentrations of oxygen which gives rise to the formation of free radicals and other strong oxidizing agents. Even under these conditions, seaweeds seldom suffer any serious photodynamic damage during metabolism. This suggests that seaweed cells have protective mechanisms and compounds (Matsukawa et al., 1997). The bioactive compounds in seaweeds include carotenoids, phlorotannins, glycolipids, polysaccharides, vitamins, sterols, tocopherol and meroterpenoids

(Cox et al., 2010; Holdt and Kraan, 2011; Liu et al., 2012; Smit, 2004). The reported biological activities of these components include, among others, antioxidant, antibacterial, antifungal and fibrinolytic activity (Liu et al., 2012; Matsukawa et al., 1997; Wu et al., 2009). Epidemiological studies comparing Japanese and Western diets have linked seaweed consumption to lower incidences of chronic diseases such as cancer, hyperlipidaemia and coronary heart disease (Brown et al., 2014). Seaweeds are used traditionally for the treatment of diseases such as arthritis, high blood pressure, gout, goitre and hypertension.

Seaweeds are rich in proteins, vitamins, minerals, fibre, carbohydrates and physiologically important fatty acids therefore they are used as vitamin and mineral supplements (Dawczynski et al., 2007; Gillesi`le et al., 1996; Ruperez, 2002). The mineral content of some seaweeds can account for up to 36% of its dry matter with some of the macronutrients being sodium, calcium, magnesium, potassium, chlorine, sulphur and phosphorus and some of the micronutrients being iodine, iron, zinc ,copper, selenium, fluorine, manganese, boron and nickel (Rajapakse and Kim, 2011). Such properties of seaweeds have the potential to contribute positively to the nutritional requirements of humans and can benefit human health. However, some seaweed may also contain high levels of toxic elements like arsenic, cadmium and lead which are potential risks to human health. Therefore, the determination of the concentration of toxic elements in seaweeds is necessary to evaluate both their nutritive potential and risks to human health. In most countries, there are no special regulations enforced for seaweed consumption except that they have to conform to the general safety regulations for food as specified by the Provisional Tolerable Weekly Intake (PTWI) recommended by the World Health Organization (WHO) (Mouritsen et al., 2013). However, France is one of the countries that have a specific list of seaweeds for

human consumption which specific upper limits of inorganic arsenic, cadmium, lead, tin, mercury and iodine (Burtin, 2003; Holdt and Kraan, 2011).

South Africa has the highest regional seaweed diversity and is considered among those countries with the richest marine flora in the world. To date, the known species of seaweeds in South Africa are estimated at around 750-800, of which 270 species (only intertidal and shallow water collections) are believed to be in KwaZulu-Natal (Bolton and Stegenga, 2002; Bolton et al., 2004). Despite their abundance, the valuable health and nutritional benefits of these seaweeds are yet to be explored and exploited in South Africa. Research on seaweeds found in South Africa focuses mostly on species that are being exploited for commercial use (hydrocolloid industry) and abalone farming from the Cape provinces (Anderson et al., 2003; Levitt et al., 2002; Rothman et al., 2010). Studies on the chemical composition, medicinal and nutritional properties of a great variety of species found in South Africa, are still lacking.

1.2 Problem Statement

South Africa has a coastline of 3650 km in length and has been shown to have an extremely rich seaweed flora of well over 800 species but remains, to a great extent, under-researched with regards to utilisation (Bolton et al., 2004; Griffiths et al., 2010). Studies from other parts of the world where seaweeds (from wild stock or cultivated) are utilised have shown seaweeds to provide great economic benefit as well as to be a healthy food resource. The KwaZulu-Natal coast supports a wide range of genera which have found uses throughout the world, but studies on the constituents of these seaweeds are still lacking. Therefore, there is need to investigate the chemical composition of these seaweeds and evaluate their nutritive potential. It is also important

to investigate, isolate and characterise the secondary metabolites found in these seaweeds to explore their ethno-medicinal properties.

1.3 Aims

The aim of this study was to analytically and phytochemically investigate different classes of seaweeds namely, *Halimeda cuneata*, *Spyridia hypnoides*, *Codium capitatum*, *Hypnea spicifera* and *Sargassum elegans* (latter three are edible), found along the east coast of KwaZulu-Natal. The analytical investigation was done to determine the nutritional value of the edible seaweeds and evaluate the effect of seasonal variation on elemental uptake by seaweeds. The phytochemical investigation was done on the brown edible seaweed, *S. elegans*, to determine if it contains any secondary metabolites. Total and inorganic arsenic concentrations in *S. elegans* were also determined to evaluate its safety for human consumption.

1.4 Objectives

- To determine the chemical composition (ash, carbohydrate, lipid, moisture and protein) of edible seaweeds.
- To determine the elemental concentrations in different classes of seaweeds as a function of seasonal variation.
- To evaluate the nutritional value of edible seaweeds by comparing their elemental concentrations to recommended dietary allowances (RDAs).
- To determine the concentration of total and inorganic arsenic in the brown edible seaweed, *S. elegans*.

- To extract, characterise and identify the compounds from *S. elegans* using spectroscopic techniques (NMR, IR, UV and MS).

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CHAPTER 2

LITERATURE REVIEW

2.1 Seaweeds

Seaweeds are marine macro-algae which are found throughout all oceans, in all climatic zones, from the warm tropics to the icy polar-regions. They grow wherever rocks, coral or some fixed structures such as shells are available for their attachment. Seaweeds contain photosynthetic pigments similar to land plants and use sunlight and nutrients from seawater to photosynthesise and produce food. However, they are not considered true plants as they lack structures such as roots, stems and leaves (Garza, 2005). Generally, seaweeds consist of a root-like holdfast which is not used to absorb nutrients but to attach to suitable substrata, a stipe (the stem of seaweed) which is used to support the seaweed and blades (leave-like) which provide a large surface for absorption. Some seaweeds have hollow gas-filled sacs which are called floats that help keep them afloat on water. The basic structure of seaweeds is represented in Figure 1.

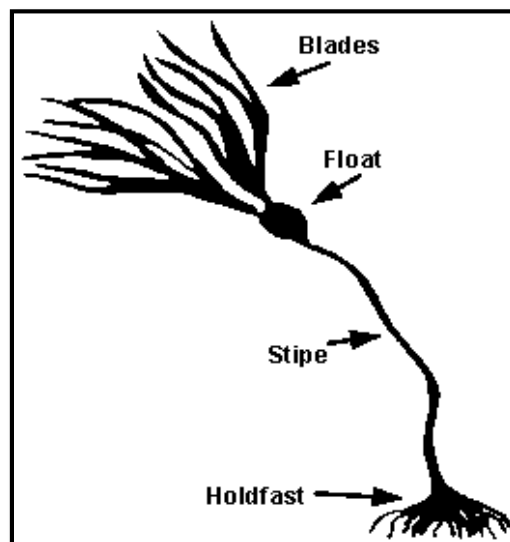


Figure 1. Basic structure of seaweeds.

Biologically, seaweeds are classified into three main groups according to their pigmentation, morphology, anatomy and chemical composition as *Phaeophyta* (brown), *Rhodophyta* (red) and *Chlorophyta* (green) (Pramanick et al., 2015). *Phaeophyta*, is the largest type of seaweed, growing up to 20 m in length. Examples include *Laminaria*, *Saccharina*, *Fucus* and *Sargassum*. The brown or yellow-brown colour in brown seaweeds is mainly due to fucoxanthin and some small amounts of chlorophyll. *Rhodophyta*, often have brilliant red and orange hues due to phycoerythrin and phycocyanin, which are dominant over other pigments such as chlorophyll α , β -carotene and a number of xanthophylls. The green colour in *Chlorophyta* is due to chlorophylls *a* and *b* which are in the same proportions as in higher plants (Mouritsen, 2013).

2.2 The seaweed industry in South Africa

In South Africa, the seaweed industry is based on *Ecklonia*, *Laminaria pallida* and red seaweeds of the genus *Gelidium*. Currently, *Gelidium* species are collected in the Eastern Cape and *Gracilaria* species are collected at Saldana Bay; these are exported for the production of agar. Large beds of kelp (*Ecklonia maxima* or “sea bamboo”) grow on Western Cape shores which, during storms, wash up on the beaches. These beach casts are picked, dried, separated and milled to various size chips and exported for the extraction of alginate (Troell et al., 2006).



Figure 2. Beach cast.

http://www.nda.agric.za/doaDev/sideMenu/fisheries/03_areasofwork/Resources.

The majority of seaweeds, harvested and collected in South Africa, are exported since it is technologically complicated and too expensive to extract and manufacture the end-products (plant-growth stimulants, cosmetics, alginate and carrageenan). Presently, only plant growth stimulants are produced from kelp in South Africa. However, large quantities of fresh kelp are harvested from the surface, using boats. This is done during low-tide when the kelp fronds are accessible from the surface. The fresh kelp is used as feed for farmed abalone (approximately 6000 tonnes fresh weight of kelp in 2003) (Troell et al., 2006). Abalones are edible sea snails (marine gastropod molluscs in the family Haliotidae) that are a delicacy in many parts of the world.



Figure 3. A farmer feeding fresh kelp to abalone (Robertson-Anderson et al., 2006).

2.3 Seaweed uses in Africa

Seaweed, as a direct source of food, appears to be largely neglected on the African continent. Along the South African coast, early Cape colonists have been reported to have used *Suhria vittata* known as “red ribbon” for jelly making (FAO, 2011). In Chad, *Spirulia* (blue-green algae) is used as food. It is particularly rich in vitamins and has been long valued by central African tribesmen as a weaning food for infants. It is also used to make biscuits and meals in conjunction with millet. *Spirulia* grows on ponds and pools and it is reported that annual yields of up to 50 tonnes per hectare can be harvested (FAO, 2011).

It is reported that the Topnaar people who lived near the Kuiseb River in Namibia dried, roasted and grounded kelp into a powder which they mixed with fat and used as a salve to prevent infection and aid the healing of wounds (Van Damme et al., 1922). Some herbalists from KwaZulu-Natal believe that the sea has extraordinary powers and they use seaweed to make tea infusions with boiling water to treat sore throats, chest pains and stomach ache. Some tribes burn

the seaweed in a pot and inhale the smoke to drive away evil spirits. To these tribes, seaweeds are commonly known as *Amakhafilithi* or *izibizolwande*.

2.4 Hydrocolloids

A hydrocolloid is a non-crystalline substance with very large molecules that dissolve in water to give a thickened viscous solution. Currently, they are used in various industries for thickening and gelling aqueous solutions, foams, emulsions and dispersions, inhibiting ice and sugar crystal formation. Red and brown seaweeds are mainly used to produce hydrocolloids namely agar, alginate and carrageenan (Phillips and Williams, 2009).

2.4.1 Agar

Agar, commonly known as agar-agar was the first hydrocolloid used as a food additive; its use began over 300 years ago in the Far East (Armisen and Gaiatas, 2009). Agar is a strong gelling hydrocolloid extracted from the red seaweeds, *Gelidium* and *Gracilaria*. The extraordinary gelling power of agar is due to the hydrogen bonds formed among its linear galactan chains that provide an excellent reversibility; they melt just by heating and gel again upon cooling (Armisen and Gaiatas, 2009). Since agar does not need any other substance to gel, it has enormous potential in applications such as the manufacture of capsules for medical applications and as a medium for cell cultures. It has been reported that agar-agar leads to a decrease in the concentration of blood glucose levels and exerts an anti-aggregation effect on red blood cells (Kraan, 2012). The anti-tumour activity of the agar-type polysaccharide obtained from a *Gracilaria* species by cold water extraction was also reported (Holdt and Kraan, 2011).

2.4.2 Alginate

Alginates, for commercial use, are mainly extracted from species of brown seaweeds (*Laminaria*, *Ascophyllum nodosum*, *Macrocystis pyrifera* and *Sargassum*) that contribute up to 40% of their dry weight (Rhein-Knudsen et al., 2015). Alginates extracted from brown seaweeds are water-soluble. In the food industry, alginates are used as stabilizers and thickeners. In addition, alginates are important in the healthcare and pharmaceutical industries and in biotechnology where they are being used as wound dressings, in dental impressions, and enzyme immobilization (Kaplan, 2013). Studies on alginic acid (acid form of alginate) have shown that it leads to a decrease in the concentration of cholesterol, exerts an anti-hypertension effect, can prevent absorption of toxic chemical substances, and plays a major role as dietary fibre for the maintenance of animal and human health (Kraan, 2012).

2.4.3 Carrageenan

Carrageenan is extracted mainly from red seaweeds (*Kappaphycus alvarezii* and *Eucheuma denticulatum*). Carrageenan dissolves in water, forms highly viscous solutions and remains stable over a wide pH range. They are used as stabilizers, gelling agents, emulsifiers, and thickeners in the food and baking industries (ice-cream, cheese, jam, bread dough). Recently, carrageenan has attracted attention in the pharmaceutical industry due to its ability to inhibit attachment of viruses such as the human papillomavirus, dengue virus and herpes virus. In addition, carrageenan is used in several drug delivery systems as matrixes to control drug release, microcapsules, and microspheres (Rhein-Knudsen et al., 2015).

2.5 Nutrition in seaweeds

Worldwide, approximately 221 species of seaweeds are commercially utilised, of which 65% are used as human food. Of the 221 species, 32 are *Chlorophyta*, 64 *Phaeophyta* and 125 *Rhodophyta* (Subba-Rao et al., 2009). From a nutritional perspective, edible seaweeds are low calorie foods with high concentrations of minerals, vitamins and proteins and low content of lipids which range from 2.3 to 4.6% based on semi-dry sample weight (Mohamed et al., 2012). Seaweeds are an excellent source of vitamins A, B1, B12, C, D, and E, riboflavin, niacin, pantothenic acid, folic acid and minerals such as calcium, potassium, sodium and phosphorus (Dhargalkar and Pereira, 2005). Seaweeds are reported to have more than 54 trace elements, most of which are required for the physiological functioning of the human body, and these are in quantities greatly exceeding vegetables and other land plants (Chapman and Chapman, 1980). The essential elements in seaweeds are in chelated, colloidal, optimally balanced form, which enhances their bioavailability in the human body (Mouritsen, 2013).

The protein content in seaweeds is reported to vary from 26.6% in red seaweeds to 12.9% in brown seaweeds (Dawczynski et al., 2007). The protein in red seaweeds contain essential amino acids with levels sufficient enough to meet dietary requirements; the protein content in *Palmaria Palmata* (Dulse) and *Porphyta Tenera* (Nori) can reach 35% and 45% of their dry weight, respectively (Burtin, 2003). These levels are comparable to those found in high protein vegetables such as soybeans (35% protein based on dry mass).

Seaweeds contain large amounts of polysaccharides in their cell walls which are not found in land plants (Ruperez et al., 2002). Algal polysaccharides are considered to be undigested by humans, hence they are considered to be an abundant source of dietary fibre (Misurcova et al., 2012). The content of total dietary fibre in seaweeds ranges from 33 to 50% dry weight and it is

higher than that found in most fruits and vegetables (Ruperez and Saura-Calixto, 2001). Human consumption of algal fibre has proven to promote growth and protection of the intestinal flora, it greatly increases stool volume and also reduces risk of colon cancer (Dawczynski et al., 2007). Anticoagulant activity is the most investigated in sulfated polysaccharides, as researchers attempt to find a substitute for heparin (Ruperez et al., 2002).

The role of the diet in human health is progressively gaining more attention over the last few years. Food is not only beneficial due to the presence of essential nutrients, but also due to the occurrence of other bioactive compounds which have been found to be important for health promotion and disease prevention. These beneficial effects can be attributed to the complex mixture of secondary metabolites which possess antioxidant, antimicrobial, anticancer and antiviral activity. The compounds responsible for these activities include phenolic compounds, terpenes, carotenoids and volatile halogenated organic compounds and seaweeds are a rich source of such compounds (Gupta and Abu-Ghannam, 2011).

2.6 Secondary metabolites in seaweeds

The secondary metabolites of seaweeds, compared to land plants, have attracted the interest of biochemists because of their diversity. The major groups of secondary metabolites found in seaweeds are isoprenoids (terpenes and steroids), pigments (carotenoids and chlorophylls), polyketides (phlorotannins), amino acid derived natural products (alkaloids) and shikimates (flavonoids). Red seaweeds (*Rhodophyta*) are richer sources of these secondary metabolites compared to the other macro-algae. Phlorotannins are found exclusively in brown seaweeds (Mendis and Kim, 2011) and their amounts can vary among species, depending on algae age, size, tissue type, nutrient level and season (Lopes et al., 2012). Phlorotannins, like other

polyphenolic compounds, exhibit numerous biological activities such as, antioxidant, anti-inflammatory, anti-allergic, antimicrobial, anticancer and antidiabetic activities (Lopes et al., 2012; Yang et al., 2010).

Carotenoids, chlorophylls and phycobiliproteins are the main classes of pigments found in algae. β -carotene, lutein and violaxanthin are among the carotenoids found in green seaweeds. Red seaweeds contain mainly α -and β -carotene, lutein and zeaxanthin (Takaichi, 2011). Brown seaweeds contain mostly fucoxanthin, a xanthophyll that possesses a wide range of biological activities (Peng et al., 2011; Rajauria and Abu-Ghannam, 2013). Chlorophylls are green lipid-soluble pigments found in all algae, higher plants and cyanobacteria which are responsible for photosynthesis. Chlorophylls are sensitive to extreme pH and temperature conditions which allow the formation of distinct chlorophyll derivatives such as phaeophytins. These derivatives have shown anti-mutagenic effects and may play a significant role in cancer prevention (Holdt and Kraan, 2011; Pangestuti and Wibowo, 2013). Phaeophytin **a**, a chlorophyll *a* derivative isolated from the brown alga, *Sargassum fulvellum* was shown to be a strong neuro-differentiating compound with strong antioxidant activity (Ina et al., 2007).

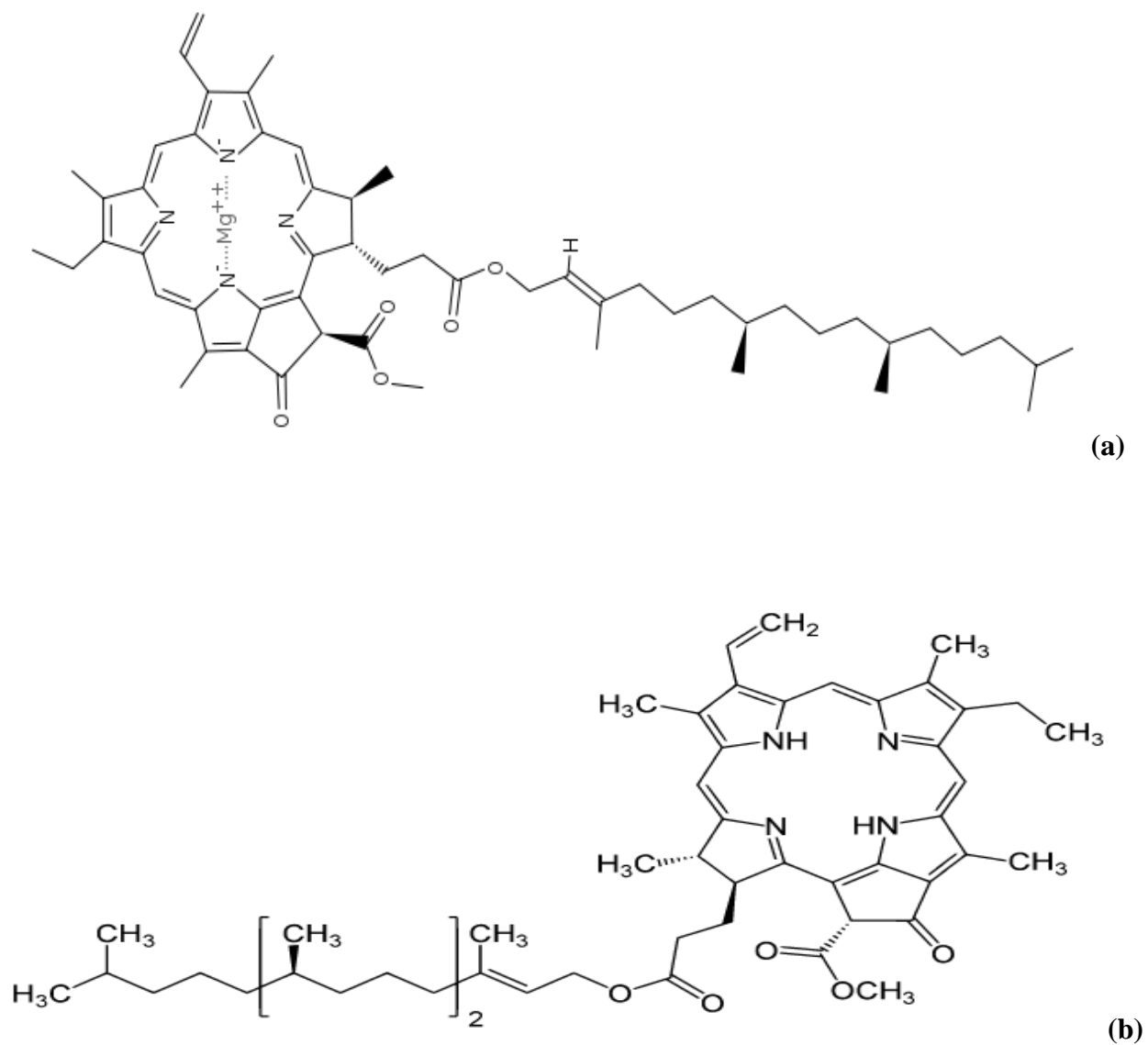


Figure 4. Chemical structures of (a) chlorophyll a and (b) phaeophytin a.

Terpenes from seaweeds are frequently observed with substituted halogenated functional groups. In brown seaweeds, terpenes are found in two main orders; fucales and dictyotales. Diterpenes, sesquiterpenes and halogenated terpenes isolated from brown seaweeds have been reported to

exhibit antiviral and anticancer properties and possibly have the potential to counteract malaria (Mouritsen, 2013).

Sterols are abundant in macro-algae, they can occur in free form, esterified with fatty acids or in glycosylated conjugates. Algal sterols are similar in structure to cholesterol (that is also found in red seaweeds), however they contain an additional alkyl group at C-24 (Figure 5) that is absent in cholesterol (Lopes et al., 2013).

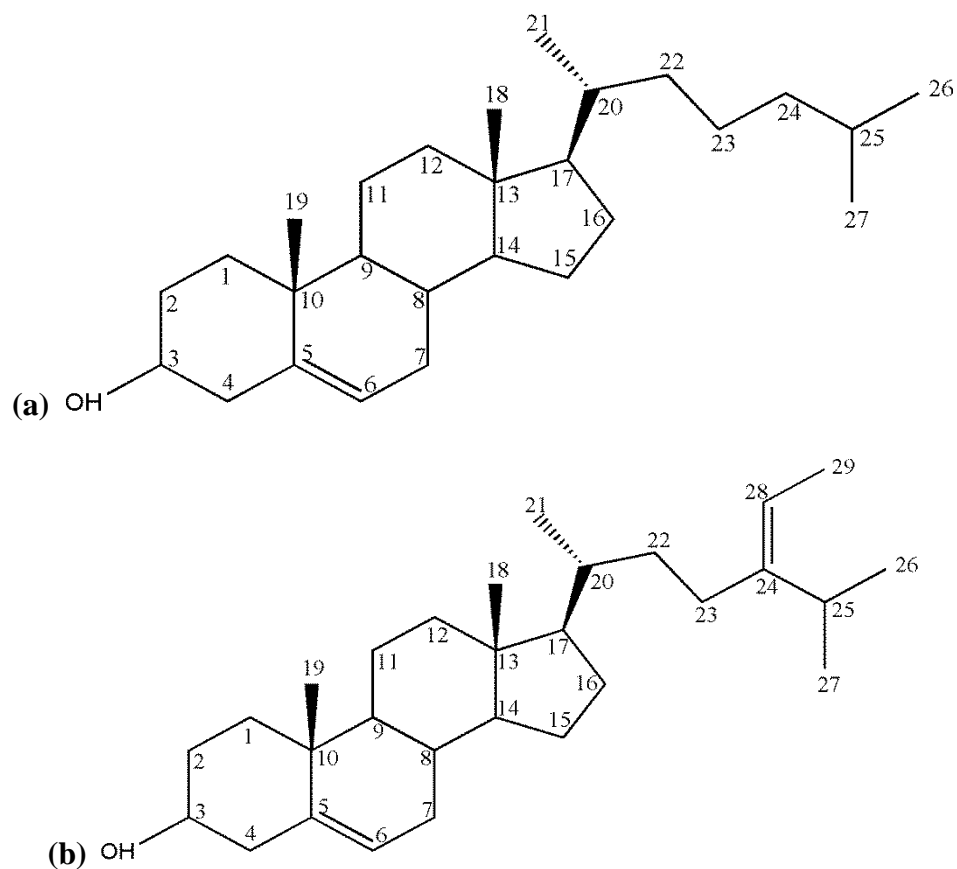


Figure 5. Chemical structures of (a) cholesterol and (b) fucosterol.

Cholesterol plays a vital role in cellular function where it affects the fluidity of the cell membrane and acts as a secondary messenger in developmental signalling. Cholesterol is the major sterol in red seaweeds. Fucosterol is the predominant sterol in green and brown seaweeds and it has been reported to have antidiabetic and anti-oxidant activities (Lee et al., 2003).

Alkaloids are relatively rare in marine algae. Phenyl ethylamine alkaloids, that are known anti-depressants, have been found in brown and red seaweeds (Güven et al., 2010). The indole alkaloid, caulerprin, isolated from *Caulerpa racemosa* was found to possess strong anti-inflammatory and anti-nociceptive activities (Tenorio de Souza et al., 2009). Halogenated indole alkaloids, which possess strong antibacterial activity, have been isolated from algae and not from land plants (Güven et al., 2010).

Many reports are available on the content of flavonoids in vegetables and fruit but not so much in seaweeds. Yumiko et al. (2003) investigated the distribution of flavonoids and related compounds in several seaweeds found in Japan. They found most red seaweeds to contain hesperidin (626 to 119000 $\mu\text{g g}^{-1}$, dry weight) and green seaweeds to contain catechol (1660 to 777000 $\mu\text{g g}^{-1}$, dry weight).

2.7 *Sargassum*

2.7.1 Scientific classification of *Sargassum*

The taxonomy of *Sargassum* species is considered difficult since *Sargassum* morphology is highly plastic, with environmental and temporal factors creating variations between and within populations of the same species. Many descriptions in the literature are not complete or based on

variable characteristics such as blade size and shape, making it difficult or impossible to determine the species (Phillips, 1995). However, the most recent classification is outlined below:

Kingdom : Protista
Division : Heterokontophyta
Class : Phaeophyceae
Order : Fucales
Family : Sargassaceae
Genus : *Sargassum*

Sargassum, a genus of brown seaweed, is well represented in both temperate and tropical waters worldwide, often being a dominant member of both the sub-tidal and intertidal flora. It is found to be the most diverse genus among Phaeophyceae and it is presented with approximately 400 species (Marimuthu et al., 2012; Liu et al., 2012). *Sargassum* is consumed as food and medicine in many cultures, with the Oriental being the largest consumers. Currently, about 200 compounds such as terpenoids, phlorotannins, fucoidans and sterols have been identified from this genus.

The English common names for *Sargassum* are gulfweed or sea holly. In Asia, where the majority of ethnopharmacological knowledge is found, *Sargassum* has numerous common names such as “Hai Zao” or “Hai Qian” in Chinese, “Hondawara” in Japanese and “Mojaban” in Korean. *Sargassum* is characterised by a holdfast (discoid or conical) that attaches to the substrate, a short stem that differentiates into numerous primary branches that mostly have leaf-like laterals, spherical air vesicles that aid flotation and reproductive structures in specialised laterals called receptacles. The shape of leaf-like thallus, vesicles and receptacles are highly

diversified. Even within the same species, *Sargassum* morphology significantly varies under different environmental conditions and at different seasons. Due to these variations it is often a difficult task to identify *Sargassum* species, especially from diverse tropical flora (Liu et al., 2012).

2.7.2 Bioactive compounds found in the family Sargassaceae

In recent years, seaweeds have caused emerging interest in biomedicine and the food industry because they possess a wealth of bioactive compounds. Many biologically active compounds have been isolated from different *Sargassum* species and some are highlighted in Table 1.

Table 1. Compounds isolated from different *Sargassum* species and their uses.

Species	Compounds	Use	Reference
<i>S. thumbergii</i>	Sargathunbergol A.	Antitumor	Youngwan et al., 2007
<i>S. siliquastrum</i>	Fucoxanthins	Antioxidant	Heo and Jeon, 2009
<i>S. wighitti</i>	Diocetyl phthalate	Antibacterial	Sastry and Rao, 1995
<i>S. vulgare</i>	Alginic acid	Anticancer	Holdt and Kraan, 2011
<i>S. micracantham</i>	Plastoquinones	Antioxidant	Mori et al., 2005

2.7.3 *Sargassum* as a food source

The use of seaweed as food has strong roots in Asian countries such as China, Japan and Korea. However, in recent decades, due to advances in understanding of the relationship between diet and health, consumers are increasingly becoming interested in foods that not only adequately meet nutritional needs but also confer health benefits, hence the growing demand for foods such as seaweeds. The well-known correlation between the diet and health demonstrates the great possibilities of food to maintain or even improve our health. Over the past few decades, there have been many changes in food habits and lifestyle. The diet of individuals in most developed and developing countries are often high in calories, saturated fats and sugars and low in dietary fibre. This, together with a decrease in physical activity, has given rise to obesity, heart disease, diabetes and hypertension.

Seaweeds, due to their phenomenal biodiversity, are a treasure house of novel healthy food ingredients and biologically active compounds. *Sargassum* is used as food in different parts of the world and can be consumed fresh, cooked or dry. Table 2 highlights some of the uses of *Sargassum* as a food source.

Table 2. Traditional uses of *Sargassum* as a food source.

Country and species	Uses	References
Japan and Phillipines <i>S. siliquosm</i>	Seaweed salad	Chennubhotla et al., 1981
Pacific Islands <i>Sargassum sp.</i>	Dry spice	Novaczek and Athy, 2001
Hawaii <i>S. echninocarpum</i> (Limu Kala)	Dry spice, fried chips	Green www.eattheweeds.com
Pacific Islands <i>Sargassum sp.</i>	Seaweed Soup	Novaczek and Athy, 2001

2.7.4 *Sargassum elegans* Suhr (1840)

This study focuses on the edible species, *Sargassum elegans*, which grows abundantly along the KwaZulu-Natal coastline, and is a dominant alga of many upper intertidal rock pools. *S. elegans* is one of the largest non-kelp brown alga often attaining lengths in excess of 1 m. The genus consists of long, highly branched fronds with prickly margins, which can make it appear almost leafy and has characteristic berrylike-gas filled bladders (Figure 6). These bladders keep *S. elegans* free floating near the surface of the water enabling it to photosynthesise. *S. elegans* tends to clump together and form mats that provide a habitat for many marine creatures including shrimps, crabs, worms and fish.



Figure 6. *Sargassum elegans*.

2.8 Seaweed species in this study

2.8.1 *Halimeda cuneata* Hering in Krauss (1846)

Halimeda cuneata is an inedible, green seaweed (*Chrolophyta*) belonging to the family Halimedaceae that is commonly known as “wedge weed”. The algal body (thallus) is composed of calcified green segments. Calcium carbonate is deposited in the tissues, making it inedible to most herbivores. *H. cuneata* is exclusively marine algae restricted to tropical water. These seaweeds colonise sand and mud substrates, where rhizoids of the plant penetrate the soft bottom to develop holdfasts.



Figure 7. *Halimeda cuneata*.

2.8.2 *Spyridia hypnoides* (Bory de Saint-Vincent) Papenfuss (1968)

Spyridia hypnoides is an inedible, red seaweed (*Rhodophyta*) belonging to the family Spyridiceae. They are most common in calm protected areas, epiphytic on *Hypnea* or on “seagrass”. They are characterised by a thallus of pink to red colour, fuzzy, filamentous, densely bushy and branching alternate in all directions.



Figure 8. *Spyridia hypnoides*.

2.8.3 *Codium capitatum* P. Silva (1959)

Codium is a genus of green seaweed belonging to the Codiaceae family; it is particularly abundant in intertidal rock pools that are also prone to sand inundation. *Codium capitatum* is forked, upright and feels velvety and spongy. It is able to regulate the movement of its chloroplasts to maximise photosynthesis. Its thallus is internally composed of interlocking filaments that end in club-like structures bearing the chloroplasts and the reproductive structures. *C. capitatum* belongs to a group of seaweeds that are unique in that their internal filaments lack cross-walls. Thus, instead of being divided into cells, each filament is a giant cell with many nuclei. The *Codium* species are edible and are eaten raw as salads in Hawaii and used as tea in Korea (Nishizawa, 2002)



Figure 9. *Codium capitatum*.

2.8.4 *Hypnea spicifera* (Suhr) Harvey (1847)

Hypnea Spicifera is an edible, red seaweed (*Rhodophyta*) belonging to the family of Cystocloniaceae and is commonly known as “green tips”. These seaweeds occur as dense green and purple clumps on the lowest parts of the shore and are only visible during low spring tide. The upper parts of the clumps are covered with numerous short, green, fleshy spines. The colour in this species is characteristically purple-brown at the base and a luscious translucent green at the tips. These seaweeds are able to form extensive mats on the lowest reaches of the shore because of its rhizomatous spreading holdfast system; hence it can be cultivated in high volumes for potential commercial uses.



Figure 10. *Hypnea spicifera*.

2.9 Essential elements in seaweeds

A nutrient or element is essential when a deficiency of the element makes it impossible for the seaweed to grow or complete its vegetative or reproductive cycle and the requirement cannot be replaced by another element (Lobban and Harrison, 1994). Nutrients such as C, H, O, N, Mg, Cu, Mn, Zn, and Mo are considered to be essential to all seaweeds. Sulphur, K, and Ca are required by all seaweeds but can be partially replaced by other elements. Sodium, Co, V, Se, Si, Cl, B and I are required only by some seaweed (Hurd et al., 2014). There are up to 21 elements required for metabolic processes in plants and seaweeds but more than double that number are present in seaweeds. However, the presence of an element in seaweed tissue does not necessarily mean it is essential.

Generally, essential and non-essential elements are accumulated in seaweed tissues to concentrations well above their concentration in the surrounding seawater. Some elements are absorbed in excess of the seaweed requirements, whereas others are taken up and not utilised. In some cases, the excess nutrients are stored for future growth (DeBoer, 1981).

2.10 Essential nutrients in humans

Nutrients are defined as chemical substances found in foods that are necessary for human life and growth, maintenance and repair of body tissues (Stipanuk, 2013). It is now commonly accepted that proteins, fats, carbohydrates, vitamins, minerals and water are the major nutritional constituents. Nutrients are classified into two broad groups: organic and inorganic. Nutrients in the organic or carbon-containing group (macronutrients) make up the bulk of our diets and provide us with energy. They include proteins, carbohydrates (sugars and starches), fats and vitamins. These organic compounds are synthesised by living cells from simpler compounds. Green plants and phytoplankton such as seaweeds and photosynthetic bacteria are able to use light energy to drive the synthesis of organic compounds.

Inorganic nutrients (micronutrients) are mainly minerals and do not need to come from living sources such as plants or animals, they are present in the earth's crust and are taken up from soil or water by plants and microorganisms, thereby making their way into the food chain. Unlike macro nutrients these are required in minute amounts.

2.10.1 Micronutrients

Micronutrients are classified into macro minerals and micro minerals or trace minerals. Micro minerals include Ca, P, Mg, Na, K, S, O, N, C and trace minerals include Fe, Cu, Co, Mn, Mo, B, Cr, F, I, Ni, Se and Zn. The recommended amounts of essential elements for individuals and the tolerable upper intake levels are presented in Table 3 and 4, respectively. Calcium and phosphorus are co-dependent nutrients and together they are essential for bone formation and resorption. Calcium and P, combine to form a calcium phosphate salt called hydroxyapatite which is a major structural component that gives teeth and bones their rigidity. A deficiency of Ca or imbalance of Ca to P ratio may result in osteoporosis (thinning

of bone tissue and loss of total bone amount) and other skeletal disorders such as rickets in children (Soetan et al., 2010).

Magnesium is required for the activation of approximately 300 enzyme systems, predominantly for those involved in energy metabolism for the activation of phosphate groups. Magnesium deficiency is associated with neuromuscular symptoms such as cramping, however it is rare and usually observed only in cases of chronic alcohol abuse or in critically ill patients. Low Mg dietary intake has also been associated with a number of chronic diseases including diabetes mellitus type II, cardiovascular disease, osteoporosis and metabolic syndrome (Bohn, 2008).

Chromium improves the efficiency of insulin and is required for normal protein, fat and carbohydrates metabolism. Insufficient dietary Cr has been linked to maturity onset diabetes and cardiovascular disease. Supplementation of Cr often leads to significant improvements in glucose tolerance (Anderson, 1986). Copper is associated with many metallo-enzymes and is necessary for proper development of connective tissue, myelin and melanin. Copper deficiency is usually the consequence of decreased Cu stores at birth and also inadequate dietary Cu intake and poor absorption. Anaemia, neutropenia and bone abnormalities are some of the common results of Cu deficiency. Copper has potential toxicity if the intake loads exceed lower tolerance levels (Uauy et al., 1998)

Iron is essential for a number of biochemical functions in the body including the transport of oxygen and energy production in the mitochondria. Iron deficiency is also one of the most common nutrient disorders in the world's population. Iron deficiency results in anaemia, while excess Fe is highly toxic and can lead to cell and organ damage. Interestingly, the human body does not possess the capacity to remove Fe, like other dietary metals that are

excreted in faeces and urine. However, a number of proteins have evolved which tightly regulate Fe homeostasis (Srai and Sharp, 2012).

Table 3. Dietary Reference Intakes (DRIs) - Recommended Intakes for Individuals*.

Life stage	Ca mg/d	Cr µg/d	Cu µg/d	Fe mg/d	Mg mg/d	Mn mg/d	Se µg/d	Zn mg/d
Males								
14-18yrs	1300	35	890	11	410	2.2	55	11
19-50yrs	1000	35	900	8	400	2.3	55	11
>51yrs	1200	30	900	8	420	2.3	55	11
Females								
14-18yrs	1300	24	890	15	360	1.6	55	9
19-50yrs	1000	25	900	18	320	1.8	55	8
>51yrs	1200	20	900	8	320	1.8	55	8

*Food and Nutrition Board, Institute of Medicine, National Academies, 2011.

Table 4. Tolerable Upper Intakes levels (UL)*.

Life stages	As µg/d	Ca mg/d	Cr µg/d	Cu µg/d	Fe mg/d	Mg mg/d**	Mn mg/d	Ni mg/d	Se µg/d	Zn mg/d
M/F	ND	3000	ND	8000	45	350	9	1	400	34
	ND	2500	ND	10000	45	350	11	1	400	40
	ND	2500	ND	10000	45	350	11	1	400	40

ND- Not Determinable,

*Food and Nutrition Board, Institute of Medicine, National Academies, 2011,

** Represent intake from a pharmacological agent only.

Manganese is associated with a number of metallo-enzymes and is important for protein and energy metabolism, bone mineralisation, metabolic regulation and cellular protection from reactive oxygen species. Manganese deficiency in humans is quite rare but toxicity is known

to mostly occur when inhaled as Mn-laden dust by miners. The brain is particularly susceptible to excess Mn and accumulation can cause a neurodegenerative disorder known as mangasm (Dobson et al., 2004).

Selenium has structural and enzymatic roles; it is needed for proper functioning of the immune system and appears to be a key nutrient in counteracting the development of virulence and inhibiting HIV progression in AIDS. A deficiency of Se has been linked to adverse mood states (Rayman, 2000).

Zinc is associated with catalytic activity of more than 200 enzymes and regulatory proteins. Zinc regulates secretion of calcitonin from the thyroid gland and has an influence on bone turnover. A deficiency of Zn is characterised by growth retardation, loss of appetite, and impaired immune function. In more severe cases, Zn deficiency results in hair loss, diarrhoea, delayed sexual maturation, impotence and hypogonadism in males (Hambridge, 2000).

2.10.2 Macronutrients

Macronutrients include carbohydrates, fats and proteins and are required in large amounts to fuel the body. The acceptable distribution range of macronutrients in percentages is shown in Table 5.

Table 5. Dietary Reference Intakes (DRI) - Acceptable Macronutrient Distribution Ranges*.

Macronutrient	Children 1-3 yrs. %	Children 8-14 yrs. %	Adults %
Fat	30-40	25-35	20-35
Carbohydrate	45-65	45-65	45-65
Protein	5-20	10-30	10-35

*Food and Nutrition Board, Institute of Medicine, National Academies, 2011.

Most of our energy is consumed as carbohydrates. The acceptable macronutrient distribution range (AMDR) for carbohydrates is estimated at 45 to 65% of energy for adults (Table 5). Carbohydrates, mainly sugars and starches, provide energy cells in the body, particularly to the brain which is a carbohydrate dependent organ (Lutz et al., 2015). Dietary carbohydrates are important to maintain glycaemic homeostasis and for gastrointestinal integrity and function. Unlike fats and proteins, high levels of dietary carbohydrates (provided they are obtained from a variety of sources) are not associated with adverse health effects (FAO, 1998).

Fat is also a source of fuel energy for the body and aids in the absorption of fat-soluble vitamins and other food components such as carotenoids. Saturated fatty acids, monounsaturated fatty acids and cholesterol are synthesised by the body and have no known beneficial role in preventing chronic diseases and thus are not termed essential even though they have vital physiological roles (DRIs, 2005). Excess dietary fat intake is detrimental to human health as overconsumption is associated with excess body weight and adipose stores.

Proteins form the major structural components of all the cells of the body. Dietary proteins provide the amino acids needed for synthesis of various body proteins such as skeletal muscle and other structural proteins, membrane carriers, enzymes and hormones (Guigoz, 2011). Proteins can also serve as an energy source when there are insufficient carbohydrates and fats to meet the body's needs. Insufficient protein intake is a common problem in poor communities and the disorder commonly occurs in a variety of pathologic states. Children are mostly affected from protein deficiency (although the deficits maybe both protein and energy) and the two main forms are marasmus and kwashiorkor (Barasi, 2003).

2.11 The toxic element arsenic (As)

Arsenic is a metalloid that occurs in different organic and inorganic forms and can arise from natural sources such as rocks and sediments and also as a result of anthropogenic activities such as coal burning, copper smelting and the processing of mineral ores (Rose et al., 2007). Marine organisms can accumulate higher levels of arsenic than terrestrial plants, as arsenic is fairly water soluble and may be washed out of arsenic bearing rocks into the sea (Zhao et al., 2014). In particular, seaweeds are known to bioaccumulate available nutrients from their environment making them highly nutritive, however they may also accumulate arsenic which may be harmful to humans. Hence, arsenic levels need to be monitored in seafood and edible seaweeds to establish their potential threat to consumers.

It is well known that arsenic toxicity depends not only on the total concentration but also on the chemical species in which this element is present. Inorganic arsenic species, arsenite (As(III)) and arsenate (As(V)), are generally considered to be more toxic, while the organic species such as, arsenocholine, arsenobetaine and arsenosugars are considered to be less toxic or non-toxic (FSANZ, 2010). Inorganic arsenic is a known human carcinogen associated especially with liver, bladder, lung and skin cancer (WHO, 2011).

Dietary exposure to inorganic arsenic is mainly from natural ground water and foods such as rice and other grain-based processed products (EFSA, 2014). Seafood is also a major contributor to arsenic dietary exposure. Borak and Hosgood (2007) estimated seafood to contribute approximately 90% towards dietary arsenic in the United States. However, the predominant arsenic was in the less toxic organic forms, with inorganic arsenic only accounting for a minor percentage. Edible seaweeds contain higher levels of inorganic arsenic in proportion to the total arsenic content compared to other foods (FSANZ, 2010). Since the available seaweed species are widely diverse, their levels of inorganic arsenic vary, thus their contribution to dietary arsenic exposure varies with species. However, specific

types of seaweeds such as brown seaweeds from the *Sargassum* family (*S. fusiforme* (*hijiki*)) have been found to consistently contain high levels of inorganic arsenic (Rose et al., 2007; Zhao et al., 2014).

Food regulators in most countries have not established specific maximum limits for inorganic arsenic in seaweed. France, New Zealand and Australia are the only countries with maximum limits for inorganic arsenic in seaweeds. In Australia and New Zealand, the maximum limit for inorganic arsenic in seaweeds is $1 \mu\text{g g}^{-1}$ calculated with respect to the mass of seaweed at 85% hydration, while in France it is set at $< 3 \mu\text{g g}^{-1}$, dry weight (Burtin, 2003; FSANZ, 2010). In 2010, the Joint Food and Agriculture Organization / World Health Organization Expert Committee on Food Additives (JECFA) determined the inorganic arsenic benchmark dose lower confidence limit for a 0.5% increased incidence of lung cancer in human (BMDL_{0.5}) to be $3.0 \mu\text{g kg}^{-1}$ body weight/day.

2.12 Seasonal variation

There may be different reasons for seasonal variation on elemental uptake by seaweeds including: environmental factors such as variations in metal concentrations in solution, interactions between metals and other elements, surface water and atmospheric temperature, tidal range, salinity and pH, and metabolic factors such as dilution of metal content due to growth (Villares et al., 2002).

Seasonal variation on elemental uptake has been linked to growth, where the metal concentration is stated to increase in dormant winter periods and decrease during periods of growth. Riget et al. (1995) observed metal concentrations in *Fucus vesiculosus* (brown) to be highest in winter and lowest in summer. Misheer et al. (2006) found elemental uptake by

Plocamium corallorhiza (red) found along the KwaZulu-Natal coastline to also increase in winter and decrease during summer.

In some studies higher metal concentrations have been found in the growth periods (summer). These changes could be due to higher rates of photosynthesis and respiration during summer which would favour the assimilation of metals (Villares et al., 2002). However, others studies have attributed high concentrations in summer (rainy season) to high concentrations of metals in water because of an increase in terrestrial inputs (Lacerda et al., 1985). Some studies suggested that high metal concentrations in algae indicated the capacity of the alga to take up the metals.

2.13 Analytical and phytochemical techniques

The following techniques were employed to achieve the objectives of this research.

2.13.1 Microwave digestion

Microwave digestion is a fast and effective technique that uses concentrated acids to decompose many kinds of samples for the determination of a wide range of elements by atomic spectrometric techniques. Microwave digestion involves placing the sample in a vial (or bomb), usually constructed of a fluorinated polymer, such as polytetrafluoroethylene (PTFE) or perfluoroalkoxy (PFA). After adding the digestion reagents, the vial is tightly sealed and placed in the microwave oven for irradiation by microwave energy, at elevated temperatures and pressure (Lamble and Hill, 1998). This technique ensures accelerated sample digestion with minimal contamination and loss of volatile elements.

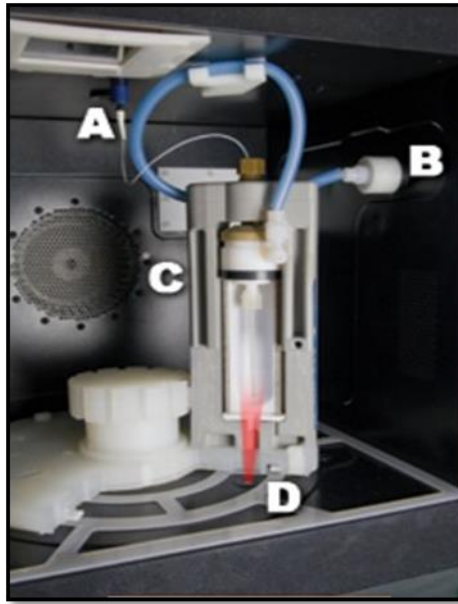


Figure 11. CEM MARS 6 microwave.

<http://www.cem.com/mars6-options.html>.

Figure 11 shows a typical closed system microwave with built in pressure and temperature controls. The microwave is designed to hold a maximum of 24 MarsXpress™ or 12 EasyPrep™ vessels (Figure 12), on a turntable that can rotate through 360 degrees to ensure uniform microwave energy to each vessel.

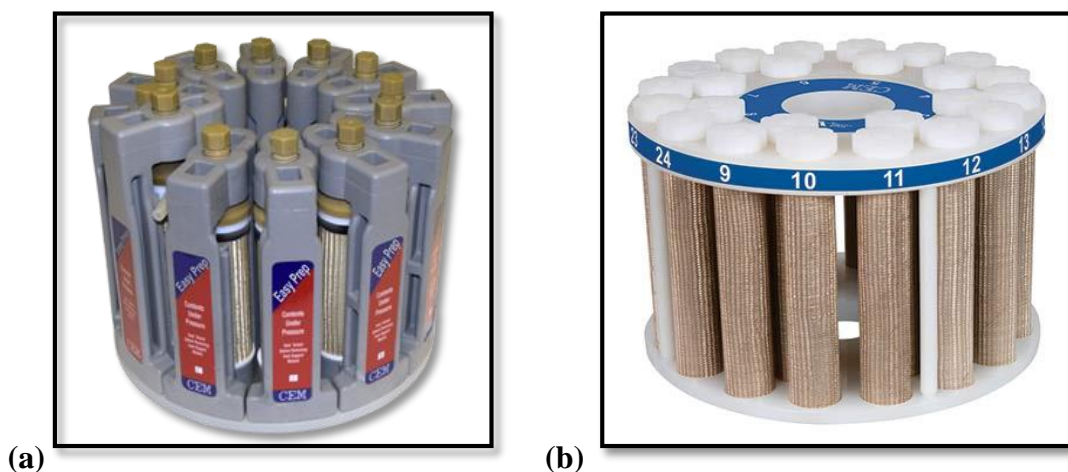


Figure 12. CEM MARS 6 (a) EasyPrep™ and MarsXpress™ vessels.

<http://www.cem.com/mars6-vessels.html>.

2.13.2 Inductively coupled plasma-optical emission spectrometry (ICP-OES)

The ICP-OES (Figure 13) is one of the most powerful and popular analytical tools for the determination of trace elements in a variety of different sample matrices. The technique is based upon the spontaneous emission of photons from atoms and ions that have been excited in a radio-frequency (RF) discharge. Liquid samples may be injected directly into the instrument, while solid samples require extraction or acid digestion so that the analytes will be present in a solution (Hou and Jones, 2000). The sample solution is converted to an aerosol and directed into the central channel of the plasma. The inductively coupled plasma (ICP) sustains a temperature of approximately 10 000 K, so the aerosol is quickly vaporised, and energised through collisional excitation to the excited states. The excited atomic and ionic species may then relax to the ground state via the emission of a photon (Ghosh et al., 2013). These photons have characteristic energies that are determined by the quantized energy level structure for the atoms or ions. Thus the wavelength of the photons can be used to identify the elements from which they originated. The total number of photons is directly proportional to the concentration of the originating element in the sample.



Figure 13. ICP-OES Optima 5300 DV at the School of Chemistry and Physics (UKZN).

The instrumentation associated with an ICP-OES system is relatively simple. A portion of the photons emitted by the ICP is usually collected by a focusing optic such as lens or a concave mirror. This optic focuses the image of the ICP discharge onto the entrance aperture of a wavelength selection device such as a monochromator. The particular wavelength exiting the monochromator is converted to an electrical signal by a photo detector. The signal is amplified and processed by the detector electronics, then displayed and stored by a computer (Wang, 2004).

2.13.2.1 Advantages and disadvantages of ICP-OES

Some of the advantages of ICP-OES include:

- Excellent detection limits for most elements (1–100 $\mu\text{g L}^{-1}$).
- Simultaneous multi-element capability (almost all the elements in the periodic table).
- Fairly simple to run the instrument.
- High stability leading to excellent accuracy and precision.
- Limited spectral interferences.
- Low matrix effects.

One major disadvantage of the ICP-OES is that it only analyses liquid samples.

2.13.2.2. ICP-OES interferences

Some of the interferences that occur when using the ICP-OES include:

- Physical interferences due to changes in viscosity of the solution.
- Chemical interferences due to the generation of compounds that have low atomization efficiency.

- Spectral interferences (also referred to as background interferences) due to the overlapping of emission or absorption lines.

Spectral interferences may be eliminated by using advanced background correction techniques or by choosing a different analytical wavelength for the element(s) of interest. The high temperature of the plasma helps to reduce chemical interferences. The temperature is high enough to break down most species into atoms or ions for excitation and subsequent emission. However, chemical interferences do exist in the ICP-OES and sometimes higher RF power and/or lower inner argon flow rates are used to reduce these interferences (Hou and Jones, 2000). Physical interferences may be minimised by use of internal standardisation and/or matrix matching.

2.13.3 Chromatography

Chromatography refers to a set of techniques used to separate compounds from different mixtures by distributing them between two phases. The stationary phase (usually a solid or bonded coating) stays fixed in one place, while the mobile phase or eluent (usually a liquid or gas) moves through the medium being used. The movement of the components in the mobile phase is controlled by the significance of their interactions with the mobile and/or stationary phases. Because of the differences in factors such as the solubility of certain components in the mobile phase and the strength of their affinities for the stationary phase, some components will move faster than others, thus facilitating the separation of the components within that mixture.

2.13.3.1 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is a simple, quick and inexpensive procedure used to determine the number of components in a mixture as well as the purity of compounds. TLC is also used to identify a compound by comparing its retention factor (R_f) to that of known compounds. TLC is performed on a sheet of glass, plastic, or aluminium coated with a thin layer of adsorbent material, such as silica or alumina. This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (mobile phase) is drawn up the plate via capillary action (Figure 14). Because different analytes ascend the TLC plate at different rates, separation is achieved.

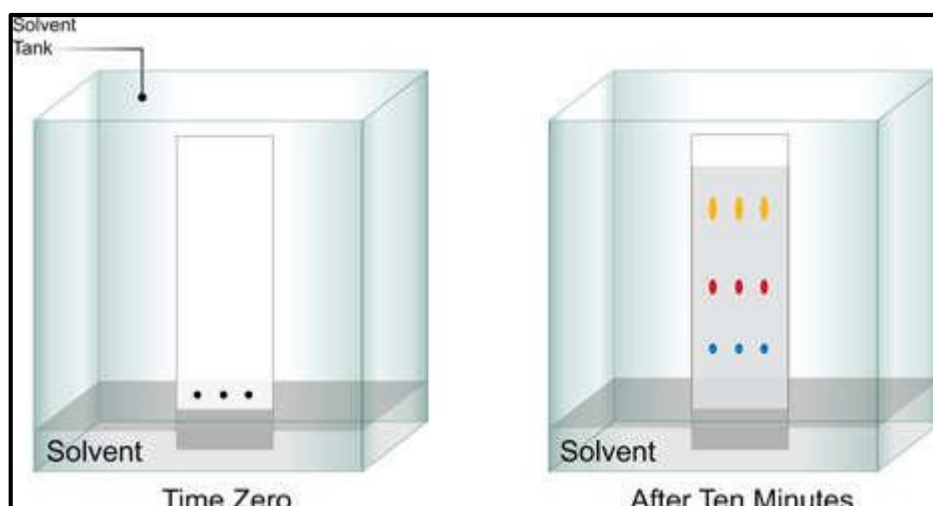


Figure 14. A schematic diagram of the TLC technique.

2.13.3.2 Column Chromatography

In column chromatography, the stationary phase (silica gel or alumina) is placed in a vertical glass column. The mobile phase, a liquid, is added to the top and flows down through the column by either gravity or external pressure (Figure 15). Column chromatography is generally used as a purification technique; it isolates desired compounds from a mixture.



Figure 15. A column used to separate compounds in this study.

Components of the sample separate from each other by partitioning between the stationary phase and the mobile eluent. Molecules with different polarity partition to different extents and therefore move through the column at different rates. The eluent is collected in fractions. Fractions are typically monitored by TLC to see if separation of the components was successful.

2.13.4 Spectroscopic techniques

Spectroscopic techniques employ light (electromagnetic radiation) to interact with matter and reveal certain features of a samples' structure. Different regions of the electromagnetic spectrum provide different kinds of information as a result of such interactions (Hofmann, 2010).

2.13.4.1 Nuclear magnetic resonance (NMR)

This technique exploits the magnetic properties of certain atomic nuclei to study physical, chemical and biological properties of matter. The NMR is frequently used by chemists and biochemists to investigate the properties of organic molecules, suitable samples range from small compounds analysed by one-dimensional proton or carbon-13 NMR spectroscopy to large and complex compounds such as proteins using two-dimensional techniques.

The ^1H -NMR spectrum provides detailed information such as;

- Chemical shifts which show differences in the hydrogens' chemical environments.
- Splitting representing the number of neighbouring hydrogens (N+1 rule).
- Integration (area under the signal) which gives the relative number of hydrogens present at each signal.
- The number of peaks shows number of different environments the hydrogen atoms are in.

This information is helpful in determining the chemical structure of organic compounds. The ^{13}C -NMR works on the same principle as ^1H -NMR (nuclei spin), however it has a wider chemical shift range from 0-230 ppm than ^1H -NMR (0-13 ppm). The signals in ^{13}C -NMR appear as singlets due to the decoupling of the attached proton.

Distortionless enhancement by polarization transfer (DEPT) is a NMR method used for determining the presence of primary, secondary and tertiary carbon atoms. The DEPT experiment differentiates between CH, CH₂ and CH₃ groups by variation of the selection angle parameter (the tip angle of the final ^1H pulse): 135° angle gives all CH and CH₃ in a phase opposite to CH₂; 90° angle gives only CH groups. Signals from quaternary carbons with no attached protons are always absent in DEPT.

Two-dimensional (2D) NMR experiments are useful in providing additional information about complex compounds whose signals overlap because their resonating frequencies are very similar, and where 1D-NMR is insufficient. Examples of 2D-NMR include: Correlation Spectroscopy (COSY), Nuclear Overhauser Effect Spectroscopy (NOESY), Heteronuclear Singlet Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlations (HMBC).

2.13.4.2 Infrared spectroscopy (IR)

Infrared spectroscopy (IR) is an absorption method in the infrared region of the electromagnetic spectrum. Absorption of infrared radiation excites vibrational and rotational motions within molecules and 'measurements' of the ways in which bonds vibrate gives rise to IR. Atom size, bond length and bond strength vary in molecules and so the frequency at which a particular bond absorbs infrared radiation is characteristic to that bond.

An infrared spectrometer (Figure 16) analyses a compound by passing infrared radiation, over a range of different frequencies, through a sample and measuring the absorptions made by each type of bond in the compound. This produces a spectrum, normally a 'plot' of % transmittance against wavenumber. Examination of the spectrum can provide information of the functional groups in the compound.

This technique is very useful in the identification and structure analysis of a variety of substances, including both organic and inorganic compounds. It can also be used for both qualitative and quantitative analysis of complex mixtures of similar compounds.



Figure 16. Infrared spectrometer at the School of Chemistry & Physics (UKZN).

2.13.4.3 Mass spectrometry

Mass spectrometry (MS) is an analytical tool used for measuring the molecular mass of a sample. A mass spectrometer determines the mass of a molecule by measuring the mass-to-charge ratio (m/z) of its ion. Ions are generated by inducing the loss of a charge from a neutral species. Once formed, ions are electrostatically directed into a mass analyser where they are separated according to m/z and detected. The detected signal is sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of an m/z spectrum. The mass or structure of the molecule is subsequently derived from careful interpretation and analysis of the spectrum. There are many types of ionization methods used in mass spectrometry, including modern techniques such as Matrix Assisted Laser Desorption Ionization (MALDI). The time-of-flight (TOF) analysers are typically used with the MALDI ionization source.

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CHAPTER 3

CHEMICAL COMPOSITION AND SEASONAL VARIATION ON ELEMENTAL UPTAKE OF SELECTED SEAWEEDS FROM THE INDIAN OCEAN, KWAZULU-NATAL, SOUTH AFRICA.

Abstract

Five seaweeds namely *Halimeda cuneata*, *Spyridia hypnoides*, *Codium capitatum*, *Hypnea spicifera* and *Sargassum elegans*, of which the latter three are edible, were collected from the KwaZulu-Natal east coast region of South Africa during four different seasons. The proximate composition of the three edible seaweeds and seasonal variation of minerals and trace elements in all five seaweeds was investigated. The edible seaweeds had a moisture level of 85.4 to 89.5%, protein of 6.1 to 11.8%, lipids of 7.5 to 13.1% and carbohydrates (which was obtained by difference) of 37.8 to 71.9%. In general, elemental concentrations in the five seaweeds varied significantly with season ($p < 0.05$) and were found to be in decreasing order of $\text{Ca} > \text{Mg} > \text{Fe} > \text{Cu} > \text{Mn} > \text{As} > \text{Zn} > \text{Ni} > \text{Cr} > \text{Pb} > \text{Co} \approx \text{Se}$. This study suggests that *C. capitatum* and *H. spicifera* could be potential sources of most essential nutrients and may contribute positively to the diet without posing the risk of adverse health effects due to low concentrations of toxic elements. However, consumption of *S. elegans* should be moderated as it could increase dietary exposure to inorganic As if too much is consumed.

Keywords: seaweeds; arsenic; trace elements; seasonal distribution

3.1 Introduction

Marine macro algae, commercially referred to as seaweeds, are commonly classified into three main groups according to their pigmentation, morphology, anatomy and nutritional composition as red (Rhodophyta), green (Chlorophyta) or brown (Phaeophyta) seaweeds (Dawczynski et al., 2007). The consumption of seaweed is common practice in Asian countries such as Japan, China and Korea and this has been so since ancient times (Burtin, 2003). Currently, the worldwide human consumption of seaweeds is 5% for green, 66.5% for brown and 33% for red seaweeds (Marinho-Soriano et al., 2006). The demand for seaweeds as food has also extended to North America, South America and Europe. In South Africa, only six of approximately 800 seaweed species known have been harvested and this is done mostly for export to the hydrocolloid industry (Amosu et al., 2013). The fresh kelp harvested in South Africa is mainly used as feed for abalone.

Seaweeds are known to take up high levels of certain nutrients from the sea, making them highly nutritive; this can make them rich in certain vitamins, proteins, minerals, fibre and essential fatty acids (El-Said and El-Sikaily, 2013). The protein and lipid content of seaweeds make them acceptable for human consumption as they contain essential amino acids and unsaturated fatty acids (Bhargyabati et al., 2011). Generally, green and red seaweeds contain higher amounts of proteins (10 to 30%, dry weight (DW)) than brown seaweeds (5 to 15%, DW). The lipid content of seaweeds ranges from 1 to 6%, DW (Benjama and Masniyom, 2011). The mineral content of seaweeds is generally high and in some calcified seaweeds it accounts for up to 36% of the dry matter, with these seaweeds being rich in essential elements. Factors such as salinity, turbidity, nutrient content and heavy metal contamination of growth medium largely influences the mineral content of seaweeds which in turn varies according to season (Marinho-Soriano et al., 2006).

The chemical composition of seaweeds from other regions of the world has been documented but information on edible seaweeds found in South Africa is lacking. Previously, we reported on the elemental concentrations in *Gellidium abbotiorum*, *Plocamium corallorhiza*, *Caulerpa racemosa* and *Ulva lactuca* collected over a period of one year from different sites along the east coast of KwaZulu-Natal (Misheer et al., 2006a, 2006b, 2006c, 2006d). In this work, the elemental concentrations in different classes of edible seaweeds, *Codium capitatum* (green), *Hypnea spicifera* (red), and *Sargassum elegans* (brown) found in KwaZulu-Natal, South Africa was investigated as a function of seasonal variation and compared to each other as well as to concentrations in inedible seaweeds, *Halimeda cuneata* (green) and *Spyridia hypnoides* (red). The chemical composition (protein, ash, lipid, moisture and carbohydrate) of *C. capitatum*, *H. spicifera* and *S. elegans* was also determined and the potential nutritive value of these edible seaweeds was evaluated.

3.2 Materials and Methods

3.2.1 Sampling

Samples of five seaweed species, *C. capitatum*, *H. cuneata*, *H. spicifera*, *S. hypnoides* and *S. elegans* were collected from Inyoni Rocks, KwaZulu-Natal (30.0479 ° S, 30.8902 ° E) during low tide in autumn, winter, summer and spring of 2014. Seaweeds were washed on-site with seawater to remove sand, epiphytes and shells, placed in polythene bags and then thoroughly washed with double distilled water in the laboratory before drying at 45 °C to constant mass. Dried samples were ground in a blender (Braun range) and stored in plastic bottles in the refrigerator until analysed.

3.2.2 Analysis of proximate composition

The protein, ash and moisture content in seaweeds were determined using standard AOAC methods with slight modifications (Benjama and Masniyom, 2012; Smith et al., 2010). Crude protein was obtained by the Kjeldahl distillation method using a conversion factor of 6.25 to convert total nitrogen to crude protein ($\%N \times 6.25$). The ash content was determined gravimetrically after heating the sample in a muffle furnace at 600 °C for 16 hr. Moisture content was determined by drying the seaweed in an oven at 105 °C to constant mass. The lipids were obtained using a soxhlet apparatus with hexane as the solvent (Marinho-Soriano et al., 2006). Carbohydrate content was calculated by subtracting the amount of protein, lipid and ash from total dry matter.

3.2.3 Reagents

All reagents were of analytical reagent grade and were supplied by Sigma-Aldrich, Germany. Double distilled water was used for all dilutions. All plastic and glassware was cleaned by soaking in dilute HNO₃ and rinsed with double distilled water prior to use. Elemental standards were prepared from 1000 mg L⁻¹ stock solutions that were supplied by Fluka Analytical, Sigma, Switzerland.

3.2.4 Determination of elemental content

Seaweed samples were digested using the CEM MARS 6 microwave (CEM Corporation, Matthews, North Carolina, USA) with Easyprep™ vessels. A 0.25 g portion of each ground seaweed and certified reference material (CRM), *white clover* BCR-402, was transferred into Teflon vessels to which 10 mL of concentrated HNO₃ was added. Samples were allowed to pre-digest for 2 hr to minimise the risk of an exothermic reaction and to allow most of the organic matrix to decompose at atmospheric pressure before sealing (Rhoades Jr, 1996). For

digestion, the microwave power was at 1050 W; the temperature was ramped to 180 °C for 15 min where it was held for 15 min and then cooled for 15 min. All digests were transferred to 25 mL volumetric flasks and made to volume with double distilled water before being analyzed (in triplicate) for As, Ca, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn using a Perkin Elmer simultaneous inductively coupled plasma-optical emission spectrometer (ICP-OES, model 5300DV) with radial viewed plasma. Analytical wavelengths were selected based on minimum spectral interferences and maximum analytical performance. Initially, the three most sensitive lines were chosen, thereafter, the line with no interfering elements was selected.

3.2.5 Statistical analysis

A Levene's test was applied to data sets to test for homogeneity of variances. For parameters that presented homogeneous variances, one-way ANOVA was employed to test for statistically significant differences within groups. For parameters that presented heterogeneous variances, Welch-ANOVA was used to test for statistically significant differences. In case of significance, the Tukey's post hoc test (homogeneous variances and equal sample numbers) or Games-Howell post hoc test (heterogeneous variances) was performed. All statistical analyses were performed using the Statistical Package for the Social Sciences (PASW Statistics 22, IBM Corporation, Cornell, N.Y.)

3.3 Results and Discussion

3.3.1 Proximate composition

The chemical compositions of the three edible seaweeds, *C. capitatum*, *H. spicifera* and *S. elegans* were significantly different ($p < 0.05$) (Table 6). The moisture content of the fresh seaweed was 85.4% in *S. elegans*, 89.4 % in *H. spicifera* and 89.5% in *C. capitatum*. On a

dry weight basis, the protein content ranged from 6.1% in *S. elegans* to 11.8% in *C. capitatum*. These results are consistent with those previously reported in brown seaweed (5-15%, DW) and red and green seaweeds (10-30%, DW) (Burtin, 2003). Variations in the protein content have been reported in different species and have been seen in the same species collected in different seasons and under different environmental conditions (Matanjan et al., 2009).

According to literature, the lipid content in most seaweeds range from 1 to 6%, DW (Ambrozova et al., 2014). However, some seaweed such as *Dictyota sandvicensis* (20.2%, DW) and *Dictyota acutiloba* (16.1%, DW) have been shown to have a higher lipid content (McDermid and Stuercke, 2003). In this study, the lipid content was significantly high in *C. capitatum* (13.1%, DW) and relatively low in *S. elegans* (7.5%, DW). These results are consistent with those reported on *Codium* species collected in Australia with lipid content ranging from 7.3% to 21.1%, DW (Xu et al., 1998). The *Sargassum* species in this study had higher lipid content than those previously reported in *S. subrepandum* (3.61%, DW) (Abou-El-Wafa et al., 2011) and *S. mcclurei* (1.2%, DW) (Hong et al., 2007).

Ash broadly represents mineral content and some studies have shown the ash content in seaweeds to range from 8 to 40%, DW (Mabeau and Fleurence, 1993; Ortega-Calvo et al., 1993). In this study, the ash content was significantly high in *H. spicifera* (40.4%) and relatively low in *S. elegans* (14.5%). These results suggest that seaweeds could be richer in their mineral content than most land vegetables that have an average ash content of 5 to 10%, DW (Tabarsa et al., 2012).

Total carbohydrates were estimated by difference, this method gives a summary value of simple sugars, complex soluble sugars and insoluble carbohydrates (Menezes et al., 2004). In this study, carbohydrates were a major component ranging from 37.8% in *C. capitatum* to

71.9% in *S. elegans*. The carbohydrate content in *S. elegans* was higher than previously reported in *S. polycystum* (34.9%) but lower than *S. vulgare* 93.3% and *S. fusiforme (hijiki)* 90.7% (Ahmad et al., 2012; El- Shafay, 2014). These variations might be due to many factors such as environment, metabolic preferences and season.

Table 6. Proximate chemical composition of the seaweed samples analysed (Mean \pm SD, n=3) at the 95% confidence interval.

Seaweeds	Protein* (%)	Lipid (%)	Ash (%)	Carbohydrate** (%)
<i>C. capitatum</i>	11.8 \pm 0.55 ^a	13.1 \pm 0.55 ^a	37.3 \pm 0.49 ^b	37.8 \pm 0.49 ^c
<i>H. spicifera</i>	10.4 \pm 0.20 ^a	5.4 \pm 0.58 ^c	40.4 \pm 0.87 ^a	43.8 \pm 0.59 ^b
<i>S. elegans</i>	6.1 \pm 0.23 ^b	7.5 \pm 0.59 ^b	14.5 \pm 0.21 ^c	71.9 \pm 0.30 ^a

* Percentage based on dry weight,

** Carbohydrate obtained by subtracting the amount of ash, lipid and protein from total DW, Different superscript letters within the column indicate significant differences between species (p < 0.05).

3.3.2 Elemental analysis

To evaluate the accuracy of the analytical method, the experimental values for the CRM (*white clover* BCR-402) were compared to certified values (Table 7). The values provided for Fe, Ni and Zn are indicative so no uncertainties were ascribed to them. The experimental values were in agreement with certified values.

Table 7. Elemental concentrations (mean \pm SD, n=3) in the certified reference material, *white clover* BCR-402.

Element	Concentration in white clover BCR-402 ($\mu\text{g g}^{-1}$)	
	Certified	Experimental
Co	0.178 \pm 0.008	0.17 \pm 0.03
Se	6.70 \pm 0.25	6.60 \pm 0.26
Fe	244	240 \pm 36.65
Ni	8.25	8.23 \pm 0.46
Zn	25.2	25.40 \pm 0.64

In all of the five seaweed species studied, the concentrations of Ca, Cu, Fe, Mg and Mn differed significantly with season ($p < 0.05$) (Table 8). In general, concentrations of Ca, Cu, Fe and Mn in green seaweed (*C. capitatum* and *H. cuneata*) were low in winter and high in summer with the lowest and highest concentrations being significantly different. Calcium uptake in *H. cuneata* was relatively high compared to all of the other species studied (88 850 $\mu\text{g g}^{-1}$ in autumn). This could be because *H. cuneata* is a typical calcareous alga; previous studies have reported different calcifying algae to have Ca concentrations as high as 18.4%, DW (Hou and Yan, 1998). The accumulation of nutrients from winter to summer was observed as in *C. capitatum*, where there was a 110% increase in Cu uptake and 9.5% increase in Mn uptake, clearly indicating seasonal variation on elemental uptake. The accumulation of nutrients from summer to winter was also observed as in *H. cuneata*, which accumulated 3960 $\mu\text{g g}^{-1}$ of Mg in winter which was significantly higher than that in summer.

The effect of seasonal variation on elemental uptake in red seaweeds (*H. spicifera* and *S. hypnoides*) was different where; *H. spicifera* accumulated high concentrations of Ca, Cu and

Fe in winter and low concentrations in summer, opposite to *S. hypnoides*. This showed variations in elemental uptake among species of the same group. In *S. hypnoides*, the concentration of Cu increased significantly (> 8.5 times) between consecutive seasons (winter and spring). The uptake of Mn by *H. spicifera* was significantly different between the seasons that took up the highest and lowest concentrations of the element. The uptake trends for Mg were similar in both species with uptake being higher in the colder seasons than warmer ones. Amongst all the seaweed species studied, *S. hypnoides* had the highest affinity for Mg (22 276 $\mu\text{g g}^{-1}$ in autumn) and Fe (3 324 $\mu\text{g g}^{-1}$ in summer).

Generally, uptake of Ca, Cu, Fe and Mn was lowest in the brown seaweed, *S. elegans*. Calcium, Fe and Mn concentrations in *S. elegans*, between seasons, were within a small range of variation. Copper uptake increased significantly from 8.43 $\mu\text{g g}^{-1}$ in winter to 43.20 $\mu\text{g g}^{-1}$ in spring.

Of the minor elements (Co, Cr, Ni, Se and Zn) in *C. capitatum*, there was no statistically significant difference in Co or Se concentration between seasons ($p > 0.05$) (Table 8). Uptake trends for Cr and Ni were similar with concentrations increasing from autumn to summer. The uptake of Zn followed no clear trend as concentrations alternated between seasons however these were significantly different ($p < 0.05$). In *H. cuneata*, Co, Cr and Se concentrations fell within a small range of variation. For Ni, uptake increased significantly from spring to summer. The uptake of Zn was higher than the other minor elements in autumn, winter and summer but uptake appeared to be significantly restricted in spring.

In *H. spicifera*, the concentrations of Co and Ni showed no significant difference through all seasons and for Cr there was no significant difference from autumn to spring. The uptake trends for Se and Zn were similar with highest concentrations in autumn and lowest concentrations in summer. Zinc uptake in autumn was significantly different from the other

seasons and was higher than Co, Cr, Ni and Se in all seasons. In *S. hypnoides*, concentrations of Co, Cr, Ni, Se and Zn varied significantly between seasons. The mean concentration of Ni in summer ($53.62 \mu\text{g g}^{-1}$) was noticeably higher than the other seasons. Zinc concentrations decreased significantly between autumn and winter and increased significantly between winter and spring.

In *S. elegans* concentrations of Co, Cr and Se in all four seasons were very low compared to the other species studied. In general, no statistically significant difference was observed between seasons for Cr and Se. For Ni, no statistically significant difference was observed from autumn to spring but there was a significant increase from spring to summer. The uptake of Zn decreased significantly from winter through to summer.

The difference in elemental concentration due to seasonal variation found in this study is attributed to the algae's inherent controls as the effect of environmental factors would be constant (since collected from the same site). *S. hypnoides* had the highest capacity to accumulate metals. The findings indicated that certain species tended to accumulate certain metals in summer whilst some tended to accumulate metals in winter. High concentrations in summer could be correlated to concentrations of metals in water which could be high due to increased terrestrial inputs as a result of the rainy season. High concentrations in winter could be linked to growth, where uptake tends to increase in dormant winter periods and decrease during periods of growth.

Table 8. Concentration (in $\mu\text{g g}^{-1}$, dry weight) of elements in seaweeds (Mean \pm SD, n = 3) for the four different seasons.

Species	Season	Ca	Cu	Fe	Mg	Mn	Co	Cr	Ni	Se	Zn
<i>C. capitatum</i> (G)	Autumn	18 125 \pm 742 ^b	8.80 \pm 0.4 ^c	748.63 \pm 20.6 ^b	8921 \pm 51.9 ^a	45.10 \pm 1.5 ^b	0,67 \pm 0.12 ^a	3.43 \pm 0.12 ^b	2.93 \pm 0.12 ^b	1.70 \pm 0.56 ^a	8.53 \pm 0.46 ^c
	Winter	8 661 \pm 644 ^c	7.76 \pm 0.7 ^c	529.93 \pm 11.4 ^c	7846 \pm 19.3 ^b	21.47 \pm 1.6 ^d	0.53 \pm 0.05 ^a	3.93 \pm 0.29 ^b	3.20 \pm 0.26 ^b	1.30 \pm 0.44 ^a	11.97 \pm 0.95 ^b
	Spring	10 580 \pm 396 ^c	200.53 \pm 16.9 ^b	841.10 \pm 32.9 ^b	8 642 \pm 33.2 ^a	26.63 \pm 0.8 ^c	0.57 \pm 0.21 ^a	7.40 \pm 0.26 ^a	8.76 \pm 0.29 ^a	0.87 \pm 0.38 ^b	5.90 \pm 0.40 ^a
	Summer	23 184 \pm 646 ^a	858.24 \pm 69.9 ^a	2 583 \pm 282 ^a	6 336 \pm 274 ^c	201.77 \pm 12.8 ^a	0.58 \pm 0.02 ^a	9.25 \pm 1.45 ^a	23.44 \pm 4.57 ^a	0.03 \pm 0.00 ^{ab}	20.22 \pm 4.42 ^{abc}
Significance		*	**	**	**	**	**	**	**	*	**
<i>H. cuneata</i> (G)	Autumn	88 850 \pm 84.9 ^a	10.27 \pm 0.3 ^c	1 166 \pm 61.1 ^a	2 317 \pm 60.1 ^b	32.67 \pm 2.3 ^a	0.87 \pm 0.06 ^a	3.60 \pm 0.17 ^c	3.13 \pm 0.42 ^b	1.23 \pm 0.06 ^b	24.17 \pm 4.01 ^a
	Winter	66 600 \pm 95.4 ^c	7.30 \pm 0.2 ^d	343.67 \pm 15.1 ^b	3 960 \pm 82.2 ^a	14.77 \pm 0.4 ^b	0.47 \pm 0.06 ^{ab}	7.30 \pm 0.32 ^a	1.57 \pm 0.06 ^b	1.93 \pm 0.06 ^a	17.63 \pm 4.70 ^a
	Spring	73 760 \pm 361.7 ^b	58.87 \pm 0.3 ^b	1 258 \pm 28.7 ^a	3 683 \pm 54.1 ^a	30.73 \pm 0.5 ^a	0.90 \pm 0.02 ^a	5.27 \pm 0.11 ^b	5.60 \pm 0.47 ^b	1.53 \pm 0.51 ^{abc}	4.13 \pm 0.12 ^b
	Summer	73 302 \pm 689 ^b	516.27 \pm 50.2 ^a	1 270 \pm 203 ^a	2 429 \pm 256 ^b	34.41 \pm 4.6 ^a	0.37 \pm 0.04 ^b	14.92 \pm 2.05 ^a	17.75 \pm 3.69 ^a	0.01 \pm 0.00 ^c	23.03 \pm 8.50 ^a
Significance		*	**	*	*	*	**	**	*	**	*
<i>H. spicifera</i> (R)	Autumn	18 436 \pm 471 ^a	7.30 \pm 0.4 ^c	262.67 \pm 22.4 ^b	3 567 \pm 48.1 ^a	18.73 \pm 2.1 ^a	0.47 \pm 0.06 ^a	3.03 \pm 0.35 ^a	6.60 \pm 1.17 ^a	1.93 \pm 0.75 ^a	22.70 \pm 3.39 ^a
	Winter	17 325 \pm 431.3 ^b	21.20 \pm 1.2 ^b	504.33 \pm 3.0 ^a	3 698 \pm 67.1 ^a	16.30 \pm 1.9 ^{ab}	0.50 \pm 0.01 ^a	3.63 \pm 0.76 ^a	7.00 \pm 0.17 ^a	1.70 \pm 0.35 ^a	14.40 \pm 1.47 ^a
	Spring	10 907 \pm 580.2 ^c	62.77 \pm 4.1 ^a	447.50 \pm 33.2 ^a	2 929 \pm 36.5 ^b	15.77 \pm 2.1 ^b	0.47 \pm 0.12 ^a	2.60 \pm 0.69 ^a	11.67 \pm 6.17 ^a	0.30 \pm 0.01 ^b	10.80 \pm 0.26 ^b

	Summer	4 522±232 ^d	10.19±1.6 ^c	206.10±7.3 ^b	2 939±1.6 ^b	11.37±2.3 ^b	0.59±0.05 ^a	0.84±0.08 ^b	6.70±0.77 ^a	0.01±0.00 ^b	8.24±0.76 ^b
Significance		*	**	*	*	*	**	*	**	*	*
<i>S. hypnoides</i> (R)	Autumn	20 117±105 ^d	12.83±0.6 ^c	2 215±46.7 ^b	22 276±214 ^a	134.20±2.9 ^a	2.93±0.06 ^a	9.37±0.47 ^b	10.30±0.79 ^a	0.4±0.06 ^a	29.15±1.06 ^a
	Winter	23 845±162.6 ^c	94.13±4.0 ^b	2 471±28.6 ^b	13 380±60.8 ^b	133.60±2.7 ^a	2.00±0.10 ^b	19.30±0.82 ^a	11.87±0.21 ^a	0.50±0.01 ^a	9.90±1.76 ^c
	Spring	43 435±572.8 ^b	801.80±39.5 ^a	2 943±18.7 ^a	8 245±12.7 ^c	74.57±2.5 ^b	1.37±0.12 ^c	6.97±0.25 ^c	6.47±0.25 ^b	0.03±0.00 ^b	22.80±0.82 ^b
	Summer	45 816±610 ^a	164.17±44.0 ^b	3 324±92.6 ^a	6 964±364 ^d	119.77±11.3 ^a	2.01±0.09 ^b	8.97±1.39 ^{bc}	53.62±12.59 ^{ab}	0.03±0.00 ^b	21.23±5.19 ^{abc}
Significance		**	**	**	*	*	*	*	**	**	**
<i>S. elegans</i> (B)	Autumn	9 270±16.3 ^a	7.43±0.8 ^c	189.77±9.6 ^a	5 130±33.5 ^a	10.27±0.6 ^a	0.47±0.06 ^b	1.20±0.26 ^a	1.83±0.31 ^b	1.13±0.40 ^a	18.70±2.69 ^a
	Winter	9 684±736.2 ^a	8.43±0.5 ^c	115.55±15.2 ^b	3 875±65.1 ^b	8.73±0.7 ^b	0.30±0.01 ^b	3.40±1.76 ^a	3.03±1.10 ^b	1.47±0.46 ^a	23.33±2.13 ^a
	Spring	8 428±392.6 ^a	43.20±2.2 ^a	111.50±14.8 ^b	3 686±50.2 ^b	7.45±0.2 ^c	0.20±0.01 ^c	0.70±0.10 ^a	1.37±0.31 ^b	0.80±0.40 ^{ab}	9.10±0.01 ^b
	Summer	10 347±303 ^{ab}	16.48±1.9 ^b	152.30±29.7 ^{ab}	4 768±320 ^{ab}	8.19±0.3 ^{bc}	1.50±0.09 ^a	0.41±0.08 ^a	9.67±1.74 ^a	0.30±0.00 ^b	7.16±0.36 ^c
Significance		*	*	*	**	*	**	**	**	*	**

G -green, R - red, B – brown,

*, ** indicates significance at $p \leq 0.05$ for one-way ANOVA and Welch ANOVA, respectively,

Different superscript letters within columns indicate mean separation by Tukey or Games-Howell at the 5% level.

3.3.3 Toxic elements

The effect of seasonal variation on uptake of As for the five studied seaweed species is presented in Figure 17.

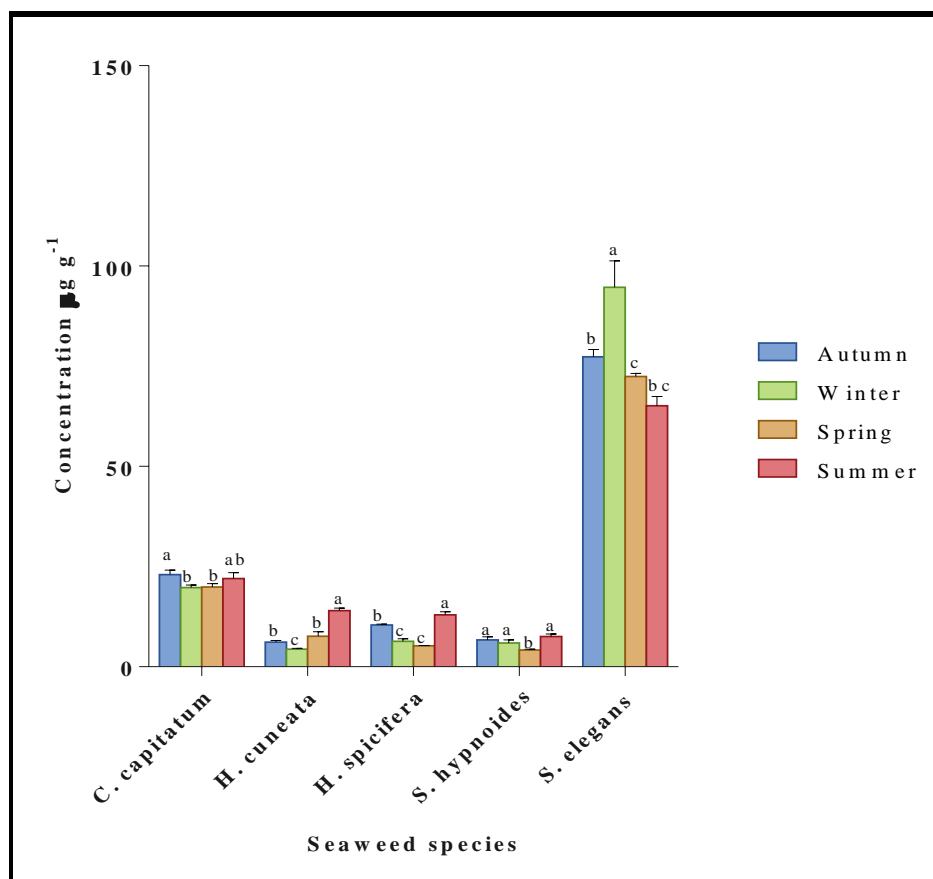


Figure 17. Mean concentrations of As in the five seaweed species studied during the four different seasons, $n = 3$. Different superscript letters within columns indicate mean separations by Tukey's or Games-Howell post-hoc tests at the 5% level.

In *S. elegans*, there was a significant difference in uptake of As from summer ($65.1 \mu\text{g g}^{-1}$) through to winter ($94.70 \mu\text{g g}^{-1}$). In *C. capitatum*, the uptake of As was comparable in all four seasons. Uptake of As in *H. spicifera* and *H. cuneata* increased significantly from winter ($6.37 \mu\text{g g}^{-1}$ and $4.37 \mu\text{g g}^{-1}$, respectively) to summer ($12.99 \mu\text{g g}^{-1}$ and $13.99 \mu\text{g g}^{-1}$, respectively). In *S. hypnoides*, uptake of As was comparable in summer, winter and autumn but concentrations decreased significantly from winter to spring. In general, the concentration

of As was highest in *S. elegans*, followed by *C. capitatum*, *H. cuneata*, *H. spicifera* and *S. hypnoides*. This was in agreement with previous studies that reported brown algae to have a high affinity for As ($77 \mu\text{g g}^{-1}$ in *Sargassum sinicola*) (Rodriguez-Castaneda et al., 2006).

The effect of seasonal variation on Pb uptake for the studied species is presented in Figure 18. The uptake trends for Pb in *C. capitatum*, *H. cuneata*, *H. spicifera*, *S. hypnoides* and *S. elegans* were similar with a significant increase from winter to spring to summer and a significant decrease from summer to autumn. In general, uptake of Pb increased with an increase in temperature (hotter seasons) and decreased with a decrease in temperature (colder seasons).

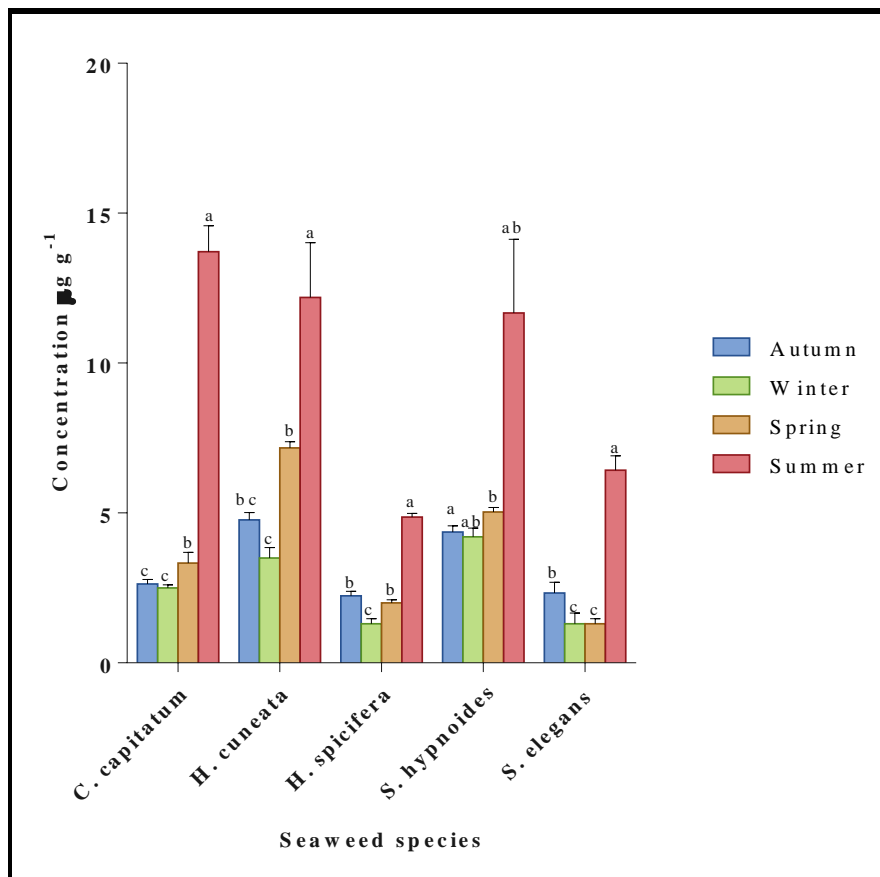


Figure 18. Mean concentrations of Pb in the five seaweed species studied during the four different seasons, $n = 3$. Different superscript letters within columns indicate mean separations by Tukey's or Games-Howell post-hoc tests at the 5% level.

3.3.4 Contribution to the diet

The mineral content of edible seaweeds makes them nutritionally valuable and they could contribute significantly to the diet of the impoverished coastal communities in South Africa. Generally, poor communities often depend upon natural resources to meet their basic nutritional needs, and these resources degrade easily and become less productive which in turn leads to inadequate nutrition and sickness caused by deficiencies in the diet. Previously, we reported on the elemental contribution of fruits and vegetables found in the east coast region of KwaZulu-Natal, South Africa, to the diet (Moodley et al., 2012; Kisten et al., 2015). The coastal communities in South Africa rely mostly on fishing for food (Stern, 2013; Young, 2013). This can be supplemented by seaweeds which could offer an alternative to vegetables for a balanced diet. The elemental distribution in the edible seaweeds studied is compared to Dietary Reference Intakes (DRIs) for most individuals (Table 9) to determine the contribution of seaweeds to the diet.

Based on dry mass, if 10 g of any of the studied edible seaweeds is consumed, it would contribute satisfactorily to the intake of most essential elements and would not exceed the tolerable upper intake levels (ULs). Iron is essential for a number of biochemical functions in the body including the transport of oxygen, and its deficiency results in anaemia. About 10 g of *C. capitatum* would contribute 78 to 146% towards the RDA for Fe and 299% towards the RDA for Cu, both of which would be considered safe as the ULs were not exceeded. Chromium improves the efficiency of insulin and is required for normal protein, fat and carbohydrate metabolism (Chowdhury et al., 2003). Chromium deficiency has been linked to maturity onset diabetes and cardiovascular disease. If about 10 g of *C. capitatum*, *H. spicifera* or *S. elegans* is consumed, it would contribute 171 to 250%, 85.7 to 125% and 28.6 to 41.7%, respectively towards the RDA for Cr. Chromium is considered one of the safest nutrients, and

is likely to be without risk of adverse health effects over a lifetime therefore no ULs have been established for this element (Krejpcio, 2001).

Copper is necessary for proper development of connective tissue, myelin and melanin; its deficiency is usually the consequence of decreased Cu stores at birth, inadequate dietary Cu intake and poor absorption. Anaemia, neutropenia and bone abnormalities are some of the common results of Cu deficiency (Uauy et al., 1998). If about 10 g of *C. capitatum* is consumed, it would contribute 299% towards the RDA for Cu; however this amount does not exceed the UL so it would be considered safe. Consumption of 10 g of *H. spicifera* and *S. elegans* would contribute 27.8% and 21.1%, respectively towards the RDA for Cu.

Lead is a toxic metal and high dosages of Pb can damage the central nervous system and the kidneys (Chamannejadian et al., 2013). The WHO/FAO Expert Committee on Food Additives has set the provisional tolerable daily limit for Pb, which can be taken over a lifetime with no adverse health effects, as 0.214 mg per day for an average body weight of 60 kg (WHO, 2000). The average concentration of Pb in 10 g of *C. capitatum*, *H. spicifera* and *S. elegans* was 0.06, 0.03 and 0.04 mg, respectively which do not exceed the daily limit for Pb.

Results from the survey of total As in the 1999 Total Diet Study showed an upper-bound daily intake of 0.1 $\mu\text{g kg}^{-1}$ body weight for a normal consuming adult (Tao and Bolger, 1999). For an average person (body weight of 60 kg), this would amount to 6 μg of As per day. Based on the United States Department of Agriculture's 1987-1988 Nationwide Food Consumption Survey, the estimated daily total As average intake is 28 to 37 μg per day for most women and 47 to 57 μg per day for most men. Uptake of total As in *S. elegans* ranged between 65.1 $\mu\text{g g}^{-1}$ and 94.70 $\mu\text{g g}^{-1}$. If 10 g of seaweed is consumed in summer (the season where uptake is lowest), this would contribute 651 μg per day to an adult which is more than

a hundredfold increase in the upper bound daily intake for a normal consuming adult and it exceeds the United States estimated daily total As average intake. However total As content alone does not fully reflect the level of hazard in consuming *S. elegans*, hence it is important to determine the various forms of As to evaluate the risk associated with too much consumption of *S. elegans*. The most toxic forms of arsenic are the inorganic ones (As(III) and As(V)). The organic forms such as arsenocholine, arsenobetaine and arsenosugars are considered less toxic or non-toxic (Rose et al., 2007).

Table 9. Dietary Reference Intake (Recommended Dietary Allowance (RDA) and Tolerable Upper Intake Level (UL)) for each essential element and estimated contribution of seaweeds (*C. capitatum*, *H. spicifera* and *S. elegans*) towards the RDA for most individuals.

Element	Average concentration			DRI*		Estimated contribution to RDA		
	(mg per 10 g)			(mg g ⁻¹)		(%)		
	<i>C. capitatum</i>	<i>H. spicifera</i>	<i>S. elegans</i>	RDA	UL	<i>C. capitatum</i>	<i>H. spicifera</i>	<i>S. elegans</i>
Fe	11.75	3.55	1.42	8-15	45	78-146	23.7-44.4	9.5-17.8
Cu	2.69	0.25	0.19	0.9	10	299	27.8	21.1
Zn	0.12	0.14	0.15	8 -11	40	1.1-1.5	1.3-1.8	1.4-1.9
Mn	0.74	0.15	0.09	1.6-2.3	11	32.2-46.3	6.5-9.4	3.9-5.6
Pb	0.06	0.03	0.04		ND	28.0	14.0	18.7
Cr	0.06	0.03	0.01	0.024-0.035	ND	171-250	85.7-125	28.6-41.7
Ni	0.10	0.08	0.04	ND	1.00	ND**	ND	ND
Se	0.01	0.01	0.01	0.055	0.40	18.2	18.2	18.2

* Dietary Reference Intake,

**ND- Not determinable.

3.4 Conclusion

The three edible seaweeds from this study, *C. capitatum*, *H. spicifera* and *S. elegans* were shown to be rich sources of proteins, lipids and carbohydrates and had sufficient amounts of essential nutrients to contribute positively to the diet. Seaweeds are therefore potential sources of these minerals and their potential as a health food should be explored especially in the impoverished coastal communities in South Africa. However, consumption of *S. elegans* should be moderated as it could increase dietary exposure to inorganic As if too much is consumed. The study also revealed the effect of seasonal variation on elemental uptake in seaweeds. In general, the concentrations of essential elements in seaweeds were found to be in decreasing order of $\text{Ca} > \text{Mg} > \text{Fe} > \text{Cu} > \text{Mn} > \text{As} > \text{Zn} > \text{Ni} > \text{Cr} > \text{Co} \approx \text{Se}$.

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CHAPTER 4

BIOACTIVE COMPOUNDS, ELEMENTAL CONCENTRATIONS, TOTAL AND INORGANIC ARSENIC IN *SARGASSUM ELEGANS* SUHR (1840).

Abstract

The brown marine macro alga, *Sargassum elegans* Suhr 1840 (Phaeophyta), collected from the coast of KwaZulu-Natal South Africa, was investigated for its secondary metabolites. Structural elucidation was performed using IR, NMR and mass spectroscopy. The compounds isolated from *S. elegans* were identified as β -sitosterol, fucosterol and phaeophytin **a**. The distribution of essential elements and the toxic element arsenic (total and inorganic) in *S. elegans* from eight different sites along the coast of KwaZulu-Natal were investigated. In general, elemental concentrations varied significantly with location ($p < 0.05$) and were found to be in the decreasing order of $\text{Ca} > \text{Mg} > \text{Fe} > \text{Cu} > \text{Zn} > \text{Mn} > \text{Ni} > \text{Pb} > \text{Co} > \text{Se} > \text{Cr}$. Total As in *S. elegans* was extremely high, ranging from 42.1 to 105.4 $\mu\text{g g}^{-1}$, of which, 21.4 to 53.0 $\mu\text{g g}^{-1}$ were in inorganic form. This study suggests that consumption of *S. elegans* could significantly increase dietary exposure to inorganic As which can cause adverse health effects, therefore its consumption should be avoided.

Keywords: fucosterol, phaeophytin **a**, inorganic arsenic, toxicity

4.1 Introduction

Sargassum is brown seaweed belonging to the family Sargassaceae and order Fucales. It has more than 400 species and is widely distributed in the tropical and temperate oceans of the world (Marimuthu et al., 2012). *Sargassum* spp. has been used in traditional Chinese medicine for nearly 2000 years to treat various diseases including thyroid diseases such as goitre (Liu et al., 2012). Chinese herbalists use powdered *Sargassum* to prepare a tea that is used to remove excess phlegm (Simoons, 1991). Cultures outside of China and Japan such as Indonesian and Hawaiian have also consumed *Sargassum* as food and medicine. The use of *Sargassum* in South Africa has not been documented; however the locals living in the coastal areas use *Sargassum* spp. to make tea infusions as a remedy for sore throat and to treat chest pains. The quantities used to make tea infusions are not consistent or properly measured which creates the risk of exposure to high levels of toxic inorganic As.

Many biologically active compounds like terpenoids, flavonoids, sterols, polyphenols, sargaquinoic acids, sargachromenol and phaeophytin have been isolated from this genus. These isolated compounds have been reported to exhibit diverse biological activities like analgesic, anti-inflammatory, antioxidant, neuroprotective, anti-microbial, anti-tumor, fibrinolytic, immune-modulatory, anti-coagulant, hepato-protective and anti-viral activity (Hur et al., 2008; Liu et al., 2012; Peng et al., 2013; Seo et al., 2007; Zhang et al., 2013). This suggests that *Sargassum* species have great potential to be used in pharmaceutical and nutraceutical industry. *Sargassum elegans* Suhr (1840) is widely distributed along the coastline of South Africa however this species is not utilized commercially in this country. Seaweed utilization is mainly focused on the larger kelps like, *Ecklonia* and *Laminaria* and the agar producing red seaweeds *Gelidium* and *Gracilaria* (Gillespie and Critchley, 1999).

Sargassum species have also been found to contain high concentrations of As to which long-term exposure especially to the inorganic form, is known to cause cancer and skin lesions, and can be fatal (Chen et al., 1992; Chiou et al., 1995). *Sargassum fusiforme* (*hijiki*) has been reported to contain high concentrations of inorganic As and its consumption has since been stopped by the Canadian Food Inspection Agency (CFIA, 2001). In a previous study, we investigated the elemental concentrations in different classes of edible seaweeds, found in KwaZulu-Natal, South Africa as a function of seasonal variation and found total As in *S. elegans* to range between 65.1 $\mu\text{g g}^{-1}$ (in summer) and 94.7 $\mu\text{g g}^{-1}$ (in winter) (unpublished results). In this study, total and inorganic As in *S. elegans* from eight different sites in KwaZulu-Natal, South Africa were determined to evaluate its safety for human consumption. We also report on the secondary metabolites present in *S. elegans* to validate its ethno-medicinal use.

4.2 Materials and Methods

4.2.1 General experimental procedure

4.2.2 Sampling

Samples of *S. elegans* were collected by hand picking during low tide from the coast of KwaZulu-Natal, in summer and identified by marine biologist, Dr Deborah V. Robertson-Anderson from the School of Life Sciences, University of KwaZulu-Natal, Westville. The sampling sites were: 1 Compensation Beach (29.55353 ° S, 31.2228 ° E), 2 Inyoni Rocks (30.0479 ° S, 30.8902 ° E), 3 Isipingo Beach (29.9974 ° S, 30.9446 ° E), 4 Winklespruit (30.0946 ° S, 30.8604 ° E), 5 Park Ryne (30.3167 ° S, 30.7333 ° E), 6 Pennington (30.3674 ° S, 30.7333 ° E), 7 Ifafa (30.4623 ° S, 30.8902 ° E) and 8 Hibberdene (30.5718 ° S, 30.5724 ° E). The collected seaweeds were washed with seawater to remove sand particles, epiphytes and shells and transported to the laboratory in polythene bags where they were thoroughly

washed with double distilled water and dried at 45 °C to constant mass. The dried samples were ground using a blender (Braun range), then stored in plastic containers in the refrigerator until analysed.

4.2.3 Preparation of extracts

About 1.100 g of ground *S. elegans* was sequentially extracted exhaustively with hexane, dichloromethane (DCM) and methanol (MeOH) on an orbital shaker at room temperature. The crude extracts were filtered using Whatman No. 1 filter paper and the filtrates were concentrated under reduced pressure using a rotary evaporator. The aqueous MeOH extract was partitioned with equal volumes of DCM followed by ethyl acetate (EtOAc) and the collected fractions were concentrated by rotatory evaporation.

4.2.4 Phytochemical screening

Phytochemical screening of the extracts was carried out according to standard methods as described by Trease and Evans (2002) and Sofowora (1993). The different extracts were tested for alkaloids, flavonoids, terpenoids, tannins and steroids.

4.2.5 Characterization and quantification methods.

The compounds were characterized using different spectroscopic techniques. Nuclear Magnetic Resonance (NMR) spectra (1D and 2D) were recorded in deuterated chloroform (CDCl₃) at room temperature using the Bruker AVANCE III 400 MHz spectrometer with tetramethylsilane (TMS) as an internal standard. Infrared (IR) spectra were obtained using the Perkin Elmer Universal ATR Spectrometer. Mass spectra were recorded using a Waters Micro-mass LCT Premier TOF-MS. Column chromatography was performed using silica gel (Merck Kieselgel 60, 0.063-0.200 mm, 70-230 mesh ASTM). The obtained fractions were

monitored by thin layer chromatography (TLC) (Merck silica gel 60, 20× 20 cm F254 aluminum sheets). The TLC plates were viewed under an ultraviolet lamp (254 nm) and developed using 10% H₂SO₄ in MeOH.

4.2.6 Isolation of compounds from *S. elegans*

The hexane (3.34 g) and DCM (5.06 g) extracts were combined due to similar retention factors, R_f. The combined extract was subjected to column chromatography and separated using a hexane: EtOAc step gradient starting with 100% hexane and gradually increased by 10% to 100% EtOAc. Ten 50 mL fractions were collected for each solvent system and fractions with similar TLC profiles were combined and concentrated using the rotary evaporator. After elution with a hexane: EtOAc (7:3) solvent system, fractions 35 and 36 afforded the isolation of compound **1** (106.3 mg). Fractions 32-34, were combined and re-chromatographed to give compound **2** (20 mg), which eluted with a hexane: EtOAc (8.5:1.5) solvent system.

The DCM fraction from MeOH extract (14.45 g) was subjected to column chromatography using 100% hexane that was gradually increased by 10% to 100% EtOAc. Ten 50 mL fractions were collected for each solvent system and fractions 40-46 were combined and purified with hexane: EtOAc (8:2) to yield compound **3** (78 mg).

Compound 1:

¹H-NMR spectral data (CDCl₃, 400 MHz) δ_H ppm: 5.33 (1H, t, J = 5.7 Hz, H-6), 3.52 (1H, m, H-3), 0.99 (3H, s, H-19), 0.91 (3H, d, J = 6.5 Hz, H-21), 0.82 (3H, s, H-29), 0.81 (3H, br s H-27), 0.78 (3H, s, H-26), 0.66 (3H, s, H-18).

^{13}C -NMR spectral data (CDCl_3 , 400 MHz) δ_{H} ppm: 140.8 (C-5), 121.7 (C-6), 71.8 (C-3), 56.8 (C-14), 56.1 (C-17), 50.1 (C-9), 45.8 (C-24), 42.3 (C-4), 42.2 (C-13), 39.8 (C-12), 37.3 (C-1), 36.5 (C-10), 36.1 (C-20), 33.9 (C-22), 31.9 (C-7, 8), 31.7 (C-2), 29.2 (C-25), 28.2 (C-16), 26.1 (C-23), 24.3 (C-15), 23.1 (C-28), 21.1 (C-11), 19.8 (C-27), 19.4 (C-19), 19.0 (C-26), 18.8 (C-21), 12.0 (C-18), 11.9 (C-29).

Compound 2:

^1H -NMR spectral data (CDCl_3 , 400 MHz) δ_{H} ppm: 5.33 (1H, br d, $J = 5.34$ Hz, H-6), 5.18 (1H, q, $J = 6.73$ Hz, H-28), 3.53 (1H, m, H-3), 2.25 (1H, m, H-25), 1.57 (3H, d, $J = 6.4$ Hz, H-29), 0.99 (3H, s, H-21), 0.96 (3H, d, $J = 1.2$ Hz, H-27), 0.95 (3H, d, $J = 1.2$ Hz, H-26), 0.66 (3H, s, H-18).

^{13}C -NMR spectral data (CDCl_3 , 400 MHz) δ_{H} ppm: 147.0 (C-24), 140.7 (C-5), 121.7 (C-6), 115.6 (C-28), 71.8 (C-3), 56.8 (C-14), 55.8 (C-17), 50.1 (C-9), 42.4 (C-13), 42.3 (C-4), 39.8 (C-12), 37.2 (C-1), 36.5 (C-10), 36.4 (C-20), 35.2 (C-22), 34.8 (C-25), 31.9 (C-7,8), 31.6 (C-2), 28.2 (C-16), 25.7 (C-23), 24.3 (C-15), 22.2 (C-26), 22.1 (C-27), 21.0 (C-11), 19.4 (C-19), 18.8 (C-21), 13.2 (C-29), 11.8 (C-18).

Compound 3:

^1H -NMR spectral data (CDCl_3 , 400 MHz) δ_{H} ppm: 9.35 (1H, s, H-10), 9.20 (1H, s, H-5), 8.52 (1H, s, H-20), 7.88 (1H, dd, $J = 11.58, 17.73$ Hz, H-3¹), 6.27 (1H, s, H-13²), 6.21 (1H, d, $J = 17.95$ Hz, H-3²), 6.10 (1H, d, $J = 11.60$ Hz, H-3²), 4.47 (1H, m, H-18), 4.21 (1H, m, H-17), 3.90 (3H, s, H-13⁴), 3.64 (2H, s, H-8¹), 3.33 (3H, s, H-2¹), 3.10 (3H, s, H-7¹), 1.81 (3H, d, $J = 7.2$ Hz, H-18¹)

^{13}C -NMR spectral data (CDCl_3 , 400 MHz) δ_{H} ppm: 189.67 (C-13¹), 172.98 (C-17³), 172.22 (C-19), 169.64 (C-13³), 161.25 (C-16), 155.60 (C-6), 150.94 (C-9), 149.66 (C-14), 145.15 (C-8), 142.86 (C-1), 142.03 (C-P3), 137.92 (C-11), 136.47 (C-3), 136.21 (C-4), 136.11 (C-7), 131.81 (C-2), 129.05 (C-13), 129.00 (C-12), 128.96 (C-3¹), 122.72 (C-3²), 117.74 (C-P2), 105.24 (C-15), 104.37 (C-10), 97.48 (C-5), 93.10 (C-20), 64.72 (C-13²), 61.49 (C-P1), 52.85 (C-13⁴), 51.16 (C-17), 50.14 (C-18), 39.80 (C-P4), 39.36 (C-P14), 37.39 (C-P6), 37.33 (C-P8), 37.26 (C-P10), 36.64 (C-P12), 32.76 (C-P11), 32.62 (C-P7), 31.22 (C-17²), 29.71 (C-17¹), 27.97 (C-P15), 24.99 (C-P5), 24.77 (C-P9), 24.42 (C-P13), 23.09 (C-18¹), 22.72 (C-P16), 22.62 (C-P17), 19.73 (C-P11¹), 19.66 (C-P7¹), 19.39 (C-8¹), 17.39 (C-P3¹), 16.29 (C-8²), 12.08 (C-12¹), 12.04 (C-2¹), 11.17 (C-7¹).

4.2.7 Reagents

All reagents were of analytical reagent grade and were supplied by Sigma-Aldrich (Germany). Double distilled water was used for all dilutions. All plastic and glassware was cleaned by soaking in dilute HNO_3 and rinsed with double distilled water prior to use. Elemental standards were prepared from 1000 mg L^{-1} stock solutions that were supplied by Fluka Analytical, Sigma, Switzerland.

4.2.8 Determination of elemental content

Ground seaweed samples of 0.25 g dry weight (DW) were quantitatively digested in Easyprep™ teflon closed vessels using 10 mL of concentrated nitric acid with a CEM MARS 6 microwave (CEM Corporation, Matthews, North Carolina, USA). The digests were transferred to 25 mL volumetric flasks and made to volume with double distilled water before being analysed (in triplicate) for As, Ca, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn using a Perkin Elmer simultaneous inductively coupled plasma-optical emission spectrometer (ICP-

OES, model 5300DV) with radial plasma observation. Analytical wavelengths were selected based on minimum spectral interferences and maximum analytical performance. Initially, the three most sensitive lines were chosen, thereafter, the line with no interfering elements was selected. Quality assurance for the measured elements was performed using the certified reference material (CRM), *white clover* BCR-402, from the Community Bureau of Reference of the Commission of the European Communities.

4.2.9 Determination of inorganic arsenic

Different methods are used for the extraction of inorganic As from algae, with MeOH and water (with or without the assistance of microwave or sonication) being among the commonly used solvents (Petursdottir et al., 2014). Given that the As compounds investigated are very polar, the method described by Zhao et al. (2014) was used. Seaweed samples (0.5 g, DW) were accurately weighed into 50 mL polypropylene vials and extracted with 38 mL of deionized water using ultrasonic irradiation (ultrasonic bath PS-30A frequency of 40 Hz) for 40 min. The samples were held at 4 °C before being centrifuged for 10 min. The supernatants were microwave digested then filtered through a 0.45 µm membrane before being analysed for As using ICP-OES. At the same time, a tea infusion using 0.5 g of seaweed and 38 mL of boiling water was also prepared, filtered and analysed for As using ICP-OES.

4.2.10 Statistical analysis

The significant differences in elemental concentrations from the different sampling sites were tested by One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test at

$p < 0.05$. All statistical analyses were performed using the Statistical Package for the Social Sciences (PASW Statistics 22, IBM Corporation, Cornell, N.Y.)

4.3 Results and Discussion

Preliminary phytochemical screening for flavonoids, terpenoids, tannins, steroids and alkaloids was conducted on three different extracts of *S. elegans*. These tests facilitated the qualitative separation of biologically active compounds using column chromatography. The screening revealed the presence of sterols in the hexane extract, flavonoids and sterols in the DCM extract and tannins in the MeOH extract (Table 10).

Table 10. Preliminary phytochemical screening of crude extracts of *S. elegans*.

Extract	Flavonoids	Terpenoids	Tannins	Steroids	Alkaloids
Hexane	-	-	-	+	-
DCM	+	-	-	+	-
MeOH	+	-	+	-	-

4.3.1 Structure elucidation of compounds from *S. elegans*

Compound **1** was isolated as a white solid with a mass of 106.33 mg. The $^1\text{H-NMR}$ spectrum of compound **1** showed six methyl resonances (δ_{H} 0.66-0.99), a resonance for a single olefinic proton at δ_{H} 5.33 (H-6) and a multiplet at δ_{H} 3.52 (C-3) confirming the presence of a hydroxyl group. The $^{13}\text{C-NMR}$ spectrum resolved twenty nine carbon resonances, of which two resonances were due to the presence of a double bond at δ_{C} 140.8 (C-5) and 121.7 (C-6). The IR spectrum of compound **1** showed absorbance bands at $3\,342\text{ cm}^{-1}$ (-OH) and 2934 cm^{-1} (-C-H). The mass spectrum of compound **1** gave molecular ion peak at m/z 414 [M^+]

corresponding to a molecular formula $C_{29}H_{50}O$. This data corresponded with that in literature (Chaturvedula and Prakash, 2012; Murade et al., 2015; Tripathee et al., 2011) and confirmed compound **1** to be β -sitosterol, (Figure 19). β -sitosterol has been reported to be an effective immune booster and has been used in the treatment of prostate enlargement and HIV (Sen et al., 2012). It has also been shown to possess anti-inflammatory and antipyretic activity (Gupta et al., 1980).

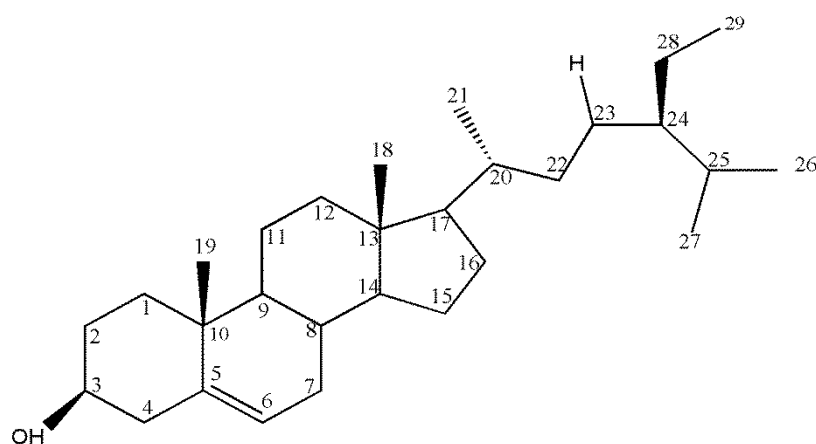


Figure 19. Structure of compound **1** (β -sitosterol).

Compound **2** was isolated as white crystalline solid (20 mg). The 1H -NMR and ^{13}C -NMR spectra of compound **2** was similar to compound **1** expect for the olefinic resonance at δ_H 5.18 (H-28). The IR spectrum showed absorption bands at 3329 cm^{-1} (-OH), 1378 cm^{-1} (C-H) and 822 cm^{-1} (H atom on a trisubstituted double bond). The mass spectrum obtained by HR-ESI-MS positive mode gave a molecular ion peak at m/z 395.33 $[M-OH]^+$ corresponding to molecular formula $C_{29}H_{48}O$. This data corresponded with that in literature (Hwang et al., 2012; Khanavi et al., 2012) and confirmed compound **2** to be fucosterol (Figure 20). Fucosterol, the most abundant sterol in brown algae (Bouzidi et al., 2008) has been reported

to be non-toxic and has the ability to reduce blood cholesterol levels (Mouristen, 2013). It has also been reported to possess anti-diabetic, antioxidant and anticancer activities (Lee et al., 2004; Kim et al., 2013).

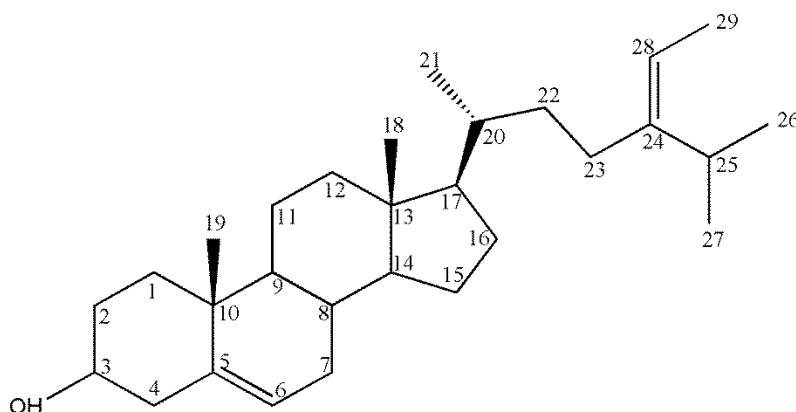


Figure 20. Structure of compound **2** (fucosterol).

Compound **3** was isolated as a brownish green amorphous solid (78 mg). The $^1\text{H-NMR}$ spectrum showed characteristic peaks for chlorophyll derivatives with an up-field shift at δ_{H} - 1.7 (pyrrole ring) and δ_{H} 6.1-7.9 (vinyl group). The $^{13}\text{C-NMR}$ (DEPT 90 and DEPT 135) spectrum of compound **3** resolved fifty five carbon resonances corresponding to, eleven methyl, fourteen methylene, nine methine and twenty-one quaternary carbons. The IR spectrum of compound **3** showed absorption bands at 3388 cm^{-1} (NH), 1618 cm^{-1} (C=C) and 1376 cm^{-1} (CN). The mass spectrum obtained by HR-ESI-MS positive mode gave a molecular ion peak at m/z 893.5530 corresponding to molecular formula $\text{C}_{55}\text{H}_{74}\text{N}_4\text{O}_5$ $[\text{M}+\text{Na}]^+$. This data corresponded with that in literature (Gonçalves de Brito Filho et al., 2014) and confirmed compound **3** to be phaeophytin **a** (Figure 21). Phaeophytin **a** was previously isolated from brown alga *Sargassum fulvellum* was found to be a strong neuro-differentiating compound that could be used to develop new therapeutic drugs for

neurodegeneration diseases such as Alzheimer's (Ina et al., 2007). Phaeophytin **a**, is also known to have antioxidant activity (Higashi-Okai et al., 2001).

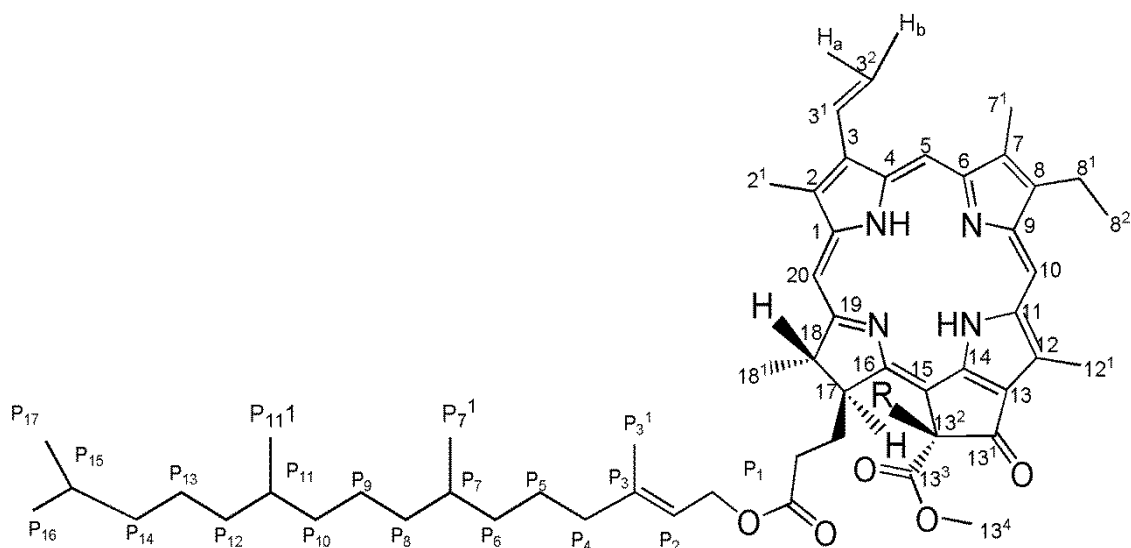


Figure 21. Structure of compound **3** (phaeophytin a).

4.3.2 Elemental analysis

To evaluate the accuracy of the analytical method, the experimental values for the CRM (*white clover* BCR-402) were compared to certified values. The values provided for Fe, Ni and Zn are indicative so no uncertainties were ascribed to them. The experimental values obtained for the CRM (n=3, p=0.05) (expressed in $\mu\text{g g}^{-1}$) were 0.089 ± 0.25 , 0.17 ± 0.03 , 240 ± 36.65 , 8.23 ± 0.46 , 6.60 ± 0.26 and 25.40 ± 0.64 for As, Co, Fe, Ni, Se, and Zn, respectively; compared to their corresponding certified values 0.093 ± 0.010 , 0.178 ± 0.008 , 244, 8.25, 6.70 ± 0.25 and $25.2 \mu\text{g g}^{-1}$.

Table 11 represents the concentrations of selected elements in *S. elegans* from the eight different sites. The variation in elemental concentrations for an element could be attributed to the different geographical locations. Concentrations of the macro-elements (Ca and Mg)

ranged from 6400 to 11113 and 4548 to 6475 $\mu\text{g g}^{-1}$, respectively. For the micro-elements (Cu, Fe and Zn) concentrations ranged from 6.91 to 34.61, 37.80 to 191.9 and 8.14 to 31.56 $\mu\text{g g}^{-1}$, respectively. Cobalt concentrations were within a small range of variation (0.23 to 3.58 $\mu\text{g g}^{-1}$) with no statistically significant difference ($p < 0.05$) observed from Compensation Beach (site 1) to Park Ryne (site 5). The concentrations of the micro-elements (Cr, Mn, Ni and Zn) from the different sites were within a small range of variation indicating the algae's control on uptake of these elements. In general the concentrations of essential elements were found to be in the decreasing order of $\text{Ca} > \text{Mg} > \text{Fe} > \text{Cu} > \text{Zn} > \text{Mn} > \text{Ni} > \text{Co} > \text{Se} > \text{Cr}$.

For the toxic element Pb, concentrations ranged from 0.89 to 4.98 $\mu\text{g g}^{-1}$. The maximum level of Pb in foodstuffs (fish and processed fish) set by the Department of Health, South Africa, is 0.5 $\mu\text{g g}^{-1}$ (Department of Health, 1994). All sites exceeded this threshold. Results for the toxic element As are presented in Table 12. The concentrations of total and inorganic As in *S. elegans* varied significantly with location ($p < 0.05$). Total As ranged from 42.1 $\mu\text{g g}^{-1}$ (site 1) to 105.4 $\mu\text{g g}^{-1}$ (site 7) and inorganic As ranged from 21.37 $\mu\text{g g}^{-1}$ (site 1) to 53.0 $\mu\text{g g}^{-1}$ (site 4). Ifafa (site 7) had the highest concentration of total As 105 $\mu\text{g g}^{-1}$ of which 31% was found to be in inorganic form. On average, about 50% of total As was found to be in inorganic form. The high levels of As in *S. elegans* are consistent with previous studies that have reported high concentrations of As in the brown seaweed genus, *Sargassum*. The levels of both total and inorganic As in the prepared tea infusion were found to be 83.60 $\mu\text{g g}^{-1}$ and 53.0 $\mu\text{g g}^{-1}$ (63% of the total), respectively.

Table 11. Concentrations (in $\mu\text{g g}^{-1}$, dry weight) of elements in *S. elegans* (Mean \pm SD, n = 3) at the eight different sites.

Elements	Site 1*	2	3	4	5	6	7	8
Ca	10147 \pm 31.8 ^c	7276 \pm 38.7 ^e	7145 \pm 29.8 ^e	7754 \pm 77.7 ^d	6400 \pm 53.9 ^f	10547 \pm 85.2 ^b	11113 \pm 41.3 ^a	7476 \pm 66.2 ^e
Mg	5516 \pm 68.8 ^b	4806 \pm 33.9 ^f	4834 \pm 66.7 ^{e,f}	4548 \pm 93.3 ^g	5044 \pm 57.8 ^d	5268 \pm 26.1 ^c	6475 \pm 31.2 ^a	4966 \pm 36.7 ^{d,e}
Fe	37.80 \pm 0.51 ^e	189.4 \pm 14.5 ^a	133.7 \pm 5.04 ^{b,c}	191.9 \pm 2.63 ^a	89.11 \pm 7.08 ^d	115 \pm 2.18 ^c	146.06 \pm 2.90 ^b	82.36 \pm 14.4 ^d
Cu	8.18 \pm 0.48 ^c	7.88 \pm 0.33 ^c	7.93 \pm 0.12 ^c	8.20 \pm 0.17 ^c	18.67 \pm 1.99 ^b	6.91 \pm 0.22 ^c	31.46 \pm 4.55 ^a	34.61 \pm 0.76 ^a
Co	0.30 \pm 0.00 ^c	0.42 \pm 0.06 ^c	0.30 \pm 0.00 ^c	0.40 \pm 0.00 ^c	0.23 \pm 0.06 ^c	3.27 \pm 0.09 ^b	3.58 \pm 0.06 ^a	3.53 \pm 0.13 ^a
Cr	0.27 \pm 0.12 ^c	1.92 \pm 0.17 ^a	0.77 \pm 0.06 ^b	0.57 \pm 0.12 ^{b,c}	2.00 \pm 0.20 ^a	0.33 \pm 0.04 ^c	0.36 \pm 0.08 ^c	0.27 \pm 0.02 ^c
Mn	6.61 \pm 0.33 ^c	9.26 \pm 0.23 ^a	8.53 \pm 0.25 ^{a,b}	9.20 \pm 0.56 ^a	6.10 \pm 0.26 ^c	5.85 \pm 0.07 ^c	7.89 \pm 0.40 ^b	6.22 \pm 0.38 ^c
Ni	1.40 \pm 0.15 ^d	1.99 \pm 0.45 ^d	3.30 \pm 0.35 ^{c,d}	1.60 \pm 0.36 ^d	3.13 \pm 0.06 ^d	5.13 \pm 0.51 ^{b,c}	8.97 \pm 1.87 ^a	6.72 \pm 0.15 ^b
Se	1.09 \pm 0.34 ^b	0.71 \pm 0.07 ^{a,b}	1.37 \pm 0.12 ^a	0.93 \pm 0.23 ^{a,b}	0.23 \pm 0.12 ^b	1.46 \pm 0.59 ^a	1.41 \pm 0.53 ^a	1.34 \pm 0.22 ^a
Zn	31.56 \pm 6.31 ^a	26.17 \pm 1.79 ^{a,b}	18.87 \pm 3.36 ^{b,c}	3.50 \pm 0.85 ^e	8.30 \pm 0.44 ^{d,e}	13.90 \pm 1.93 ^{c,d}	8.93 \pm 0.18 ^{d,e}	8.14 \pm 0.21 ^{d,e}
Pb	0.89 \pm 0.19 ^d	1.03 \pm 0.39 ^{c,d}	1.47 \pm 0.29 ^b	1.57 \pm 0.12 ^b	1.23 \pm 0.25 ^c	4.34 \pm 0.22 ^a	4.92 \pm 0.19 ^a	4.98 \pm 0.24 ^a

Difference superscript letters within rows indicate significantly different means (Tukey, post hoc comparisons, $p < 0.05$).

*Sites: 1 Compensation Beach, 2 Inyoni Rocks, 3 Isipingo Beach, 4 Winklespruit, 5 Park Ryne, 6 Pennington, 7 Ifafa and 8 Hibberdene.

Table 12. Concentrations (in $\mu\text{g g}^{-1}$, dry weight) of total and inorganic arsenic in *S. elegans* (Mean \pm SD, n = 3).

Sites	Total arsenic ($\mu\text{g g}^{-1}$)	Inorganic arsenic ($\mu\text{g g}^{-1}$)	Inorganic arsenic %
1	42.10 \pm 2.92 ^d	21.37 \pm 0.40 ^f	51
2	71.28 \pm 2.25 ^c	42.68 \pm 0.29 ^b	60
3	63.87 \pm 1.63 ^c	37.15 \pm 0.33 ^c	58
4	83.60 \pm 5.91 ^b	53.00 \pm 0.38 ^a	63
5	72.70 \pm 1.32 ^c	40.69 \pm 1.83 ^b	56
6	87.49 \pm 3.34 ^b	27.91 \pm 0.28 ^e	32
7	105.4 \pm 4.28 ^a	32.89 \pm 1.01 ^d	31
8	90.52 \pm 1.58 ^b	34.45 \pm 0.47 ^{c,d}	38
Mean	77.12 \pm 17.9	36.27 \pm 9.62	50

Difference superscript letters within columns indicate significantly different means (Tukey, post hoc comparisons, $p < 0.05$).

*Sites: 1 Compensation Beach, 2 Inyoni Rocks, 3 Isipingo Beach, 4 Winklespruit, 5 Park Ryne, 6 Pennington, 7 Ifafa and 8 Hibberdene.

In South Africa, no specific regulatory limits for heavy metals in algae foods have been set however in some countries regulations or maximum limits for total and inorganic As have been established. In 2010, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) set the health based-guidance value for inorganic As (lower limit) as $3 \mu\text{g kg}^{-1}$ body weight per day (FSANZ, 2010). Also, the UK independent Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) stated that As is a

genotoxic carcinogen so no Provisional Tolerable Weekly Intake (PTWI) would be set and exposure should be 'as low as reasonably practicable' (ALARP) (Rose et al., 2007).

In those parts of the world where seaweed is regularly consumed, an average serving size would be between 5-10 g, DW (Mouristen et al., 2013; Smith et al., 2010). Consumption of the minimum suggested serving size (5 g) of *S. elegans* with a concentration greater than 36 $\mu\text{g g}^{-1}$ of inorganic As would lead to an intake of more than 180 μg of inorganic As. This would exceed the limit set by JECFA for inorganic As (3 $\mu\text{g kg}^{-1}$ body weight per day) for an average individual weighing 60 kg. The French legislation permits less than 3 $\mu\text{g g}^{-1}$ (DW) of inorganic As in edible seaweeds (Burtin, 2003). According to French limits, consumption of *S. elegans* would be prohibited as the inorganic As content exceeds the maximum limit by more than thirty-fold.

Previous studies have consistently found high concentrations of inorganic As, in *S. fusiforme* (*hijiki*). The concentrations of inorganic As in *hijiki* have been reported to be as high as 88 $\mu\text{g g}^{-1}$ (DW), up to 72% of the total As content (Kraan, 2013). In another study, the mean concentration of total As in *hijiki* was found to be 109 $\mu\text{g g}^{-1}$ (DW), of which, 77 $\mu\text{g g}^{-1}$ (71%) was in the toxic inorganic form (Rose et al., 2007). In the same study, the concentrations of inorganic As in other seaweed species were found to be below 0.3 $\mu\text{g g}^{-1}$. In this study, the mean concentration of total As in *S. elegans* was found to be 77.12 $\mu\text{g g}^{-1}$ and 50% of this was in inorganic form. It is evident from these results that seaweed species of the *Sargassum* family concentrate large amounts of inorganic As.

4.4 Conclusion

The phytochemical investigation led to the isolation of three compounds (β -sitosterol, fucosterol and phaeophytin **a**). These compounds were not previously isolated from *S.*

elegans (Suhr). This study showed that *S. elegans* could be a potential and alternate source of the bioactive compounds isolated. However, consumption of *S. elegans* for nutritional or medicinal purposes could increase exposure to inorganic As which could cause adverse health effects therefore it should be avoided. This study confirms that seaweed species from the *Sargassum* genus concentrate high levels of inorganic As. The effect of geographical location on the distribution of essential elements was also evident. In general, the concentrations of essential elements in *S. elegans* were found to be in the decreasing order of Ca > Mg > Fe > Cu > Zn > Mn > Ni > Pb > Co > Se > Cr.

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CHAPTER 5

5.1 Summary

This study focused on different seaweed species found along the coast of KwaZulu-Natal namely *H. cuneata*, *S. hypnoides*, *C. capitatum*, *H. spicifera* and *S. elegans*, of which the latter three are edible. Studies from other regions of the world have shown that seaweeds are a good source of essential nutrients and bioactive compounds, but information on nutritional and medicinal properties of the seaweeds found in KwaZulu-Natal is still lacking. Therefore, this study aimed at investigating the seaweeds as a source of essential dietary elements and secondary metabolites. The total and inorganic arsenic in the brown seaweed, *S. elegans*, was also determined to evaluate its safety for human consumption.

5.2 Findings from the chemical analysis of the edible seaweeds

The edible seaweeds (*C. capitatum*, *S. elegans* and *H. spicifera*) were shown to be rich sources of proteins, lipids and carbohydrates therefore they could be potential sources of these nutrients and could be explored as a health food, especially in the impoverished coastal communities in South Africa.

5.3 Findings from the elemental analysis

The effect of seasonal variation on elemental uptake of the five studied seaweeds was investigated. In general, elemental concentrations in the five seaweeds varied significantly with season ($p < 0.05$) and were found to be in decreasing order of $\text{Ca} > \text{Mg} > \text{Fe} > \text{Cu} > \text{Mn} > \text{As} > \text{Zn} > \text{Ni} > \text{Cr} > \text{Pb} > \text{Co} \approx \text{Se}$. The concentrations of essential elements in the edible seaweeds were compared to RDAs to assess their nutritional value. The results showed that

C. capitatum and *H. spicifera* could contribute to the health and nutritional needs of most individuals for most elements, without causing adverse health effects. However, *S. elegans*, showed very high concentrations of the toxic element As, therefore it was further investigated for elemental distribution from eight different geographic locations in KwaZulu-Natal, South Africa. The elemental concentrations in *S. elegans* varied with location and were found to be in decreasing order of Ca > Mg > Fe > Cu > Zn > Mn > Ni > Pb > Co > Se > Cr. Total As in *S. elegans* was found to be extremely high, of which 50% was in the more toxic, inorganic form.

5.4 Findings from the phytochemical study of *S. elegans*

Three compounds β -sitosterol, fucosterol and phaeophytin **a**, were isolated from *S. elegans*. These compounds were not previously isolated from *S. elegans* (Suhr). This showed that *S. elegans* could be a potential and alternate source of the bioactive compounds isolated.

5.5 Overall conclusion

In general, this research has contributed significantly to the scientific knowledge base on seaweed. More specifically, this research can contribute positively to the Nutrient and Food Database for edible seaweeds. This study has also shown that the consumption of *S. elegans* for nutritional or medicinal purposes could increase exposure to inorganic As which could cause adverse health effects therefore it should be avoided. The phytochemical analysis revealed that *S. elegans* could be a potential and alternate source of secondary metabolites.

5.6 Recommendations for further study

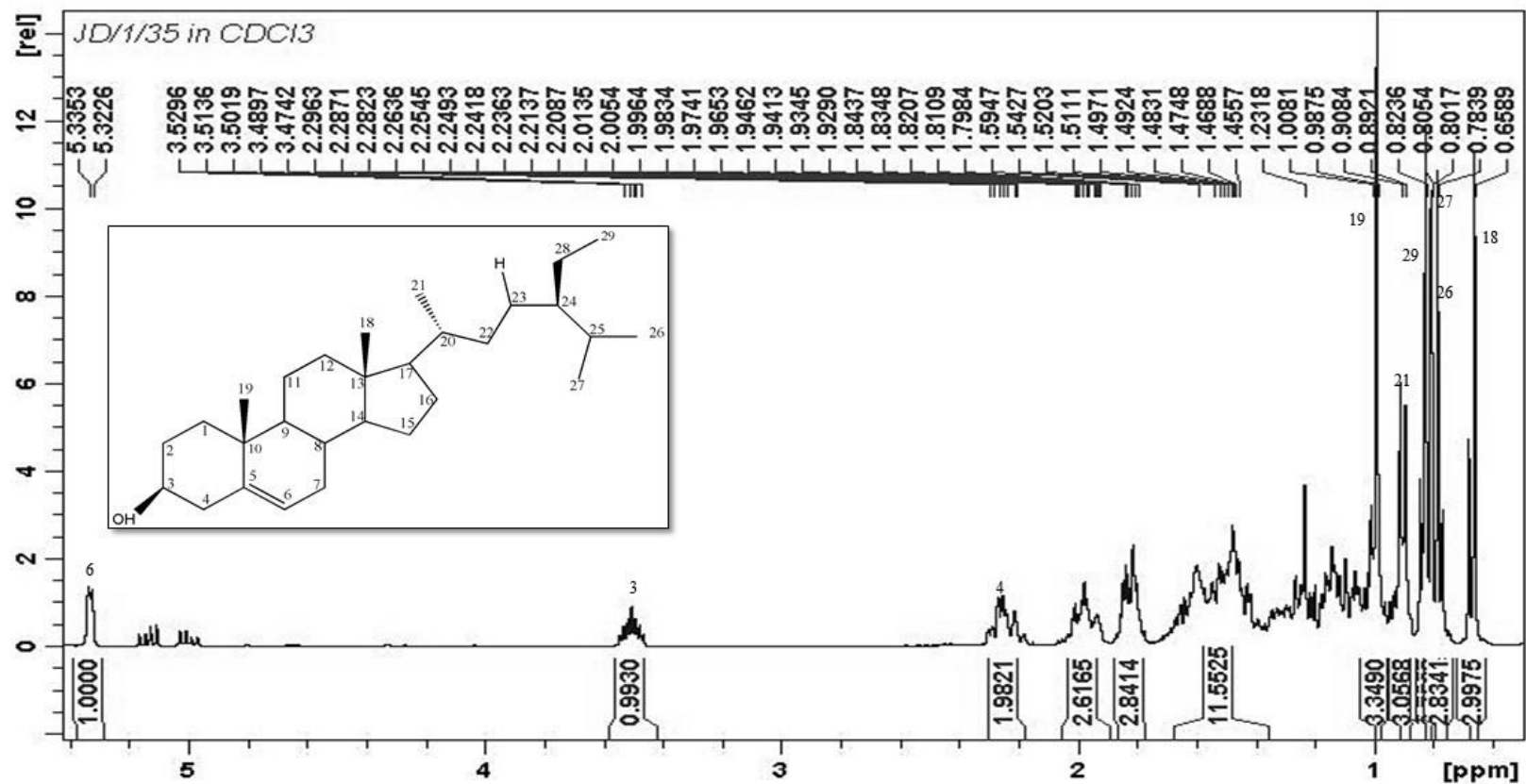
- Isolation and identification of the secondary metabolites in the other edible seaweeds species investigated.
- Speciation of inorganic arsenic in brown seaweeds.
- Research on the other species of seaweeds found on the coast of KwaZulu-Natal to assess their potential for exploitation.

APPENDIX

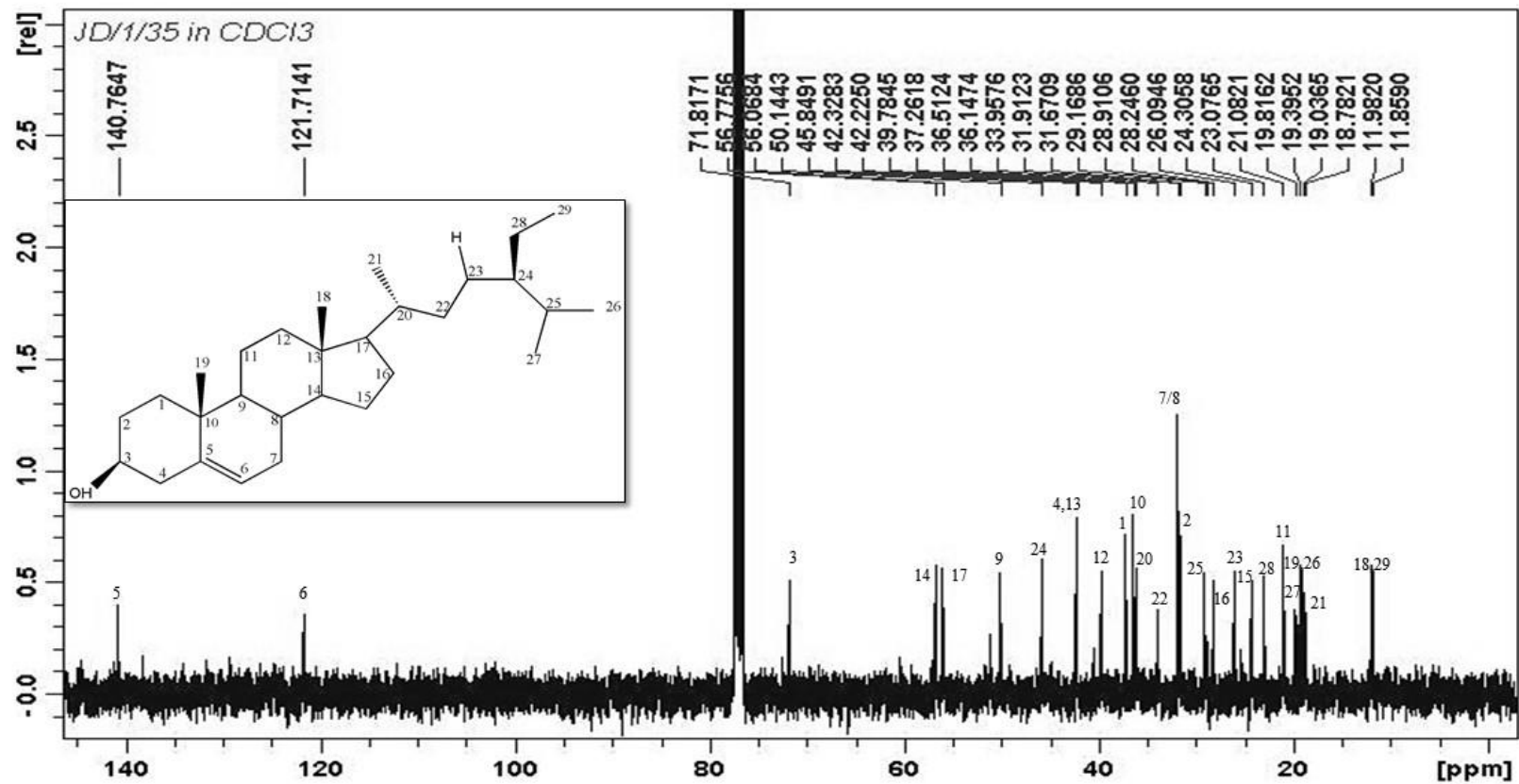
Supporting information

Supporting information includes NMR, IR and MS data.

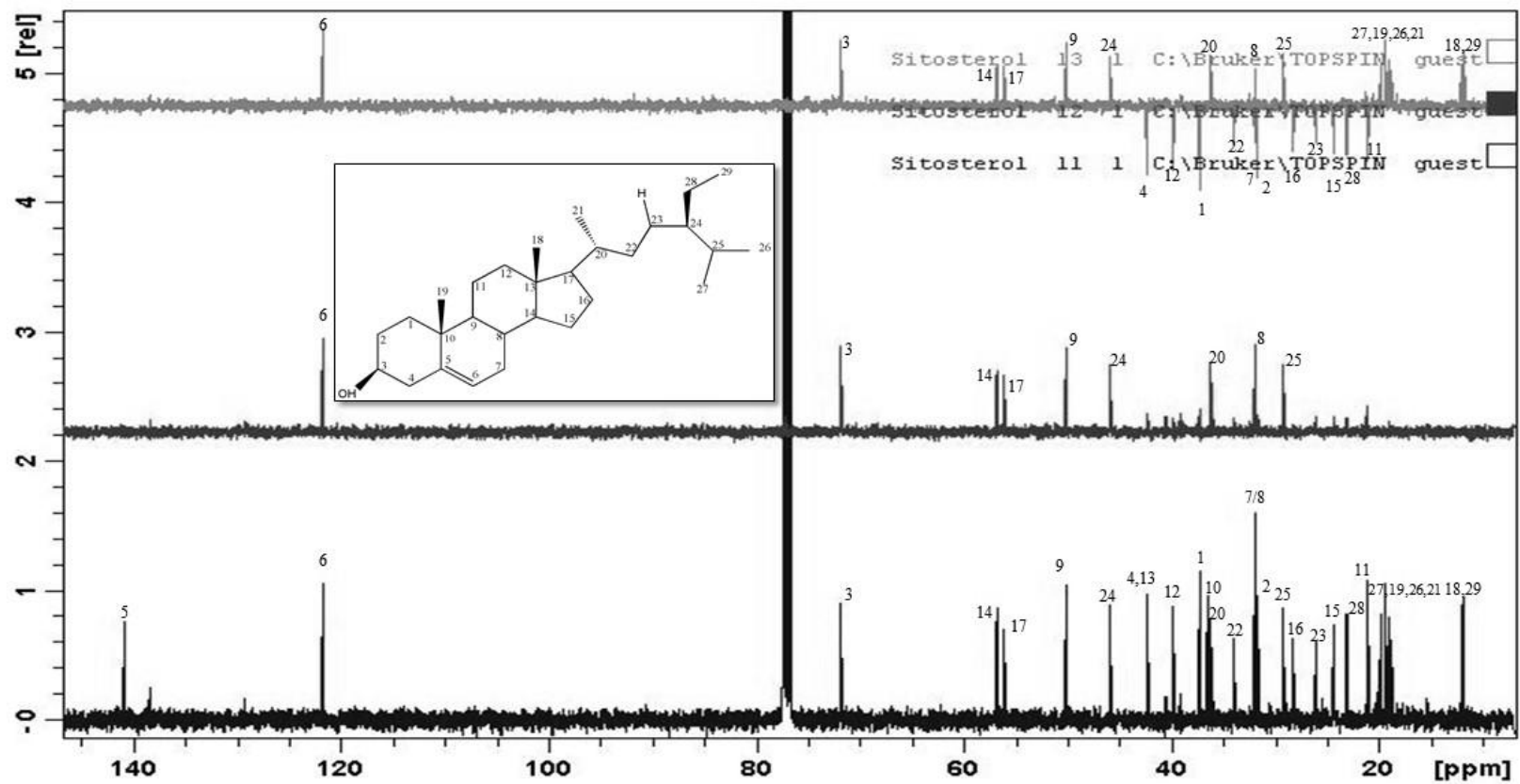
The CRM certificate is also attached.



¹H-NMR spectrum for β -sitosterol

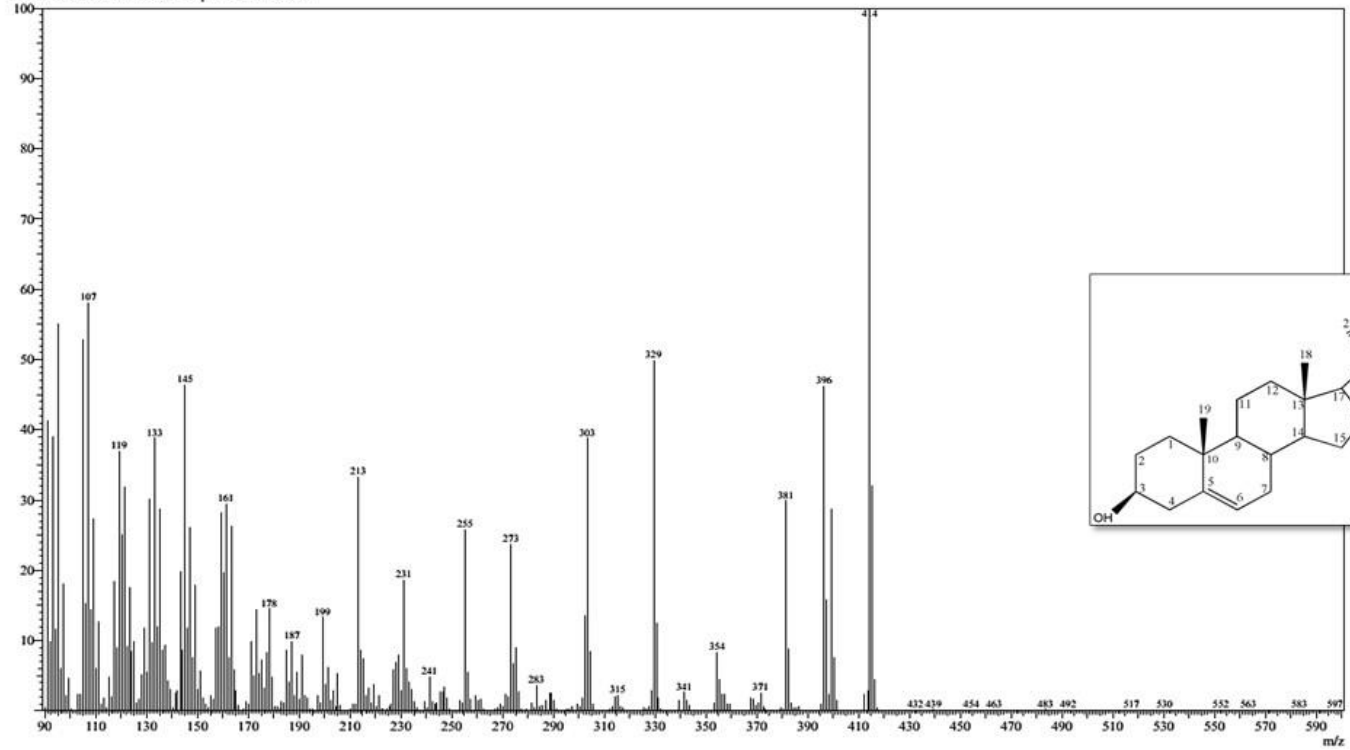


¹³C-NMR spectrum for β-sitosterol

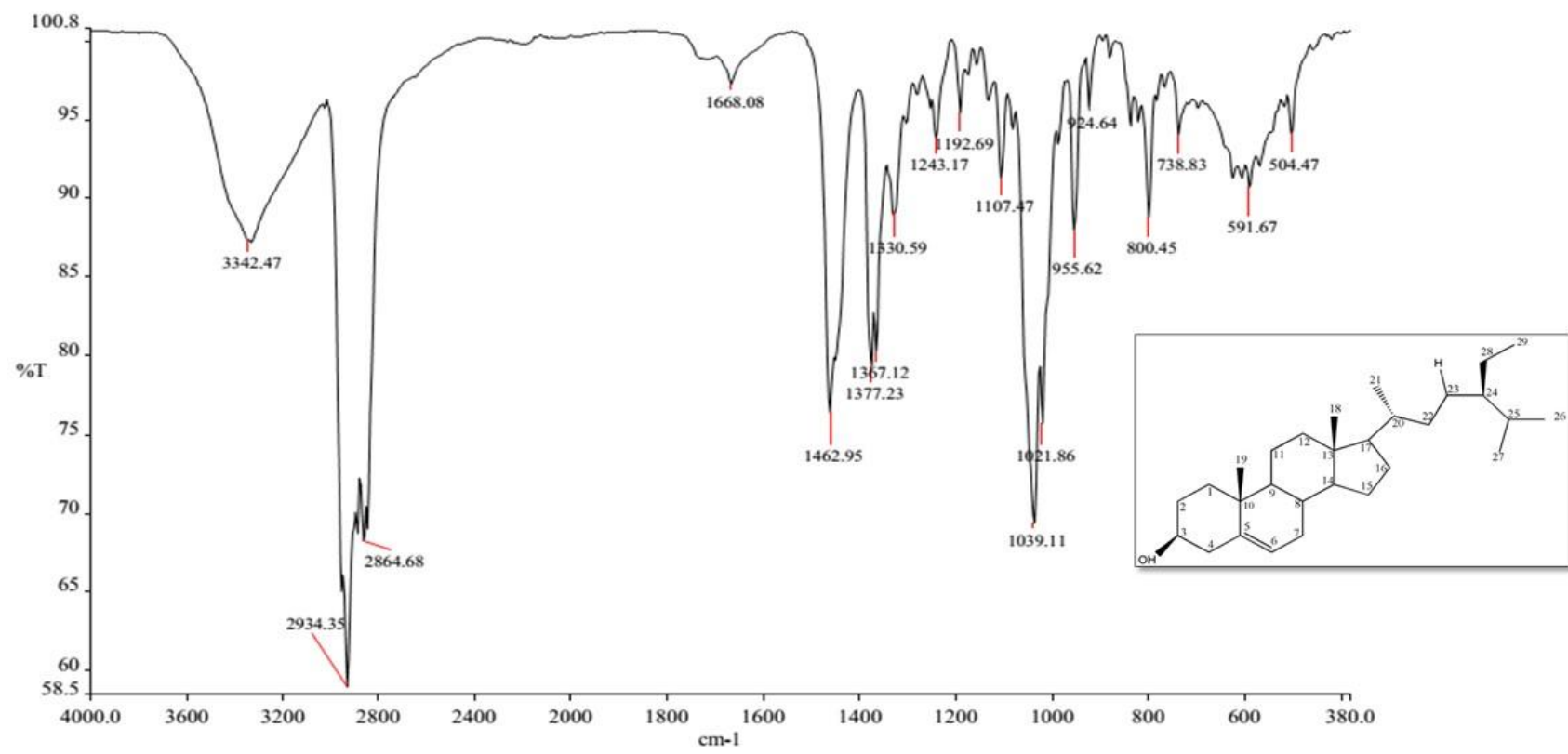


Spectrum

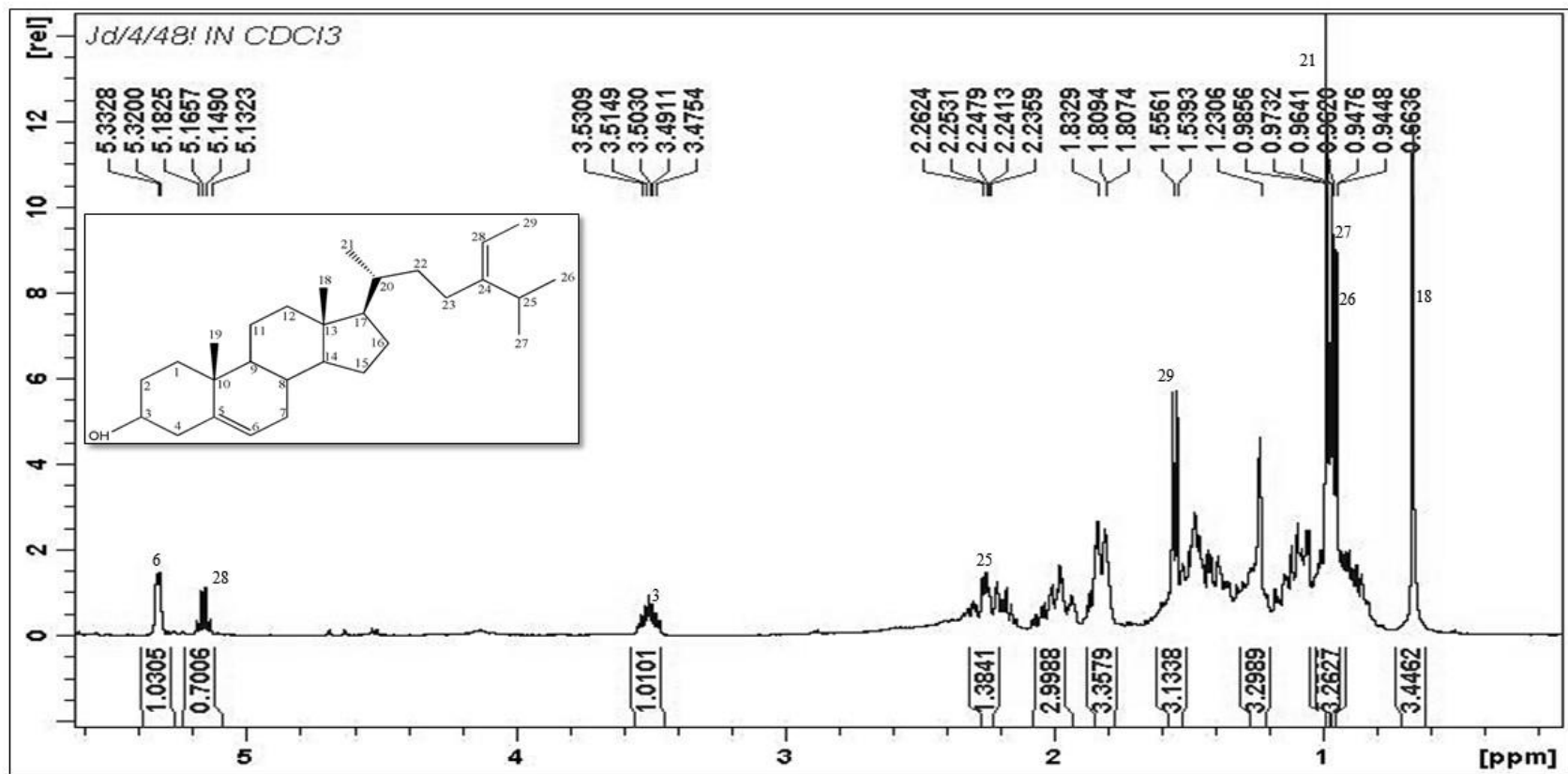
Line#:1 R_Time:24.600(Scan#:4321)
MassPeaks:524
RawMode:Averaged 24.595-24.605(4320-4322) BasePeak:414(39761)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



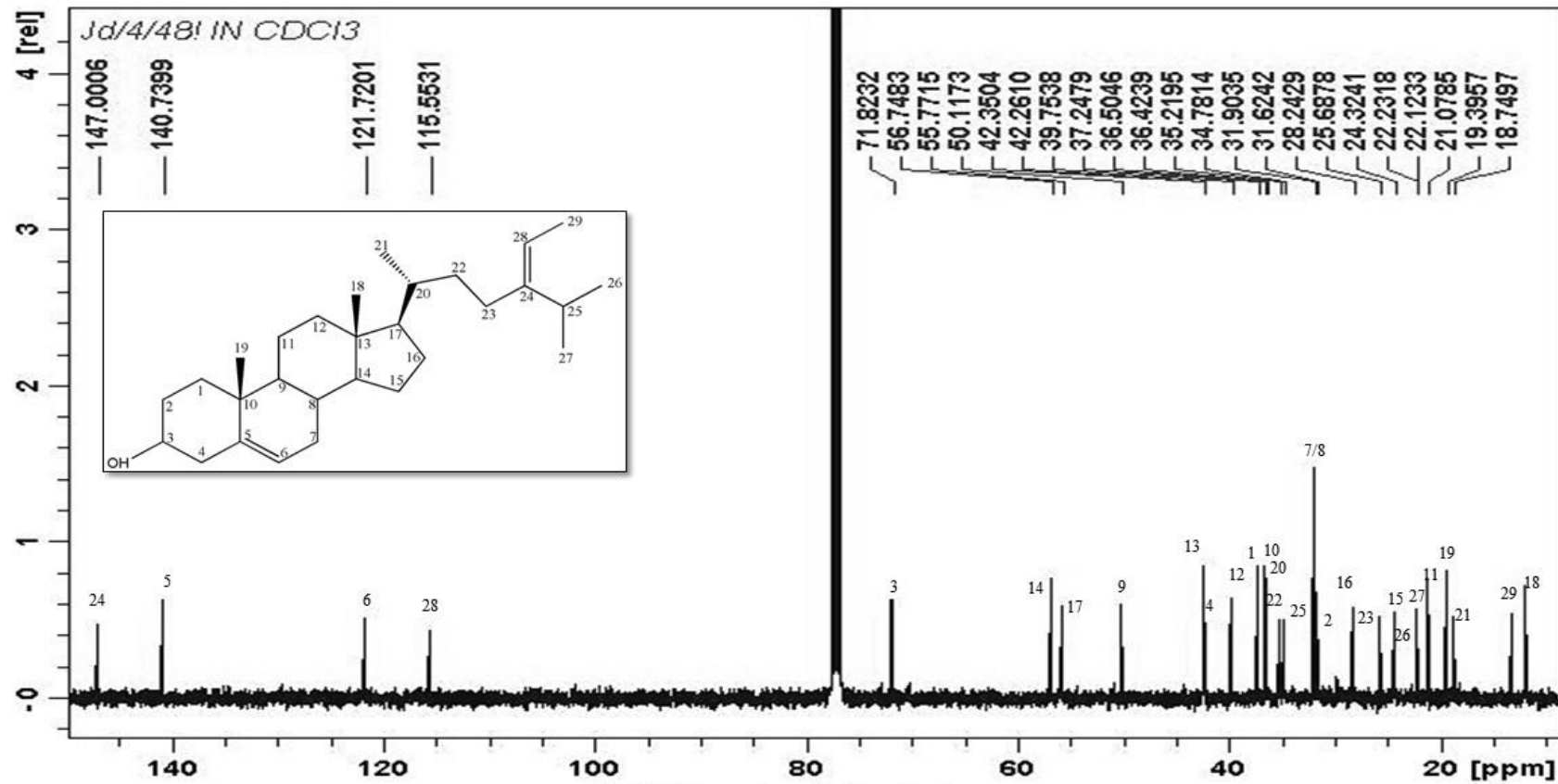
Mass spectrum for β -sitosterol

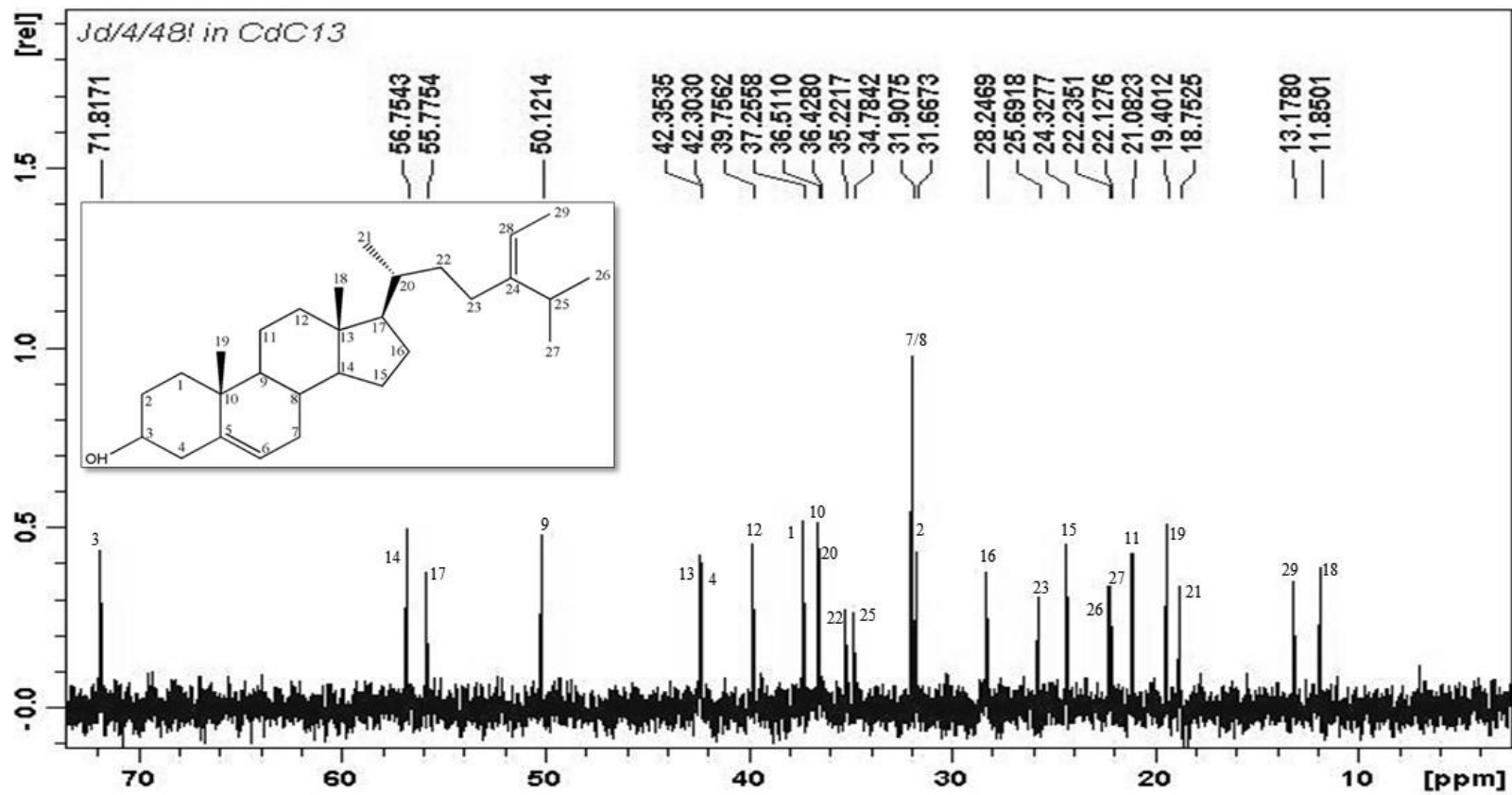


IR spectrum for β -sitosterol

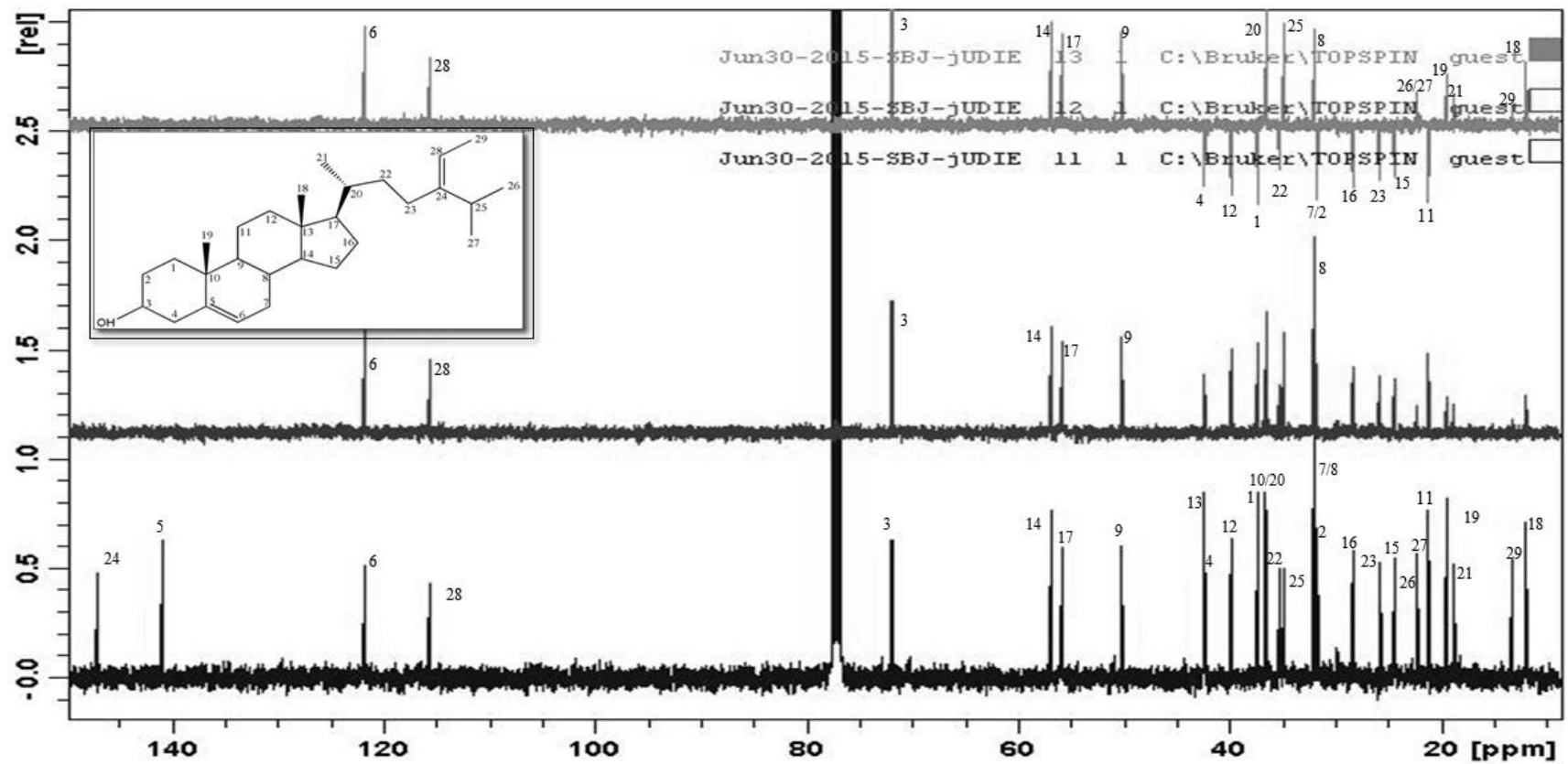


¹H-NMR spectrum for fucosterol





¹³ C-NMR spectrum for fucosterol expanded 70-10 ppm



DEPT spectrum for fucosterol

Elemental Composition Report

Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

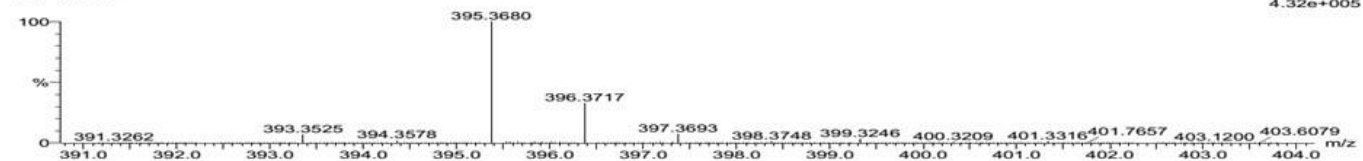
1 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)

Elements Used:

C: 25-30 H: 45-50

JD-3-35 7 (0.203) Cm (1:61)

TOF MS AP+



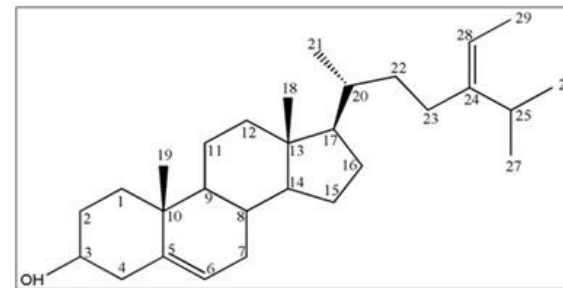
Minimum:

Maximum:

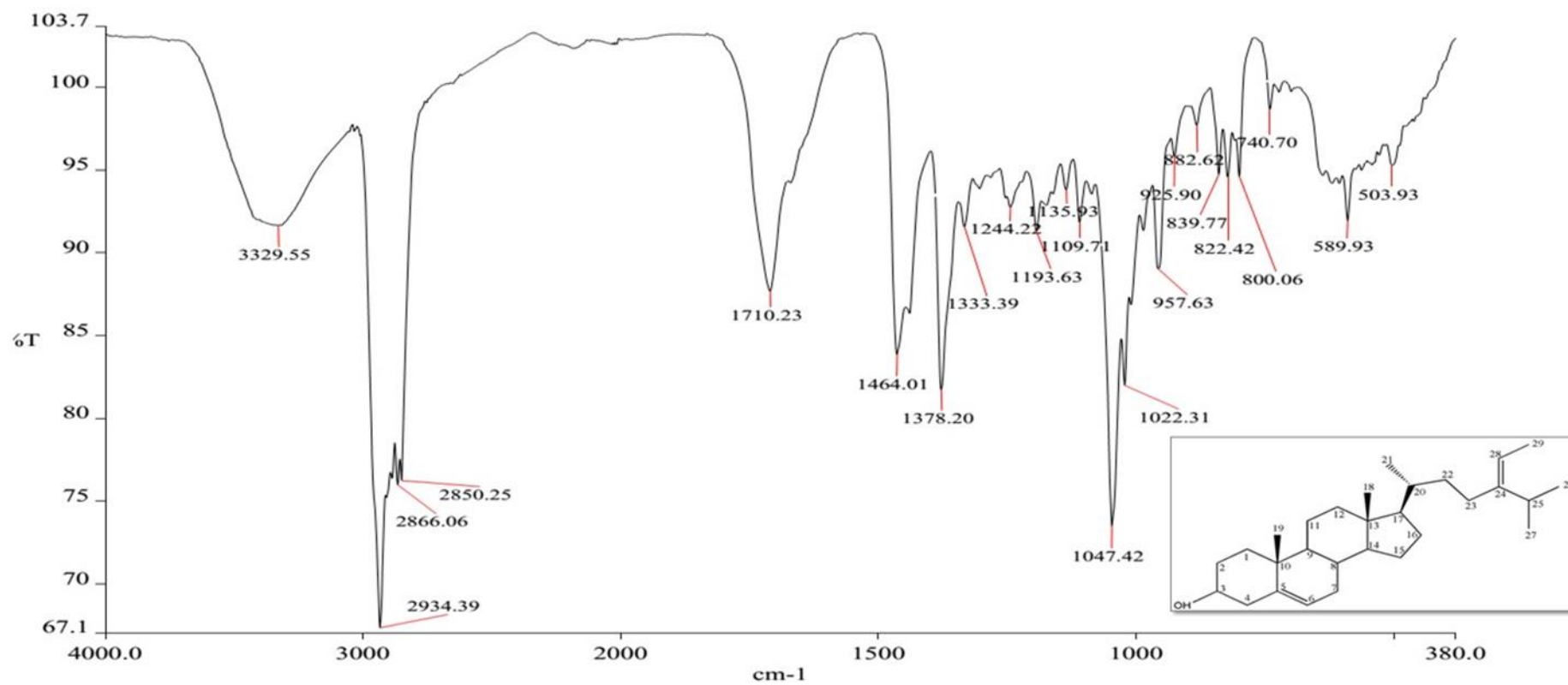
5.0 5.0 -1.5 100.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
395.3680	395.3678	0.2	0.5	6.5	702.3	0.0	C ₂₉ H ₄₇

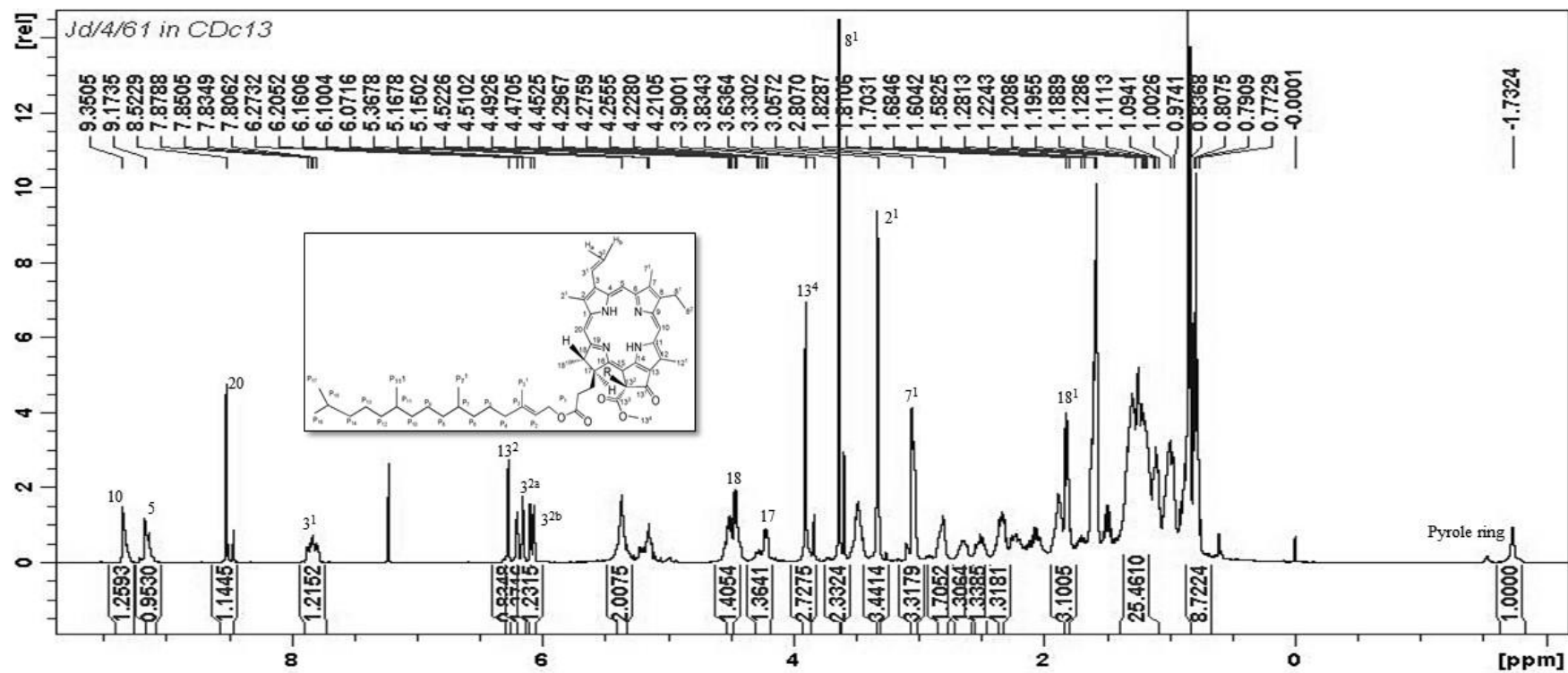
loss of OH- to produce [M]⁺ ion peak



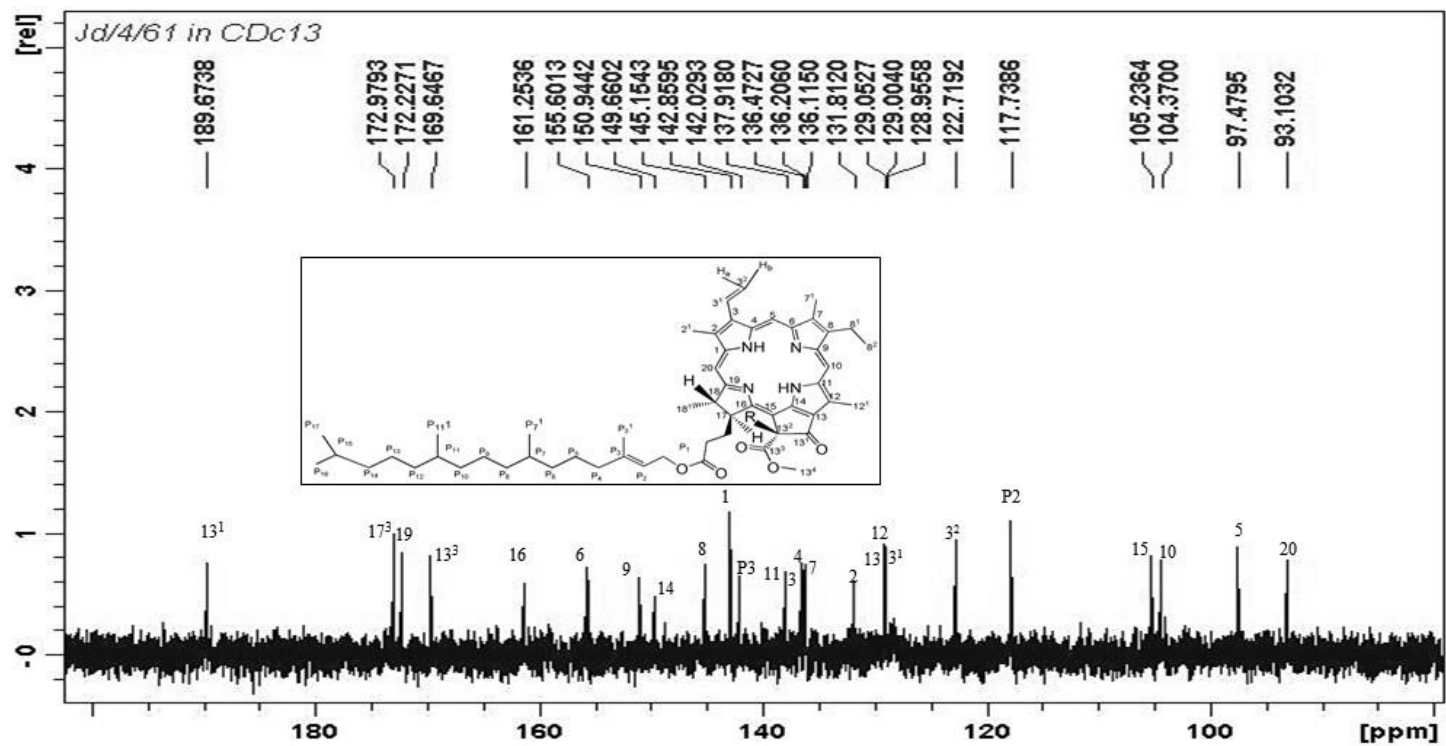
Mass spectrum for fucosterol



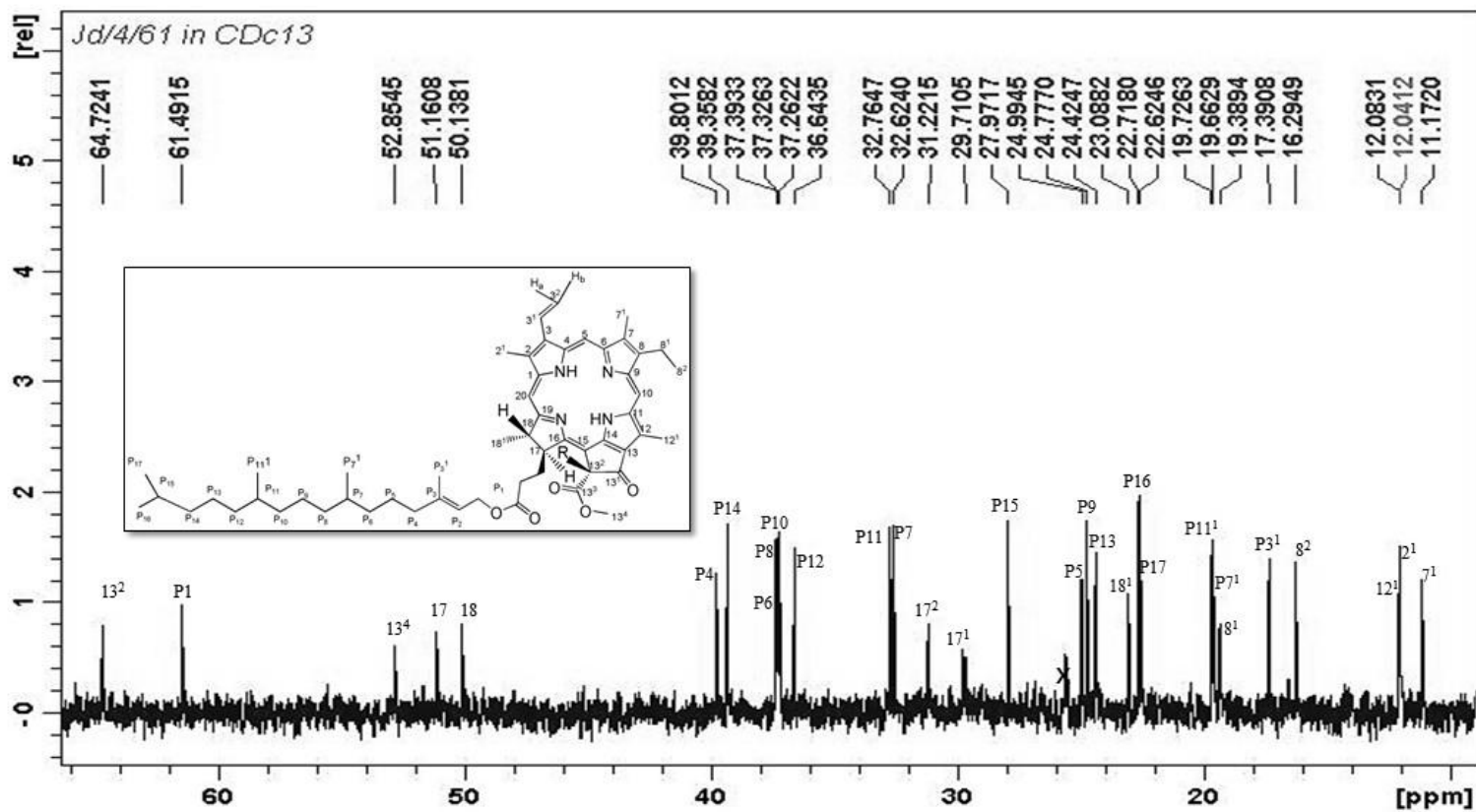
IR spectrum for fucosterol



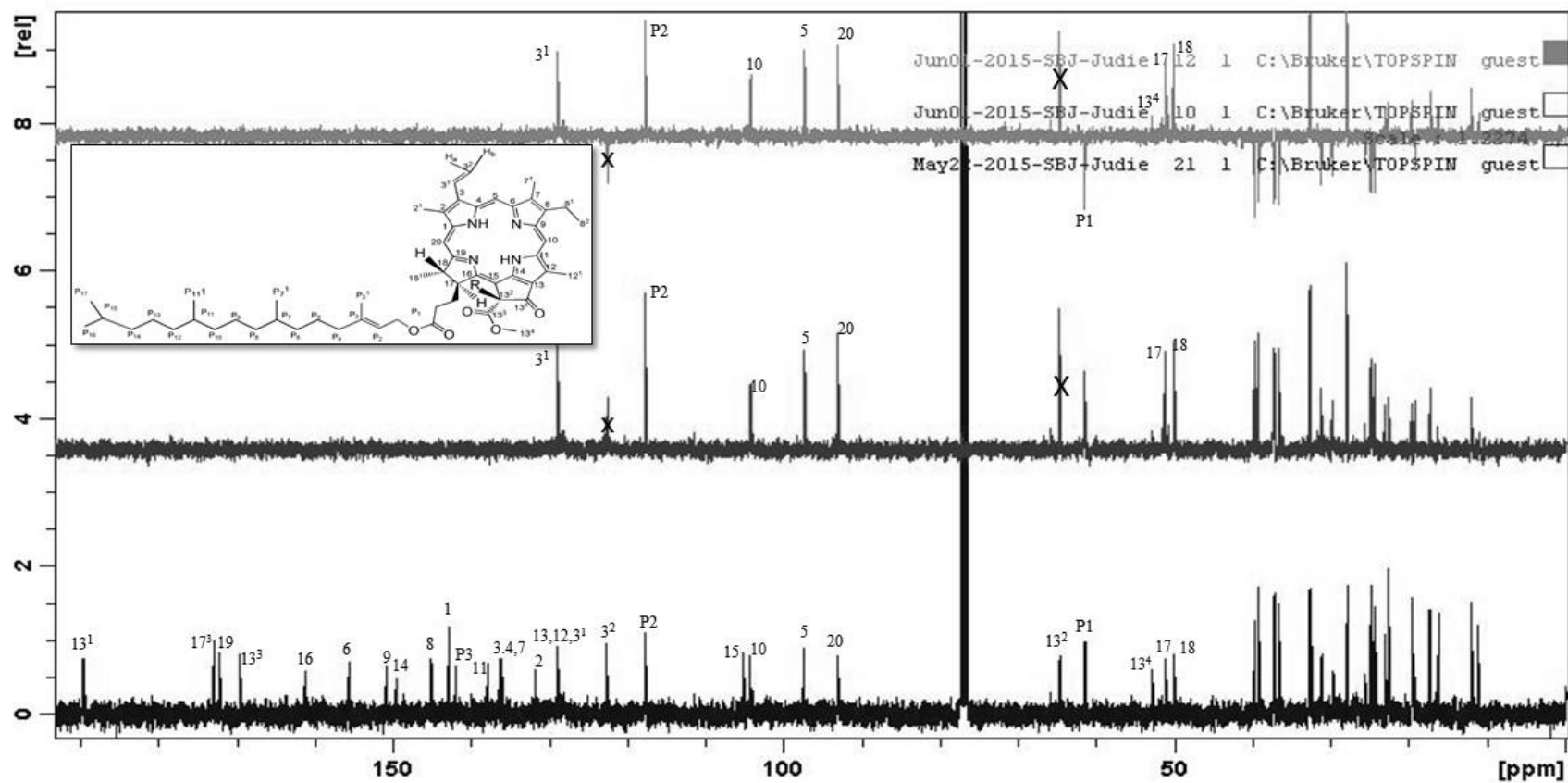
¹H-NMR spectrum for phaeophytin a



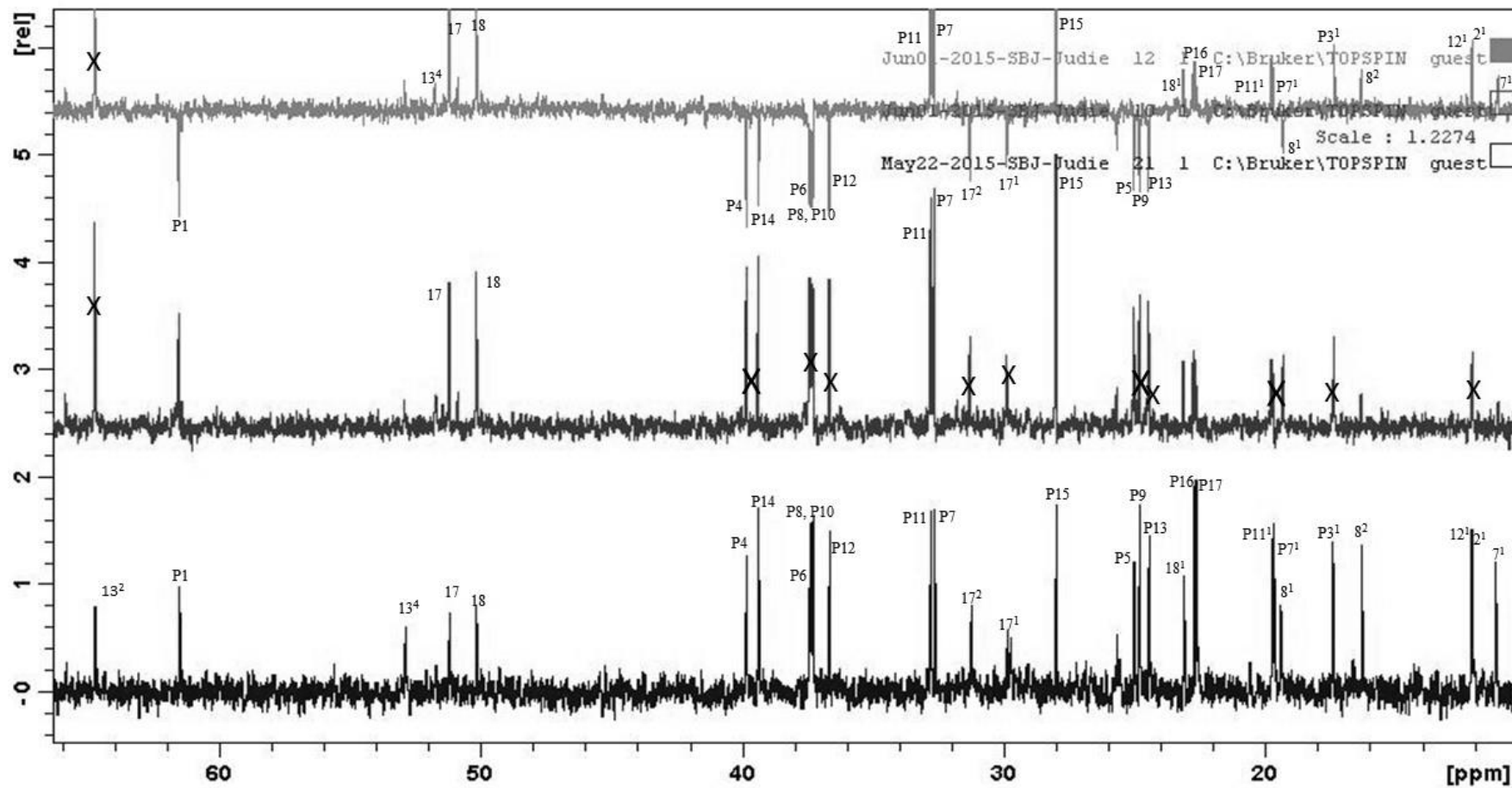
¹³C-NMR spectrum for phaeophytin **a**, expanded (δ_c 189.67- 93.10)



¹³C-NMR spectrum for phaeophytin **a**, expanded (δ_c 65-10)



DEPT spectrum for phaeophytin a



DEPT spectrum for phaeophytin a expanded (δ_c 65-10)

Elemental Composition Report

Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

13 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)

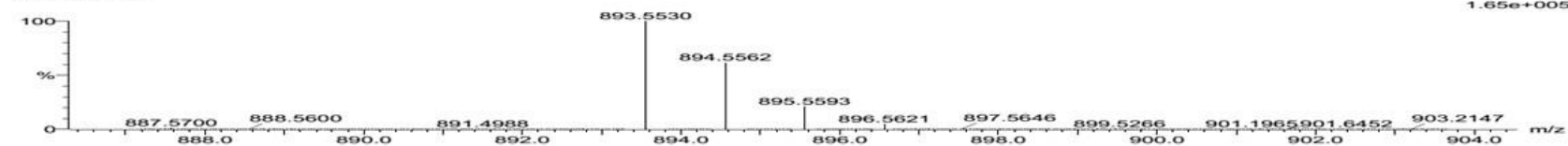
Elements Used:

C: 50-55 H: 70-75 N: 0-5 O: 0-5 Na: 0-1

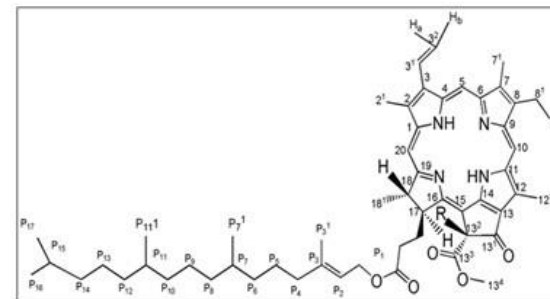
05-3-B-29-42 53 (1.755) Cm (1:61)

TOF MS ES+

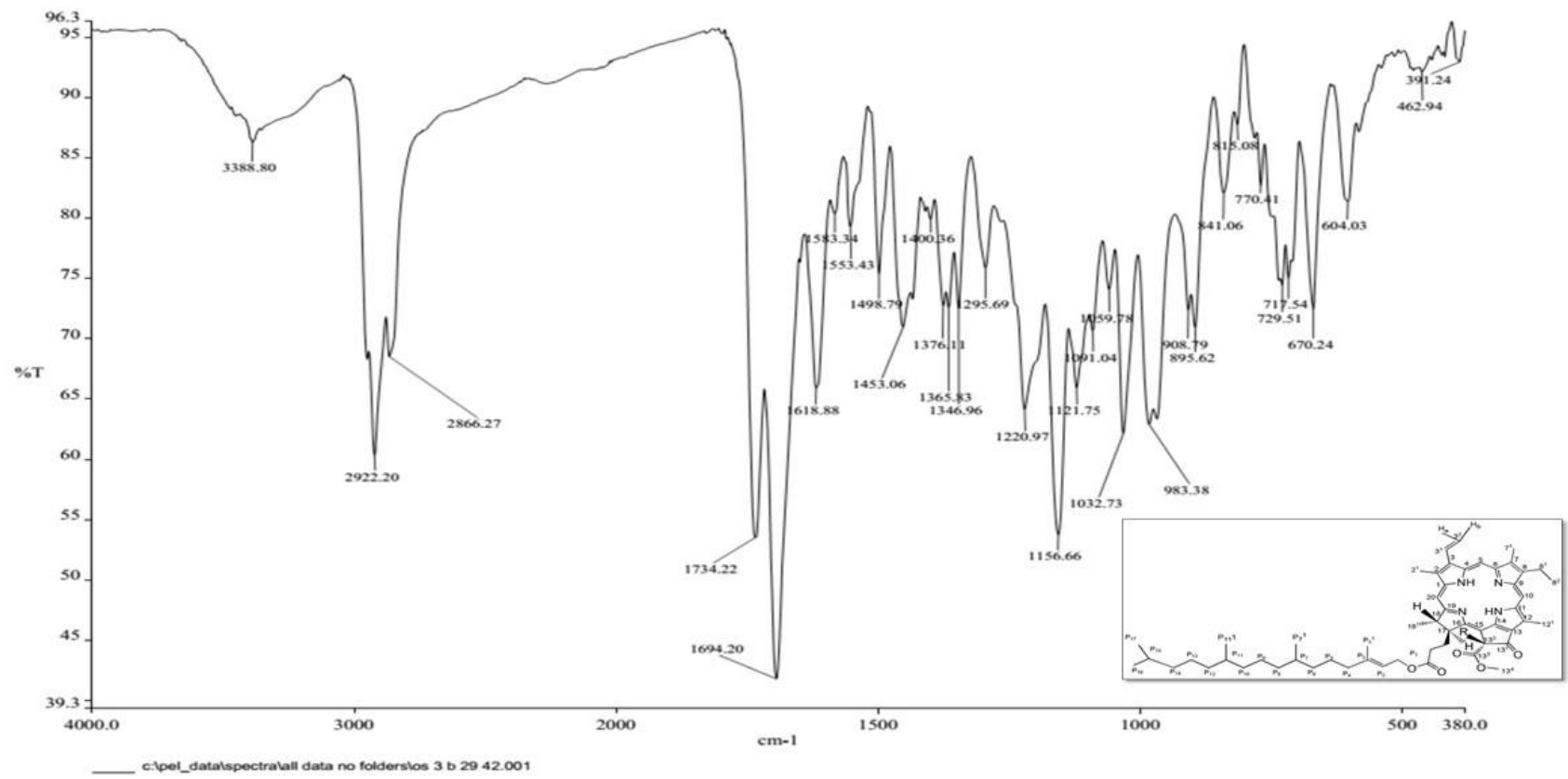
1.65e+005



Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
893.5530	893.5557	-2.7	-3.0	20.5	361.3	0.0	C55 H74 N4 O5 Na



Mass spectrum for phaeophytin a



IR spectrum for phaeophytin a



CERTIFIED REFERENCE MATERIAL BCR[®] – 402

CERTIFICATE OF ANALYSIS

WHITE CLOVER			
	Mass fraction based on dry mass		Number of accepted sets of data p
	Certified value ¹⁾ [mg/kg]	Uncertainty ²⁾ [mg/kg]	
As	0.093	0.010	15
Co	0.178	0.008	7
Mo	6.93	0.19	13
Se	6.70	0.25	15

¹⁾ Unweighted mean value of the means of p accepted sets of data, each set being obtained in a different laboratory and/or with a different method of determination. The certified value is traceable to the SI.
²⁾ Half-width of the 95 % confidence interval of the mean defined in ¹⁾.

This certificate is valid for one year after purchase.

Sales date: 30. Juni 2014

The minimum amount of sample to be used is 100 mg.

NOTE

This material has been certified by BCR (Community Bureau of Reference, the former reference materials programme of the European Commission). The certificate has been revised under the responsibility of IRMM.

Brussels, November 1991

Revised: May 2007

Signed: 

Prof. Dr. Hendrik Emons
Unit for Reference Materials
EC-JRC-IRMM
Retieseweg 111
2440 Geel, Belgium

Additional Material Information	
	Mass fraction based on dry mass
	Value ¹⁾ [mg/kg]
Cr	5.19
Fe	244
Ni	8.25
Zn	25.2

1) The value is traceable to the SI.

DESCRIPTION OF THE SAMPLE

The material consists of a white clover powder in a glass bottle. The bottle contains about 25 g of powder and a small PTFE ball which has been added to facilitate the homogenisation prior to use.

ANALYTICAL METHOD USED FOR CERTIFICATION

- Direct current plasma atomic emission spectrometry
- Energy dispersive X-ray fluorescence
- Electrothermal atomic absorption spectrometry
- Electrothermal atomic absorption spectrometry with Zeeman background correction
- Hydride generation atomic absorption spectrometry
- Hydride generation inductively coupled plasma emission spectrometry
- Inductively coupled plasma emission spectrometry
- Inductively coupled plasma mass spectrometry
- Instrumental neutron activation analysis
- Neutron activation analysis with radiochemical separation
- Visible light or U.V. spectrometry

PARTICIPANTS

- Agriculture and Food Development Authority, Wexford (IE)
- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)
- Labor für Spurenanalytik, Bonn (DE)
- Forschungszentrum für Umwelt und Gesundheit, Neuherberg (DE)
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- Centre de Recherches Forestières, Nancy (FR)
- Ecole Européenne des Hautes Etudes des Industries Chimiques, Strasbourg (FR)
- E.C.N. Energieonderzoekcentrum, Petten (NL)
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- Labor für Spurenanalytik, Bonn (DE)
- Laboratory of the Government Chemist, Teddington (GB)
- Landesanstalt für Ökologie, Recklinghausen (DE)
- Natural Env. Research Council, Swindon (GB)
- NLR "Demokritos", Aghia Paraskevi Attikis (GR)
- Rijksinstituut voor de Volksgezondheid, Bilthoven (NL)
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- Service Central d'Analyse, CNRS, Vernaison (FR)
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- Universidad Complutense, Facultad de Química, Madrid (ES)
- Università di Pavia, Chimica Generale, Pavia (IT)
- Universität Ulm, Sektion Analytik und Höchstreinigung, Ulm (DE)