ELEMENTAL AND PHYCOCHEMICAL ANALYSIS OF VARIOUS SEAWEED SPECIES FOUND ALONG THE EAST COAST OF KWAZULU-NATAL, SOUTH AFRICA

by

Kimona Kisten

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University of KwaZulu-Natal, Durban, South Africa.

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

Signed_____ Name_____ Date_____

Signed_____ Name_____ Date_____

PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Chemistry, School of Chemistry and Physics of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa. The research was financially supported by Deutscher Akademischer Austauschdienst (DAAD) National Research Foundation (NRF) and the College of Agriculture, Engineering and Science.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Signed: Prof. S.B. Jonnalagadda

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Signed: Dr. R. Moodley

Date: December 2016

DECLARATION 1: PLAGIARISM

I, Kimona Kisten, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

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DECLARATION 2: PUBLICATIONS

Publication 1

Title: Elemental Analysis and Nutritional Value of Seaweed from KwaZulu-Natal, South Africa

Authors: Kimona Kisten, Dr. Roshila Moodley and Prof, Sreekantha Babu Jonnalagadda

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Publication 2

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Authors: Kimona Kisten, Dr. Roshila Moodley and Prof. Sreekantha Babu Jonnalagadda

Journal: Submitted to International Journal of Food Science and Nutrition (currently under review)

All laboratory experiments and data interpretation content in the above publications were carried out by me. The co-authors assisted in the scientific verification process and editing of the manuscript.

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ABSTRACT

Seaweed, biologically and phycologically referred to as algae, can be classified as Rhodophyta (red), Chlorophyta (green) and Ochrophyta (brown). In this study, the concentrations of thirteen elements (As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn) were investigated for 14 seaweed species of varying classes (*Amphiroa bowerbankii, Ceramium virgatum, Dichotomaria tenera, Ellisolandia (Coralline) elongata, Gelidium abbottiorum, Gracilaria canaliculata, Jania rubens* and *Jania (Haliptylon) squamata* of Rhodophyta (red); *Caulerpa filiformis, Codium capitatum, Halimeda cuneata* and *Ulva rigida* of Chlorophyta (green); and *Cystoseira myrica* and *Sargassum elegans* of Ochrophyta (brown)). This was done to determine the nutritional value of seaweed due to their edible nature, medicinal properties, industrial importance and natural abundance.

The elemental distribution in seaweed was found to be in decreasing order of Ca > Mg > Fe > Cu > Mn > Zn > Cr > Co > Se > As > Pb > Ni > Cd. All edible species contained high levels of both macro and micro-elements with the corali species accumulated high levels of Ca. Of the fourteen edible seaweed species studied, only three (*G. abbottiorum, E. (Coralline) elongata* and *C. virgatum*) are suitable for human consumption due to high levels of toxic elements (As, Cd and Pb) being present in the other species. These three species are also rich in essential nutrients, specifically *C. virgatum*, which is rich in Cu and Se.

Codium capitatum, a seaweed species belonging to the phylum Chlorophyta (green seaweed) was also investigated to evaluate the effect of geographical location on elemental uptake and a phycochemical analysis was conducted to identify secondary metabolites which impart medicinal value to the species. Concentrations of elements in *C. capitatum* were found to be in decreasing

order of Ca > Mg > Fe > Mn > Zn > Cu > As > Cr > Se > Co > Ni > Pb > Cd, with As concentrations at most sites being higher than threshold values set by the South African Department of Health however, these are known to be mostly in organic form. The effect of site did not influence elemental uptake of essential micronutrients in *C. capitatum* but seemed to have affected uptake of the essential macro-elements, Ca and Mg. The phycochemical analysis revealed the presence of Clerosterol, an algal sterol known to possess anticancer properties. This study confirms the nutritional and medicinal benefits of *C. capitatum* however, the study also highlights the risk associated with excessive consumption due to potential toxicities of high As intake.

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ABBREVIATIONS

ANOVA	Analysis of variance
br s	Broad singlet
^{13}C-NMR	Carbon 13 nuclear magnetic resonance
COSY	Correlated spectroscopy
CRM	Certified reference material
d	Doublet
DEPT	Distortionless enhancement by polarization transfer
DRI	Dietary reference intake
DW	Dry weight
EU	European Union
FAO	Food and agriculture organization of the United Nations
HMBC	Heteronuclear multiple bond coherence
^{1}H-NMR	Proton nuclear magnetic resonance
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
ICP-OES	Inductively coupled plasma optical emission spectroscopy

m	Multiplet
MS	Mass spectrometry
NOESY	Nuclear overhauser effect spectroscopy
q	Quartet
RDA	Recommended dietary allowance
s	Singlet
t	Triplet
TLC	Thin layer chromatography
UL	Upper tolerable intake level
WHO	World health organization

CHAPTER 1: INTRODUCTION

1.1 Introduction

Seaweed contains approximately 40% inorganic matter that comprise mainly of macro-elements (Ca and Mg), microelements (Cu, Fe, Mn, Ni, and Zn) and toxic elements (As, Cd, Hg and Pb) (Rupérez, 2002). Due to it being rich in essential nutrients and due to the ease of acquiring large amounts of it along the coastline, seaweed is an affordable source of food especially to people living in coastal areas (Abirami and Kowsalya, 2011). Whilst the nutritional benefits of seaweed are being investigated, their toxicity as a result of As, Cd, Hg and Pb accumulation also needs to be investigated. This is imperative due to the nature of the environment in which seaweed grow and the ability of aquatic life to accumulate toxic elements to such elevated levels that, if consumed, they would be detrimental to human health. This is the case with many Ochrophyta species that consist of high levels of As, even higher than maximum permissible limits set by health organisations.

The organic content of seaweed consists of proteins, carbohydrates, fatty acids, fibre, vitamins (A, C, D, E, K, B6 and B12), thiamine, riboflavin, niacin, folate, pantothenic acid, choline and betaine (Sˇkrovánková, 2011). Natural products research has gradually gained popularity due to secondary metabolites (such as terpenoids, phenolics, alkaloids, tannins and sterols) that have been isolated from plant species exhibiting biological activity (such as antibacterial, antifungal, anti-tuberculosis, antioxidant and antidiabetic activity) thereby favouring plant extraction over synthetic methods of developing these medicinally beneficial compounds (Gupta and Abu-Ghannam, 2011). Seaweed, similar to plant species, also possesses secondary metabolites that are known to be medicinally beneficial. In traditional medicine, certain seaweed species are utilised

to combat and act against influenza, intestinal infections and allergies. Some seaweed species are used for their curative properties against arthritis, cancer, diabetes and even removal of radioactive toxins (Smit, 2004). In alternate medicine, certain seaweed species have already been formulated and developed for use as energy supplements.

While South Africa is gradually moving towards large scale industrial importance of seaweed, a few species of Chlorophyta and Ochrophyta have already been used for direct food consumption and some Rhodophyta species have been used in the production of agar. However, the main use of seaweed in the west coast of South Africa remains in the production of Midae meal, also known as kelp feed, in abalone farming (Erasmus et al., 1997; Troell et al., 2006). Although seaweed has found use in South Africa, these are for only a few species, compared to the diverse range of indigenous seaweed species found in South Africa especially along the east coast of KwaZulu-Natal (Bolton and Stegenga, 2002). Further studies on other seaweed species available in South Africa needs to be conducted in order to increase their commercial use as well as to increase the likelihood of discovering novel compounds that may potentiate the activity of currently available drugs or that may possess biological activity themselves.

1.2 Problem statement

The importance of seaweed has developed throughout the world in many industries stemming from food and cosmetic to agriculture and medicine. Whilst a few species in South Africa have been identified and utilised as a healthy food source and for economic importance, there are still a number of indigenous species which have not yet been identified and analysed for nutritional value or secondary metabolites for potential use in health and medicine. Further analysis on the various species found along the coast of South Africa needs to be carried out in order to determine the benefits that a new species would provide.

1.3 Aims

The aim of the study was to perform an analytical investigation on fourteen different species of seaweed of various classes found along the east coast of KwaZulu-Natal, South Africa, to do a comparative study on elemental composition of different species and to evaluate their nutritional value. These are the Rhodophyta (red) species *Amphiroa bowerbankii, Ceramium virgatum, Dichotomaria tenera, Ellisolandia (Coralline) elongata, Gelidium abbottiorum, Gracilaria canaliculata, Jania rubens* and *Jania (Haliptylon) squamata*, the Chlorophyta (green) species *Caulerpa filiformis, Codium capitatum, Halimeda cuneata* and *Ulva rigida*, and the Ochrophyta (brown) species *Cystoseira myrica* and *Sargassum elegans*. Of the fourteen species studied, *Codium capitatum* was selected for elemental analysis as a function of geographical location and phycochemical analysis to determine the effect of site variation on elemental uptake and to determine the secondary metabolites contained within the species as it is used in traditional medicine.

1.4 Objectives

- ✓ To determine the elemental composition of 14 different seaweed species found along the KwaZulu-Natal coast of South Africa using inductively coupled plasma-optical emission spectrometry (ICP-OES).
- ✓ To perform method validation using certified reference materials and to compare elemental concentrations of selected elements in different seaweed species using statistical analysis.
- ✓ To compare experimental results for selected nutrients to recommended dietary allowances in order to determine the nutritional value of seaweed.
- ✓ To compare experimental results to maximum permissible limits for toxic elements to determine the accumulation potential by seaweed and to assess for toxicities.
- ✓ To perform elemental analysis on *C. capitatum* as a function of site and to evaluate the effect of site on uptake by the species.
- ✓ To determine the proximate chemical composition (moisture, dry ash, carbohydrates, fatty acids and proteins) of *C. capitatum*.
- ✓ To extract, isolate and identify the secondary metabolites found in *C. capitatum* using column chromatography, thin layer chromatography (TLC), nuclear magnetic resonance (NMR) spectrometry and gas chromatography-mass spectroscopy (GC-MS).

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CHAPTER 2: LITERATURE REVIEW

Seaweeds are eukaryotic, multicellular, macroscopic algae found in marine environments and are grouped into classes as green, red and brown seaweed. The study of seaweed is referred to as phycology.

2.1. Structure of seaweed

The basic structure of seaweed, from the bottom up, includes the holdfast which is needed to attach the base to the rock bed, a haptera which may be present as an extension of the holdfast to attach to other algae, a stipe which acts as a stem-like structure which may or may not be present in the species, a thallus or float which forms the major part of the body of the algae, a lamina or blade which acts as a 'leaf' and a sorus, also known as fucus or kelp, which is a floatation assisting organ between the blade and the stem (Fig. 2.1) (Morrison et al., 2009).

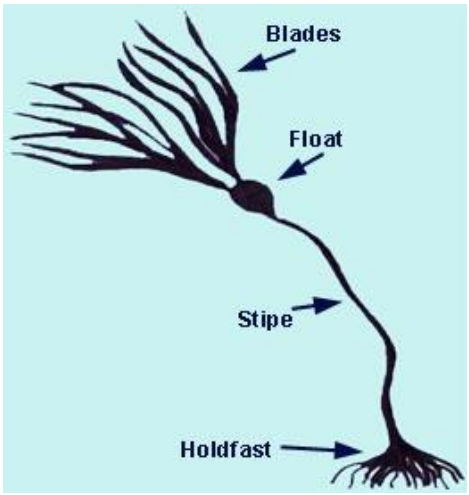

Figure 2.1: Structure of seaweed

http://mesa.edu.au/marine_algae/default.asp

2.2. Growth of seaweed

Seaweed have two prerequisites for production and growth; the first being sunlight which is required for photosynthesis and the second being fresh seawater which is required for nutrient uptake. Seaweed are found at different locations in the environment with most preferring growth on rock beds close to the seashore and a few preferring growth either far out to sea, at greater depths or in tidal pools. Species growing in tidal pools would be considered resistant due to their ability to withstand drying out, the change in salinity of the seawater and changes in temperature. Seaweed locations are referred to as littoral zones and they extend from shorelines to submerged rock formations. At times, various classes of seaweed prefer certain parts of the littoral zone with red algae growing at the deepest parts (Lu̎ning et al., 1990).

2.3. Uses of seaweed

Whilst the benefits of seaweed have been realised and this advantage utilised in other countries, the potential and economical value of seaweed in South Africa is underexploited. South Africa is found in the centre of the two merging currents, the warm Mozambique current and the cold Benguela current (Wysokiński, 1986). These currents are only present at the tip of Africa and have led to the existence of seaweed whose benefits are still unknown and yet to be analysed. Different countries have different climates and currents which could lead to the growth of different species and which could lead to differences in their chemical composition thereby broadening their commercial benefits.

2.3.1 Bioindicators

A bioindicator is an organic or biological species having the ability to provide information on the environment it is present in. Plant bioindicators have the ability to absorb and accumulate nutrients and pollutants at a particular rate. This rate is proportional to the concentrations present in the growth medium. Therefore, a high concentration of a particular nutrient in the plant would mean a high concentration in the growth medium. As bioindicators, seaweed species are able to give a quantitative, and somewhat qualitative, information on biological compounds, nutrients and pollutants present in the growth medium (Conti and Cecchetti, 2001). Environmentally, due to the bioaccumulation factors of aqueous vegetation in free flowing bodies of water, seaweed can be used as bioindicators of heavy metals in industrial areas such as oil factories, ports and harbours, and vacationing areas.

2.3.2. Scrubbers

Scrubbers are objects with the ability to attract and adsorb unwanted particulates or nutrients. After adsorption, separation of the unwanted nutrient is carried out thereby removing from the medium. Whilst the term was initially used with regards to air pollution, it is now widely used as a general cleaning mechanism term (Boll, 1973). Seaweed have a high affinity for unwanted nutrients embedded in the water. Ammonia, phosphates and carbon dioxide are a few examples of substances that seaweed species are able to remove after which separation becomes a straight forward process. This filtration process using algae scrubbers is a technique used to treat wastewater and discourage eutrophication (Adey, 1982).

2.3.3. Food

The most common use of seaweed is in the food industry with the most basic use being its consumption either raw or cooked. The nutritional value of seaweed was first discovered by the Chinese, Japanese and Korean population; seaweed contributed approximately 30% to their total diet (mostly red algae known as *Porphyra* used for sushi, rice and noodles) (Fleurence, 1989). Kelp was also used where it was dried, powdered and added to dishes, sauces, soups and tea as a flavour enhancer or spice as well as a monosodium glutamate contributor (Nisizawa, 1987). Populations of Ireland, Wales, France and many other European countries realised the benefits and made use of the various species found in their vicinity. Due to its nutritional value, certain species e.g. *Laver* have been formulated into a medicinal tablet that is recommended for use as an energy supplement (MacLean, 1993). These countries exposed the benefits of seaweed and eventually they were consumed worldwide.

Whilst most seaweed are edible and beneficial due to their high nutritional value (including trace elements, proteins, carbohydrates, vitamins and folic acid), other more industrially important uses include the production of agar (gelatinous substance extracted from red seaweed and used as a food thickener or substitute for gelatin), carrageenan (sulfated polysaccharides from red seaweed used as a food thickener) and alginate (viscous substance from brown seaweed used as a food thickener) (MacArtain et al., 2007). These products make use of physical properties including the capacity to retain water and emulsification ability of gel like substances known as hydrocolloids (compound forming a gel-like substance in the presence of water and used as a gelling agent in food) and phycocolloids (polysaccharides: agar, carrageenan and alginate all extracted from seaweed). Alginate and agar are used as food additives in food items rich in sugar and

carbohydrates, beverages, meat and poultry, and desserts while carrageenan is utilised in dairy and baked products, sauces, salad dressings, diet manufactured items and meat and fish preservatives (Dhargalkar and Pereira, 2005).

2.3.4. Agriculture

In agriculture and commercial gardening, seaweed can be used as a mulch in order to protect crops from dry and hot weather by discouraging evaporation and keeping the soil moist and aerated, discouraging the sprouting of weeds, providing frost resistance, repelling pests due to its dry and salty nature, enriching the soil and stimulating plant growth (increased crop yields) due to the presence of trace minerals, carbohydrates, hormones (gibberellins) and low cellulose content. It can also be utilised as sheet composting, as a fertilizer and even chicken and fish feed (Verkleij, 1992).

2.3.5. Skincare

In skincare and cosmetics, seaweed are used for their anti-inflammatory properties. It is known to improve the elasticity of the skin and reduce the formation of cellulite (Capitanio et al., 2012). Brown seaweed is reported to strengthen and nourish the scalp and promote hair growth by fighting off bacteria eating away at the hair follicles (Capitanio et al., 2012).

2.3.6. Medicine

In medicine and herbalism, seaweed have been used to combat influenza, sore throat, urinary infections, goitre, worm infestations, malnutrition, obstructions of the gall bladder (Liu et al.,

2012), swelling of the legs, intestinal infections, congestion, headaches, constipation, water stools, cataracts, allergies, abdominal wall abscesses, clammy skin, dry skin and hair, obesity, oedema, ulcers, testicular pain and swelling, colitis, scrofula, sluggishness of prostate and cervical dysmenorrhea causing irregular menstruation (Smit, 2004). It has also been shown to possess curative properties towards tuberculosis, arthritis, cancer, diabetes, tumours and growths, and cardiovascular diseases (Bocanegra et al., 2009). It acts as a disinfectant towards external wounds due to the alginate present, encourages heavy metal chelation, promotes proper functioning of the liver, kidneys, pancreas, thyroid and nervous system, aids in the removal of radioactive toxins present in the body, and regulates blood pressure by arterial cleansing (Tan et al., 2008).

2.3.7. Uses in South Africa

The areas in South Africa where seaweed are generally used are Saldanha Bay, Langebaan Lagoon, Table Bay, False Bay, St. Helena Bay and Lamberts Bay. One of the largest commercial uses of seaweed in South Africa involves the use of *Ecklonia maxima* and *Laminaria pallida* (kelp feed), for the production of agar and emulsifying alginate, as a fertilising growth stimulant for agricultural crops and as a feed (Midae meal) in Abalone farming for large sea snails (*Haliotis midae)* (Troell et al., 2006). Kelp pellets are also developed using seaweed species of the genera *Gracilaria*, *Gelidium* (Saldanha Bay), *Porphyra* and *Ulva*. Green seaweed are used primarily as a food source in South Africa, while red and brown seaweed are harvested, dried, exported and used for alginate and agar production used to encapsulate tablets, moulds and casts (Maneveldt, 2010). The edible green seaweed (*Caulerpa lentillifera* and *Codium tomentosum)* and red seaweed (*Eucheuma denticulatum* and *Kappaphycus alvarezii)* are consumed directly in salads and

Gelidiella and *Gracilaria* species are used as the main ingredient in jams (Nedumaran and Arulbalachandran, 2015).

Sargassum tea is ingested to treat certain illnesses by normalisation of the thyroid gland due to its high iodine content (Gillespie et al., 1996). It is also known to promote hair growth and healthy nails and reduce blood glucose which is beneficial to diabetes sufferers (Dhargalkar and Pereira, 2005). *Fucus vesiculosus* (bladder weed) and *Laminaria digitata* (oarweed) are used as the main additives in weight loss gels (Dweck Article).

Kelp sludge is used to prevent soil erosion while dried kelp is exported rather than processed in the country (Davey, 1984). Red seaweed have been proven to possess phycocolloids such as carrageenan and agar and are being used in the food, paint, dye and cosmetic industries (Abowei and Ezekiel, 2013). South African carrageenophyte seaweed species (*Hypnea spicifera, Gigartina, Sarcothelia* and *Aoedes)* are currently being investigated for industrial significance (Anderson et al., 1989). Coralline rubble known as maerl (red algae) are used as food for livestock, calcium supplements for humans and even to offset acidic waters (Maneveldt, 2006).

Research conducted on seaweed in South Africa have shown *Caulerpa taxifolia* to display antifungal properties, *Chorella vulgaris*, anticoagulant properties, *Vidalia obtusiloba*, anti-inflammatory properties (Stirk, 2003), *Amphiroa ephedraea, Corraline sp., Gigartina clathrata* and *Sarcothalia scutellata*, anticancer properties and *Caulerpa filiformis* and *Halimeda cuneate*, antimicrobial properties (Smit, 2004). Although seaweed have been shown to exhibit potential medicinal properties, large scale pharmaceutical production has not yet been commercially explored in this country. Table 2.1 shows the main seaweed species used commercially in South Africa.

Table 2 1: The main seaweed species used commercially, worldwide (McHugh and Lanier, 1983).

Seaweed Species	**Uses**
Ochrophyta	
Laminaria	Gelling, emulsifying and stabilising agents, food, paper, textiles, welding rods, pharmaceuticals, fertiliser and animal feed, human consumption (Ecklonia).
Ecklonia	
Rhodophyta	
Gracilaria	Gelling agents, food such as jelly and sweets, bacteriological culture base.
Gelidium	
Gigartina	Stabiliser and gelling agent, confectionary, ice cream, meats, sauces, cosmetics and silk industry.
Porphyra	Human consumption.
Not yet commercially based	
*Macrocystis (*Ochrophyta*)*	Available in South Africa but require mariculture to produce in economic quantities.
*Suhria (*Rhodophyta*)*	

The six species, namely *Laminaria, Ecklonia, Gracilaria, Gelidium, Gigartina* and *Porphyra*, economically used in South Africa are used mostly for alginate, agar and carrageenan production. The only seaweed species used for human consumption in South Africa are *Laminaria* and *Porphyra*.

2.4. Classification of seaweed

The different classes Chlorophyta (green), Rhodophyta (red) and Ochrophyta (brown) can be distinguished by colour alone but have other differences associated with them such as the compounds that they contain. Chlorophyta and Rhodophyta fall within the kingdom Plantae while Ochrophyta falls within the kingdom Chromista (Minge et al., 2009). Species belonging to Chromista are different in that they contain chlorophyll c but also additional pigments which contribute to their golden colour. They also do not contain energy in the form of starch as is the case with plants. While some may say all seaweed belong to the kingdom Protista (which refers to a class other than animals, fungi or plants due to no complex organ structures, organised tissue structures and adaptation patterns), seaweed have been shown to be eukaryotic due to the presence of mitochondria, chloroplasts, paired chromosomes, a nucleus and organelle cells, causing them to be referred to as plants (Corliss, 2002).

2.4.1. Chlorophyta

Chlorophyta, commonly referred to as green seaweed, gets its green colour from the chloroplasts that contain chlorophyll a and b (Domozych, 1980). They also contain accessory pigments including beta carotene, xanthophylls and thylakoids as well as cellulose making up the cell walls and starches being the carbohydrate present (Paul and Fenical, 1987). Green algae have 8000 known species with the majority not being seaweed species, but rather single celled species.

2.4.2. Rhodophyta

Rhodophyta, commonly referred to as red seaweed, with an ancient Greek meaning of rose plant, gets its red colour from phycobiliproteins and phycoerythrin, the accessory pigments. The cell walls are made up of carrageenan, a sulfated polysaccharide. Red algae also produce tannins known as phlorotannins while some produce porphyran (Xu and Gao, 2008). Red algae are the basis of coralline algae known as corali which are highly calcinated species containing calcium carbonate (Woelkerling, 1993). Red algae are the oldest identified seaweed species with over 10000 known species and 5000-6000 of those being Rhodophyta seaweed.

2.4.3. Ochrophyta

Ochrophyta, commonly referred to as brown seaweed, gets its brownish colour from the pigment known as polysaccharide fucoxanthin. Brown algae contain compounds of P700 with chlorophyll a, chlorophyll c, carotenoids and phlorotannins (more than that of red algae) where the cell walls are made up of cellulose, alginic acid and aragonite (Zubia et al., 2008). Brown algae are different from green and red algae as it adopts different metabolic pathways that cause more rapid growth. Approximately 1500-2000 species of brown algae have been identified, worldwide.

2.5. Species in this study

The fourteen species selected in this study are *Amphiroa bowerbankii, Ceramium virgatum roth, Dichotomania tenera, Ellisolandia coralline elongate, Gelidium abbottorium, Gracilania canaliculata, Jania rubens* and *Jania (Haliptylon) squamata* of the phylum Rhodophyta (red); *Caulerpa filiformis, Codium capitatum, Halimeda cuneater* and *Ulva rigida* of the phylum

Chlorophyta (green); and *Cystoseria myrica* and *Sargassum elegans* of the phylum Ochrophyta (brown).

2.5.1. *Amphiroa bowerbankii* (Rhodophyta)

Amphiroa bowerbankii (Fig. 2.2) is a species belonging to the family Corralinaceae, class Florideophyceae, phylum Rhodophyta, kingdom Plantae and empire Eukaryota. It is a marine species found in subtidal zones (only visible during low tide and submerged during high tide) in South Africa and Madagascar (WoRMS, 1849). It is an edible, light pink, segmented species with flattened branches and rounded tips and is found during low tide either growing on the rocks in large clumps, floating in the water or washed onshore as small branches ripped off by the constant gushing of water.

Figure 2.2: *Amphiroa Bowerbankii*

https://www.ispotnature.org/species-dictionaries/sanbi/Amphiroa%20bowerbankii

2.5.2. *Ceramium virgatum* (Rhodophyta)

Ceramium virgatum (Fig. 2.3) also known as *Ceramium rubrum* or *Ceramium nodulosum* is a species belonging to the family Ceramiaceae, class Florideophyceae, phylum Rhodophyta, kingdom Plantae and empire Eukaryota. It is an edible, versatile and abundant marine species found on rocks and intertidal and subtidal zones in Belgium, France, Ireland, Italy, Mediterranean Sea, Netherlands, North Atlantic Ocean, North Sea, Mauritius, South Africa, Sweden and United Kingdom (WoRMS, 1797). It is a tree-like structure consisting of reddish brown, dichotomous branches running through to a hooked greenish stem. There is a huge variety in size and intensity of colour in existence.

Figure 2.3: *Ceramium virgatum*

http://www.seaweed.ie/descriptions/Ceramium_virgatum.php

2.5.3. *Dichotomaria tenera* (Rhodophyta)

Dichtomaria tenera (Fig. 2.4) also known as *Dichotomaria marginata* is a species belonging to the family Galaxauraceae, class Florideophyceae, phylum Rhodophyta, kingdom Plantae and empire Eukaryota. It is an edible marine species found in intertidal zones, on rocks and between coral reefs in Australia, Djibouti, Mexican Gulf, Indian Ocean, Kenya, Madagascar, Mozambique, Mauritius, South Africa, Taiwan and Tanzania (WoRMS, 1816). It has a visible, greenish holdfast connected to reddish stems that develop into brownish or pinkish, short, flat branches. It usually occurs as small clumps of branches covered in gelatinous mucilage.

Figure 2.4: *Dichtomaria marginata*

http://www.boldsystems.org/index.php/Taxbrowser_Taxonpage?taxid=263671

2.5.4. *Ellisolandia (Coralline) elongata* (Rhodophyta)

Ellisolandia (Coralline) elongate (Fig. 2.5) is a species belonging to the family Corallinaceae, class Florideophyceae, phylum Rhodophyta, kingdom Plantae and empire Eukaryota. It is an edible marine species found on rocks, lower intertidal and coastal areas in France, Ireland, Italy, Mediterranean Sea, North Atlantic Ocean, North Sea, South Africa and United Kingdom (WoRMS, 1786). It is a light pink, segmented large mass of flattened branches consisting of rounded and white tips.

Figure 2.5: *Ellisolandia (Coralline) elongata*

http://www.sb-roscoff.fr/fr/ecogeochimie-et-fonctionnement-des-ecosystemes-benthiques/publications/2011-2015

2.5.5. *Gelidium abbottiorum* (Rhodophyta)

Gelidium abbottiorum (Fig. 2.6) belongs to the family Gelidiaceae, class Florideophyceae, phylum Rhodophyta, kingdom Plantae and empire Eukaryota. It is an edible marine species found on rocky areas in low intertidal to high subtidal habitats in South Africa (WoRMS, 1990). It is a reddish tree-like species with a central column and branches sprouting out like leaves with dichotomous, smaller branches sprouting off those.

Figure 2.6: *Gelidium abbottiorum*

http://surialink.seaplant.net/HANDBOOK/Genera/reds/Gelidium/Gelidium.htm

2.5.6. *Gracilaria canaliculata* (Rhodophyta*)*

Gracilaria canaliculata (Fig. 2.7) is a species belonging to the family Gracilariaceae, class Florideophyceae, phylum Rhodophyta, kingdom Plantae and empire Eukaryota. It is an edible species found on rocky shores and coral reefs in Australia, Indian Ocean, Kenya, Madagascar, Mozambique, Mauritius, South Africa, Taiwan and Tanzania (WoRMS, 1871). It consists of a central holdfast with singular branches, being either red or greenish yellow, with hair follicles emanating from the central apex.

Figure 2.7: *Gracilaria canaliculata*

http://biogeodb.stri.si.edu/pacificalgae/liferesults/red/wiry

2.5.7. *Jania rubens* (Rhodophyta)

Jania rubens (Fig.2.8) is a species belonging to the family Corallinaceae, class Florideophyceae, phylum Rhodophyta, kingdom Plantae and empire Eukaryota. It is an abundant, calcinated, edible marine species found in low intertidal areas in Djibouti, Somalia, Mexican Gulf, Indian Ocean, Ireland, Kenya, Madagascar, Mediterranean Sea, North Atlantic Ocean, North Sea, Mauritius, South Africa, Sweden and Tanzania (WoRMS, 1816). It consists of a large mass of dichotomous, thin, pink branches with white tips connected to a central disc, held down by a solid substratum.

Figure 2.8: *Jania rubens*

https://seaweedindustry.com/seaweed/type/jania-rubens

2.5.8. *Jania (Haliptylon) squamata* (Rhodophyta)

Jania (Haliptylon) squamata (Fig. 2.9) is a species belonging to the family Corrallinaceae, class Florideophyceae, phylum Rhodphyta, kingdom Plantae and empire Eukaryota. It is a calcinated, edible marine species occurring in wave exposed shorelines and rocky areas in Britain, Canary Islands, Ireland, Mediterranean, North Atlantic Ocean, Portugal, Senegal and South Africa (WoRMS, 2007). It is a light pink, white-tipped mass of arrow-shaped, patterned branches more regularly branched and longer than that of *Jania rubens*.

Figure 2.9: *Jania squamata*

http://www.seaweed.ie/descriptions/Jania_squamata.php

2.5.9. *Caulerpa filiformis* (Chlorophyta)

Caulerpa filiformis (Fig. 2.10) is a species belonging to the family Caulerpaceae, class Ulvophyceae, phylum Chlorophyta, kingdom Plantae and empire Eukaryota. It is an edible marine species which can only be found at low tide levels growing on the edges of rocks or in gutters in Australia, Mozambique, South Africa and Tasman Sea (WoRMS, 1841). It consists of thin, long, green, flat branches protruding from a singular stem, creating a large mass.

Figure 2.10: *Caulerpa filiformis*

http://www.ispotnature.org/node/638418

2.5.10. *Codium capitatum* (Chlorophyta)

Codium capitatum (Fig. 2.11) belongs to the family Codiaceae, class Ulvophyceae, phylum Chlorophyta, kingdom Plantae and empire Eukaryota. It is an edible marine species given the nickname ‘dead man’s fingers’ due to the texture and shape of the seaweed. It is found inside rock pools and on the side of rocks in intertidal and subtidal zones and are visible at low tide in areas including the Indian Ocean, Kenya, Madagascar, Mozambique and South Africa (WoRMS, 1959). It is a dark green species, which looks black, connected to a steady substratum acting as the holdfast of the system. It consists of protruding branches multiplying as it gets closer to the top. The braches themselves and edges are thick and rounded with a scaly, gelatinous and elastic texture.

Figure 2.11: *Codium capitatum*

https://www.researchgate.net/publication/236161333_Codium_capitatum

Phytochemical studies on *Codium* species, to date, have been preliminary. No secondary metabolites have been isolated from *Codium* species; studies conducted thus far have been on extracts only.

Preliminary investigations on *Codium decorticatum* species have shown positive results for the presence of amino acids, carbohydrates, saponins and glycosides in petroleum ether, chloroform and methanol crude extracts (Sunilson et al., 2009). Studies on *Codium duthieae* indicated the presence of phenolics, flavonoids and tannins (Rengasamy et al., 2015); *Codium tomentosum* indicated the presence of carotenoids, terpenoids, alkaloids and polysaccharides and *Codium dwarkense* indicated the presence of sulfated polysaccharides (Wang et al., 2014). Crude extracts of *Codium capitatum*, used for the synthesis of silver nanoparticles and analysis of biological activity tested positive for the class of secondary metabolites terpenoids, alkaloids and polyphenolics (Kannan et al., 2013).

2.5.11. *Halimeda cuneata* (Chlorophyta)

Halimeda cuneata (Fig 2.12) belonging to the family Halimedaceae, class Ulvophyceae, phylum Chlorophyta, kingdom Plantae and empire Eukaryota is an inedible marine species found on rocks and in shaded crevices in the Indian Ocean, Kenya, Madagascar, Mozambique, Somalia, South Africa and Tanzania (WoRMS, 1846). It consists of green branches connected to a light green to yellow stem. The branches are made up of connected coin-shaped segments. The inedible nature is due to the cell walls bioaccumulating calcite (calcium carbonate) within the tissues. This calcite is released back into the beach sand once death of the organism occurs (Gibson et al., 2006).

Figure 2.12: *Halimeda cuneata*

http://www.marbef.org/wiki/diversity_and_classification_of_marine_benthic_algae

2.5.12. *Ulva rigida* (Chlorophyta)

Ulva rigida, (Fig. 2.13) commonly known as sea lettuce, belongs to the genus *Ulva*, family Ulvaceae, class Ulvophyceae, phylum Chlorophyta, kingdom Plantae and empire Eukaryota. It is an abundant, versatile, edible marine species grown in areas rich with ammonium phytoplankton and phosphates. It is commonly found growing on rocks and other plants (epiphytes) in Belgium, France, Indian Ocean, Ireland, Kenya, Madagascar, Mauritius, Mediterranean Sea, Mexican Gulf, Mozambique, North Atlantic Ocean, North Sea, Seychelles, Somalia and South Africa (WoRMS, 1823). It is a green species consisting of a thick thallus at the base but becoming thin and very brittle, towards the tips. The base is usually connected to another plant on which the *Ulva* commences growth.

Figure 2.13: *Ulva rigida*

http://www.seaweed.ie/descriptions/ulva_rigida.php

2.5.13. *Cystoseira myrica* (Ochrophyta)

Cystoseira myrica (Fig. 2.14) belonging to the family Sargassaceae, class Phaeophyceae, phylum Ochrophyta, kingdom Chromista and empire Eukaryota is an edible marine species found in warm waters either on rocky substrates, coral or *Jania*, in sublittoral or sheltered zones in the Indian Ocean, Kenya, Madagascar, Mauritius, Mozambique, Mexican Gulf, Red Sea, Somalia, South Africa and Tanzania (WoRMS, 1820). It is a bushy, tree-like species consisting of a base disc from which thallus axes protrude giving rise to yellowish-brown, short, elastic-like branches that are covered in shells and sand providing shelter to plants and animals.

Figure 2.14: *Cystoseria myrica*

http://waste.ideal.es/cystoseirамediterranea.htm

2.5.14. *Sargassum elegans* (Ochrophyta)

Sargassum elegans (Fig. 2.15) is a species belonging to the family Sargassaceae, class Phaeophyceae, phylum Ochrophyta, kingdom Chromista and empire Eukaryota. It is an edible species found growing in intertidal rock pools in Madagascar, Mozambique and South Africa (WoRMS, 1840). It is diversely used brown seaweed with long branches with various sizes of leaf-like structures protruding from the stem. It also contains pneumatocysts, being gas filled bladders allowing movement and buoyancy of the seaweed along the water (Draisma et al., 2010).

Figure 2.15: *Sargassum elegans*

http://oceanexplorer.noaa.gov/explorations/04etta/logs/aug25/aug25.html

2.6. Nutrients

Nutrients are essential substances needed to maintain system functioning, nourishment and growth within a plant and in the human body. These nutrients are either organic or inorganic. Seaweed are rich in omega 3 and 6 fatty acids, vitamins (A, C, D, E, K, B6 and B12), thiamine, riboflavin, niacin, folate, pantothenic acid, choline and betaine (Chipponi et al., 1982). They are also rich in the essential elements calcium, chromium, cobalt, copper, iodine, iron, magnesium, manganese, phosphorous, potassium, selenium, sodium and zinc (O'Dell and Sunde, 1997). Table 2.2 shows the uses of some of the nutrients in the human body.

Table 2 2: Uses of nutrients in the human body.

Compounds	Uses in the body
Carbohydrates	- Converted to glucose, stored in liver and transported to the circulatory system, tissues, muscles, brain, organs and bloodstream and used as energy (Lloyd et al., 1978).
Fatty acids	- Used for half the energy of the body (McGarry and Foster, 1980). - Omega 3 and 6 are used to decrease inflammation in the body (Gittleman, 2004).
Proteins	- Used to transport biological molecules, aiding communication between cells and acting as catalysts for chemical reactions in the body (Linton, 2007). - Stored in organs, skin, membranes and hair and used as building blocks of enzymes and hormones responsible for cellular repair and immune system response (Fersht, 1999). - Converted by liver and kidney to energy in the absence of carbohydrates and fats (Schwarcz, 2002).

Calcium in the human body clots the blood, stabilizes blood pressure, helps with brain function as well as strengthens the bones and teeth. However, if in excess, it can cause muscle and joint weakness, fatigue, vomiting, constipation, abdominal pains and extreme urination and thirst (Bronner, 1964). This can be tied in with why *Halimeda cuneata* is rendered inedible due to the high Ca content being available in carbonate precipitates which tend to accumulate in the soft tissue of the body.

Magnesium helps with the functioning of muscles, nervous and immune system; it strengthens bones, regulates blood sugar and pressure and is used in the synthesis of proteins. Adverse effects of high Mg intake include lowering of blood pressure, diarrhoea, reduced kidney function, lethargy, cardiac arrest, weak muscles and breathing problems (Jahnen-Dechent and Kettleler, 2012).

Chromium helps insulin regulate blood sugar levels, helps build body muscle mass, lowers cholesterol and regulates blood pressure levels. An excess of Cr can cause nostril and skin irritation, stomach ulcers, male reproductive defects and breathing problems (Nriagu, 1988). Chromium has however not been seen to cause toxic poisoning in the body as tests have proven that expulsion of Cr occurs before reaching detrimental effects (Thompson and Manore, 2012).

Cobalt is a source of vitamin B12 and vitamin C, helps with cardiac functioning of the body and helps to absorb Fe into the body even though present in small concentrations. An excess of Co can cause liver damage, neurological disorders, the impairing of senses, cancer, seizures, headaches, tissue death and hip problems (Kazantzis, 1981).

Copper has been seen to produce pigment melanin in the skin, maintains the sheath covering the nerves, helps synthesise phospholipids, helps with haemoglobin formation, helps maintain healthy

hair and forms enzymes needed to oxidise fatty acids but can also cause organ cancers, renal failure and reduced kidney function if at high concentrations (Uauy et al., 1998).

Iron is used in the body to produce red blood cells, and transport oxygen but can also be the cause of liver damage, heart disease and increased risk of cancer if at elevated levels (Hallberg et al., 2000).

Manganese is used to help tissue and bone formation, control blood sugar levels, normalise brain and nerve functions and prevent arthritis, diabetes, epilepsy and osteoporosis (Nielsen, 1999). It can however affect the nervous system, cause behavioural changes, reproductive effects, lung irritation and brain effects if at elevated levels (Levy and Nessetta, 2003).

While Ni has been seen as a toxic element due to its low concentrations and serious causes of eczema, cancer, nasal effects, sinusitis, bronchitis, asthma, kidney toxicity and shortness of breath, it is also utilised in the body in on low concentrations to help build strong skeletal frames, strengthen bones, absorb iron to prevent anaemia, break down glucose and produce enzymes (Nielsen, 1999).

Selenium, present at low concentrations, contributes to protein synthesis, prevention of cell damage, prevention of cancer and heart disease and helps the body recover from harmful metal toxicity (Holben and Smith, 1999). High concentrations of Se cause hair loss, irritability, fatigue, nerve damage, gastrointestinal pains, kidney and liver problems and blood clotting (Mehdi et al., 2013).

Zinc promotes growth of the body, helps in the synthesis of DNA, keeps the immune system healthy and helps heal wounds. Extreme concentrations can however cause vomiting, diarrhoea,

increased urine output, gastrointestinal pains, low blood pressure and convulsions (Maret and Sandstead, 2006).

Toxic elements are elements that, even if present at very low concentrations in the human body, can be detrimental to human health. This does however depend on the speciation of the element. These elements are not needed in the body for proper functioning. Expulsion or non-ingestion and non-inhalation of these elements is therefore encouraged as acute toxic poisoning can lead to death.

Arsenic, although not essential, can be used in the human body to help produce red and white blood cells and to help hair, nails, skin, bones and teeth to grow (Pérez-Granados and Vaquero, 2002). Arsenic needs to be in trace amounts as slightly higher concentrations can cause vomiting, throat and stomach pain, bloody diarrhoea, thickening and blotching of skin, cancer, diabetes, reproductive problems, nervous system defects, seizures, coma and even death (Chen et al., 2009).

Cadmium, although not essential, can help with kidney and liver functioning. However, it is considered to be one of the most toxic elements, causing damage to lungs, liver and kidneys, vomiting, diarrhoea, weakening of bones, anaemia and brain damage. Cadmium can accumulate in the kidneys and cause hypertension and it can displace Zn thereby lowering Zn levels and causing the body to no longer perform certain organ and enzymatic functions. It can also cause mental illness, cardiovascular and skin problems (Bernard and Lauwerys, 1986).

Lead, although not essential, has been seen to help with bone, muscle and brain functions but has also been the cause of anaemia, irritability, seizures, comas, heart attacks and kidney failure (Schroeder and Tipton, 1968).

While a few benefits to these toxic elements are noted, the detrimental effects to the body greatly outweigh the benefits. Avoiding exposure to these elements is therefore recommended.

2.6.1. Bioavailability and bioaccumulation

Bioavailability, most commonly referenced in human nutrition, are nutrients readily absorbed from the diet, circulated and utilised in bodily functions. A nutrient utilised to a higher extent in the body is said to be more bioavailable (Sunda, 2001). Bioavailability is also used similarly in aspects of plant nutrition, where it refers to the concentration or amount of a nutrient easily and readily accessible for uptake by the plant from the growth medium. The bioavailability of an aquatic plant is therefore the percentage of nutrient taken up by the plant from the water (Naidu et al., 2008).

Bioaccumulation in the plant is its ability to accumulate a specific nutrient in the soft tissue thereby increasing the total concentration present in the plant. This is represented by the bioaccumulation factor determined by comparing bioavailable (nutrients taken up by the plant) to total concentrations (nutrients present in the water).

$$BF = \frac{Concentration\ in\ plant}{Concentration\ in\ water}$$

The bioaccumulation factor is directly proportional to the concentration present in the plant due to the fact that a higher ability to uptake results in a higher amount being present (Arnot and Gobas, 2006). Plants have a tendency to differentiate between nutrients resulting in preference or accumulation of one nutrient to another causing antagonistic and/or synergistic effect, where the uptake of one either positively or negatively affects the uptake of another nutrient (Havlin et al., 2005).

Aquatic plants in the ocean tend to have higher bioaccumulation factors due to the continuously free flowing water rather than a stagnant body of water. The free flow results in constant replenishment of nutrients in the growth medium to be taken up by the plant (DeForest et al.,

2007). Higher pollution in an area also increases the amount of nutrients present resulting in a higher bioaccumulation factor. Aquatic plants, such as seaweed can therefore be used as a bioindicator of pollution and toxicity, due to its ability to give an accurate reading of excess nutrients, if tested (Förstner and Wittmann, 2012).

2.6.2. Dietary reference intakes (DRIs)

Recommended dietary allowances (RDAs) (Table 2.3) indicate the concentrations required to be ingested to allow a significant beneficial effect to be observed while tolerable upper intake levels (ULs) (Table 2.3) indicate the threshold values above which there would be an onset of harmful effects to the body (Hellwig, 2006). Toxic elements do not have an RDA value as they are considered non-essential (Trumbo et al., 2001). No UL has been determined for Cr as no harmful effects have been observed even at high concentrations (Thompson and Manore, 2012).

Table 2 3: Dietary reference intakes (DRIs) for selected elements.

	Element	As	Ca	Cd	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Se	Zn
DRI (mg/day)	**RDA**	ND	1000-1300	ND	ND	0.024-0.035	1	8-18	310-320	1.6-2.3	ND	ND	0.055	8-11
	UL	0.1(SA)	2500	7	1.4	ND	8	45	350	11	1	0.1(SA)	0.4	34

[a] Institute of Medicine of the National Academies: Dietary Reference Intakes (2001)
[b] SA = Department of Health, South Africa (2014)
[d] ND = not determined/determinable.

2.7. Instrumentation and techniques

There are three main types of instrumentation and three techniques used in the determination of the concentration of the elements as well as the phytochemical constituents contained within the seaweed species studied. These instruments included the microwave digestor, inductively coupled plasma-optical emission spectrometer and nuclear magnetic resonance spectrometer while the techniques included extraction, thin layer chromatography (TLC) and column chromatography.

2.7.1. Microwave digestion

Microwave digestion, is an accurate instrumental method which is used to dissolve metals in a sample when there are other constituents other than the inorganic metals present. It is a fast technique compared to open vessel techniques and it elevates to high temperatures resulting in the complete digestion of a sample.

It separates the inorganic constituents from the organic constituents contained within the plant or water. This is done by irradiating the sample in a closed beaker of acid (low pH) with microwaves. By having a closed vessel system, it decreases the contamination error to the sample as well as the loss of any volatile compounds compared to an open vessel technique such as hot plate digestion. This in turn increases the pressure and temperature rapidly to allow decomposition of the sample as well as dissolving of the metals. The sample would then be ready for quantitative and qualitative analysis.

In this study, the Microwave Accelerated Reaction System CEM MARS 5 (MARS CEM Corporation) (Fig. 2.16) with infrared temperature sensors that can measure each vessel and that can reach temperatures of 260°C, as well as Teflon liners which regulated pressure was used. The

microwave was used due to the rapid dissolution of the matrix as well as little to no contamination of the sample.

Easyprep™ vessels were used rather than Xpress™ vessels due the instability and pressure build up causing expansion and rising of the vessels from the protective sleeves. Easyprep™ vessels use a control vessel which can monitor temperature and pressure which allows the analyst to stop the analysis if conditions become too harsh. Easyprep™ vessels can also withstand much higher temperatures and pressures without getting damaged (MARS CEM Corporation).

Figure 2.16: A microwave digestor instrument

http://de.cem.com/pdf/Mars_Configuration.pdf

2.7.2. Inductively coupled plasma-optical emission spectrometry (ICP-OES)

The benefits of inductively coupled plasma-optical emission spectrometry (ICP-OES) are that it can detect trace concentrations as well as analyse up to 70 elements, simultaneously. In this study, the Perkin Elmer ICP-OES with radial plasma viewing was used. ICP-OES is the instrument of choice due to its multi-element analysis capabilities, high sensitivity and high linear range. In the analysis, usually the three most sensitive lines with no interfering elements are selected and used for quantification.

In this instrument, plasma is used as the excitation source. It consists of three quartz tubes and a tesla coil (Fig. 2.17). A transmitter sends radio frequencies through to the coil creating an electromagnetic field where the incoming argon gas is ionised.

The ionised argon gas flows towards the radio frequency in a set path and heating sets in as it couples with the field created by the radio frequency (Bradford and cook, 2011).

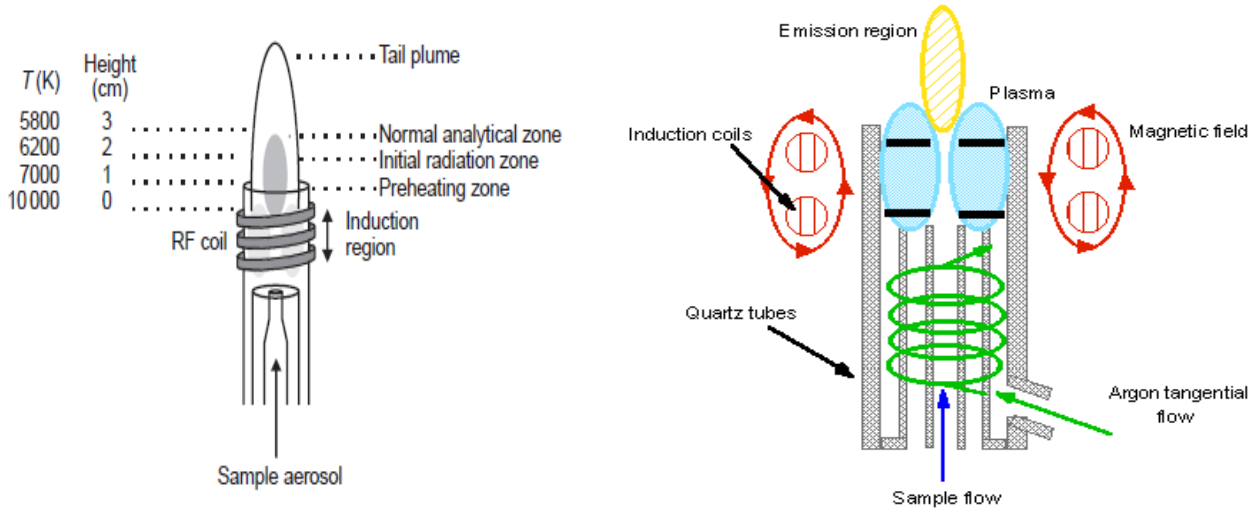

Figure 2.17: Plasma from the ICP

http://www.chemicool.com/definition/inductively_coupled_plasma_excitation.html

The sample is then introduced into the ICP where it is nebulized and turned into an aerosol. This is then carried by the argon gas to the plasma. The aerosol is then desolvated by removing all solvent contained within the droplets creating a dry particulate, decomposition of the particulate then occurs to form a gas molecule after which atomization breaks the gas molecule into atoms. The atoms are then excited and then radiated (Bradford and Cook, 2011).

This emitted radiation of light is then separated by a monochromator to obtain the desired wavelengths. The wavelength of the light emitted is characteristic of certain elements. This gives the quantitative analysis. The detector then picks up the signals after which the photomultiplier intensifies the signals. The intensities of the emitted light are then compared to the intensities of standards of known concentration and the concentrations of the elements in the sample can be determined by use of calibration curves.

Since it is a simultaneous detection technique, it can analyse a range of elements at the same time which reduces the downtime of the instrument as different lamp sources are not required for the analysis of each element. It is also one of the most sensitive techniques and is able to detect elements in trace amounts due to the low limits of detection reaching ppb ($\mu g\ L^{-1}$) levels. Another advantage is that if one of the wavelengths becomes inaccurate due to chemical or background interferences resulting from the overlap of an atomic wavelength with the signal of the analyte or interferences from the instrument itself such as stray light causing baseline shift of an analyte, another one of the sensitive wavelengths can be chosen. Therefore method validation is carried out with a sample with known concentrations to optimize the instrument settings and wavelength. Matrix interferences are removed by a process called matrix matching which uses the same matrix in the standards as is in the sample. This allows viscosity and flow rate differences to be resolved (Bradford and Cook, 2011).

2.7.3. Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy works on the property and principle of magnetic field effects. NMR spectroscopy determines the chemical structure of a compound by the effect of electromagnetic radiation on matter. All atoms consisting of nuclei contain an angular moment also known as spin, acting as a small magnet. These nuclei are randomly aligned when placed in an external magnetic field where they are able to align with or against the field. Alignment with the field is preferred by most nuclei (alpha). The alpha aligned nuclei are bombarded with electromagnetic radiation changing the alignment to beta (orientation against magnetic field) then allowing relaxation back to alpha orientation generating an electric field in the receiver coil found around the sample. The magnetic field fluctuation during this period is known as resonance which is the data recorded and converted to peaks seen in an NMR spectrum (Proton NMR basics, 1995).

Due to the presence of local magnetic fields created by lone pair electrons, sigma and pi bonds, other nuclei nearby are shielded or deshielded causing the peaks to occur at different positions along the spectrum. The integral under each peak can be used to determine the number of hydrogens present at that frequency (Proton NMR basics, 1995).

The coupling constant (J coupling) is a constant given to the interaction between bonds where the value will be larger between atoms directly next to each other compared to atoms 2 to 3 bonds away. There are different experiments which can be run using NMR with the most common ones being 1D proton (^{1}H) NMR which shows coupling between hydrogens in a sample, 1D carbon (^{13}C) NMR which shows carbon resonances in a sample, 1D Dept (distortionless enhancement by polarization transfer) 90 and 135 which allows the determination of CH, CH_2 and CH_3 bonds in the sample, Cosy (homonuclear correlation spectroscopy) sequence, a 2D experiment used to

correlate spins that are coupled to each other, Noesy (nuclear overhauser effect spectroscopy), a 2D experiment used to correlate protons that are spatially close rather than those that are bond coupled to each other, HSQC (heteronuclear single quantum correlation), a 2D experiment correlating carbons with protons that are one bond away (attached), and HMBC (heteronuclear multiple-bond correlation), a 2D experiment correlating carbons and protons 2 bonds away (neighbours) from each other (Fesik and Zeiderweg, 1988).

2.7.4. Extraction

Solvent extraction is a technique used to remove compounds from within a plant into a solvent. It involves adding solvent to a mass of dried plant sample and allowing it to shake on an orbital shaker (Fig. 2.18) for approximately 3 days. The solvent is filtered and evaporated in order to obtain the crude extract after which another solvent of a higher polarity (e.g. methanol) is then added to the same mass extract to remove compounds of a higher polarity. The crude extract is then a solid left behind after solvent evaporation allowing for further analysis (Sultana et al., 2009).

Figure 2.18: Orbital shaker http://www.onlinelabsupplies.com/category.jhtm?cid=26

2.7.5. Chromatography

2.7.5.1. Thin layer chromatography

Thin layer chromatography is a technique that involves using an aluminium plate covered with a layer of adsorbent (silica gel) known as the stationary phase. The sample is dissolved in minimum solvent in which it is soluble and then spotted on the bottom of the plate and put in a solvent bath in which the solvent is allowed to run from the bottom to the top of the plate. Different compounds move up the silica gel at different rates according to their various functional groups and polarity (Fig. 2.19). The mobile phase solvent system is then altered (mixture of various polar and non-polar) in order to elute and separate different compounds as much as possible on the plate. This optimum solvent system can then be used to determine the presence of suspected compounds and to separate and isolate compounds using column chromatography.

Figure 2.19: Thin layer chromatography plates after solvent elution

2.7.5.2. Column chromatography

Column chromatography is utilised to clean up crude extracts removed from the plant by solvent extraction. A tempered glass column (size dependent on sample size) is fitted with a porous filter above the tap. The column is then packed with silica gel (either wet or dry packed), a layer of sand to discourage mixing, a layer of the sample and lastly a piece of cotton wool to discourage disturbing of the sample. Solvent is then added into the top of the column and allowed to run until movement of sample through the silica gel is observed. The solvent system (mixture of polar and nonpolar solvents dependent on compound needed to be separated) is then altered with discretion in order to elute cleaner compounds separately. Extracts of a specific volume are collected and the solvent is evaporated in order to analyse the newly separated extract (Fig. 2.20).

Figure 2.20: Column chromatography and extracts eluted

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CHAPTER 3: ELEMENTAL ANALYSIS AND NUTRITIONAL VALUE OF SEAWEED FROM KWAZULU-NATAL, SOUTH AFRICA

Abstract

Seaweed, biologically and phycologically referred to as algae, can be classified as Rhodophyta (red), Chlorophyta (green) and Ochrophyta (brown). In this study, the concentrations of 13 elements (As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se and Zn) were investigated for 14 seaweed species of varying classes (*Amphiroa bowerbankii, Ceramium virgatum, Dichotomaria tenera, Ellisolandia (Coralline) elongata, Gelidium abbottiorum, Gracilaria canaliculata, Jania rubens* and *Jania (Haliptylon) squamata* of the Rhodophyta (red); *Caulerpa filiformis, Codium capitatum, Halimeda cuneata* and *Ulva rigida* of the Chlorophyta (green); and *Cystoseira myrica* and *Sargassum elegans* of the Ochrophyta (brown)). This was done to determine the nutritional value of seaweeds due to their edible nature, medicinal properties, industrial importance and natural abundance. The elemental distribution in seaweeds were found to be in decreasing order of Ca > Mg > Fe > Cu > Mn > Zn > Cr > Co > Se > As > Pb > Ni > Cd. All edible species contained high levels of both macro and micro-elements with the corali species accumulating high levels of Ca. Of the thirteen edible seaweeds studied, only three (*G. abbottiorum, E. (Coralline) elongata* and *C. virgatum*) are suitable for human consumption due to high levels of toxic elements (As, Cd and Pb) being present in the other species. These three species are also rich in essential nutrients, specifically *C. virgatum*, which is rich in Cu and Se. Hierarchical cluster analysis showed a clear similarity in distribution of essential and toxic elements in similar classes of seaweeds.

Keywords: Tolerable upper intake levels, Chlorophyta, Rhodophyta, Ochrophyta.

3.1 Introduction

South African ports, linking the Middle East to Europe and America and being along the world's busiest shipping route, have accelerated the economic growth of the country. However, this together with ocean outfalls that discharge sewage and industrial effluent into the sea every day has exposed the coastline to the risk of marine pollution (Weigand 1958). Seaweeds are algae species that can be classified by their pigments as Rhodophyta (red), Chlorophyta (green) or Ochrophyta (brown) species (Minge et al. 2009). Algae are very important in the food chain as they are a source of food to marine and fresh water species. The marine species that are consumed by humans usually form the link between the aquatic environment and humans in the human food chain. Seaweeds enter the human food chain through direct consumption of edible varieties, or are themselves consumed by fish and other marine species that are then consumed by humans (Spolaore et al. 2006).

The industrial uses of seaweeds are numerous including their use as additives in cosmetics and toothpaste, their use in the production of hydrocolloids which can be used to make gelatine and agar that are used to preserve certain food products (Bixler and Porse 2011), and as gastric binding agents to make the body feel more full in weight loss procedures (Fleurence 1999; Smit 2004). The beneficial effects of marine algae have been recognized in many places around the world making it a multi-million dollar industrial business. However, South Africa has not really placed any emphasis on exploiting this market or investigating these organisms, especially those varieties found on the East Coast (Isaac 1937).

Seaweeds have the capacity to accumulate trace metals several thousand times higher than their concentrations in seawater. As a result, seaweeds have been used as biodetectors and bioindicators of radioactive metals from surrounding waters. Due to the role of seaweeds in the human diet and their potential to accumulate toxic metals, the metal content in seaweeds should be monitored. The biosequestering capacity of seaweeds to different metals under the influence of effluent should also be investigated. This can provide vital information on the suitability of seaweeds for bioremediation, it can indicate areas of pollution in the marine environment and it can also provide nutritional information on seaweeds (Vasquez and Guerra 1996).

Seaweeds are considered among the World's Healthiest Foods because of their rich mineral content however due to toxicity risks, studies need to be carried out on their elemental concentrations (Fraga 2005). Studies on seaweed, which reported high concentrations of inorganic As in *Sargassum fusiforme* (hijiki), have resulted in the Canadian Food Inspection Agency warning against its consumption (Rose et al. 2007). Whilst seaweed might not be consumed on a daily basis, health risks may ensue with routine consumption if toxic elements are at elevated levels. Despite there being well documented information on the nutritional value and toxicity risks in seaweed from other regions of the world, there is a dearth of information on those found in South Africa. Previously, we reported on the elemental concentrations as a function of seasonal variation in *Gellidum abbottiorum, Plocamium corallorhiza, Caulerpa racemosa* and *Ulva lactuca* found in KwaZulu-Natal, South Africa (Misheer at al. 2006a; 2006b; 2006c; 2006d). In this work, we investigate the elemental concentrations in 14 seaweed species of varying classes (Rhodophyta, Chlorophyta and Ochrophyta) found along the coast of KwaZulu-Natal, to determine their nutritional value and toxicity risks.

3.2 Materials and Methods

3.2.1 Sampling

Samples of various species of seaweed were collected from three different sampling sites along the east coast of KwaZulu-Natal, South Africa in June and July 2014. The sampling sites were 1 - Brighton Beach, Ansteys, Wentworth (29.560° S, 31.00° E), 2 - Scottsburgh (30.2833° S, 30.7500° E) and 3 - Rocky Bay (30. 20120° S, 30.43923° E), South Coast. Fourteen species, namely *Amphiroa bowerbankii, Caulerpa filiformis, Ceramium virgatum, Codium capitatum, Cystoseira myrica, Dichotomaria tenera, Ellisolandia (Coralline) elongate, Gelidium abbottiorum, Gracilaria canaliculata, Halimeda cuneata, Jania rubens, Jania (Haliptylon) squamata, Sargassum elegans* and *Ulva rigida*, were collected. Approximately one Ziplock® plastic bag of each species was collected from each site. Approximately 1000 mL of the surrounding water (growth solution) was collected from each site and stored in plastic bottles in the refrigerator at 4°C.

3.2.2 Reagents and standards

All chemicals used were supplied by Sigma Aldrich (St. Louis, USA) or Merck (Kenilworth, USA) chemical companies. Analytical reagent grade chemicals were used for samples and spectroscopic grade were used for standards. Glassware was properly washed and oven dried at 40°C. Double distilled water was used throughout the experiments for preparation of solutions as well as to rinse out glassware.

3.2.3 Sample preparation

Seaweeds were dried in an oven at 40°C, for 72 h then finely ground using a mill and stored in plastic bags until analysed.

3.2.4 Digestion and elemental analysis of samples

Microwave-assisted closed vessel digestion was used for digestion of plant samples due to its superior digestion capability and sample throughput. Digestions were performed using the CEM Microwave Accelerated Reaction System (MARS 6) (CEM Corporation, Matthews, NC, USA) with patented EasyPrep^{TM} technology that consists of EasyPrep™ vessels with integrated thermowell (Kisten et al. 2015).

Approximately 0.25 g of dried seaweed samples and certified reference material (CRM), were placed in the vessels with 10 mL of HNO_3 and allowed to pre-digest for 2 h to avoid damage to vessels from resulting high pressures of new samples (Kisten et al. 2015). Thereafter, vessels were placed in the carousel which was placed in the microwave to allow for digestion of samples. The microwave was set at 1600 W at 100% power after which it was ramped to 170°C for 15 min, held for 15 min and cooled for 15 min. Digests were filtered under gravity into 50 mL volumetric flasks using fluted filter paper and made up to the mark with double distilled water. Digestions for all samples were done in triplicate. This was transferred to plastic bottles and stored in the refrigerator at 4°C until analysed.

All digests were analysed for As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, and Zn by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) due to its multi-element determination capability, dynamic linear range and low detection limits. Measurements were

carried out using the Perkin Elmer® Optima™ 5300 Dual View ICP-OES (Billerica, Massachusetts, USA) with axial 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 lines with no interfering elements and that which produced the best CRM results were selected (Quevauville et al. 1992).

3.2.5 Statistical analysis

Comparison and similarity groupings of data was determined by one way ANOVA and a follow up posthoc analysis (Tukey's) was performed to determine significant differences between means. Hierarchical cluster analysis with between-groups linkage, measuring the Euclidean distance between elemental concentrations, was also performed. All statistical analyses were performed using the Statistical Package for the Social Sciences (PASW Statistics 23, IBM Corporation, Cornell, New York)

3.3 Results and Discussion

3.3.1 Quality assurance

Method validation was performed by analysis of the CRM (White clover, BCR - 402) from the Community Bureau of Reference of the Commission of the European Communities, by comparing measured results with certified values (Table 3.1). For As, Co and Se certified values with standard deviations (which stated the uncertainty level within a level of confidence) were provided whilst

the values for Cr, Fe, Ni and Zn were indicative and no standard deviations were provided. Measured values compared well with certified values.

Table 3.1: Comparison of measured and certified/indicative values (Mean ± SD, at 95% confidence interval, n = 3), based on dry mass, in the certified reference material (White clover, BCR - 402).

Element	Wavelength (nm)	Certified/Indicative[a] concentration (μg g^{-1})	Measured concentration (μg g^{-1})
As	197.197	0.093 ± 0.010	0.10 ± 0.014
Co	228.616	0.178 ± 0.008	0.184 ± 0.006
Cr	283.563	5.19	4.98 ± 0.248
Fe	238.204	244	235 ± 0.74
Ni	231.604	8.25	8.02 ± 0.312
Se	196.026	6.70 ± 0.25	6.90 ± 0.03
Zn	206.200	252	255 ± 2.68

[a] Indicative values are those without uncertainties.

3.3.2 Elemental distribution

Of the seaweed species studied, *A. bowerbankii*, *C. virgatum*, *D. tenera*, *E. (Coralline) elongata*, *G. abbottiorum*, *G. canaliculata*, *J. rubens* and *J. (Haliptylon) squamata* belong to the Rhodophyta (red) class; *C. filiformis*, *C. capitatum*, *H. cuneata* and *U. rigida* belong to the Chlorophyta (green) class and *C. myrica* and *S. elegans* belong to the Ochrophyta (brown) class. The elemental distribution of the macro-elements (Ca and Mg) and the micro-elements (Co, Cr, Cu, Fe, Mn, Ni, Se and Zn) is presented in Table 3.2.

Table 3 2: Concentrations (Mean ± SD, n = 3, in μg g^{-1}) of macro and micro-elements in the 14 different seaweed species.

	Concentration (μg g^{-1})													
Species	***AB***	***CF***	***CV***	***CC***	***CM***	***DT***	***ECE***	***GA***	***GC***	***HC***	***JR***	***JS***	***SE***	***UR***
Macro-elements														
Ca	59785±373 (bc)	40420±113 (de)	32860±300 (ef)	24960±167 (f)	26123±537 (f)	23258±504 (f)	39401±314 (de)	27532±630 (f)	21948±301 (f)	39741±296 (de)	70238±384 (a)	60041±379 (b)	24254±914 (f)	49345±128 (cd)
Mg	33356±183 (ab)	6691±87 (de)	16483±146 (ef)	19062±180 (f)	16454±336 (cd)	7592±150 (e)	18195±102 (cd)	12134±630 (de)	7023±90 (e)	12091±943 (e)	35024±144 (a)	35946±215 (a)	15831±517 (cd)	26170±388 (b)
Micro-elements														
Co	1.4±0.5 (de)	1±0.2 (ab)	1.9±0.2 (de)	1.2±0.4 (e)	4.8±0.7 (cd)	8.4±2.2 (fg)	5.6±0.3 (g)	9.3±0.8 (g)	6.7±1.8 (b)	4.1±0.5 (ef)	3.9±0.4 (a)	3.2±3.2 (c)	3.2±0.3 (g)	10.9±0.3 (f)
Cr	1.6±0.6 (i)	17±2 (a)	3.5±0.5 (h)	5±1.1 (fg)	10±1.8 (b)	6.2±0.5 (e)	3.7±0.7 (gh)	6.5±0.7 (de)	7.4±0.8 (cd)	8.1±1.2 (c)	4.7±0.7 (gh)	3.4±1 (h)	5.6±5.6 (ef)	5.2±0.2 (ef)
Cu	25±12 (def)	24±4 (ef)	228±39 (def)	11±2 (cd)	39±8 (cde)	78±67 (a)	47±12 (f)	14±1 (def)	110±32 (c)	25±2 (ef)	52±17 (def)	17±6 (b)	32±17 (def)	34±5 (def)
Fe	274±57 (f)	388±41 (f)	288±35 (f)	584±67 (def)	1976±14 (a)	1033±41 (cd)	732±28 (def)	1319±93 (bc)	1592±16 (ab)	1624±128 (ab)	568±60 (ef)	326±46 (f)	632±25 (def)	973±30 (cde)
Mn	39±2.4 (de)	97.8±7 (a)	14.3±1.4 (h)	33.5±3.8 (ef)	76.7±5.3 (b)	38.7±14 (de)	31.7±3.4 (ef)	39±2.7 (de)	44.3±4 (cd)	48.2±3.5 (c)	30.3±3.6 (ef)	18.4±2.2 (gh)	27.2±11.2 (g)	27.1±0.6 (fg)
Ni	0±0 (d)	2.1±0.4 (a)	0.8±0.8 (bc)	1.5±0.6 (abc)	1.9±0.7 (ab)	1.3±0.7 (abc)	0.4±0.2 (cd)	0.4±0.1 (cd)	0.8±0.5 (bc)	1.1±0.8 (abc)	0.9±0.6 (bc)	0.6±0.4 (bc)	0.6±0.9 (bc)	0.2±0.2 (cd)
Se	4.4±1.5 (bc)	4.8±0.2 (bc)	7.8±1.3 (a)	4.2±1.2 (bc)	3.6±1.5 (cd)	4.6±1.4 (bc)	4.9±0.4 (b)	2±0.4 (e)	6.1±1.7 (a)	1.8±0.6 (e)	2.2±0.2 (de)	3.6±0.4 (bcd)	4.4±1.3 (bc)	2.3±0.3 (de)
Zn	19.3±7 (g)	25.4±6.3 (efg)	53.5±28.1 (b)	68±2.8 (a)	38.9±5.1 (cd)	15.8±6.8 (gh)	7.1±4.7 (h)	7.4±2.1 (h)	24.1±6 (efg)	41.7±20 (c)	29±8.5 (def)	17±8.7 (gh)	19.8±11.2 (fg)	31.5±8.8 (cde)

Different letters in a row indicate significantly different means (Tukey's posthoc comparisons, $p < 0.05$).

AB - Amphiroa bowerbankii, CF - Caulerpa filiformis, CV - Ceramium virgatum, CC - Codium capitatum, CM - Cystoseira myrica, DT - Dichotomaria tenera, ECE - Ellisolandia (Coralline) elongata, GC - Gracilaria canaliculata, GA - Gelidium abbottiorum, HC - Halimeda cuneata, JR - Jania rubens, JS - Jania (Haliptylon) squamata, SE - Sargassum elegans, UR - Ulva rigida.

Of the macro-elements, Ca was found to be in highest concentrations in all species, ranging from 21948 μg g^{-1} in *G. canaliculata* to 70238 μg g^{-1} in *J. rubens*. *Jania* species are corali species which are known to be highly calcinated. Calcium concentrations were highest in Rhodophyta (red), followed by Chlorophyta (green) then Ochrophyta (brown). Magnesium, ranged from 6691 μg g^{-1} in *C. filiformis* to 35946 μg g^{-1} in *J. (Haliptylon) squamata*. Corali species appear to accumulate Mg similar to Ca. Magnesium concentrations in Ochrophyta were not significantly different ($p < 0.05$).

Of the micro-elements, Fe, which is used for the production of red blood cells and transportation of oxygen to cells (Adamson 1994), ranged from 274 μg g^{-1} in *A. bowerbankii* to 1976 ug g^{-1} in *C. myrica.* Iron was found to be highest in Ochrophyta *(C. myrica)* and Chlorophyta *(H. cuneata)* and lowest in Rhodophyta *(C. virgatum* and *A. bowerbankii)*. Copper, which is used for the production of enzymes and reparation of connective tissues (Uauy et al. 1998), ranged from 11 μg g^{-1} in *C. capitatum* to 228 μg g^{-1} in *C. virgatum*. Manganese ranged from 14.3 μg g^{-1} in *C. virgatum* to 97.8 μg g^{-1} in *C. filiformis.* Zinc, used for growth and division of cells, healing of wounds, functioning of the immune system and breakdown of carbohydrates (Burch et al. 1975), ranged from 7.1 μg g^{-1} in *E. coralline elongate* to 68 μg g^{-1} in *C. capitatum.*

Of the micro-elements required in small concentrations in the body, Co, which is an integral part of B12, itself aiding the formation of red blood cells and preventing the onset of anaemia (Mertz 1981), ranged from 1 μg g^{-1} in *C. filiformis* to 10.9 μg g^{-1} in *U. rigida*. Both of these species belong to Chlorophyta, thereby indicating that Co uptake is not dependent on the class of species. Chromium, utilised for digestion of food and prevents loss of Ca and movement of blood sugar from the bloodstream to the cells (Mertz 1969), ranged from 1.64 μg g^{-1} in *A. bowerbankii* to 17.11 μg g^{-1} in *C. filiformis.* Selenium, which is used to produce antioxidant enzymes that protect the

body from certain cancers, heavy metal poisoning and cardiovascular disease (Arvilommi et al. 1983), ranged from 1.8 μg g^{-1} in *H. cuneata* to 7.8 μg g^{-1} in *C. virgatum*. Selenium concentrations were not significantly different ($p < 0.05$) in the studied seaweed species. Nickel concentrations were lowest in *A. bowerbankii* (< 0.1 μg g^{-1}) and highest in *C. filiformis* (2.1 μg g^{-1}).

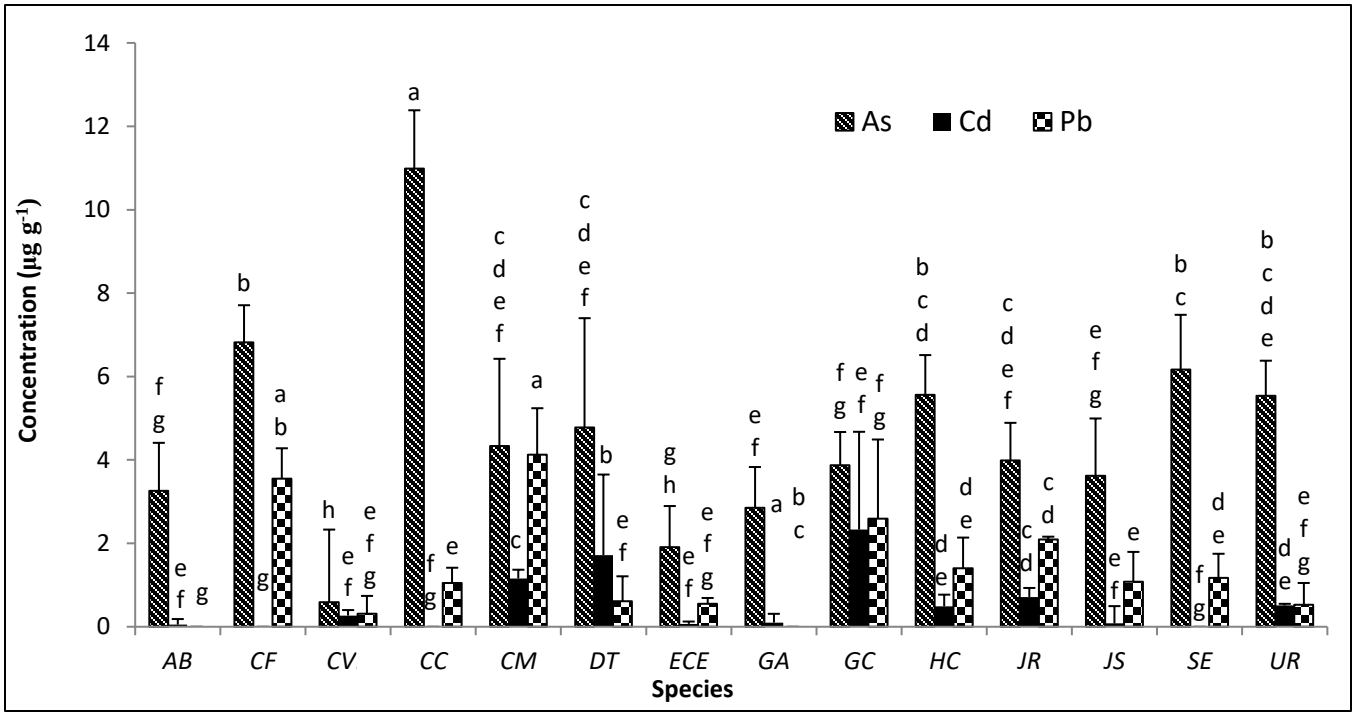

Figure 3.1: Graph showing mean concentrations of toxic elements (As, Cd and Pb, μg g^{-1}, n=3) in the 14 different seaweed species. Different letters for an element indicate significantly different means (Tukey's posthoc comparisons, $p < 0.05$).

AB - Amphiroa bowerbankii, CF - Caulerpa filiformis, CV - Ceramium virgatum, CC - Codium capitatum, CM - Cystoseira myrica, DT - Dichotomaria tenera, ECE - Ellisolandia (Coralline) elongate, GC - Gracilaria canaliculata, GA - Gelidium abbottiorum, HC - Halimeda cuneata, JR - Jania rubens, JS - Jania (Haliptylon) squamata, SE - Sargassum elegans, UR - Ulva rigida.

The concentration of the toxic elements As, Cd and Pb in the fourteen different seaweed species is presented in Figure 3.1. For As, concentrations ranged from 0.59 μg g^{-1} in *C. virgatum* to 10.99

μg g^{-1} in *C. capitatum*. Concentrations of As in *C. capitatum* were significantly higher than the other seaweed species studied ($p < 0.05$). Cadmium concentrations were lowest in *C. capitatum, C. filiformis* and *S. elegans* and highest in *G. canaliculata* (2.33 μg g^{-1}) which was significantly higher than the other species studied ($p < 0.05$). Lead concentrations were lowest in *G. abbottiorum* and *A. bowerbankii* (< 0.1 μg g^{-1}) and highest in *C. myrica* (4.125 μg g-1) which was significantly higher than the other species ($p < 0.05$).

For As and Pb, the highest concentrations were found in Chlorophyta and the lowest were found in Rhodophyta. For Cd, an opposite trend was observed with highest concentrations in Rhodophyta and lowest in Chlorophyta. Although all toxic elements studied were present in small concentrations in the seaweed, As concentrations were slightly elevated suggesting uptake and bioaccumulation of As by seaweed. The concentrations of the toxic elements were found to be in decreasing order of As > Pb > Cd. Except for *H. cuneata,* all studied seaweed species are edible. The maximum level in foodstuffs (fish and processed fish) set by the Department of Health, South Africa (in μg g^{-1}) is 1.0 for Cd, 0.5 for Pb and 3.0 for As (Staatskoerant 2014). Arsenic, Cd and Pb levels in most of the edible species were above those considered toxic in fish which is a cause for concern. However, organic As is commonly found in most seaweed and marine species and is considered to be relatively non-toxic (Hanaoka et al. 2001). Based on the results, the only edible species with low levels of As, Cd and Pb were *G. abbottiorum, E. coralline elongate* and *C. virgatum*. The data shows concentrations of the studied elements in the seaweeds (both essential and toxic) to be present in decreasing order of Ca > Mg > Fe > Cu > Mn > Zn > Cr > Co > Se > As > Pb > Ni > Cd.

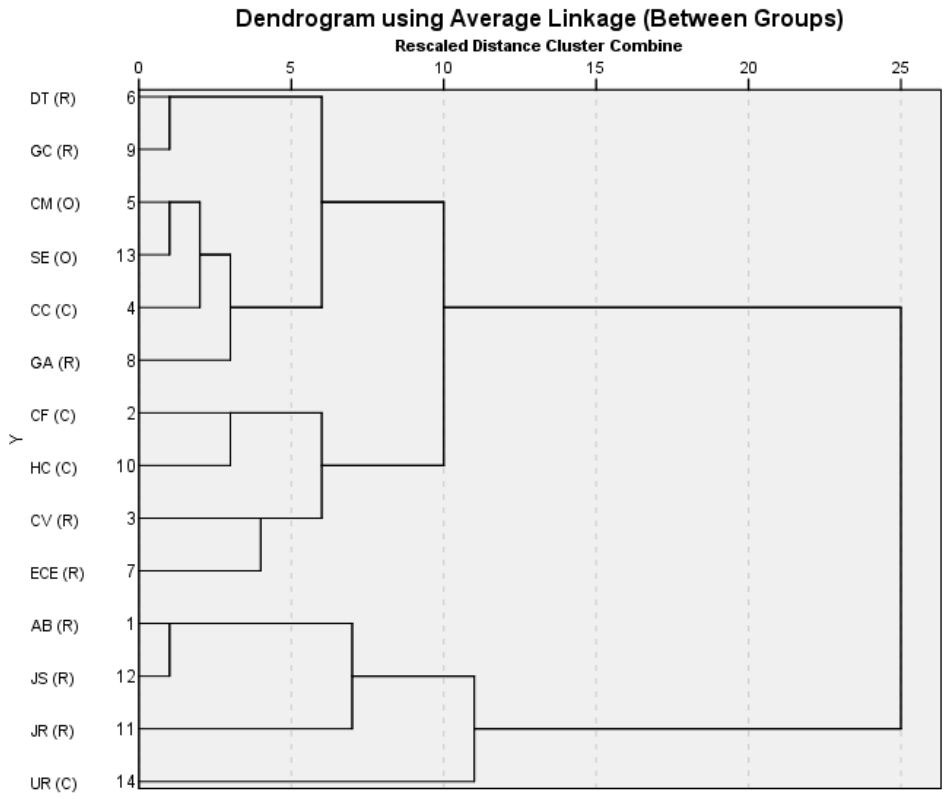

Figure 3.2: Cluster analysis showing the relationship in elemental distribution of essential elements in the three classes of seaweed species, Rhodophyta (R) (red), Chlorophyta (C) (green) and Ochrophyta (O) (brown).

AB - Amphiroa bowerbankii, CF - Caulerpa filiformis, CV - Ceramium virgatum, CC - Codium capitatum, CM - Cystoseira myrica, DT - Dichotomaria tenera, ECE - Ellisolandia (Coralline) elongate, GC - Gracilaria canaliculata, GA - Gelidium abbottiorum, HC - Halimeda cuneata, JR - Jania rubens, JS - Jania (Haliptylon) squamata, SE - Sargassum elegans, UR - Ulva rigida.

Hierarchical cluster analysis measuring dissimilarity, based on the Euclidean distance between mean elemental concentrations for essential and toxic elements was evaluated in the different classes of seaweeds. A dendrogram using average linkage (between groups) for essential and toxic elements is presented in Figure 3.2 and 3.3, respectively. The proximity in the dendrogram between species of the same class for essential elements (Fig. 3.2), for instance, *D. tenera* and *G. canaliculata* of the Rhodophyta, *C. myrica* and *S. elegans* of the Ochrophyta and *C. filiformis* and

H. cuneata of the Chlorophyta, shows a clear similarity in distribution of essential elements in the same class of seaweeds. The proximity in the dendrogram (Fig. 3.3) also shows a clear similarity in distribution of toxic elements in similar classes.

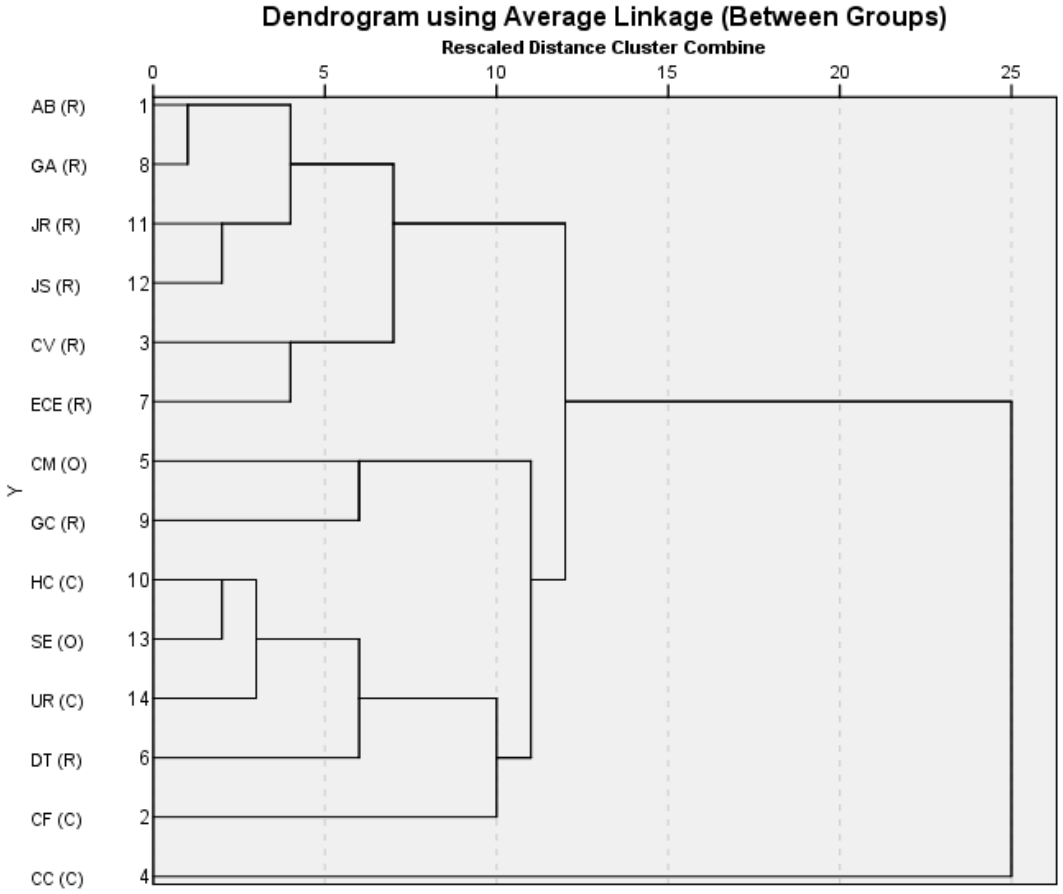

Figure 3.3: Cluster analysis showing the relationship in elemental distribution of toxic elements in the three classes of seaweed species, Rhodophyta (R) (red), Chlorophyta (C) (green) and Ochrophyta (O) (brown).

AB - Amphiroa bowerbankii, CF - Caulerpa filiformis, CV - Ceramium virgatum, CC - Codium capitatum, CM - Cystoseira myrica, DT - Dichotomaria tenera, ECE - Ellisolandia (Coralline) elongate, GC - Gracilaria canaliculata, GA - Gelidium abbottiorum, HC - Halimeda cuneata, JR - Jania rubens, JS - Jania (Haliptylon) squamata, SE - Sargassum elegans, UR - Ulva rigida.

3.3.3 Estimated contribution of metals in seaweed to the diet

The elemental concentrations reported for the edible seaweed species were compared to Dietary Reference Intakes (DRIs) standardized by the Institute of Medicine of the National Academies (Table 3.3). The data revealed the contribution to the health and nutrition of an individual to be significant. With an average serving size of 10 g per day (dry mass), based on estimated normal consumption, all elements for all species excluding *H. cuneata* (inedible species) were investigated. The percentage contribution of each element in 10 g of sample was compared to recommended dietary allowances (RDAs), and tolerable upper intake levels (ULs), which are threshold values not to be exceeded to avoid toxic effects.

Chromium concentrations in all seaweed species exceeded the RDA for the element. However, this is no cause for concern as Cr does not have a UL due to the general consensus that Cr does not cause toxic effects despite the concentration ingested, as expulsion of the element is carried out by the body before adverse effects ensue (Levine et al. 1968).

All seaweed species were found to be rich in Ca (contributes > 19% towards the RDA), Cr, Cu (> 12%), Fe (> 21%), Mg (>21%), Mn (> 7%) and Se (>36%). *C. virgatum* was extremely rich in Cu (253% towards RDA) and Se (141% towards RDA). This implies that this species can be used to overcome nutrient deficiencies and malnutrition especially in areas close to coastal regions where seaweeds are more readily available. This species also had low levels of toxic metals. *G. canaliculata* was also found to be rich in Cu, Fe and Se however, it was also found to contain high levels of toxic elements.

Table 3.3: Dietary Reference Intake (DRI), Recommended Dietary allowance (RDA) and Tolerable Upper Intake Level (UL) for an element for most individuals and average concentration of elements (n = 3) in 10 g of seaweed (dry mass, DM).

Element	DRI (mg/day)		% Contribution to RDA (10 g/day)												
	RDA	UL	*AB*	*CF*	*CV*	*CC*	*CM*	*DT*	*ECE*	*GA*	*GC*	*JR*	*JS*	*SE*	*UR*
Ca	1000-1300	2500	52	35	29	22	23	20	34	24	19	61	32	21	45
Co	ND	1.4	1	0.7	1.4	0.8	3.4	6	4	6.6	4.8	2.8	2.7	2.3	7.8
Cr	0.024-0.035	ND	56	580	119	168	340	212	125	221	252	161	116	191	177
Cu	0.9	8	28	27	253	12	43	87	52	15	122	57	18	36	38
Fe	8-18	45	21	30	22	45	152	79	56	101	122	44	25	49	75
Mg	310-320	350	106	21	52	61	52	24	58	39	22	111	114	50	83
Mn	1.6-2.3	11	20	50	7	17	39	20	16	20	23	16	9	14	14
Ni	ND	1	0	2.13	0.83	1.49	1.85	1.34	0.44	0.42	0.82	0.9	0.65	0.6	0.2
Se	0.055	0.4	79	87	141	76	65	84	88	36	111	41	66	80	42
Zn	8-11	34	2	2.7	5.6	7.2	4.1	1.7	0.7	0.8	2.5	3.1	1.8	2.1	3.3

[a] Institute of Medicine of the National Academies: Dietary Reference Intakes (2001).
[d] ND = not determined/determinable.

AB - Amphiroa bowerbankii, CF - Caulerpa filiformis, CV - Ceramium virgatum, CC - Codium capitatum, CM - Cystoseira myrica, DT - Dichotomaria tenera, ECE - Ellisolandia (Coralline) elongata, GC - Gracilaria canaliculata, GA - Gelidium abbottiorum, HC - Halimeda cuneata, JR - Jania rubens, JS - Jania (Haliptylon) squamata, SE - Sargassum elegans, UR - Ulva rigida.

Calcium and Mg are supplements taken to strengthen bones, regulate muscle and nerve function and control blood pressure. Statistics from the World Health Organization (WHO) show that South Africans are consuming only half of their RDA for Mg (WHO 2009). This is due to a poor diet or growth soil of agricultural crops being depleted in Mg. Hypocalcaemia (Ca deficiency) is a common nutritional problem in South Africa. A study conducted in 1979 in rural South Africa showed 13.2% of children to have abnormally low levels of Ca in their blood (Pettifor et al. 1978). To overcome this, food fortification strategies against malnutrition were implemented. Staples such as maize meal and bread flour are fortified with Fe and Zn to overcome its deficiency however for Ca and Mg intake, a balanced diet is recommended. Seaweeds, being a rich source of these nutrients can contribute significantly towards the RDA for these elements especially to rural South Africans and those living in the coast, where the nutritional status is still of concern. Therefore, the consumption of seaweeds, rich in essential nutrients and low in toxic elements, should be promoted.

3.4 Conclusion

The elemental distribution in seaweeds were found to be in decreasing order of Ca > Mg > Fe > Cu > Mn > Zn > Cr > Co > Se > As > Pb > Ni > Cd. All edible species contained high levels of both macro and micro-elements with the corali species accumulating high levels of Ca. For the toxic elements, As and Pb, highest concentrations were found in Chlorophyta and lowest concentrations were found in Rhodophyta. For Cd, highest concentrations were found in Rhodophyta and lowest concentrations were found in Chlorophyta. Of the thirteen edible species studied, only three (*G. abbottiorum*, *E. (Coralline) elongata* and *C. virgatum*) are suitable for human consumption due to high levels of toxic elements being present in the other seaweed

species. These three species are also rich in essential nutrients, specifically *C. virgatum* which is rich in Cu and Se.

3.5 Acknowledgements

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CHAPTER 4: ANALYTICAL AND PHYCOCHEMICAL ANALYSIS OF THE GREEN SEAWEED SPECIES, CODIUM CAPITATUM

Abstract

Codium capitatum, a seaweed species belonging to the phylum Chlorophyta (green seaweed) and indigenous to the Indian Ocean, was investigated. The effect of geographical location on elemental uptake by the seaweed was evaluated by sampling along the east coast of KwaZulu-Natal, South Africa, and thirteen different analytes were analysed using inductively coupled plasma-optical emission spectrometry. A phycochemical analysis was also conducted to identify secondary metabolites which impart medicinal value to the species. Concentrations of elements in *C. capitatum* were found to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cu > As > Cr > Se > Co > Ni > Pb > Cd, with As concentrations at most sites being higher than threshold values set by the South African Department of Health however, these are known to be in organic form. The phycochemical analysis revealed the presence of clerosterol, an algal sterol known to possess anticancer properties. This study confirms the nutritional and medicinal benefits of the seaweed species however, the study also highlights the risk associated with excessive consumption due to potential toxicities of high As intake.

Keywords: Phycocompounds, arsenic toxicity, clerosterol, anti-cancer agent

4.1 Introduction

Metal toxicity is a serious health concern with As, Cd and Pb ranking among the priority elements due to their high degree of toxicity and presence in virtually all environmental matrices. These systemic toxicants classified as human carcinogens, are known to affect the central nervous system and can induce multiple organ damage, even at low levels of exposure (Sharma and Agrawal 2005). Toxicity of these elements depend on dose, route of exposure and chemical speciation which is further influenced by age as well as nutritional and physiological status of exposed individuals. The largest route of human exposure to As is the diet. This is also true for Cd with highest sources of exposure being mushrooms, shellfish, mussels, cocoa powder and dried seaweed. The greatest source of exposure to Pb is lead-contaminated dust particles, food, water, and paint.

Edible aquatic life is an intermediate reservoir through which toxic elements from rivers or oceans can be transferred to humans and may threaten human health if at elevated concentrations. Some seaweed species are edible and are consumed for their nutritional value; others have potential medicinal value and are utilised for their therapeutic effectiveness. Whatever the use, intake of one form (for instance, inorganic nutrients) does not preclude intake of the other (for instance, organic constituents).

Seaweed can be classified into three classes as Chlorophyta, Rhodophyta and Ochroophyta. These classes are based on colours of seaweed where Chlorophyta indicates green, Rhodophyta indicates red and Ochrophyta indicates brown (Minge et al. 2009). Chlorophyta (green seaweed), are named as such due to their green colour obtained from the chloroplasts that contain both chlorophyll a

and b (Domozych 1980). They also contain accessory pigments such as ß-carotene, xanthophylls and thylakoids (Paul and Fenical 1987).

Codium capitatum is a eukaryotic algae belonging to the empire Eukaryota, kingdom Plantae, phylum Chlorophyta, class Ulvophyceae, family Codiaceae and genus *Codium*. *C. capitatum,* more commonly known as "dead man's fingers", is an edible species indigenous to the Indian Ocean with distribution in South Africa, Kenya, Mozambique and Madagascar (WoRMS 1959). It is a dark green, almost black, species connected to a steady substratum acting as the holdfast of the system. It consists of protruding branches multiplying as it gets closer to the top of the seaweed. The braches themselves and edges are thick and rounded with a scaly, gelatinous and elastic texture.

Besides pigments, accessory pigments, sugars and carbohydrates commonly found in the phylum Chlorophyta, the class Ulvophyceae is also known to contain mycosporine-like amino acids that are utilised for osmotic regulation and damage defence. Other compounds such as ulvans, galactans and sulfated mannans that have been shown to possess significant biological activity have specifically been found in the genus *Codium* (Wang et al. 2014).

Due to the daily consumption of seaweed by many people living along the coast, their role in traditional medicine and potential for accumulating toxic metals from the growth environment, the accumulation potential of toxic elements and the phycochemical constituents in seaweed needs to be investigated. Previously, we reported on the nutritional composition and seasonal variation on elemental uptake of selected seaweeds from KwaZulu-Natal, South Africa (Magura et al. 2016). Herein, we report on elemental concentrations in the seaweed species, *Codium capitatum*, as a

function of geographical location and we also extract and isolate the major secondary metabolites from the species that provide its therapeutic effectiveness.

4.2 Materials and Methods

4.2.1 Sampling and sample preparation

Samples of *Codium capitatum* were collected at six sites along the east coast of KwaZulu-Natal. Sampling sites included Brighton Beach Ansteys (29°55′29″S 31°0′14″E) , Margate (30.8500° S, 30.3667° E) , Park Rynie (30.3167° S, 30.7333° E), Rocky Bay (S30° 20.120' E30° 43.923'), Scottsburgh (30°17′S 30°45′E) and Treasure Beach (17.8760° N, 77.7572° W). Samples were collected from within rock pools in the intertidal littoral zone during low tide, stored in plastic bags, rinsed to remove solid particles, dried at 40°C for 72 h, ground in a mill and stored in plastic bags for analysis.

4.2.2 Reagents and standards

All standards and reagents were supplied by Sigma Aldrich (St. Louis, USA) and Merck (Kenilworth, USA). Glassware was thoroughly rinsed and oven dried at 40°C, double distilled water was used throughout the analysis. Analytical grade reagents were used for samples and spectroscopic grade was used for the preparation of standards. Analytical grade solvents were used for extraction, HPLC grade was used for instrumental analysis and deuterated solvents were used for Nuclear magnetic resonance (NMR).

4.2.3 Organic content

The moisture content (percentage, %) was determined by weighing a portion of wet sample, drying in the oven at 40°C for 72 h and weighing the dried portion of sample.

$$\frac{Wet\ sample - Dried\ sample}{Wet\ sample}\ x\ 100$$

Dry ash content was determined by obtaining the mass of dried seaweed sample and crucible that was then incinerated in a furnace for 6 h at 600°C, placed in a desiccator for 2 h then re-weighed to obtain the mass of ash. The percentage (%) ash was calculated by dividing the initial mass by the final mass and multiplying by 100.

Fatty acid content was determined by obtaining the mass of hexane extract. Protein content was determined using a back titration method. Approximately 0.5 g sample was added to 10 g K_2SO_4, 25 mL concentrated H_2SO_4 and 1 $CuSO_4$ crystal and heated in a heating mantle to allow for complete digestion. The digest was diluted to 250 mL and added to a 500 mL round bottom flask where 45 g NaOH, 75 mL H_2O, Zn granules and litmus paper was added. The solution was distilled with the receiver flask already containing 50 mL HCl till one third remained. The residual HCl in the receiver flask was titrated with 0.1 M NaOH using a bromocresol green as an indicator. By assuming all nitrogen content in the sample was protein and calculating the number of residual moles that reacted in the titration, the number of moles (of protein present) which reacted with the H_2SO_4 was calculated and therefore the mass and percentage.

Carbohydrate content was determined by difference:

Carbohydrate % = Total mass dry weight (100%) - Dry ash (inorganic) - Protein % - Fatty acid %

4.2.4 Extraction, separation and isolation of compounds

Approximately 1.55 kg of the dried and ground sample was placed in a 2 L round bottom flask, immersed in dichloromethane (DCM) and allowed to shake on an orbital shaker for approximately 2 days. Filtrates were filtered through Whatman No. 1 filter papers, concentrated using a rotary evaporator and allowed to evaporate in the drying room to obtain 11.64 g of crude DCM extract. The same procedure was followed using methanol (MeOH) to give 20.16 g of extract.

Column chromatography was done using a wet silica gel (Merck 0.063-0.200 mm) packed column on the DCM extract. Fractions of 50 mL were collected using a hexane: ethyl acetate (EtOAc) (9:1, v/v) solvent system. Fractions were profiled using thin layer chromatography (TLC) (Merck aluminum sheets F254), and visualized using the Ultraviolet (UV) lamp and a 10% H_2SO_4/MeOH acid bath dye. Compound **1** was obtained from fraction 33 which produced a single spot on the TLC plate. This fraction was dissolved in deuterated chloroform ($CDCl_3$) and taken for 1D and 2D NMR spectroscopy using the Bruker Avance III 400 MHz spectrometer.

4.2.5 Digestion and elemental analysis of samples

Digestion of samples was carried out using a closed vessel digestion technique with the aid of the CEM Microwave Accelerated Reaction System (MARS 6) (CEM Corporation, Matthews, NC, USA) with patented EasyPrep™ technology consisting of EasyPrep™ vessels fitted with an integrated thermowell coupling system.

Approximately 0.25 g dried seaweed sample as well as certified reference material (CRM), BCR 402 white clover, was weighed out into the vessels with 10 mL HNO_3 and allowed to pre-digest for 1.5 h before microwave digestion. The power was set at 1600 W, ramped to 170°C for 15 min,

held for 15 min and cooled for 15 min. Digests were gravity filtered into 25 mL volumetric flasks with Whatman No. 1 filter paper to remove residual particulate matter and made up to the mark with double distilled water (Kisten et al. 2015). This was done in triplicate. Samples were stored in plastic bottles in the refrigerator at 4°C until analysed.

Samples were analysed using the Perkin Elmer® Optima™ 5300 Dual View Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) (Billerica, Massachusetts, USA) with axial plasma viewing for 13 elements (As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, and Zn). This was the method of choice due to simultaneous elemental determinations and low detection limits. Wavelength selection was based on CRM data; the wavelength that produced the most accurate results with no interferences was chosen (Quevauville et al. 1992).

4.2.6 Statistical analysis

Comparisons and similarity groupings of data was observed by one way ANOVA as well as posthoc analysis (Tukey's) to determine significant differences amongst means using three replicates. Statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS version 23, IBM Corporation, Cornell, New York).

4.3 Results and Discussion

In *C. capitatum* species, the average moisture content was 88.3 ± 0.4% and dry ash was 47.0 ±0.1%. The organic content, based on dry mass, constituted of proteins (10.9 ± 0.2%), fatty acids (12.5 ± 0.3%) and carbohydrates (29.6 ± 0.2%). Generally, the ash content of seaweeds, including all Chlorophyta, Rhodophyta and Ochrophyta, ranges from 7 to 46% (Marinho-Soriano et al.,

2006; Matanjun et al., 2009), with Chlorophyta and Ochrophyta having higher ash content than Rhodophyta (Ruperéz, 2002). These percentages are consistent with the moisture content determined for *C. capitatum*. Dry ash content is indicative of inorganic minerals present in the species. For green leafy vegetables found in KwaZulu-Natal, South Africa, as indicated in previous studies, the ash content ranged from 1.3 to 3% (Odhay et al. 2007; Afolayan and Jimoh 2009; Schönfeldt and Pretorius 2011). This illustrates that the mineral content of seaweed is much higher than that of land plants.

Generally, the protein content ranges from 7-13% in Rhodophyta species (Norziah and Chio 2000), 11-24% in Ochrophyta species (Matanjun et al. 2009) and 15-20% in Chlorophyta species (Lahaye et al. 1999). The exception is the blue-green algae known as Spirulina (used as a supplement) with protein content as high as 70% (Burtin 2003). The lipid content of most seaweed species range from 1-5% (Burtin 2003); this indicates that *C. capitatum* produces more than double the normal amount. Carbohydrates in seaweed range from 4.5 to 40% (McDermid and Brooke 2003); carbohydrates in *C. capitatum* are closer to the upper range.

4.3.1 Elemental analysis

4.3.1.1 Quality assurance

Method validation for the analytical method was carried out using the certified reference material, BCR-402 - white clover. The results show experimentally obtained values to fall within the stipulated certified ranges for As, Co and Se and within acceptable ranges for Cr, Fe, Ni and Zn. These values are presented in Table 4.1.

Table 4.1: Comparison of measured and certified/indicative values (Mean ± SD, at 95% confidence interval, n = 3), based on dry mass, in the certified reference material (White clover, BCR - 402).

Element	Wavelength (nm)	Certified/Indicative[a] concentration (μg g^{-1})	Measured concentration (μg g^{-1})
As	197.197	0.093 ± 0.010	0.10 ± 0.014
Co	228.616	0.178 ± 0.008	0.184 ± 0.006
Cr	283.563	5.19	4.98 ± 0.248
Fe	238.204	244	235 ± 0.74
Ni	231.604	8.25	8.02 ± 0.312
Se	196.026	6.70 ± 0.25	6.90 ± 0.03
Zn	206.200	252	255 ± 2.68

[a] Indicative values are those without uncertainties.

4.3.1.2 Elemental distribution in various seaweed samples

The elemental concentrations in *C. capitatum* found at the eight different sites are presented in Table 4.2. The major elements, Ca and Mg, were found to range from 10998 - 38921 μg g^{-1} and 14852 - 21499 μg g^{-1}, respectively while the ranges (in μg g^{-1}) for the minor elements were Fe (501 – 613), Mn (30.85 - 34.78), Ni (0.18 – 2.8), Zn (17.19 - 30.76), Cu (10 - 12.58), Cr (4.21 - 5.67), Se (3.57 - 4.7) and Co (0.98 - 1.69). In general, the elements were found to be in decreasing order of Ca > Mg > Fe > Mn > Zn > Cu > As > Cr > Se > Co > Ni > Pb > Cd. The data shows concentrations of major elements to have a large range of variation whilst concentrations of minor

elements have a small range of variation. This indicates that *C. capitatum* species control uptake of minor essential elements to meet physiological requirement levels but external inputs appear to influence uptake of major essential elements. These inputs could be wastewater from industrial effluent found in nearby factories or domestic effluent from residential properties situated just above the tide line.

Due to seaweed falling within the Plantae kingdom, it can be compared to nutrient content, mechanisms and general trends of edible land plants. Generally, in leafy green vegetables, Ca concentrations are extremely high and much higher than that of Mg. Calcium has structural roles in plant cell walls and membranes whilst Mg has structural roles in chlorophyll molecules and ribosomal aggregates. Despite their unique physiological roles in plants, Ca and Mg uptake, transport and localisation is likely to be controlled by common regulatory networks due to their chemical similarity (Loneragen and Snowball 1969). This also appears to be true for *C. capitatum* however, unlike leafy green vegetables, Ca and Mg concentrations are more comparable.

For the toxic elements, As, Cd and Pb, concentrations (in $\mu g\ g^{-1}$) ranged from 6.98 - 15, 0.01 - 0.96 and 0.56 - 1.68, respectively. Ochrophyta species are known to contain high concentrations of As with *Hizikia fusiforme* (Hijiki) (109 $\mu g\ g^{-1}$, 71-12% in inorganic form) and *Sargassum elegans* (105 $\mu g\ g^{-1}$, 31% in inorganic form) having the highest concentrations (Magura et al. 2016; Rose et al. 2007). Nori, of the Rhodophyta, is also known to contain high concentrations of As (24 $\mu g\ g^{-1}$) but still lower than Ochrophyta (Rose et al. 2007). Chlorophyta species are not known to contain high levels of As.

Table 4.2: Elemental concentrations (μg g^{-1}) in *Codium capitatum* species found at 8 different sites.

	1	2	3	4	5	6	7	8
As	6.98±1.39	15±1.4	11.12±0.16	9.78±0.87	10.88±0.21	8.34±0.09	7.86±0.15	9.11±0.20
Ca	38921±1751	10998±1595	22955±1000	19873±1212	28741±975	20512±1123	21897±623	23951±146
Cd	0±0	0±0	0.58±0.11	0.96±0.08	0.23±0.16	0.10±0	0.12±0.03	0±0
Co	1.34±0.52	1±0.31	1.25±0.15	1.69±0.25	0.98±0.21	1.41±0.16	1.56±0.18	1.32±0.25
Cr	4.56±0.80	5.38±1.40	4.98±0.06	5.11±0.14	4.21±0.19	5.67±1.23	5.11±0.18	4.96±0.63
Cu	11.2±3	10±1.2	10.96±2.31	12.13±2.15	11.74±1.87	10.53±1.89	12.58±1.57	11.65±1.84
Fe	581±38	587±46	501±48	589±42	603±31	564±29	568±31	613±25
Mg	21499±821	16625±794	18987±621	17563±578	17200±402	20632±523	14852±426	15236±521
Mn	33.1±2	33.8±5.5	33.88±4.5	34.78±3.7	32.96±2.9	31.58±1.6	32.56±2.9	30.85±1.7
Ni	0.18±0.15	2.8±1.11	1.56±0.18	2.20±0.26	2.67±1.56	1.99±0.95	2.23±1.24	1.69±0.87
Pb	0.56±0.19	1.54±0.54	1.21±0.32	1.68±0.65	0.75±0.54	0.94±0.28	1.24±0.56	1.53±0.27
Se	3.65±1.36	4.7±1	3.68±1.23	4.12±1.58	4.69±1.27	3.57±1.45	4.23±1.65	3.97±1.52
Zn	30.76±3.55	17.19±2.12	19.56±3.17	22.54±2.54	25.87±3.24	27.65±2.47	20.16±2.01	24.38±2.62

1=Brighton Beach, 2=Margate, 3=Park Rynie, 4=Rocky Bay, 5=Scottsburgh, 6=Treasure Beach, 7=St. Lucia, 8=Zinkwazi.

The maximum limit for As in shellfish products set by the Department of Health, South Africa is 3.0 μg g^{-1} (Department of Health, 2003). In this study, the concentration of As in *C. capitatum* ranged from 6.98 - 15 μg g^{-1}, which is just over double to triple the threshold value. However, all forms of As are not toxic; inorganic As is considered more toxic than organic As. Unlike Ochrophyta species, Chlorophyta species are not known to have high levels of inorganic As but are known to contain more of the harmless organic forms (arsenobetaine, arsenocholine and arsenosugars).

Cadmium and Pb are heavy metals which are extremely toxic to human health. Cadmium concentrations are higher in Rhododphyta species, *Porphyra* (0.089 - 3.19 μg g^{-1}), *Palmaria palmata* (0.079 - 0.877 μg g^{-1}) and *Chrondus crispus* (0.418 - 0.722 μg g^{-1}) and Ochrophyta species, *Laminaria* (0.074 - 0.908 μg g^{-1}), *Hizikia fusiforme* (0.511 - 1.16 μg g^{-1}) and *Durvillaea antartica* (2.46 μg g^{-1}) (Caliceti et al. 2002; Almela et al. 2006). The highest concentration of Cd found in this study was 0.96 μg g^{-1}, with concentrations at most sites being below the instrument detection limit, thereby indicating a possibly polluted site (Förstner and Wittmann 2012). Previous studies showed Pb concentrations to be approximately 0.205 μg g^{-1} in Chlorophyta, to range from 0.123 μg g^{-1} (*Porphyra*) to 0.595 μg g^{-1} (*Palmaria*) in Rhodophyta, and to range from 0.14 μg g^{-1} (*Undaria pinnatifida*) to 0.885 μg g^{-1} (*Hizikia fusiforme*) in Ochrophyta (Van Netten et al. 2000; Lozano et al. 2003). Lead concentrations in *C. capitatum* ranged from 0.56 - 1.68 μg g^{-1} with the average concentration being 1.18 μg g^{-1}. The maximum limit for Cd and Pb in shellfish products set by the Department of Health, South Africa is 3.0 μg g^{-1} and 4.0 μg g^{-1}, respectively (Department of Health, 2003). *C. capitatum* is therefore safe for human consumption as it does not accumulate Cd or Pb.

4.3.2 Structure elucidation of compounds from Codium capitatum

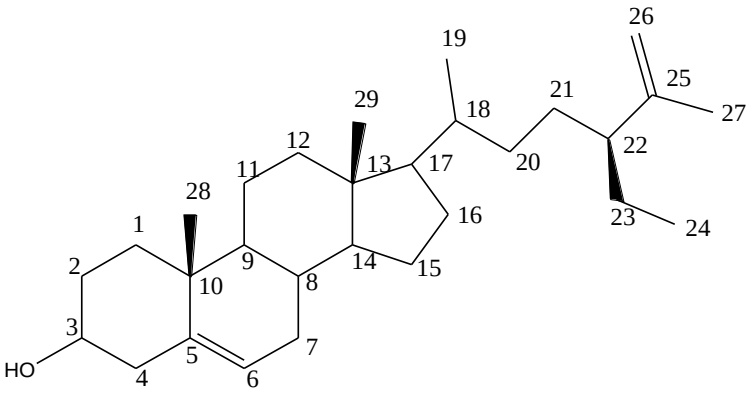

Figure 4.1: Structure of isolated clerosterol

^{1}H-NMR spectral data ($CDCl_3$, 400 MHz) δ_H ppm: 5.32 (IH, br d, J = 4.92 Hz, H-6), 4.70 - 4.61 (2H, br d, J = 1.8 Hz, H-26a, 26b), 3.50 (1H, m, H-3), 1.54 (3H, s, H-27), 1.04 (3H, s, H-19), 0.98 (3H, m, H-21), 0.87 (3H, t, H-29), 0.64 (3H, s, H-18).

^{13}C-NMR spectral data ($CDCl_3$, 400 MHz) δH ppm: 147.5 (C-25), 140.7 (C-5), 121.7 (C-6), 111.4 (C-26), 71.8 (C-3), 56.8 (C-14), 56.0 (C-17), 50.1 (C-9), 49.5 (C-22), 42.3 (C-10), 39.8 (C-13), 37.2 (C-12), 36.5 (C-1), 35.6 (C-20), 33.7 (C-24), 31.9 (C-7,8), 31.6 (C-2), 29.7 (C-23), 29.4 (C-4), 28.4 (C-16), 26.5 (C-28), 24.3 (C-15), 21.1 (C-11), 19.4 (C-21), 18.6 (C-19), 17.7 (C-27), 12.0 (C-18), 11.8 (C-29).

Compound 1 (37 mg) was isolated as a white, crystalline solid. The ^{1}H-NMR spectrum indicated the presence of a double bond at δ_H 5.33 (s, H-6), a hydroxyl group at C-3 due to the downfield shift of the resonance at δ_H 3.50 (m, H-3) and a terminal double bond due to the two geminal, vinylic proton resonances at δ_H 4.61- 4.70 (H-26a, 26b). The ^{13}C-NMR spectrum resolved twenty-nine carbon resonances with resonances at δ_C 147.5 (C-25), δ_C 140.7 (C-5), δ_C 121.7 (C-6) and δ_C 111.4 (C-26) in the double bond region. The GC-MS spectrum showed a base peak at m/z 394

[M^+] indicating the tendency of the compound to fragment and cleave off water. The molecular ion peak at m/z 412 [M^+] corresponded to the molecular formula for clerosterol ($C_{29}H_{48}O$). Based on the spectral data and in comparison to literature values, compound **1** was identified as clerosterol (Fig. 4.1) (Kwon et al. 2003; Marín-Álvarez et al. 2013). Clerosterol was previously isolated from the green algae, *Codium fragile*, and was shown to possess anticancer activity through apoptosis of melanoma cells (Kim et al. 2013).

4.4 Conclusion

Elemental analysis of *C. capitatum* showed the seaweed species to contain adequate levels of essential nutrients. The effect of site did not influence elemental uptake of essential micronutrients but seemed to have affected uptake of the essential macro-elements, Ca and Mg. The levels of the toxic elements, Cd and Pb, were found to be within acceptable limits however, concentrations of As were high enough to necessitate caution when it comes to its over consumption. The phycochemical analysis revealed the presence of clerosterol, which has previously been shown to possess anti-cancer potential. This is the first phycochemical report on *C. capitatum*. The results herein confirm the nutritional and medicinal value of this green seaweed species.

4.5 Acknowledgements

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WoRMS: world register of marine species

http://marinespecies.org/aphia.php/aphia.php?p=taxdetailsandid=211628

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

5.1 Summary

The aim of this study was to conduct an elemental analysis on different seaweed species found along the KwaZulu-Natal coastal region. Fourteen different species were chosen for the study. Of these, eight were Rhodophyta, four were Chlorophyta and two were Ochrophyta species. The nutritional value of the edible seaweed species (thirteen) studied was assessed by comparing elemental concentrations of essential nutrients in them to RDAs to determine their potential as a source of nourishment. Metal toxicity is a serious health concern with As, Cd and Pb ranking among the priority elements due to their high degree of toxicity and presence in virtually all environmental matrices. The seaweed species studied were also evaluated for accumulation of these systemic toxicants due to the nature of the environment in which they grow. The suitability for human consumption was evaluated by comparing levels of toxic elements to maximum permissible limits. The impact of geographical location on elemental uptake in seaweed was determined with focus on *C. capitatum*, a Chlorophyta species. Further, a phycochemical investigation on *C. capitatum* was conducted by extracting, isolating and identifying the major secondary metabolites in the species to provide a scientific basis for its ethno-medicinal use.

5.2 Findings from Elemental and Phycochemical Analysis

The elemental distribution in the fourteen seaweed species studied were found to be in decreasing order of Ca > Mg > Fe > Cu > Mn > Zn > Cr > Co > Se > As > Pb > Ni > Cd. Of the thirteen edible species studied, the elemental distribution showed higher levels of essential nutrients compared to

toxic elements. However, *C. capitatum* showed a tendency to accumulate higher levels of As and this was confirmed by evaluating elemental concentrations at different sites. Although the elemental distribution showed lower levels of toxic elements in edible seaweed, when compared to maximum permissible limits, these levels were found to be excessive. Only three edible species (*G. abbottiorum*, *E. (Coralline) elongata* and *C. virgatum*) had levels of toxic elements below maximum permissible limits which make them suitable for human consumption without the fear of elemental toxicity; these three species also had adequate levels of essential nutrients which could contribute significantly to the diet and if consumed by vulnerable communities living along coastal regions of South Africa, it would go a long way in combating the fight against malnutrition. The phytochemical analysis of *C. capitatum* resulted in the isolation and identification of one sterol, clerosterol, an algal sterol known to possess anticancer properties.

5.4 Conclusion

The study conducted has contributed to the base of knowledge on seaweed species found along the east coast of KwaZulu-Natal, South Africa. This study highlighted the potential of seaweed as a food source; it highlighted the ability of certain seaweed species to accumulate toxic elements and the potential of others to exclude them. The study showed that all edible seaweed species studied can contribute positively to the diet however; caution should be exercised when consuming certain species as overconsumption could result in metal toxicities.

5.5 Further Studies Recommended

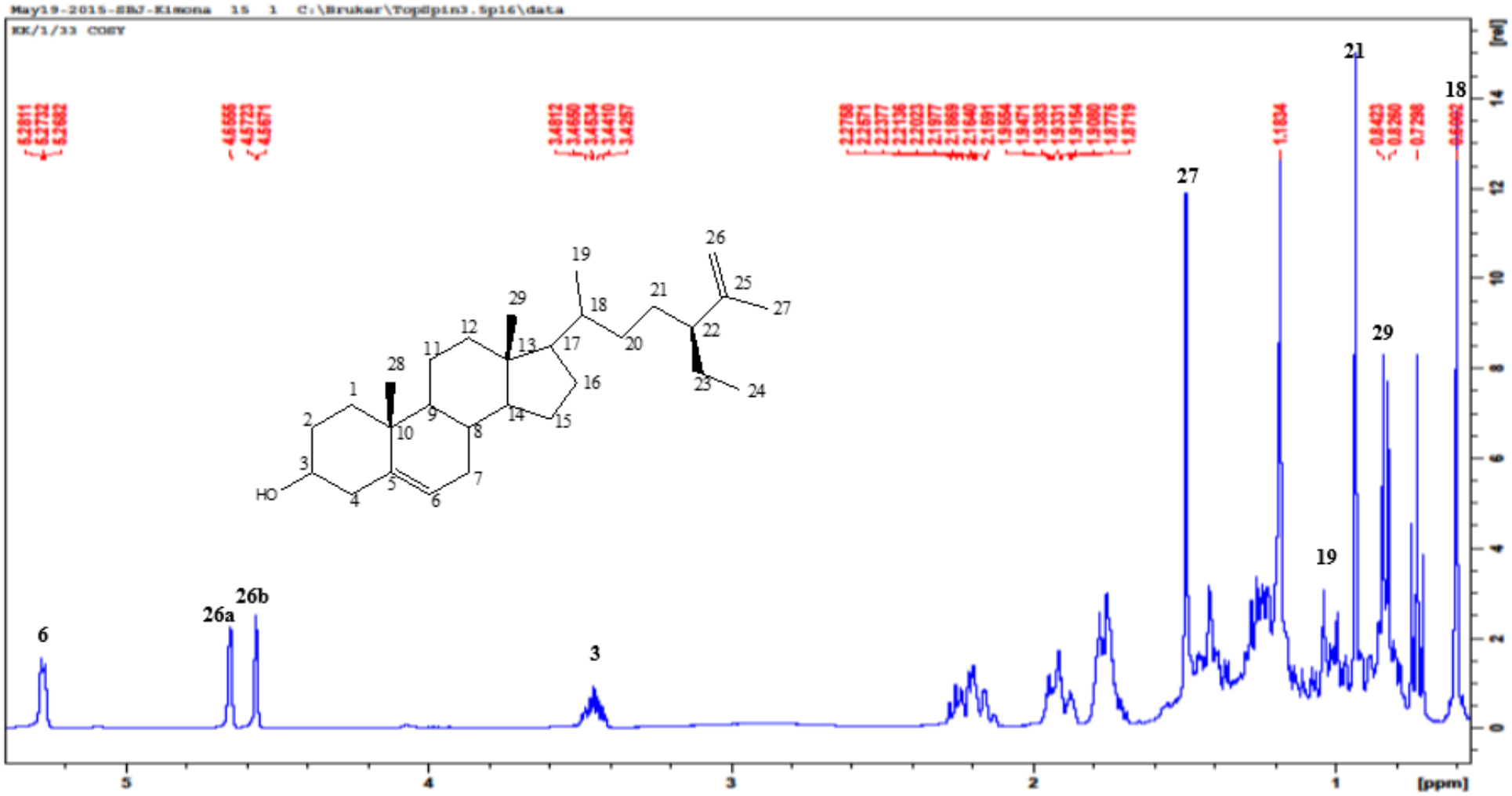
- Phycochemical analysis on other indigenous seaweed species found in South Africa.
- Biological testing of the isolated phycocompounds.
- Speciation studies of toxic elements.

APPENDIX

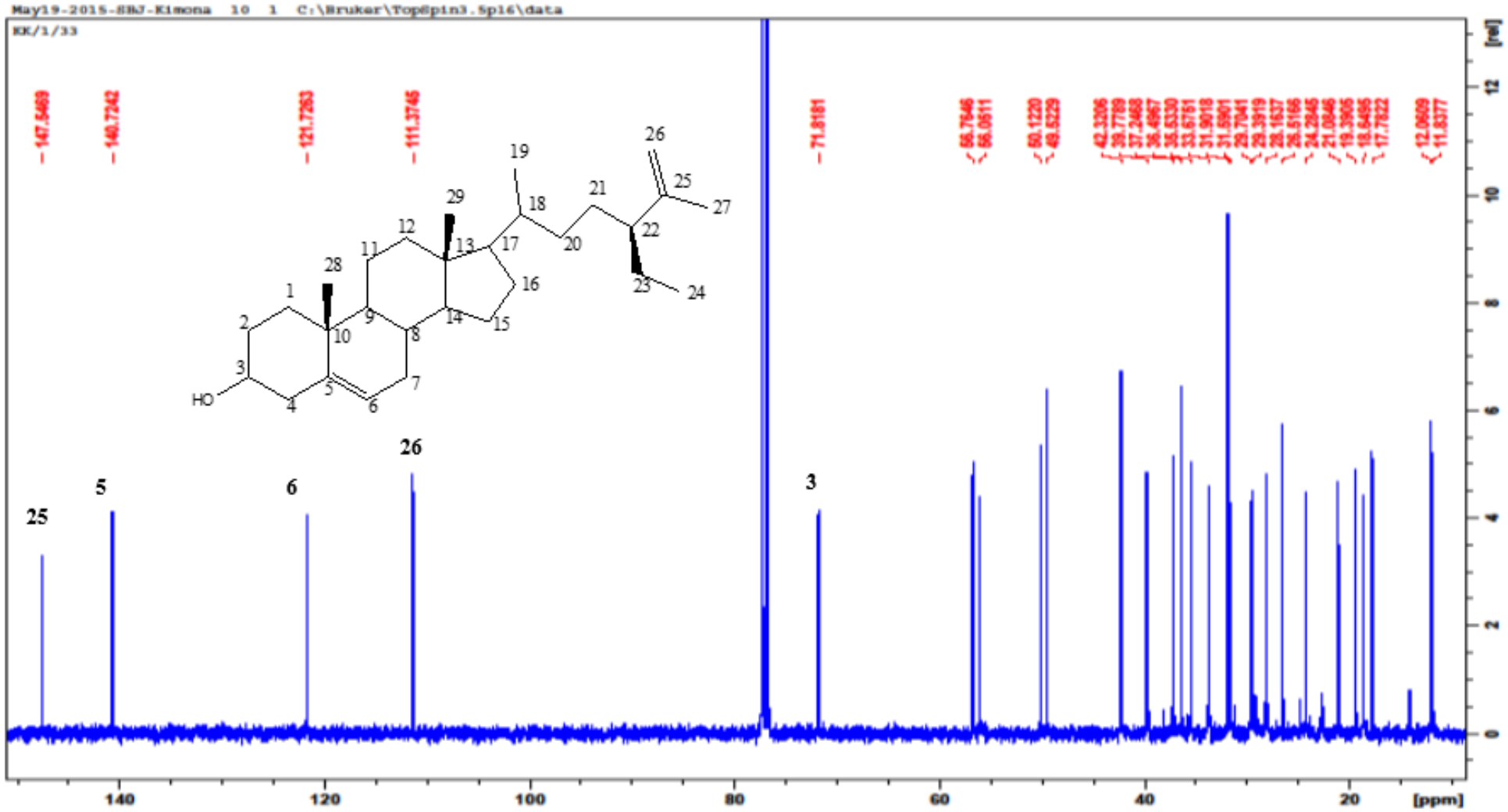
Supporting data information

NMR and GC-MS spectra

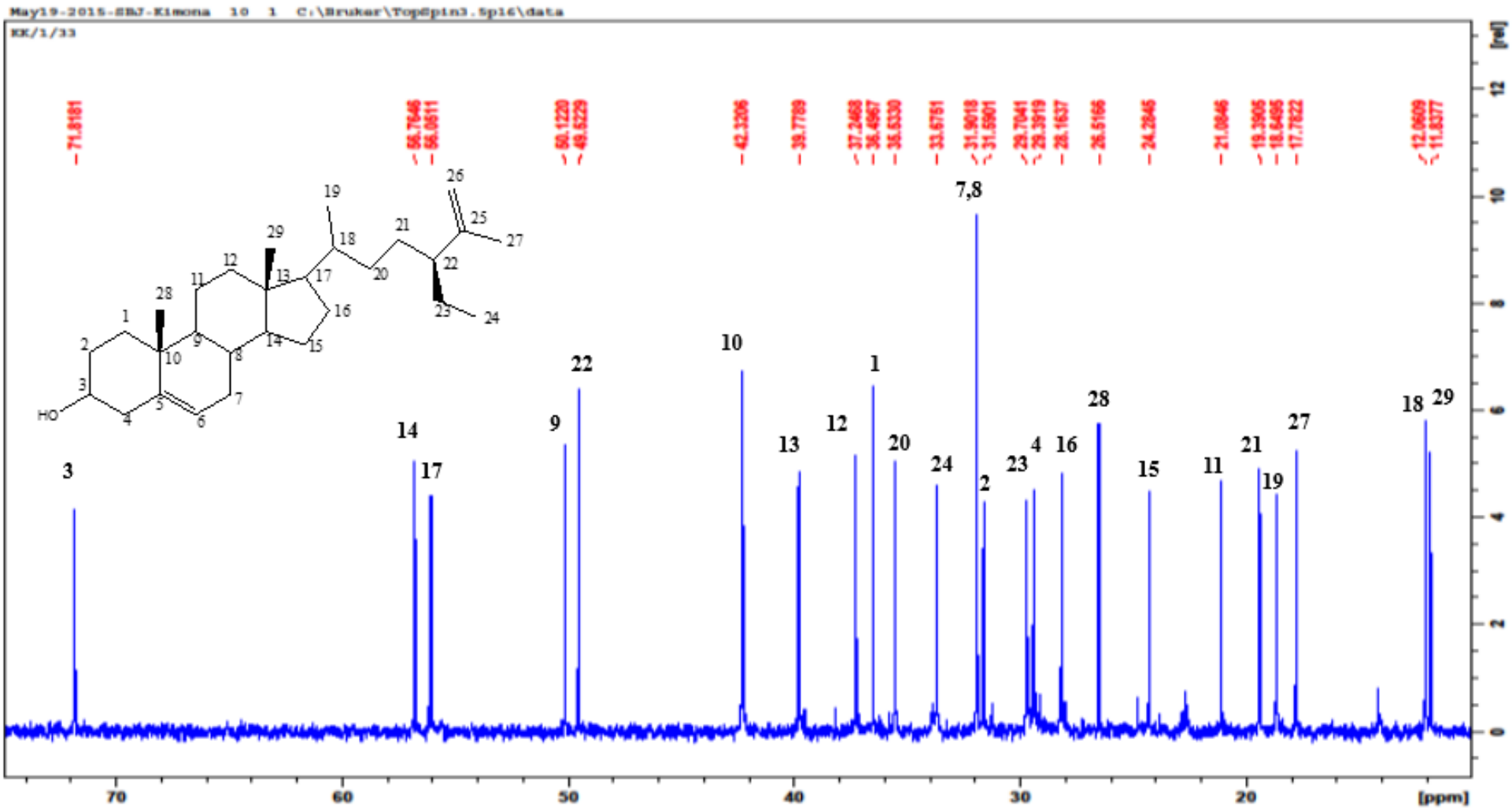
CRM certificate



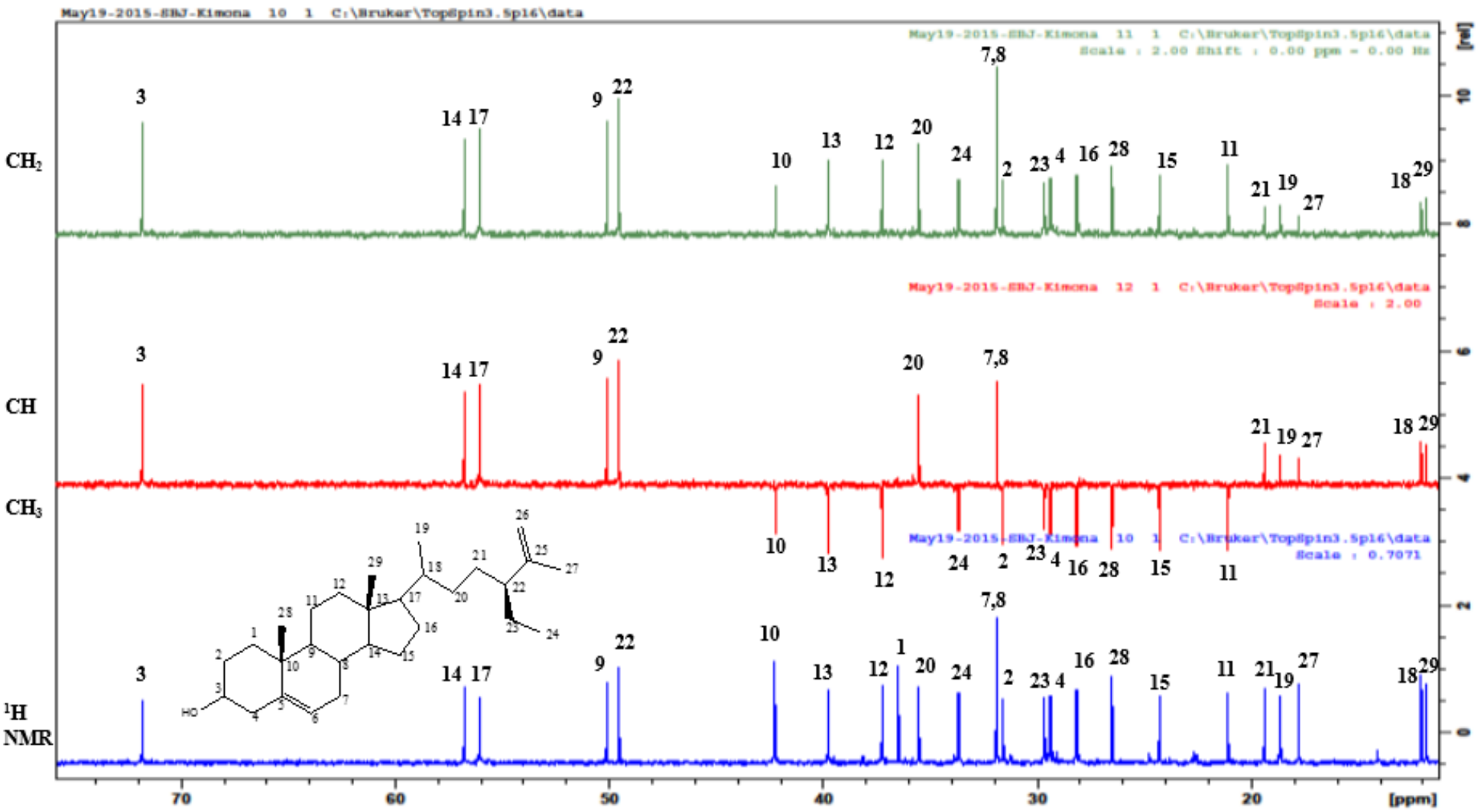
^{1}H NMR spectrum for Clerosterol



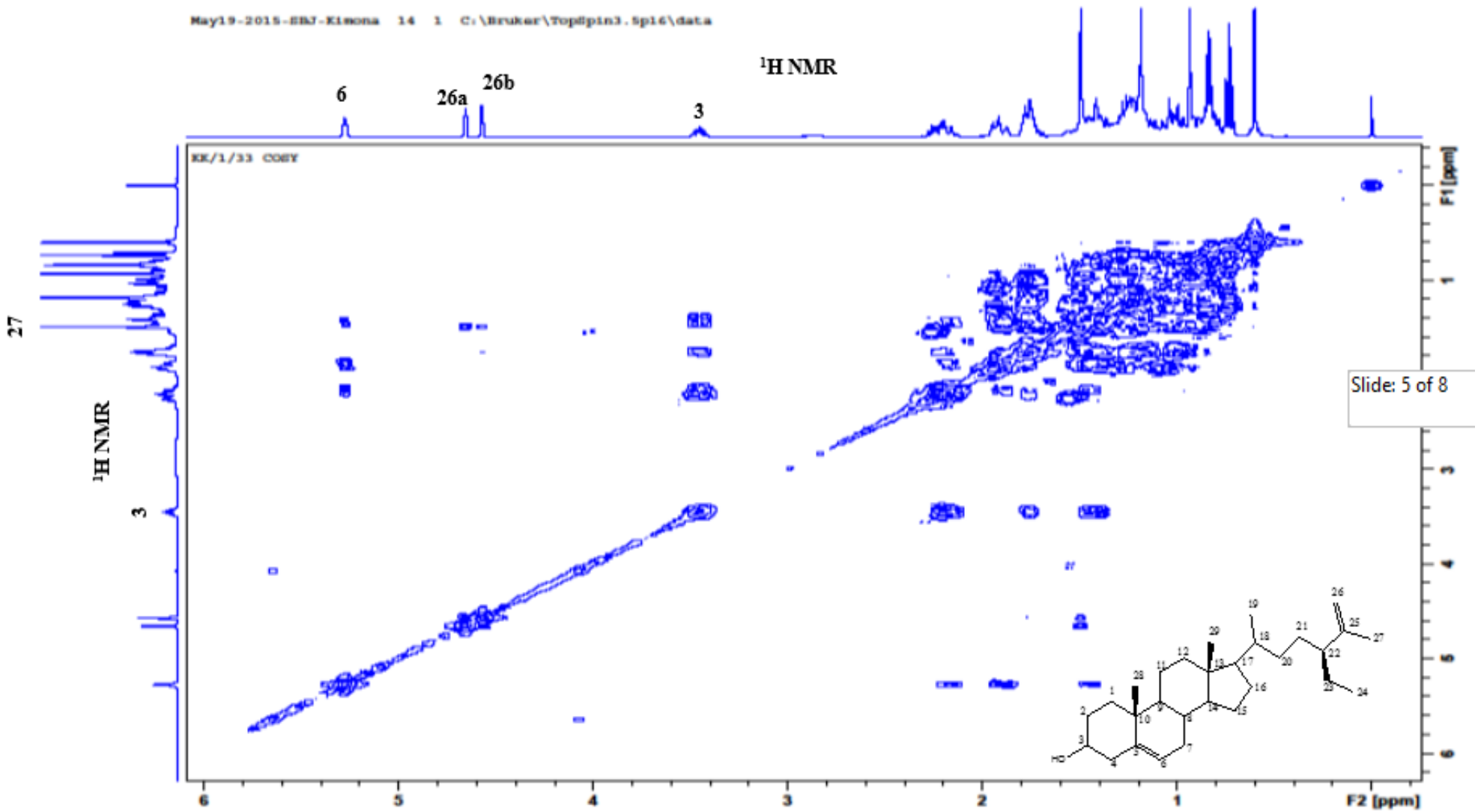
^{13}C NMR spectrum of Clerosterol



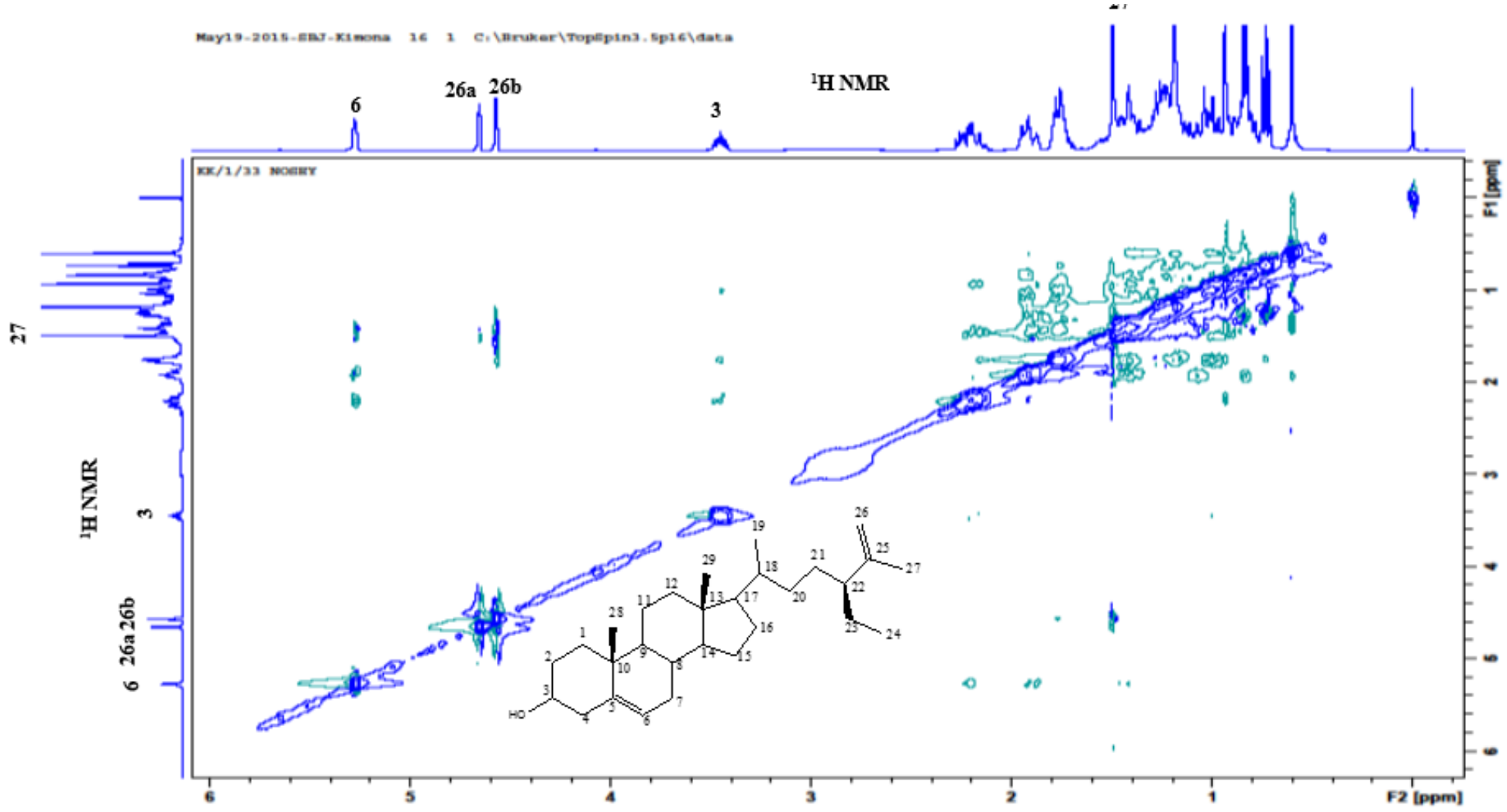
Expanded ^{13}C NMR Spectrum of Clerosterol



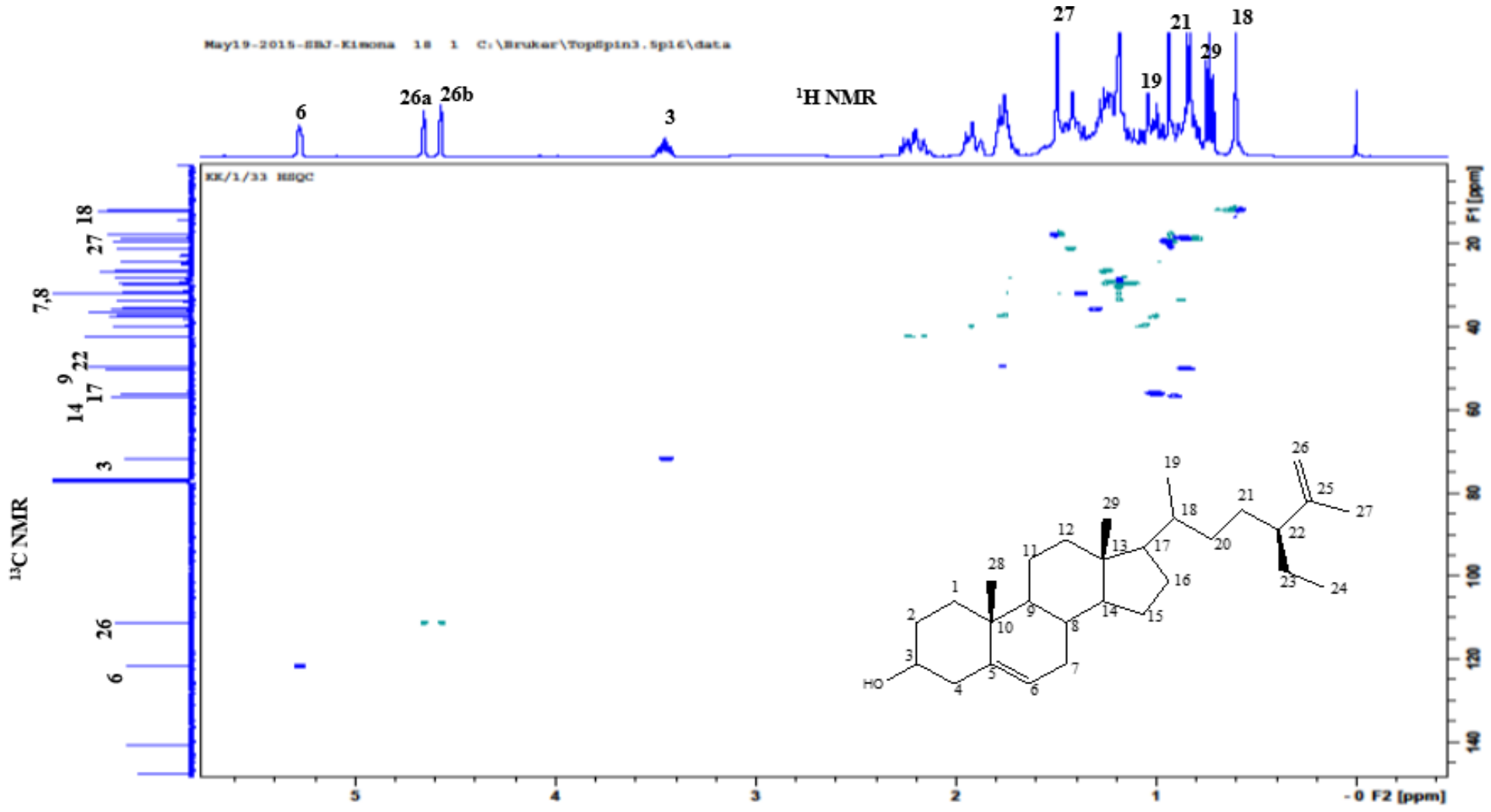
Expanded DEPT 90 and 135 spectrum for Clerosterol



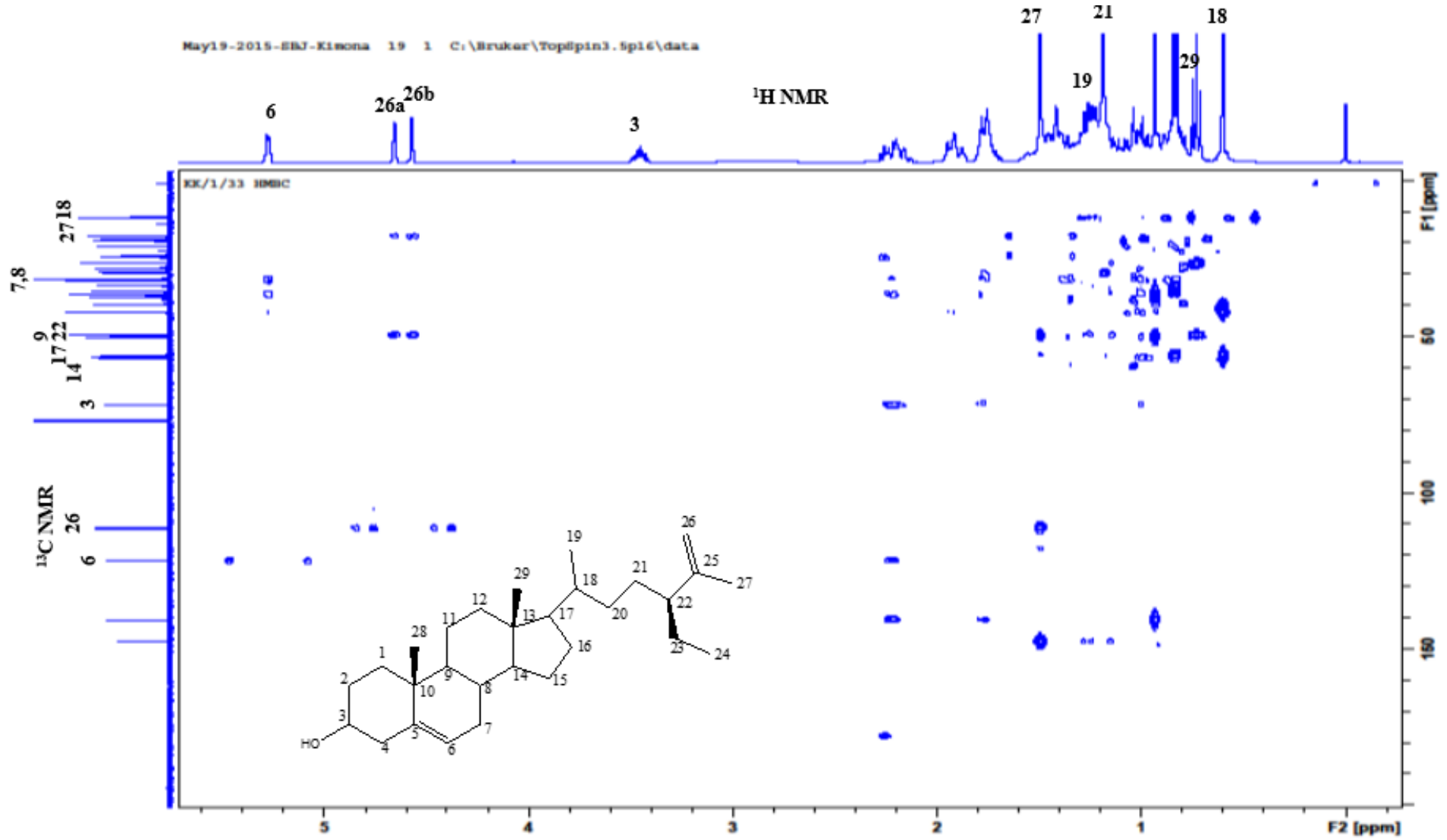
COSY spectrum of Clerosterol



NOESY spectrum of Clerosterol



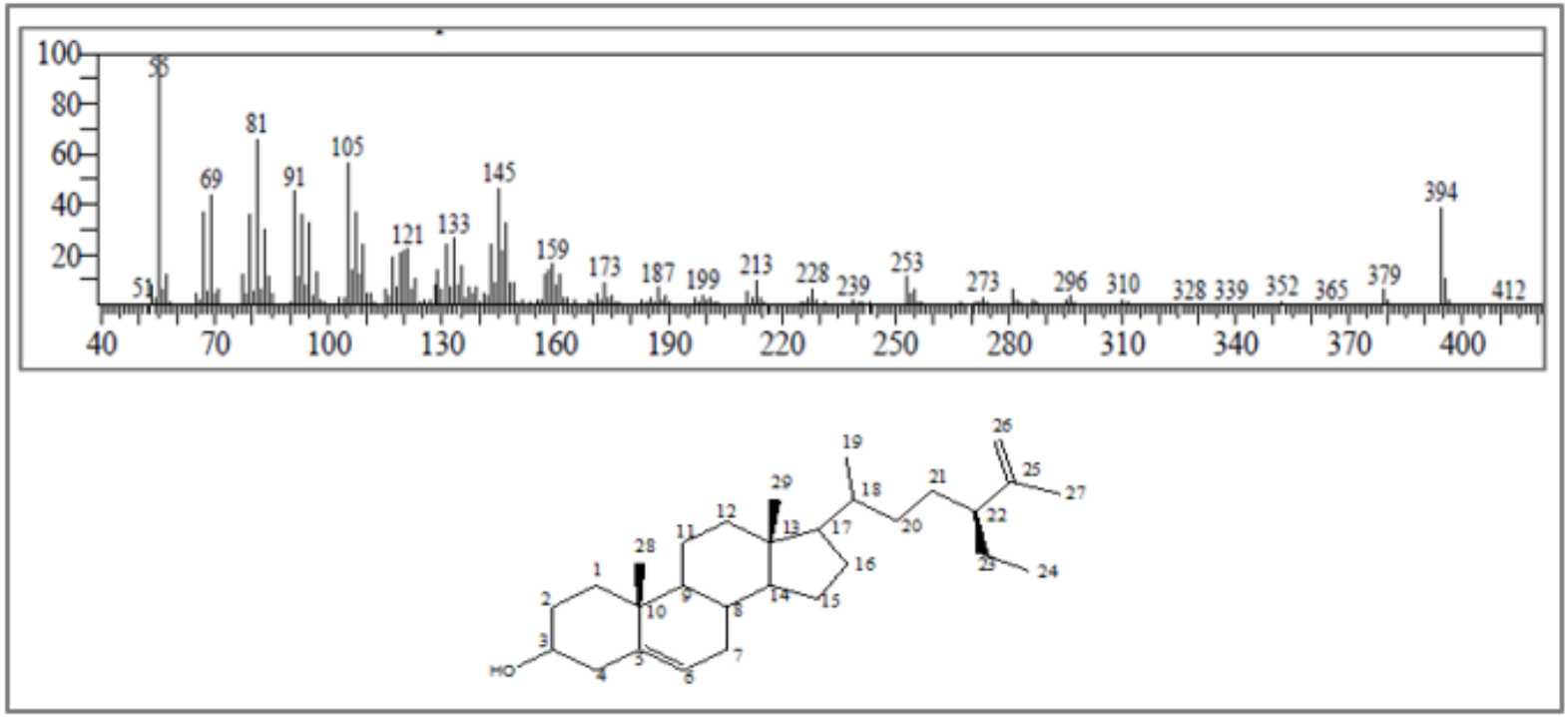
HSQC spectrum of Clerosterol



HMBC spectrum of Clerosterol

Sample Information

Analyzed by	: Neal
Analyzed	: 2016-10-05 12:46:31 PM
Sample Type	: Unknown
Level #	: 1
Sample Name	: Plant extrct isolated compound
Sample ID	: Plant extrct isolated compound
IS Amount	: [1]=1
Sample Amount	: 1
Dilution Factor	: 1
Vial #	: 2
Injection Volume	: 0.50
Data File	: C:\GCMSsolution\Data\Kimona Kisten\05102016\Plant extract Isolated compound.
Org Data File	: C:\GCMSsolution\Data\Kimona Kisten\05102016\Plant extract Isolated compound.
Method File	: C:\GCMSsolution\Methods\Kimona Plant Extract.qgm
Org Method File	: C:\GCMSsolution\Methods\Kimona Plant Extract.qgm
Report File	:
Tuning File	: C:\GCMSsolution\System\Tune1\2014Tune files\2016\28092016.qgt
Modified by	: Neal
Modified	: 2016-10-05 01:57:32 PM



GC-MS spectrum of clerosterol

EUROPEAN COMMISSION

JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements

Institute for Reference Materials and Measurements

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 couples 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)
- An Forais Taluntais, Wexford (IE)
- Aristotelian University, Lab. Anal. Chemistry, Thessaloniki (GR)
- Centre de Recherches Forestières, Nancy (FR)
- Ecole Européénne des Hautes Etudes des Industries Chimiques, Strasbourg (FR)
- E.C.N. Energieonderzoekcentrum, Petten (NL)
- Forschungszentrum für Umwelt und Gesundheit, Neuherberg (DE)
- Istituto Superiore di Sanità, Roma (IT)
- Institut National de Recherches Agronomiques, Pont-de-la-Maye (FR)
- Instituut voor Nucleaire Wetenschappen, Rijksuniversiteit Gent (BE)
- Instituut voor Scheikundig Onderzoek, Tervuren (NL)
- 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)
- Risø National Laboratory, Roskilde (DK)
- Service Central d'Analyse, CNRS, Vernaison (FR)
- Universidad de Barcelona, Faculdad de Quimica, Barcelona (ES)
- Universidad Complutense, Faculdad de Quimica, Madrid (ES)
- Università di Pavia, Chimica Generale, Pavia (IT)
- Universität Ulm, Sektion Analytik und Höchtsreinigung, Ulm (DE)