A HPLC-ESI-MS/MS Study of Hydroxybenzoic Acids and Related Derivatives in Commercial Seaweed Biostimulants and their Plant Growth Bioactivity

Thesis submitted in fulfilment of the requirements of the degree of
Doctor of Philosophy

by

Daniel Collins

BSc (Hons) (Chemical Sciences)

Institute for Sustainable Industries and Livable Cities

Victoria University

May 2022

ABSTRACT

The rapidly growing world population, increasing severity of climate change, and constantly evolving environmental pressures have drawn into question whether current agricultural practices can meet the growing food demands healthily, equitably and sustainably. This has resulted in the rising popularity of natural biostimulants, particularly seaweed extracts, to increase crop productivity in an eco-friendly and safe manner. To better understand the complex modes of action underpinning the well-reported benefits of seaweed biostimulants to crops, their phytochemical composition requires further characterisation. Hydroxybenzoic acids, a subclass of phenolic acids, are an important class of phytochemicals and the aim of this study was to characterise their profile in commercial seaweed biostimulants. This work used modern analytical technologies to investigate salicylic acid and other benzoic acid derivatives in a commercial seaweed biostimulant, and then assessed the biological activity of the monohydroxybenzoic acids using plant growth assays.

Qualitative HPLC-ESI-MS/MS methods were developed for the analysis of hydroxybenzoic acids and related derivatives. The various benzoic acid derivatives investigated include monohydroxybenzoic acids, dihydroxybenzoic acids, trihydroxybenzoic acids, methoxylated hydroxybenzoic acids, methoxylated benzoic acids, and an amino substituted benzoic acid. The HPLC-ESI-MS/MS methods for the analysis of the various derivatives were then employed to investigate the presence of these compounds in the commercial seaweed biostimulant. The compounds found to be present were the monohydroxybenzoic acids, 2,3- and 3,4-dihydroxybenzoic acid, syringic acid, and anthranilic acid.

A HPLC-ESI-MS/MS method for the analysis of the monohydroxybenzoic acids was optimised and partially validated for the quantification of salicylic acid and its isomers in a commercial seaweed biostimulant. Sample preparation employed acidified acetonitrile partitioning of the seaweed biostimulant before mixed-mode solid-phase extraction. The three isomers were successfully separated using a reversed-phase biphenyl stationary phase with a methanol/water mobile phase acidified with formic acid. The MS/MS detection employed the characteristic MRM transition of m/z 137 → 93 of the monohydroxybenzoic acids. The concentrations of 2-, 3- and 4-hydroxybenzoic acid in a commercial seaweed biostimulant were found to be 137, 3409, and 1748 μg/L, respectively.

Tomato seedling plant growth bioassays were conducted to investigate the biological effects of salicylic acid and its isomers on plant growth. Fresh and dry root and shoot weight data along with longest root length data were assessed to evaluate the biological effects of the various treatments on tomato seedling growth. It was found that a significant increase in root growth was observed when the commercial seaweed biostimulant was fortified with a combination of the three monohydroxybenzoic acids, using dosages that correlate to the concentrations determined in the seaweed biostimulant in this study.

DECLARATION

"I, Daniel Lewis Collins, declare that the PhD thesis entitled "A HPLC-ESI-MS/MS Study of Hydroxybenzoic Acids and Related Derivatives in Commercial Seaweed Biostimulants and their Plant Growth Bioactivity" is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

"I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures."

Signature: 

Date: 26/5/22

DEDICATION

I would like to dedicate this thesis in loving memory of the friends and family that are no longer with us; with whom I will sadly not be able to share this achievement.

ACKNOWLEDGEMENTS

It is extraordinarily difficult to find the words to thank all those who have helped make this achievement a reality. If I have missed anyone, I do apologise.

First and foremost, I would like to express my sincere thanks and gratitude to my principal supervisor, Associate Professor Domenico Caridi, for his unyielding support of me throughout my PhD journey. His immeasurable contributions of time and energy along with his extensive knowledge of analytical chemistry were essential to the completion of this research project and publication of this thesis. I am indebted to him for his devotion and compassion throughout my research journey, which was not without its hard times. It has truly been an honour learning at your side.

I am very grateful to my industry research supervisor, Dr. Tony Arioli, at Seasol International, Australia, for his ongoing support, positivity, and great knowledge of plant biology and agriculture. Without your patience, dedication and encouragement the completion of this project would not have been possible. Additionally, I would like to extend my thanks to Seasol for facilitating the bioassay studies at their research laboratories and greenhouses. Special thanks also to Marianne Weisser for her technical assistance with the bioassays.

My thanks to my associate supervisor, Professor Andrew Smallridge, for his willingness to assist and provide advice during the research project, and for the countless hours spent revising and editing this thesis. You had high expectations and were demanding, but only to ensure that I continued to strive to be the best possible researcher I could be, for that I am thankful.

Special thanks to Associate Professor Randall Robinson and the other science academics for the morale-boosting chats. Thank you to those at the research office whose support and creativity in the final stages of the PhD were priceless. Though I was not fortunate enough to work alongside many other chemistry researchers in the laboratory, there were other research students whose support I am incredibly thankful for, in particular, Sam, Gayle, Amber and Silke. Special thanks to the technical staff at Victoria University including Nishantha, Melinda, Larry, Min, and Stacey, as well as Joseph Pelle formally of Victoria University.

Thanks to the team at Shimadzu for all their technical assistance and support throughout the PhD, in particular, Rudi, Hung and Peter, as well as Dr. Paul Wynne formally of Shimadzu. Thanks also to the team at Analytical Consulting Services, Kensington, Australia, for valuable discussions, in particular, Sandy and Vince.

Thanks to my friends and family for all their love and support. This collective of irreplaceable people in my life give me the confidence to be me and provide me strength when I need it. I unconditionally love every last one of you and look forward to sharing this achievement with you. Special thanks to Dr. Kaylene Evers for her role in nurturing my mental health.

Finally, my loving partner Natalie. I can't thank you enough for your unwavering support, love and patience throughout this difficult and time-consuming period of my life. I adore you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionisation
API	atmospheric pressure ionisation
C18	octadecylsilane-bonded silica
C8	octylsilane-bonded silica
CE	collision energy
CID	collision-induced dissociation
DC	direct current
DHBA	dihydroxybenzoic acid
d-SPE	dispersive solid-phase extraction
EAE	enzyme-assisted extraction
EIC	extracted ion chromatograph
ESI	electrospray ionization
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
HBA	hydroxybenzoic acid
HMDB	human metabolome database
HPLC	high performance liquid chromatography
LC	liquid chromatography
LC-MS	liquid chromatography mass spectrometry
LSD	least significant difference
MAE	microwave-assisted extraction
MALDI	matrix-assisted laser desorption ionisation
MHBA	monohydroxybenzoic acid
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSDS	material safety data sheet
NATA	National Association of Testing Authorities, Australia
NMR	nuclear magnetic resonance
ODS	octadecylsilane-bonded silica
PDA	photodiode array detection (also referred to as DAD)
PI	product ion scanning analysis mode
Q	quadrupole mass analyser (often followed by a number denoting quadrupole position)
QqQ	triple quadrupole mass spectrometry
Quechers	"quick, easy, cheap, rugged and safe"
RF	radio frequency
RIC	reconstructed ion chromatograph
RP-LC	reversed phase liquid chromatography
SFE	supercritical fluid extraction
SIM	selective ion monitoring
SPE	solid-phase extraction
THBA	trihydroxybenzoic acid
TIC	total ion chromatograph
TOF	time-of-flight mass spectrometry
UAE	ultrasound-assisted extraction
UHPLC	ultra high performance liquid chromatography
UV	ultraviolet
UV/Vis	ultraviolet-visible

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 The Problem: Agriculture is Currently Not Meeting Global Demands.

The rapidly growing world population, increasing severity of climate change, and constantly evolving environmental pressures have drawn into question whether current agricultural practices can meet the growing food demands healthily, equitably and sustainably [1, 2]. The Food and Agricultural Organization of the United Nations (FOA) estimates that around one in every nine people in the world were undernourished in 2018 [2]. This indicates that global agricultural productivity is currently inadequate to meet demands; therefore modern agricultural systems require new and innovative solutions to meet future demands with the global population set to rise to approximately 9.7 billion by the year 2050 [3]. Current and past agricultural practices have been a significant contributor to environmental change whilst simultaneously being heavily impacted on by climate change in a chain of negative impacts that only reinforce themselves [4]. Therefore, a paradigm shift is required away from practices deemed 'unclean' and towards mitigation and adaptation strategies that can ensure global food security in a sustainable manner [3, 4]. The FOA outlines sustainable agricultural practices (SAP) that have five major attributes: conserves resources, environmentally non-degrading, technically appropriate, economically viable, and socially acceptable [5].

1.1.2 Past Solutions: What Can We Learn from Past Strategies?

Since the 1960s the agricultural industry has been undergoing a 'Green Revolution' that has seen an increase in global food security [2, 6]. The greatly improved agricultural capacity was achieved by high agronomic research investment rates, high-yielding crops, mechanization, heavy irrigation, and the use of synthetic fertilizers and pesticides [2, 6]. These agricultural practices resulted in the world population being better fed, but at what cost? One negative aftereffect was the over-reliance on synthetic nitrogen based fertilizers as well as pesticides for the increase in crop yield [7, 8]. The over-application of these synthetic fertilizers has had negative implications such as: degradation of soils due to processes such as leaching causing acidification, pollution of water ways by run-off causing eutrophication, and broader environmental pollution such as increase emissions of nitrous oxide in the atmosphere, a seriously potent greenhouse gas [6, 8]. Likewise, the negative impacts of the misuse and excessive use of pesticides has consequences; with the World Health Organization (WHO) estimating at least 3 million cases of pesticide poisoning each year [9]. Whilst past strategies greatly increased agricultural capacity,

they have failed to meet increasing global food demands whilst inadvertently neglecting their impact on the environment and human health [7].

1.1.3 Emergent Strategies: What Are They and What Are Their Limitations?

Current and future strategies for increasing agricultural productivity should follow sustainable agricultural practices guidelines whilst remaining adaptable to changing climate patterns, widespread pests, and evolving pathogens [3]. This highlights the complexity of agricultural systems and why multidisciplinary research utilizing diverse and complementary technologies is the best way to understand how future global food demands can be met sustainably [2]. Advances in science continue to broaden the horizons of what research is possible, allowing for discoveries important to agricultural systems involving: more efficient resource use, plant stress management and tolerance, plant-microbe interactions, as well as other novel enhancements for productivity [6, 10]. These advances allow for new innovative technologies to assist in improving agricultural productivity in more sustainable ways. For example, the use of genomics and other 'omics technologies to help elucidate the genetic basis for desirable agricultural traits has assisted in crop breeding strategies and the targeted production of improved varieties of crops [3]. Unfortunately though, the genome editing tools require lengthy breeding programs and suffer from poor public perception limiting their overall effectiveness [3, 11]. Scientific advances can also help to provide a biological basis to existing strategies, prompting their further development. A sustainable strategy that has seen a resurgence is the move to biological methods for the regulation of plant growth and development, and mitigation of environmental stressors to improve productivity [12, 13]. This has resulted in the rising popularity of natural biostimulants to increase crop productivity in an eco-friendly and safe manner [8]. While the number of scientific papers reporting the benefits of biostimulants is increasing, there are still challenges facing their further development; these can be categorized as scientific, technical and regulatory in nature [13, 14].

1.1.4 Biostimulants: Their Research and Development for Future Prospects.

The challenges that biostimulants face are largely due to their complex composition: which is a result of their diverse sources, and the processes used for production and formulation [13, 14]. The continued use and optimization of biostimulants relies on the characterization of their chemistry and their modes of action, but biostimulants are often highly complex mixtures which makes them particularly challenging to characterize [13, 14]. With advances in modern analytical equipment and techniques, namely separation science combined with spectrometric/spectrophotometric analysis, a more complete characterization of biostimulants and their possible modes of action may be achieved [9, 13]. Furthermore,

the ability to be able to separate individual compounds from these complex mixtures, identify them and quantify them will help to elucidate their biochemical composition and modes of action, and allow us to further optimize them [13]. Ultimately, the use, certification and registration of biostimulants will continue to be hindered without a collaborative multidisciplinary effort to identify active components and modes of action [13]. This includes specialist chemistry research to supplement the work being conducted by plant physiologists, biologists and agronomists [13]. The benefits of research and experimentation with biostimulants is not limited just to their efficacy, there is also a significant biological case that it may help to identify novel compounds and biological processes that might have otherwise have gone undiscovered [13]. The discovery of novel biologically active molecules and modes of action in biostimulants along with novel plant physiological processes are both important in achieving the goal of meeting global food production demands [13].

1.1.5 This Research Project's Focus and Position in The Literature.

A class of biostimulants that has shown great potential for enhanced agricultural productivity are seaweed extracts [1]. Seaweed extracts benefits to crops have been widely reported in scientific literature although they, like many other multicomponent biostimulants, are complex and require further characterization of their compositions and modes of action [1, 13]. Seaweeds composition shares many similarities with plants: containing many important plant growth promoting molecules as well as bioactive secondary metabolites [15]. These secondary metabolites are more often referred to as phytochemicals: naturally occurring chemical compounds found ubiquitously in plants that are important defence molecules [16]. There are many classes of phytochemicals which are classified in reference to their molecular structure [16]. One class of phytochemicals known as phenolic acids, contains salicylic acid (2-hydroxybenzoic acid) which is a particularly important plant molecule found ubiquitously throughout the plant kingdom [17]. Salicylic acid is well researched: it is involved in many plant physiological processes from seed germination to disease resistance [17]. Characterizing salicylic acid and similar phenolic acids in seaweed extracts may help to better understand some of the modes of action of the observed benefits of seaweed extracts. This work aimed to use modern analytical technologies to identify salicylic acid and related derivatives in seaweed extracts with the goal to provide invaluable information for producers and manufacturers, farmers and the broader agricultural industry, as well as the scientific community. All of which is working towards the end goal of improving agricultural practices and productivity in order to meet global food demands sustainably and equitably. A detailed review of the research on biostimulants, phytochemicals, analytical instrumentation and methods, and agriculturally focused bioactivity studies will be presented in the following sections of this chapter.

1.2 BIOSTIMULANTS

1.2.1 What Are Biostimulants?

Biostimulants are currently poorly defined and include an array of products that have been described as 'hormone-containing products', 'metabolic enhancers', 'plant conditioners', 'biogenic stimulants', and 'biofertilizers' to name a few [13, 14]. This is mostly due to the diversity in the nature of biostimulants, their physiological functions or 'modes of actions', their agricultural/horticultural functions, and their economic and environmental benefits [14]. The nature of biostimulants refers to the physical product, usually being either substances or microorganisms [14]. Substances can be individual compounds but are more often complex mixtures of chemicals extracted from biological materials [13, 14]. The 'modes of actions' refers to any interactions with plant cellular mechanisms that affect whole-plant processes or illicit a physiological response [14]. Any modes of action that can scientifically demonstrate increased plant productivity and crop performance therefore give the biostimulant agricultural function [14]. These agricultural functions include enhanced crop quality, nutrition efficiency, and stress tolerance [14, 18]. Finally these agricultural functions translate into economic and environmental benefits such as enhanced nutritional value, higher crop yields, and reduced environmental degradation etc. [14].

1.2.2 Biostimulants' Definition and Distinction from Fertilizers.

What is clear is the acceptance that biostimulants are defined by what they are not; biostimulants are not simply fertilizers nor pesticides [13, 14]. Fertilizers provide essential plant nutrients such as nitrogen (N), phosphorus (P) and potassium (K) which play a pivotal role in plant growth and development [19]. Biostimulants' effects should be distinct from any that arise due to the nutrient content in order to draw a clear line between them and fertilizers [14]. For this reason Patrick du Jardin proposes the following definition: "A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrient content" [14]. This definition of biostimulants is focused on the agricultural functions and doesn't address the complications that arise due to the complex composition of many biostimulant products whose components are not fully defined and characterized [13, 14]. Yakhin et al adds to this by acknowledging that biostimulants may contain molecules that act synergistically in one or more plant physiological process, and furthermore that biostimulants may have properties that simply cannot be elucidated by identification of the individual components and their combinations [13]. Such is the complexity of biostimulants that it is suggested that they are emergent and cannot be understood by a reductionist method alone, and thus they are more than the sum of their components [13]. This is integrated into the definition that is proposed by Yakhin et al: "a formulated product of biological origin that improves plants productivity as a consequence of the novel, or emergent properties of the

complex constituents, and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds" [13].

1.2.3 Biostimulant Classifications.

The diversity in the nature of biostimulants as well as their modes of action has made defining biostimulants disputable: with no universally legal or regulatory definition accepted anywhere in the world [14]. These same characteristics have made the development of defined biostimulant categories also difficult, however there are now some common categories being recognised [14]. Proposed categories have in the past been based on: the natural raw materials, the origin of the active ingredient, the mode of action, and even their use or mode of application [13]. What is evident is the categorization of biostimulants by their origin has become the most widely accepted, and although this method of categorization does not provide information on their mode of action it still facilitates the discovery process and provides the framework for comparison for related products [13, 14]. Yakhin *et al* presented an extensive analysis of the majority of reported biostimulants in the literature, which was divided into 6 categories based on origin: bacteria, fungi, algae, higher plants, animal raw materials, and humate-containing raw materials [13]. A summary of the biostimulant classification table presented by Yakhin *et al* is shown below in Table 1.0, it includes the following key details: sources, production, composition, and activities [13].

Origin/source of raw material	Methods of production	Composition and bioactive compounds	Hypothesised modes of action	Biological effects
Bacteria Living microorganisms. Non-living microorganisms and their metabolites.	Cultivation. Cultivation, acid or alkali or enzymatic hydrolysis, fermentation.	Auxin like substances, cytokinins, betaines, gibberellins, amino acids, oligopeptides, peptidoglycans, lypopolysaccharides, melatonin.	Stimulate nitrogen uptake, maintaining soil fertility, hormonal influence, stimulate amino acid synthesis, increase pigments, increase antioxidants, abiotic and biotic stress resistance, activation of systemic resistance.	Increased germination rate, improved growth and plant quality, increased productivity and yield.
Fungi Living microorganisms. Non-living microorganisms and their metabolites.	Cultivation. Fermentation, lyophilisation.	Amino acids, auxin like substances, betaines, carbohydrates, chitosan, cytokinins, exopolysaccharides, gibberellins, melatonin, minerals, nucleic acids, oligopeptides, polyglucuronic acid, siderophores, vitamins.	Increase nutrient uptake, stimulate nitrogen uptake, increase enzyme activity, hormonal influence, stimulate amino acid synthesis, increase total carbohydrates, protein and phenols, increase pigments, induce plant defence, enhance stress tolerance both abiotic and biotic, influence rhizosphere.	Increased germination rate, improved growth and plant quality, increased number of flowers and fruit, increased productivity and yield.
Algae Various species of algae, primarily large biomass kelps.	Acid or alkali hydrolysis, aqueous extraction, cell burst/rupture, pressure and/or temperature treatment, enzyme-assisted extraction (EAE), fermentation, microwave-assisted extraction (MAE), solvent extraction, supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE).	Abscisic acid, alginic acid, auxins, auxin like substances, betaines, carbohydrates, cytokinins, gibberellins, carrageenans, lipids, melatonin, minerals (Na, Ca, Cu, Fe, I, K, Mg, P, S, B, Mn, Zn, Co, N, Cl etc.), oligosaccharides, pepsin, phenolic compounds, polysaccharides, proteins, sterols.	Increase nutrient absorption and fertilizer efficiency, increase mineral uptake, efficient water uptake, auxin-, cytokinin-, gibberellin-like activity, modulation of phytohormones, regulation of gene expression, increase photosynthetic efficiency, increase total carbohydrate, protein, phenolic content, enhance antioxidant activity, delay senescence, reduce transpiration, enhance stomatal conductance, alter root architecture, modulation of root exudates, strengthen cell walls, enhance abiotic and biotic stress resistance, enhance locally plant immunity.	Increased number of fruit and fruit quality, enhanced root development, improved growth, stimulate growth, increased productivity and yield.

Origin/source of raw material	Methods of production	Composition and bioactive compounds	Hypothesised modes of action	Biological effects
Higher Plants Parts of higher plants include: seed, leaves, and roots and exudates from various species.	Alkaline hydrolysis, aqueous extraction, fermentation, solid-liquid extraction, enzymatic hydrolysis, solvent extraction, microwave extraction, pressure and/or temperature treatment.	Amino acids, auxins, carbohydrates, catalase, cytokinins, elements (N, P, K, Na, S, Ca, Mg, P, B, Fe, Zn, Cu, Mn, Ni, Cl, Mo, Co, etc.), ellagitannins, enzymatic antioxidants, flavonols, flavonoids, flavanones, enzymatic proteins, gibberellins, glycosides, humic acids, lignin, lipids, polyphenols, melatonin, oligosaccharides, organic acids, phenolic acids, polyphenols, tannins, vitamins, triglycerides, volatile compounds.	Increase nitrogen assimilation, increase phosphate uptake, induce changes in root architecture, auxin-, cytokinin-, gibberellin-like activity, hormonal regulation, gene expression regulation, improve photosynthetic rate and efficiency, increase chlorophyll, simulate metabolism, increase biochemical contents, increase in osmolytes, regulation of enzyme activity, modulating antioxidant systems, improved water use efficiency, regulation of stomata, enhance resistance to abiotic and biotic stress, antimicrobial and antifungal activity.	Stimulated growth, increased seed germination, increased growth, improved rooting, increased number of flowers and fruits, positive effects on development, increased food quality, productivity and yield.
Animal Raw Materials Wastes and by-products from industries such as leather processing and seafood industry, etc.	Acid hydrolysis, chemical hydrolytic processes, enzymatic hydrolysis, thermal hydrolytic processes.	Elements (Na, S, K, Ca, Mg, P, Fe, Zn, Cu, Mn, Ni, B, etc.), fat, amino acids, melatonin, organic matter, peptides, protein.	Improve nutrient utilization, induce morphological changes in root architecture, auxin-, cytokinin-, gibberellin-like activity, change hormonal levels, effects on biosynthesis of plant growth regulators (PGR), synergistic effect with exogenous PGR, induction of gene expression, increase enzymatic activity, accelerate metabolic rates, accelerate photosynthesis rate, increase pigment, protein, phenolic, and key elemental content, enhance abiotic and biotic stress resistance, stimulate growth and activity of beneficial microbes, improve antioxidant activity.	Improved growth and development, better root formation, induction of flowering, reduced fruit drop, better fruit uniformity and quality, greater yields.

Origin/source of raw material	Methods of production	Composition and bioactive compounds	Hypothesised modes of action	Biological effects
Humate-Containing Raw Materials Composts, agro-industrial wastes, leonardite, lignin, peat, soil, vermicompost, volcanic soil, and waste materials.	Extraction, thermochemolysis.	Amino acids, cellulose, elements (C, H, N, O, Ca, Cu, Fe, K, Na, P, S, Si, Zn, etc.), fatty acids, flavonoids, humic substances, lignins, lipids, microorganisms, peptides, phenolic acids, phenols, plant hormones, auxins, brassinosteroids, cytokinins, gibberellins, proteins.	Induce nitrous oxide synthesis, increase nitrate uptake, enhance nutrient uptake, increase translocation of elements (root-to-shoot), auxin-, cytokinin-, gibberellin-like activity, hormonal regulation, regulate gene expression, stimulate various metabolic pathways, alter primary and secondary metabolism, increased chlorophyll and carotene content, regulation of photosynthesis, increase assimilation of N, C and S, increase protein, phenol content, stimulate enzymatic activity, enhance phenylpropanoid metabolism, alter REDOX homeostasis, enhance stress tolerance, change root architecture, stimulate chloroplast division, alter rhizosphere communities.	Activated growing processes, increased growth and biomass, increased root size and branching, increased yields.

1) **Table 1.2** A summary of biostimulant classifications including origin/source material, methods of production, composition and bioactive compounds, hypothesised modes of action, and biological effects [13].

1.3 ALGAL BASED BIOSTIMULANTS

1.3.1 Introduction to Algal Based Biostimulants.

Algal based biostimulants, namely seaweed extracts represent much of the commercial biostimulant market [20]. There are dozens of commercial seaweed products that are widely used to promote agricultural productivity [14, 21]. Seaweed extracts are promising natural products that maintain environment integrity and are generally regarded to be: biodegradable, non-toxic, non-polluting and non-hazardous [13, 22]. They are a sustainable solution for improving and protecting agricultural systems that is eco-friendly and even suitable for organic farming practices [20, 22]. Research into algal based biostimulants has shown a plethora of benefits from the promotion of plant growth and development, to increased resistance to both abiotic and biotic stressors that only stand to increase in severity due to the changing climate [1, 23]. Whilst the modes-of-action of these benefits are not well understood there is a consensus that it is not simply supplementation of the macronutrients that is their driving force and instead; it is the diverse range of biologically active components that are responsible for the physiological effects in plants [15, 24].

1.3.2 Seaweed and Seaweed Extracts in Agriculture.

There are nearly 10,000 different species of seaweed; they range in size from microscopic phytoplankton to the large biomass kelps [15]. Seaweeds or macroalgae as they are often referred to are plant-like organisms that are classified based on their pigmentation into green, red, and brown algae [24]. Their diversity in both species and habitats has resulted in the synthesis of a vast range of bioactive secondary metabolites with interesting activities, some with structures that cannot be found in other organisms [25]. Seaweeds are most commonly found in intertidal zones on rocks and other hard substrata and are consequently exposed to extremely variable environmental conditions such as temperature, salinity and light [22]. Consequently, the production of different stress related compounds essential for their survival [21, 22]. Studies have revealed the use of seaweeds throughout history in diverse cultures, for purposes including but not limited to: food, medicine, and agriculture [15]. The direct application of seaweeds on crops to improve soil nutrition and stimulate plant growth was employed for centuries, however, since the 1950s the use of whole seaweeds has been superseded by the use of liquid seaweed extracts [1, 15]. The ease of use of the liquid seaweed extracts pioneered the establishment of a commercial seaweed extract industry in the late 1950s; subsequently the scientific benefits of seaweed extracts have been extensively investigated [1, 15, 21, 23, 24, 26, 27].

1.3.3 Evidence for Improved Plant Growth and Development.

The scientific investigation into the benefits of seaweed extracts have been reported in peer-reviewed literature for decades; significantly, it was this mounting evidence that resulted in seaweed extracts gaining acceptance as plant biostimulants [1, 15]. Numerous benefits have been reported on a wide range of plant species using various seaweed extracts [28]. Consequently there are several review articles each with an extensive list of reported growth and development benefits that include but are not limited to: promotion of budding and flowering, increased nutrient uptake, improved germination rates, increases in quality (size/taste/yield) of fruit, increased root and leaf development, and enhanced photosynthetic activity [1, 15, 22, 24, 27]. In order to highlight the types of experiments being conducted to test for these benefits a few specific examples will be provided. An Australian study testing the impacts of a seaweed extract on broccoli establishment conducted two field experiments; the study used a seaweed extract made from *Durvillaea potatorum* and *Ascophyllum nodosum* in which seedling were soaked before being planted in two contrasting soil types and receiving three more applications of the seaweed extract over approximately three weeks [29]. The broccoli seedlings establishment was measured using leaf number, stem diameter and leaf area all of which showed significant increases; demonstrating that the extract had the capacity to improve the establishment of broccoli seedlings in Australian farm settings [29]. Another study in Australia investigated the effect of two seaweed extracts on the growth and development of lettuce growing under glasshouse conditions [30]. The first seaweed extract used is made from *A.nodosum* and the second is made from *D.potatorum, Durvillaea antarctica* and *A.nodosum* [30]. The effect of application rates of the two seaweed extracts on increasing lettuce crop performance was measured using plant height, shoot biomass, root biomass and leaf chlorophyll content; and it was shown that both seaweed extracts could increase crop performance [30].

1.3.4 Evidence for Increased Resistance to Abiotic Stressors.

The application of seaweed extracts are not limited only to improved growth and development, there is also a considerable volume of evidence in support of their effects on abiotic stress tolerance. Abiotic stresses adversely affect agricultural productivity, accounting for considerable loss in crop production globally [15]. The intensive agricultural practices of the past contributed to unfavourable conditions that are being exacerbated by the extreme weather patterns brought on largely due to the changing climate; consequently, abiotic stress mitigation strategies are an area for significant concern for future agricultural productivity [1, 15]. Seaweed extracts have been demonstrated to reduce the impacts of abiotic stress across a wide variety of plant species in various growing environments; these abiotic stresses include: freezing, drought, water logging, salinity, and extreme temperatures [1, 15, 21, 24]. For example, a study conducted by Elansary *et al* investigated the role of a seaweed extract in enhancing the growth

and phytochemical composition of medicinal shrubs during drought stress simulated in controlled greenhouse conditions [31]. Two medicinal shrubs *Spiraea nipponica* and *Pittosporum eugenioides* were treated with a seaweed extract of *A.nodosum* weekly over 8 weeks in drought conditions; a reduction in the impacts of drought due to seaweed extract application were determined by enhanced morphological, physiological, and biochemical performances [31]. One of the most common abiotic stresses is soil salinity which is amongst the most concerning globally; however, studies have demonstrated that the application of seaweed extracts can increase plant tolerance to salinity stresses [24]. An experiment investigating salinity tolerance in *Arabidopsis* after treatment with a commercial seaweed extract showed that extract-treated plants were more tolerant of salt stress; furthermore, this research highlights the multi-disciplinary approach necessary to elucidate the molecular mechanisms involved [24]. Due to the complexity of abiotic stress resistance in plants, often involving a large number of genes to mediate the plant response to stress; the study analysed a whole genome transcriptome of *Arabidopsis* which revealed the upregulation of genes known to be involved in alleviating salt, drought and cold stresses, whilst downregulating other genes that are negative regulators of salt stress [15, 24].

1.3.5 Evidence for Increased Resistance to Biotic Stressors.

Agricultural productivity is constantly being reduced by various biotic stressors including pests and pathogens such as bacteria, fungi and viruses [21]. Currently, in the agricultural industry the most common disease control method involves the application of pesticides; however, this has a number of disadvantages including: development of resistant strains, off-target effects, high cost, bioaccumulation, as well as environmental and health hazards [32]. Thankfully, plants have evolved inducible defence mechanisms that are capable of being activated by various elicitors [21, 24]. Seaweed extracts present a crop protection strategy without the associated disadvantages the current disease control methods possess; this is due to seaweed extracts containing important bioactive compounds capable of inducing defence responses against pathogens by acting as a primer or elicitor [21]. As a result there are many studies that demonstrate the ability of seaweed extracts to suppress pathogenic infections [1, 21, 24]. For example, Jayaraman *et al.* published two significant studies in 2008 and 2011, which looked at the efficacy of a commercial seaweed extract on reducing the incidence of pathogens in greenhouse carrots and cucumbers [32, 33]. The greenhouse-grown carrots were treated with a seaweed extract of *A.nodosum* and inoculated with two fungal pathogens: *Alternaria radicina* and *Botrytis cinerea*; it was shown that the seaweed extract enhanced disease resistance and increased the activity of well-known defence-related enzymes [33]. The greenhouse cucumber plants were treated with a seaweed extract of *A.nodosum* before being inoculated with four fungal pathogens: *Alternaria cucumerinum, Didymella applanata, Fusarium oxysporum,* and *B.cinera* [32]. The disease incidence of all four pathogens tested

was significantly reduced, activities of defence-related enzymes were enhanced, and a higher level of phenolic phytochemicals were accumulated in treated plants [32]. As for the role of seaweed extracts in insect management, the extracts themselves do not appear to directly have insecticidal activity; instead the biologically active components of the extracts seem to induce insect defence responses in the plants [24]. It has been postulated that the application of seaweed extracts can help to modulate plant defence mechanisms, some of which may protect against insect pests [24].

1.3.6 Evidence for Influencing Plant Rhizosphere.

Interestingly, recent studies have revealed that seaweed extracts not only increase plant resistance to biotic stressors but conversely, may potentially promote the colonisation of commensal soil-bound bacteria effectively influencing the rhizosphere [1, 21, 24]. The rhizosphere is *"the soil surrounding a root in which physical, chemical and biological properties have been changed by root growth and activity"* and includes the coexistence of a *"large number of microorganisms such as bacteria, fungi, protozoa and algae"* [34]. The application of seaweed extracts to the plant or soil has been reported to alter the rhizosphere via direct and indirect methods [21, 24]. Direct application of seaweed extracts to soils can improve the soils ability to support microbial populations; as has been observed in soils with alginate present, the benefits of which alters the soil structure into a more conducive environment for microbial growth [21]. Another example of direct soil treatment with a seaweed extract is a study that observed the increased activity of nod-forming bacteria in plants treated with an extract of *A.nodosum*; this improved plant growth presumably due to increased nitrogen fixation [35]. There are also complex interactions between the microbial population and the roots mediated through root exudates that can be influenced by seaweed extract treatment [21]. Seaweed extracts can induce production of root exudates in the plant that interact with bacteria, indirectly influencing the rhizosphere microbiome [21].

1.3.7 Seaweed Extracts' Composition: Known and Postulated.

Seaweed extracts are heterogeneous in nature, containing a diverse range of organic and inorganic components [15]. Their chemical composition depends largely on two main variables: the species of origin of the extract, and the method of extraction used during the manufacturing process [15]. The commercial seaweed extract industry uses a diverse range of extraction procedures in its manufacturing processes; including acid or alkali hydrolysis, and cellular disruption *via* pressure or fermentation in order to release the beneficial components into the extract [1, 21]. Whilst seaweeds are known to improve the levels of important soil nutrients for plant growth such as N, P and K; research into the chemical composition of seaweed extracts revealed that the extracts contained insufficient concentrations of

these common macronutrients to explain the observed benefits [15, 27]. Therefore, it was proposed that the benefits of seaweed extracts were predominantly mediated by plant growth-promoting compounds and elicitors [15]. Various seaweeds and seaweed extracts have been shown to have a diverse array of minerals that enhance nutrition and have critical roles in plant development such as Na, B, Fe, Se, Si, Ca, S, Mg, Zn, Mn, Cu, Ni, Co, F, Cr, and Cd [27]. Seaweeds are known to contain diverse polysaccharides that constitute cell walls and are sometimes used as storage components within a cell; these have varying chemical structures and are often specific to different algae taxa, for example alginates, fucoidans and laminarins in brown algae [21, 27]. Of the natural bioactive compounds reported in seaweeds, the plant growth promoting compounds with cytokinin-like, auxin-like and gibberellin-like activities have received plenty of attention due to their potential for elucidating the modes-of-action of the extracts benefits on plants [1, 24]. Due to the important role that betaines play in plant stress tolerance, they are often reported in seaweed extracts [1, 15]. Seaweeds, in particular brown seaweeds which are the most common source for commercial seaweed extracts, are rich in phenolic secondary metabolites [15]. Brown seaweeds have been shown to have a high total phenolic content as well as a diverse range of phenolic compounds; these phenolic secondary metabolites are often synthesized under stress to protect cells and cellular components via antioxidant activity for example and are critical to plant metabolic processes [15]. The complexity of seaweed extracts is such that there are many other important molecules yet to discover and characterise [1]. Furthermore, the effects of commercial seaweed extracts may be attributed to single, additive, synergistic or even antagonistic action of these important compounds; thus further investigation into their composition is critical [24].

1.4 PHENOLIC PHYTOCHEMICALS

1.4.1 What Are Phytochemicals?

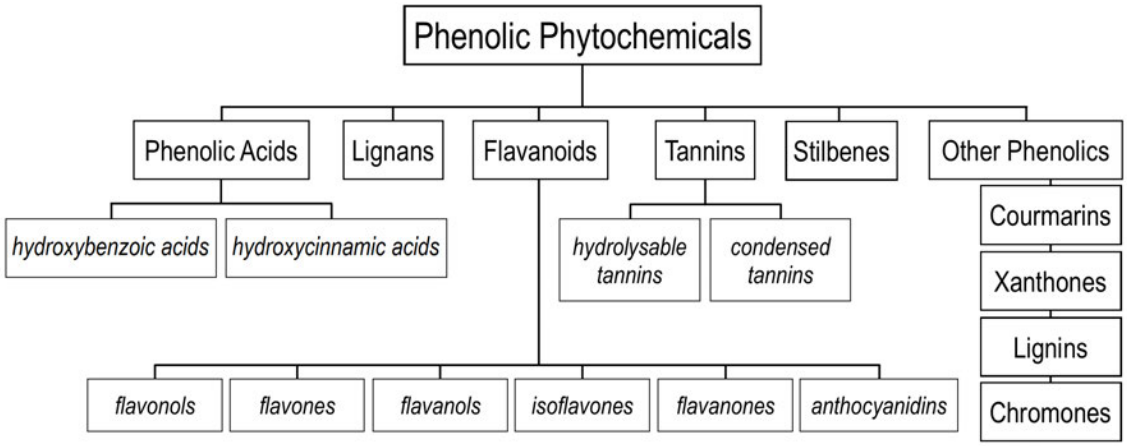
Phytochemicals are a large group of naturally occurring chemical compounds found in plants; 'phyto' meaning plant in Greek [16, 36]. Nevertheless, the term phytochemicals is often used to denote plant secondary metabolites [16, 36, 37]. Plants just like all living things produce chemicals to survive: these organic compounds are divided into two large groups: primary and secondary metabolites [38, 39]. Primary metabolites are chemical compounds such as carbohydrates, proteins, and lipids; they are essential for growth and development as well as cell maintenance and are generally considered to be ubiquitous throughout the plant kingdom [38, 39]. Plant secondary metabolites are a diverse group of non-nutrient compounds thought primarily to be involved in defence and overall survival; they are often associated with plants organoleptic properties such as colour, taste, smell and texture [37, 39]. Secondary metabolites are commonly found in low levels and vary in their distribution in plants; with some molecules being species specific [39, 40]. Then there are phytohormones: simple endogenous signalling molecules that regulate the physiological functions in plants [40, 41]. Like primary metabolites phytohormones are involved in plant growth and development, and are considered ubiquitous; however, similar to secondary metabolites phytohormones are non-nutrients involved in defence and responses to stress, and are active in relatively low concentrations [40, 41].

1.4.2 Secondary Metabolites' Biosynthesis, Diversity, and Classifications.

There are thousands of secondary metabolites that have been identified with upwards of 100,000 predicted to exist [40, 42]. The biosynthesis begins from basic pathways, such as glycolysis or shikimic acid pathways from primary metabolite precursors and subsequently diversifies [38]. The secondary metabolite biosynthetic pathways are primarily dependent on the cell type and developmental stage; additionally, studies have shown that their production is highly susceptible to environmental cues such as climate and soil conditions as well as environmental stress [39, 42]. Much of the stepwise pathways, genes, and enzymes involved in the production of secondary metabolites have been well represented and catalogued in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [43]. Based on their biosynthetic pathways and their chemical composition secondary metabolites are most often classified into three groups: nitrogen-containing compounds (alkaloids), phenolic compounds, and terpenes (terpenoids) [38, 42]. In conjunction with the three classifications mentioned above some extend the number of groups to include phytosterols and sulphur-containing compounds [16, 37].

1.4.3 Phenolic Class of Phytochemicals and Their Classification.

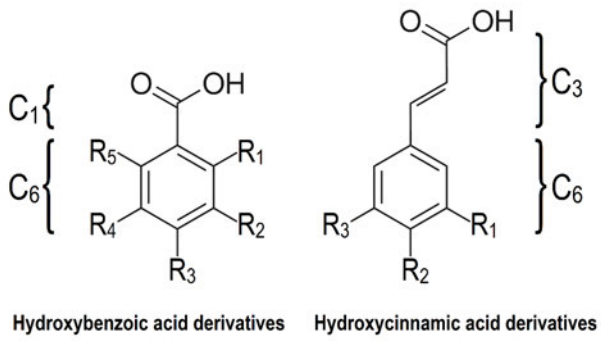
Phenolic compounds are the most abundant of secondary metabolites in plants; particularly in fruits, vegetables, spices, teas and grains [44-46]. In plants, phenolic compounds are involved in many physiological functions including growth, development, and defence against UV light, insects, viruses, bacteria and even other plants [46, 47]. Not just limited to plants, phenolic compounds are also found in fungi and bacteria [47, 48]. Phenolics are prominent in the human diet and are a major contributor to the organoleptic characteristics of many plant based foodstuffs; furthermore, some phenolics can be used as food additives that help to prolong shelf-life [45]. They have a wide range of biological and pharmacological properties, most notably as strong natural antioxidants; additional important properties include: anti-inflammatory, anticancer, antimicrobial, antiviral, antiallergenic, antithrombotic, immunoregulatory, anti-diabetic, cardio protective and more [45, 46]. Phenolics are diverse, with an extensive range of structures from the simplest phenol comprised of one hydroxyl functional group attached to an aromatic ring, to complex polymeric compounds [40, 49]. Defined by their chemical structure, phenolics can be classified into 'simple and complex' or 'flavonoids and non-flavonoids' but are often reported as three main classes: phenolic acids, flavonoids, and other phenolics (e.g. lignans, tannins, stilbenes) [16, 44, 50]. An outline of phenolic phytochemical classification is presented below in Figure 1.4.3.



1) **Figure 1.4.3** Phenolic phytochemicals classification adapted from Martinez *et al.* and Shahidi *et al.* [44, 51]

1.4.4 Phenolic Acids.

One of the main classes of phenolic compounds is phenolic acids; these simple aromatic acids have one carboxylic acid group attached to a benzene ring with one or more hydroxyl or methoxyl groups attached [46, 50]. Phenolic acids are most abundant in sources such as the seeds and skins of fruits, and the leaves of vegetables where they are mostly present in conjugated forms (as glycosides, amides and esters) but are also found in their free form (organic acids) and bound form (attached to cell wall constituents) [45, 46]. As presented in Figure 1.4.3 phenolic acids are classified into two sub-classes: hydroxybenzoic acids and hydroxycinnamic acids; with hydroxycinnamic acids being more prevalent in nature than their counterparts [45, 46]. The two subclasses are distinctive from one another due to the carbon skeleton of their chemical structure; hydroxybenzoic acids have a C_6-C_1 framework where as hydroxycinnamic acids have a C_6-C_3 framework as shown in Figure 1.4.4 [45]. Moreover, the structural difference is that in hydroxycinnamic acid the carboxylic acid moiety is attached to the benzene ring as an unsaturated propionic acid [50]. Though the two sub-classes have different carbon skeletons they both arise naturally through the same biosynthesis pathway: the shikimate pathway [46, 47].

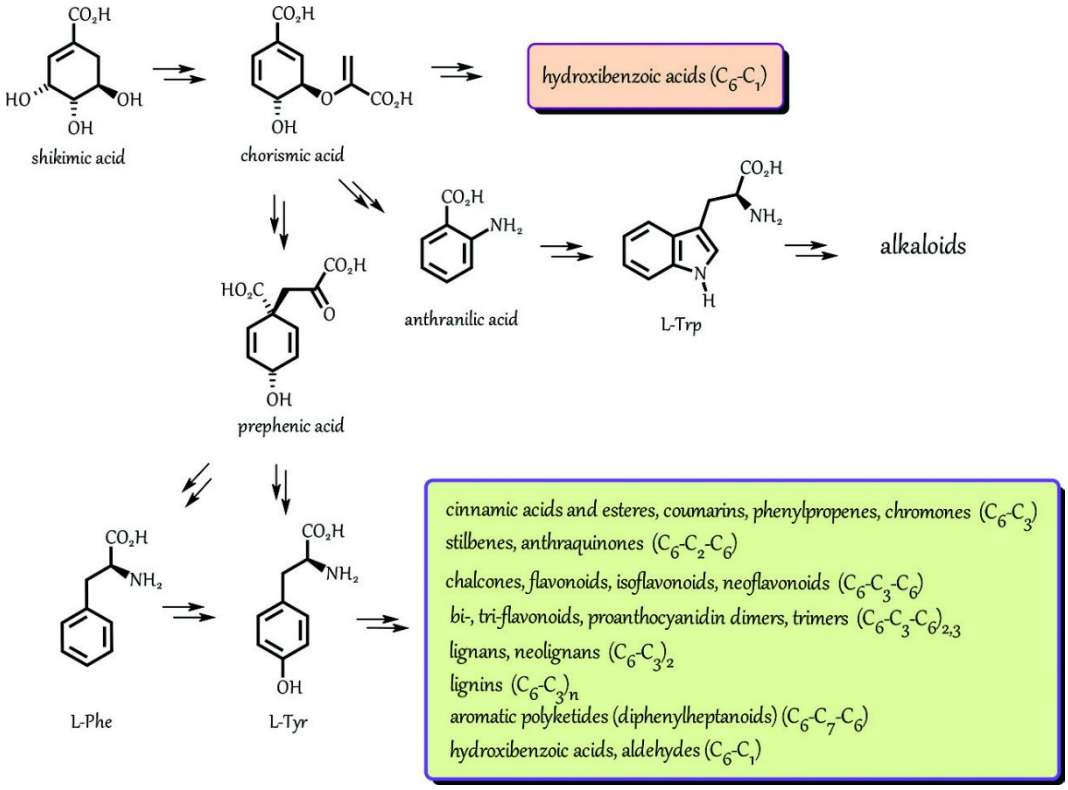


2) **Figure 1.4.4** The structural framework for the two classes of phenolic acids: hydroxybenzoic acids (C_6-C_1) and hydroxycinnamic acids (C_6-C_3).

1.4.5 Phenolic Acid Biosynthesis.

Phenolic acids like most of the phenolics in plants, bacteria and fungi are biosynthesised by the shikimate or shikimic acid pathway; a biochemical pathway that is a major link between primary and secondary metabolism particularly in higher plants [46, 52]. Since the molecules that are produced through the shikimate pathway play an important role in plant defence and adaptation to their environment; the shikimate pathway is upregulated when secondary metabolite biosynthesis is needed [52, 53]. The shikimate pathway is responsible for the conversion of simple carbohydrate molecules that arise from other metabolic pathways such as glycolysis, into shikimic acid and then finally chorismic acid [46, 52]. This pathway consists of seven sequential enzymatic steps, which have been well characterised along

with the seven enzymes involved [52, 53]. Chorismic acid is the key branch point to post-chorismic acid pathways that produce the aromatic amino acids: L-phenylalanine (L-Phe), L-tyrosine (L-Tyr), and L-tryptophan (L-Trp) [52]. These aromatic amino acids are crucial components of protein biosynthesis as well as precursors for many secondary metabolites including phenolic acids [52]. As seen in Figure 1.4.5 many phenolic compounds are synthesised from L-Phe and L-Tyr; including hydroxybenzoic acids. Additionally, hydroxybenzoic acids and other C_6-C_1 derivatives are also biosynthesised via other chorismic acid branch points [52, 53].



3) **Figure 1.4.5** Shikimic acid pathway in biosynthesis of phenolic compounds; showing shikimic and chorismic acids as the common precursors for the synthesis of L-Phe, L-Tyr, and L-Trp and a diverse array of phenolic compounds. Reproduced from Francenia Santo *et al.* [41, 52].

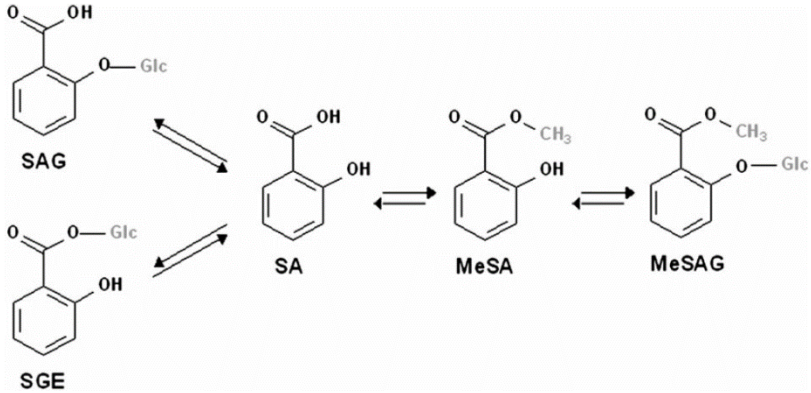
1.4.6 Hydroxybenzoic Acids and Other (C_6-C_1) Derivatives.

A variety of natural hydroxybenzoic acid derivatives exist. These derivatives include benzoic acid derivatives that have undergone aromatic hydroxylation such as: salicylic acid, *p*-hydroxybenzoic acid (4-hydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), and gallic acid (3,4,5-trihydroxybenzoic acid) [45, 47]. There are also those derivatives that have undergone aromatic methoxylation that include: vanillic acid (4-hydroxy,3-methoxybenzoic acid), isovanillic acid (3-hydroxy,4-methoxybenzoic acid) and syringic acid (4-hydroxy,3,5-dimethoxybenzoic acid) [45, 50]. Finally

there are benzoic acid derivatives that are lacking a hydroxyl moiety and are therefore not strictly hydroxybenzoic acid derivatives, but are often analysed in the same collective due to structural similarities. These benzoic acid derivatives include molecules like veratric acid (3,4-dimethoxybenzoic acid) and amino-substituted derivatives such as anthranilic acid (2-aminobenzoic acid) [47, 54]. Many of these various benzoic acid derivatives are often found methylated, glycosylated or conjugated with amino acids or Coenzyme A (CoA) [55]. The structural differences and modifications of these benzoic acid derivatives alters their critical properties including: volatility, stability, and activity, which has direct implications on their functional roles [55]. The plants ability to make small modifications to these compounds provides it control over their active forms, regulating specific functions including signalling and/or storage [55]. The importance of control over the activity of these small molecules is most evident in the most common benzoic acid derivative: salicylic acid, which is a potent phytohormone involved in regulating many plant functions most notably defence against pathogens [56].

1.4.6.1 Salicylic Acid (2-hydroxybenzoic acid).

Salicylic acid is a plant growth regulator which is present ubiquitously throughout the plant kingdom [57]. It is a phenolic acid that was first extracted nearly two centuries ago; its chemical properties have been well characterised as well as its *in vivo* biosynthesis and metabolic pathways in plants [17]. Salicylic acid's basic structure is a benzene ring with a carboxylic acid group (COOH) and a single hydroxyl group (OH) at the 2 position (*ortho*) of the aromatic ring; with salicylic acid mainly found as methylated or glycosylated conjugates, as shown in Figure 1.4.6.1 below [58].

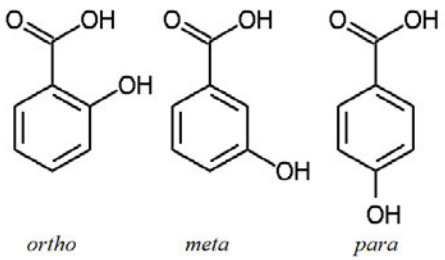


4) **Figure 1.4.6.1** The structures of salicylic acid and some of its common conjugates. SA: salicylic acid, SAG: SA 2-O-β-D-glucoside, SGE: SA glucose ester, MeSA: methyl salicylate, and MeSAG methyl salicylate 2-O-β-D-glucose. Reproduced from Rivas-San Vincente *et al.* [58].

Salicylic acid is an endogenous signal molecule considered to be a potent plant hormone, due to its role in regulating a variety of plant development processes during the entire lifespan of the plant [17, 57]. Best known for mediating immunity in plants, endogenous salicylic acid also plays a crucial role in regulating processes such as: thermogenesis, respiration, transpiration, seed germination, cell growth, photosynthesis, stomatal closure, ion uptake and transport [17, 59, 60]. Besides regulating plant growth and developmental processes, endogenous salicylic acid also modulates plant responses to various abiotic stresses including: salinity, heat shock, drought, UV light, heavy metal toxicity, and osmotic stress [17, 61]. Endogenous salicylic acid serves as a critical signal molecule in regulating plant defence response to various pathogens (biotic stressors); activating local immune responses at the site of infection [17, 59, 60]. Along with the localised response to a pathogenic attack, salicylic acid can induce a systemic defence response; this type of response to pathogenic attack is known as systemic acquired resistance [59, 62]. Recent research has shown that salicylic acid is essential for systemic acquired resistance establishment and therefore, it plays a crucial role in the coordination of the plant immune response during plant-pathogen interactions [63]. During recent years salicylic acid's role in modulating plant-microorganism interaction has received particular attention. Recently, it has been found that plant immune signalling drives selection from the available soil microbial communities to form the soil microbiome; and more significantly, that endogenous salicylic acid plays a major role in modulating such colonisation of the root [10]. In the same study the effect that exogenous salicylic acid application would have on the composition of the soil microbiome was investigated; concluding that exogenous salicylic acid significantly alters the composition of the soil microbiome [10]. Exogenous salicylic acid's role in plant pathogen defence was first realised when tobacco plants were treated with aspirin (acetyl salicylic acid); salicylic acid and aspirin induced the expression of the pathogenesis-related proteins which is a key marker for the induction of systemic acquired resistance [64]. These studies conclude that the application of salicylic acid mediates plant response to biotic stress; this is critical because it suggests that the presence of salicylic acid in seaweed biostimulants may be play a role in their observed attributes of increased plant productivity and agricultural functions.

1.4.6.2 3-hydroxybenzoic acid and 4-hydroxybenzoic acid (*m*-HBA and *p*-HBA).

Salicylic acid has two structural isomers, these monohydroxybenzoic acids (MHBAs) differ from salicylic acid by the location of the hydroxyl substitution on the benzene ring; located at either position 3 (*meta*) or 4 (*para*); as shown in Figure 1.4.6.2 [65].



5) **Figure 1.4.6.2** The structures of 2-hydroxybenzoic acid (*o*-hydroxybenzoic acid or *o*-HBA), as well as its two isomers 3- or *m*-HBA, and 4- or *p*-HBA [65]

Of the two isomers, less is known about 3-HBA; it has been reported in avocados, blueberries, cranberries, medlar, cluster beans, grapefruits, and olive oil [66, 67]. It is a major phenolic compound found in various melons; these include the peels of maazoun cultivar of *Cucumic melo* and Sharlyn melons, and the rinds of watermelons [67]. Some potential health benefits of 3-HBA include its usage in dyslipidemia treatment and glucose metabolism regulation; although pharmacologically 3-HBA remains the least explored of the MHBAs [45, 68]. Due to the anti-inflammatory and analgesic properties of salicylic acid and its synthetic derivative acetyl-salicylic acid (aspirin), both 3- and 4-HBA have been investigated for similar properties in animal models [68]. Khan *et al* found that 3- and 4-HBA did possess aspirin like activity in rodent assays, however they were quantifiably lower and there was some other inhibitory activity that required further investigation; furthermore low daily doses of both 3- and 4-HBA were effective in stress resistance in laboratory rodents [68].

There have been more investigations into 4-HBA than 3-HBA. It has been reported in fungi and plants and many dietary sources such as carrot, eggplant, parsley, beans, mustard and lettuce [45, 47]. It is well known for its anti-fungal and anti-microbial properties [67, 69]. This is due to 4-HBA being the backbone for a class of compounds known as parabens; ester derivatives of 4-HBA with various alkyl chains or benzyl groups attached to the hydroxyl group [69]. Parabens have been used for decades as preservatives in products such as pharmaceuticals, foodstuffs and cosmetics; they have more recently been shrouded in controversy due to their estrogenic activity and that they may act as weak endocrine disruptors [69]. In plants, 4-HBA is best known for being an allelochemical; a chemical that is released by one plant or organism that affects the germination, growth, development, or reproduction of other plants or organisms [70, 71]. For example Huang *et al.* reported that 4-HBA suppressed the root growth of cucumber by reducing meristem activity and cell length [70]. Interestingly, in that same study Huang *et al.* found that the exogenous application of a salicylic acid derivative salicylhydroxamate (*N*,2-dihydroxybenzamide) was able to partially restore the root growth inhibited by 4-HBA; this requires further investigation but suggests potential synergistic activities between these compounds with similar

structures [70]. Another study that highlights the importance of investigating these derivatives was conducted by Kamaya *et al.* [72]. Using unicellular green algae as bioassays to determine aquatic environmental toxicity, Kamaya *et al.* observed the varying toxicity levels of benzoic acid and the three MHBAs; with 4-HBA being the least toxic of the three [72]. Noting that 4-HBA exhibited hormesis-like effects; inhibiting growth at high concentrations but stimulating growth at lower concentrations [72]. Additionally, they observed that 4-HBA had diminishing effects on the toxicity exhibited by 2-HBA, which was the most toxic of the three MHBAs [72]. Finally it is worth noting that the concentrations employed in this study were significantly higher than known environmental concentration levels.

1.5 CHEMICAL ANALYSIS

1.5.1 Introduction to Metabolite Analysis.

Phenolic acids are just one group of the complex mixture of different metabolites present within an organism. A whole set of metabolites for an organism or biological sample at a specific time point is considered their metabolome [73]. The time element of the metabolome reflects the dynamic nature of the interaction between an organism's genome and its environment [73, 74]. The metabolome includes endogenous metabolites that are produced by the organism such as the primary and secondary metabolites, as well as exogenous metabolites that are not produced by the organism such as contaminants or additives [73]. The comprehensive qualitative and quantitative analysis of a subset or all of the metabolites in biological systems and samples is known as metabolomics [73, 75]. Metabolomics attempts to identify and detect changes in metabolites in biological systems in order to characterise biochemical pathways and better understand the biology of an organism and its response to environmental stimuli [74, 75].

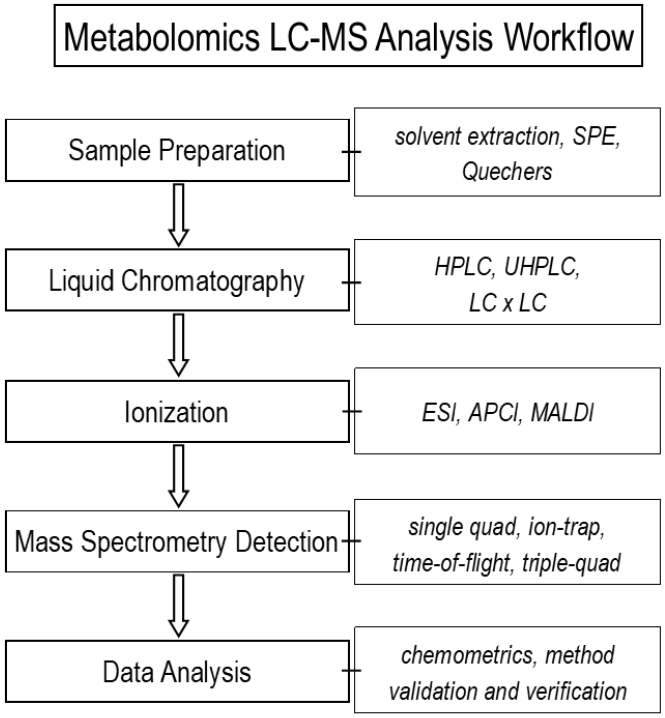
1.5.2 Metabolomics: Analytical Methodologies.

Metabolomics encompasses several conceptual analytical approaches that when combined provide a holistic profile of the metabolome; with the specificity of the approach being dependent on the objectives of each individual study [74]. An approach known as metabolomic profiling aims at simultaneously analysing as many metabolites in a biological sample as possible to provide a holistic view of the metabolism of the sample or to characterise new metabolites and metabolic pathways [74]. A combination of targeted and untargeted analyses are employed for metabolomic profiling [74]. Targeted analyses focus on a set of selected, known metabolites and aims for identification and quantification often using authenticated pure standards and/or databases [74, 76]. Untargeted analyses best reflect the aims of metabolic profiling as the objective is to reproducibly detect as many metabolites as possible. In this approach the chemical identity of detected metabolites does not need to be hypothesized prior to data acquisition and any quantification performed is relative as the goal is the widest metabolic coverage achievable [74, 76]. A different conceptual approach to metabolic profiling is metabolic fingerprinting, which aims not to identify or quantify any individual peaks but instead provide metabolic signatures or patterns for the characterisation of phenotypes [73, 74].

1.5.3 Metabolomics: Analytical Techniques and Instrumentation.

Much of the advancement in metabolomic research can be attributed to the improvement of analytical techniques and instrumentation, further enabled by the emerging data handling tools and software

platforms that allow for adequate handling, storage, normalization, and evaluation of the complex metabolic data generated by the subsequent techniques [73, 74]. A well-conceived analytical method should have a systematic plan to extract, purify, separate, detect and identify the metabolites in even the most complex sample matrices [73]. The most common technologies and instrumentation employed in metabolomic analyses are the hyphenated chromatography-mass spectrometry techniques [74, 76]. Combinations of liquid or gas chromatography separation techniques coupled with mass spectrometry provide powerful tools for metabolite profiling with good reproducibility and wide metabolome coverage [74, 75]. Liquid chromatography-mass spectrometry (LC-MS) is the most commonly used technique; this is due to LC not requiring the derivatisation steps often seen in GC-MS, and mass spectrometry having higher sensitivity and more widespread availability than nuclear magnetic resonance spectroscopy (NMR) [75]. Consequently, there exists a standard analytical workflow for LC-MS metabolomic analysis as shown in Figure 1.5.3.



6) **Figure 1.5.3** The standard analytical workflow for LC-MS metabolomics analysis adapted from Aszyk *et al.* including common examples and variations for each major step [41, 75, 77].

Excluding sampling and sample storage protocols, the workflow begins at sample preparation which can involve extraction, purification, and/or enrichment techniques [75, 77]. Following sample preparation is chromatographic separation where the chosen method must have high resolving power in order for high selectivity and sensitivity [73]. Next is the most crucial step in mass spectrometry, ionization; the

ionization efficiency dictates the number and abundance of metabolites that can be detected [75]. LC-MS analysis gives rise to the collection of enormous sums of chromatographic and spectral data that can then be processed and subjected to chemometric data analysis for statistical significance and robustness [75]. Finally the identification and quantification of metabolites can be interpreted in the context of metabolic pathway models [73, 75].

The remainder of Section 1.5 will discuss the key techniques and instrumentation used for metabolomic LC-MS analysis following the keys steps in Figure 1.5.3; throughout which the discussion will focus on the techniques and instrumentation used in this study including: solid-phase extraction, Quechers (partitioning), liquid chromatography electrospray ionisation tandem mass spectrometry, and method validation.

1.5.4 Sampling and Sample Storage.

When studying metabolites, sampling methods, sample processing and sample storage require consideration [73, 78]. Sampling methods need to be representative of the organism or sample and its environment; randomised sampling methods help to reduce sampling biases for example [73]. Additionally sampling must remain as fast as possible to mitigate the speed with which organisms respond to their surrounding environmental conditions; which additionally helps to reduce biochemical activity [73]. Moreover, sampling methods often involve risks of contamination and degradation which must be pre-emptively assessed and solved [78]. After washing the sample free of contaminants the next step is to rapidly inactivate all biochemical and enzymatic activities in a process known as quenching [73]. There are different methods of quenching that often involve drying, sudden drops in temperature, and the removal of water with one of the best methods for adequate quenching being freeze-drying [73, 78]. After the removal of water, algal samples for example are often homogenised into a powder that is then suitably stable for long-term storage in dry and preferentially water free environments [73, 78].

1.5.5 Sample Preparation: Extraction and Purification Methods.

Following on from the initial sampling/storage biological samples are not yet suitable for instrumental analysis, nor would they be optimal for application as a biostimulant because the metabolites are not released yet and an effective extraction process is required [73]. The first step is cell disruption, rupturing the cell walls to release the contents of the cells [73, 79]. Then there are various extraction techniques that can be used individually or in combination in order to recover the target metabolites and obtain the desired type of extract [21, 73]. Some of these extraction/purification techniques are selective for specific

types of molecules and others involve extracting the largest total sum of molecules whilst reducing any breakdown or enzymatic degradation to avoid altering the metabolite profile [73]. The various common extraction/purification techniques include: solvent extraction, acid or alkaline hydrolysis, ultrasonic-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, enzyme-assisted extraction, solid-phase extraction, and the Quechers method [21, 75, 80, 81].

1.5.5.1 Solvent Extraction.

The traditional sample preparation method that is sometimes referred to as liquid extraction or liquid-liquid extraction when performed with two immiscible phases, solvent extraction uses the solubility of the target molecules in various extraction solvents to separate or extract the target molecules from the sample [80, 81]. An example of a study that employed solvent extraction as a method of sample preparation was the simultaneous analysis of salicylic acid and jasmonic acid by Liquid Chromatography Electrospray Ionisation Mass Tandem Mass Spectrometry (LC-ESI-MS/MS) in crude extracts of cucumber [82]. Segarra *et al* chose a methanol/water solvent combination acidified with 1 % v/v acetic acid for the extraction of quick-frozen cucumber roots; the extraction was centrifuged and repeated, supernatants collected and dried, and resuspended in an acetonitrile/water solution acidified with 0.05 % v/v acetic acid [82]. Finally the resuspended extracts were filtered and analysed by LC-ESI-MS/MS; the solvent extraction was successful in providing an extract suitable for the fast and highly sensitive quantitation of salicylic and jasmonic acids [82]. Due to the lack of specificity of solvent extractions it is common to obtain extracts containing many compounds from the sample including unwanted interfering compounds, therefore solvent extraction often serves as the first step in sample preparation methods that target only a specific type of molecule [75]. For whole metabolome (untargeted) studies it is more common to use a gradient of solvents increasing in polarity successively where collecting the fractions from various solvents offers a higher recovery of all the metabolites and therefore a more total representation of the metabolome [73].

1.5.5.2 Acid and Alkaline Hydrolysis.

Widely used hydrolysis processes that involve the use of a strong acid or base to produce an extract can be performed on an industrial scale; for example, alkaline hydrolysis is the most common extraction method for commercial seaweed extracts made from *A.nodosum* [21, 83]. Acid hydrolysis is commonly performed with sulfuric or hydrochloric acid and is most well-known for breaking glycosidic bonds, and reducing polysaccharides to their monosaccharide building blocks and solubilizing sugars [83]. Acid hydrolysis is reported to result in the loss of some phenolics though, particularly complex phenolics as

it causes the degradation of various types of molecules [21, 83]. This loss of phenolic compounds is reduced when using alkaline hydrolysis and this is the sample treatment method of choice for recovery of phenolic compounds, particularly the more stubborn 'bound' phenolics [83]. Alkaline hydrolysis is known to break down glycosidic and ester bonds, breaking down complex polysaccharides and liberating glycosylated metabolites; furthermore, alkali treatment has been documented to produce novel compounds as a result of degradation, rearrangement, condensation and base catalysed synthetic reactions [21, 83]. The crude extracts obtained from acid and alkaline hydrolyses have proven to have many benefits, hence their use in the production of many commercial biostimulants; however, further sample treatment would be required to yield an extract suitable for instrumental analysis [21].

1.5.5.3 Ultrasonic-assisted Extraction.

An eco-friendly method of extracting bioactive metabolites from many different samples that utilises common laboratory equipment is ultrasound- or ultrasonic-assisted extraction (UAE) [81, 84]. A study that demonstrates the promise and efficacy of UAE was conducted by Oniszczuk *et al.* aiming to optimise a ultrasound-assisted extraction procedure for the LC-ESI-MS/MS analysis of phenolic acids from kale [85]. Their study found that UAE offered a higher yield of the compounds in a shorter time using less solvent; which proved to be repeatable [85].

1.5.5.4 Microwave-assisted Extraction.

Another extraction method considered to be eco-friendly is microwave-assisted extraction (MAE); commonly used for the isolation of polysaccharides, proteins, and polyphenols it has been shown to increase extraction efficiency of phenolic compounds from various brown algae species [21, 84, 86]. The study conducted by Yuan *et al* investigated the phenolic compound extraction efficiency using MAE in four brown algae species: *A.nodosum*, *Laminaria japonica*, *Lessonia trabeculate*, and *Lessonia nigrecens* [86]. The algal samples were subjected to a conventional solid-liquid extraction at room temperature for four hours as a baseline phenolic extraction; then separate algal samples were extracted using MAE [86]. A higher crude yield and total phenolic content was obtained for all four brown algae species using MAE when compared to the conventional solid-liquid extraction method, which resulted in the algae extracts produced though MAE having higher free radical scavenging ability and higher ferric reducing power amongst other important activities measured [86]. The extracts were also subjected to LC-DAD-ESI-MS/MS analysis whereby 17 phenolic compounds were tentatively identified [86].

1.5.5.5 Supercritical Fluid Extraction.

An efficient method that is also considered eco-friendly due to its lack of toxic solvents is supercritical fluid extraction (SFE) [21]. Used primarily to selectively isolate heat-sensitive compounds such as pigments and fatty acids; SFE protects the sample material from thermal or biochemical degradation, as seen most commonly in the decaffeination of coffee beans [21, 84, 87]. A study that demonstrates both the application and optimisation of SFE is the investigation of the antioxidant capacity of extracts of the red algae *Gracilaria mammillaris* produced by SFE [88]. Using CO_2 with ethanol as a co-solvent, Ospina *et al* investigated various pressures, temperatures, and co-solvent concentrations on the extraction yield, antioxidant activity, and total content of phenols and carotenes [88]. Their results demonstrated SFE's capabilities as an effective means for extracting important molecules and revealed *G.mammillaris* as a promising source of antioxidants [88].

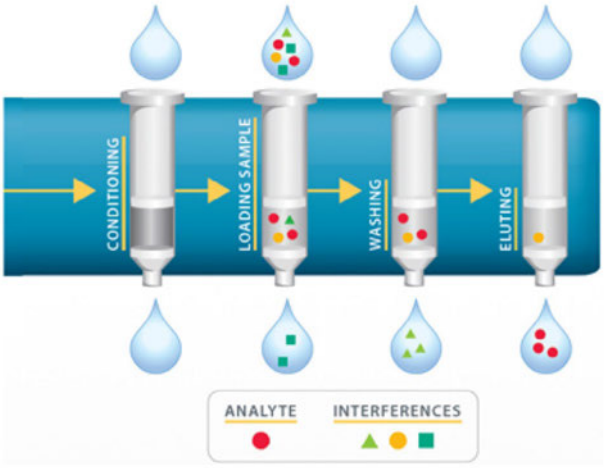
1.5.5.6 Enzyme-assisted Extraction.

An extraction method for the release of bioactive molecules that is a little more specific is enzyme-assisted extraction (EAE) [21, 83]. A study that demonstrates the comparative investigations currently being performed with EAE is the study of antioxidant activities of the brown seaweed *Ecklonia radiate* extracts prepared by microwave-assisted enzymatic extraction [89]. The study conducted by Charoensiddhi *et al* investigated EAE by comparing it to a more traditional acidic hydrolysis extraction and combining it with a MAE; the extraction of phlorotannins and antioxidant compounds from the *E.radiata* samples would then be evaluated by two antioxidant activity assays [89]. Three carbohydrases and three proteases were selected for the investigation which saw significantly higher yields in total phlorotannin content and antioxidant activity of the extracts obtained by EAE and microwave-assisted enzymatic extraction [89].

1.5.5.6 Solid-phase Extraction.

Solid-phase extraction (SPE) is the most frequently used method for the extraction of organic compounds from environmental, clinical, biological, food, and beverage samples [80, 90]. There are a wide variety of SPE materials and sorbents available with different selective applications [80, 90]. The typical SPE procedure consists of four main steps as shown in Figure 1.5.5.7a:

1. Conditioning the SPE materials to increase the effective surface area and reduce interferences,
2. Loading a liquid sample onto a solid phase capable of retaining the target analytes,
3. Washing away interfering and undesired molecules, and
4. Eluting the target analytes.



7) **Figure 1.5.5.7a** The typical SPE procedure reproduced from Andrade-Eiroa *et al* critical review article titled: Solid-phase extraction of organic compounds: a critical review (Part 1) [90].

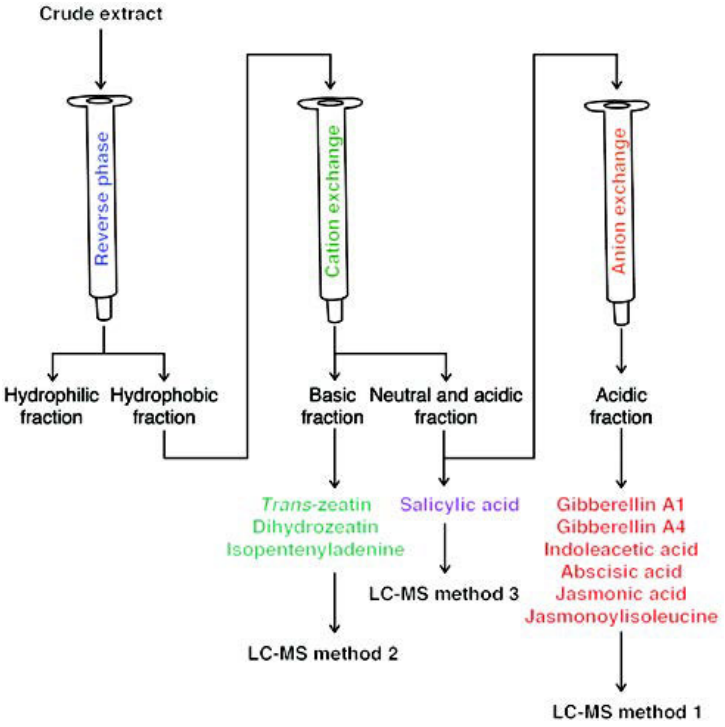
Depending on the SPE sorbent there are a number of retention mechanisms that are assumed to facilitate the process; the current theories consider simultaneous contributions from both the retention mechanisms observed in LC, as well as the partitioning observed in liquid-liquid extraction [90]. As in LC, there are various SPE materials and sorbents that are commercially available, covering a wide range of analytes and consequently countless applications [90]. The most commonly used mechanisms are: reversed-phase, normal-phase, ion-exchange, mixed-mode (ion exchange + reverse-phase), adsorption, and more recently magnetic SPE [90]. Different SPE mechanisms have varying procedures specific to the type of retention; therefore, a few examples will be provided to showcase the efficacy and variability of SPE, with particular focus on the analysis of phenolic acids and/or seaweed extracts.

A study with a conventional SPE procedure was conducted by Rajauria *et al* with the aim of optimising and validating a qualitative and quantitative HPLC method for the analysis of polyphenols in seaweed [91]. A HPLC-DAD-ESI-MS/MS method was used for the analysis of 7 phenolic compounds in the brown seaweed *Himanthalia elongata* using a sample extraction method involving a methanol solvent extraction followed by SPE [91]. The crude methanol extract of the seaweed sample was extracted using a C-18 SPE cartridge, with the developed method being validated and deemed satisfactorily sensitive and reproducible for the identification and quantitation of the phenolic compounds in the seaweed [91].

An example of the miniaturization of the SPE technique is the study that aimed to develop a method for the isolation of phenolics from sea algae before rapid resolution (RR) LC-ESI-MS/MS [92]. In that study Klejdus *et al* used the newer micro-elution SPE (μ-SPE) plate technology to analyse phenolic acid derivatives in the four sea algae species: *Sargassum muticum, Undaria pinnatifida, Chondrus crispus* and *Cystoseira abies-marina* [92]. They developed and optimised a range of sample preparation techniques, evaluated the μ-SPE plate technique and compared it to conventional SPE, and identified and

quantified benzoic and cinnamic acid derivatives by RRLC-ESI-MS/MS [92]. The μ-SPE plates were filled with different cartridges with 5 different sorbents which provided a great insight into the comparative efficacies of the various extraction procedures, as well as the exciting new possibilities of μ-SPE for the isolation of bioactive phenols from algal samples [92].

In a study conducted by Mikami *et al.* they used multiple SPE sorbents in succession on a single sample to profile nine phytohormones in the two red algae species: *Bangia fuscopurpurea* and *Pyropia yezoensis* [93]. Acidified acetonitrile extracts of the seaweeds were fractionated using the successive SPE procedure which began with the reversed-phase sorbent, from which the hydrophobic eluate was evaporated and reconstituted in acidified water before being loaded onto a ready mixed-mode cation-exchange and reversed-phase sorbent. The basic fraction from the Oasis MCX was used for the analysis of three phytohormones while the acidic fraction from the Oasis MCX was split in two; one portion of it being used for the analysis of salicylic acid, and the other portion being subjected to the mixed-mode weak anion-exchange and reversed-phase SPE sorbent. The eluate from the Oasis WAX was used for the analysis of the remaining acidic phytohormones. Figure 1.5.5.7b shows the successive SPE procedure that was used to identify the four phytohormones indoleacetic acid, *N*-isopentenyladenine, abscisic acid and salicylic acid in red seaweeds [93].



8) **Figure 1.5.5.7b** The SPE procedure for the profiling of phytohormones by Mikami *et al* reproduced from Mori *et al* critical review article titled: Phytohormones in red seaweeds: a technical review of methods for analysis and a consideration of genomic data [41, 93]

1.5.5.8 The Quechers Method.

The final sample preparation method that will be discussed is a widely recognized method developed for pesticide residue analysis in agricultural products that has subsequently been applied in other fields of research [75, 94]. The 'QuEChERS' technique (Quick, Easy, Cheap, Effective, Rugged and Safe) employs partitioning prior to dispersive SPE (d-SPE) and is rapidly being adopted due to its low cost, high throughput, easy performance and high efficiency [75, 94]. The original method which employed a 1:1 sample to solvent ratio, acetonitrile as the extraction solvent and magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) salts in a 4:1 ratio was shown to be efficient for many analytes in a variety of samples; however, adjustments have since been made to optimise the method for even more analytes, samples matrices, and instrumental analysis techniques [94, 95]. Most modifications to the Quechers method centred around three key variables: the extraction solvent, the salt, and the d-SPE sorbent; as a result there are now several iterations of the Quechers method commercially available with many companies offering to accommodate the preferences of customers [94]. A study that demonstrates the use of Quechers-based extraction methods in marine samples was conducted by Cunha *et al*; in which they developed and validated a method for the simultaneous quantification of bisphenol A and tetrabromobisphenol A in fish, mussels and seaweed samples [96]. They developed a method using the partitioning extraction following the Quechers procedure but then opted for a liquid-liquid extraction instead of the classic Quechers approach of d-SPE; finding that it promoted a better removal of interferences, better recoveries, and greater sensitivity [96].

1.5.6 Chromatographic Separation.

Even with the most sophisticated and selective sample preparation procedure, the resultant extract of a biological sample is still a complex mixture of chemical compounds [97]. Therefore, separation methods are required to chemically analyse individual compounds in these complex mixtures [76, 97]. It is common for the different chromatographic methods to be categorised by their mobile phase; the two most prevalent methods being liquid chromatography (LC) and gas chromatography (GC) [98, 99]. For the analysis of phenolic compounds the LC method is more commonly employed, as no time-consuming derivatisation process is required [80, 100].

1.5.6.1 Liquid Chromatography.

The various LC methods include normal-phase, reversed-phase, ion-exchange, and size-exclusion; however, the most suitable for the analysis of phenolic compounds is reversed-phase chromatography (RP-LC) [98, 100]. Reversed-phase liquid chromatography uses a polar mobile phase and non-polar

stationary phase; therefore, the elution order is based on increasing molecular hydrophobicity, with the most polar compounds eluted first [99]. Stainless steel columns with an internal diameter of 1 – 5 mm are packed with silica particles ranging in size from 2 – 5 μm for High-Performance Liquid Chromatography (HPLC) systems and <2 μm for Ultra High-Performance Liquid Chromatography (UHPLC) systems [97, 100]. The silica particles act as a support for the functional bonded-phase usually consisting of organic functional groups such as straight-chain octyl- (C8) or octadecyl-groups (C18), and aromatic phenyl-groups [98, 101]. The mobile phase selection for RP-LC depends on the nature of the target analyte(s); however, it is often water mixed with common solvents such as methanol or acetonitrile, and for the analysis of phenolics often includes small concentrations of an acidic modifier such as acetic or formic acid [76, 100]. The most widely used detectors for HPLC were spectrophotometric detectors such as UV/Vis or Photodiode Array that are based upon absorption of ultraviolet and/or visible radiation [98]. There are also detectors available that are based upon fluorescence, refractive index, and electrochemical detection to name a few; however, they are more analyte specific and are less universal [98]. In more recent years mass spectrometric (MS) detection began to replace absorption detection methods as the preferred system; MS not only offers increased selectivity and sensitivity, but also provides structural information for the target analytes as well as any well resolved unknown compounds within the sample [102].

1.5.7 Mass Spectrometry.

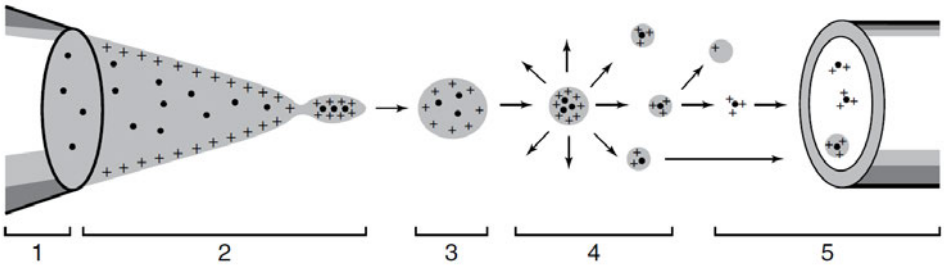
Mass spectrometry is a technique that allows for qualitative identification and quantitative determination of analytes in an expansive range of samples [103]. Modern hyphenated techniques include but are not limited to: GC-MS, LC-MS, capillary electrophoresis mass spectrometry (CE-MS), and inductively coupled plasma mass spectrometry (ICP-MS) [104]. Of all of the hyphenated analytical techniques it is LC-MS that has had the most success, consequently there is an ever-growing wide range of fields where LC-MS is applied from medical/clinical settings to environmental research, and more recently the emergent fields of "omics science"; most notably metabolomics [75, 102]. There are a number of different modern mass spectrometers often categorised by the techniques they employ for ionisation and mass analysis; for example matrix-assisted laser desorption ionisation – time-of-flight mass spectrometry (MALDI-TOF) or electrospray ionisation – tandem mass spectrometry (ESI-MS/MS) [104, 105].

1.5.7.1 MS Ionisation.

For a substance to be analysed by mass spectrometry, it needs to be ionisable; hence why many consider ionisation the most important step in MS [104]. There are many ionisation techniques that transform samples into the gas-phase ions required for mass analysis; these are classified into 'soft' or 'hard' ionisation techniques according to the amount of energy they impart on the analyte [103, 105]. The most important and popular techniques in many fields of study including metabolomics are the 'soft ionisation techniques such as electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI); these soft ionisation techniques, along with MALDI produce little to no fragmentation as the formed ions have little excess energy [77, 102, 103].

1.5.7.1.1 Electrospray Ionisation (ESI).

Electrospray ionisation (ESI) is the most commonly used technique among the group of API methods and is the leading ionisation technique when coupling LC with MS [105]. It is a soft ionisation technique that is able to transfer ions from solution to the gas phase at atmospheric pressure; owing to its ability to produce multiply charged ions, ESI allows for the mass spectrometric analysis of larger molecules with high molecular masses typically not amenable to MS such as proteins [104]. The principle of operation for ESI is based upon the basic technique of electrospray; where a solution in pumped through a capillary that has a high electric potential applied to it [103, 104]. As a result the solution forms a cone-shaped structure at its end, from which droplets are emitted; this is known as the Taylor cone named after Sir Geoffrey Taylor who described the influence of electric potential on liquid [104]. The generation of the Taylor cone facilitates the release of very fine droplets that depending on the potential applied to the capillary, would contain either an excess of positive or negative charge [103]. The concentration of the charge at the droplet's surface coupled with the evaporation of the solvent leads to electrostatic repulsion that exceeds the Rayleigh limit and overcomes the surface tension of the droplets; this droplet instability leads to a Coulomb explosion [104]. Coulomb explosions create many more fine droplets, until complete evaporation of the solvent is achieved and 'dry' ions are released [104]. This process from the Taylor cone formation to the successive Coulomb explosions that create the dry analyte ions is shown in Figure 1.5.7.1.1 below taken from Smoluch *et al* [104].



9) **Figure 1.5.7.1.1** A schematic depicting the Electrospray principle including: (1) the capillary, (2) the Taylor cone, (3) aerosol droplet, (4) Coulomb explosion generating finer droplets, and (5) complete desolvation and generation of dry analyte ions. The black dots represent analyte ions. Reproduced from Smoluch *et al.* [104].

Modern ESI interfaces have come a long way since the early beginnings of electrospray and now include/implement different features to improve the efficiency and therefore the sensitivity [105]. This includes the use of an inert gas such as nitrogen along with heating in various arrangements to improve the efficiency of ion formation by assisting in solvent evaporation [103]. Furthermore, ESI is well known to be affected by the flow rate of the solvent through the capillary, with lower flow rates having a higher efficiency of electrospray ionisation; the use of heating and gases is particularly important at higher flow rates in supporting efficient evaporation [104]. The final interesting feature built in to most modern ESI interfaces is an orthogonal, or close to orthogonal capillary axis; the capillary axis being from the electrospray capillary (component 1 in Figure 1.5.7.1.1 above) to the heated capillary inlet (component 5 in Figure 1.5.7.1.1 above) that leads to the internal parts of the MS under vacuum [104]. That capillary axis is often tilted close to 90º which initially seems unfavourable as the spray is not directed towards the instrument; however, the spray is directed, almost independently of that angle by the electric potential difference as well as the higher vacuum [104]. As it is the electric potential difference that directs the spray and guides the ions into the analyser, an orthogonal arrangement sees only charged ions enter the inlet capillary; causing less non-ionised components and impurities to enter the system, decreasing background noise and preventing system contamination [104].

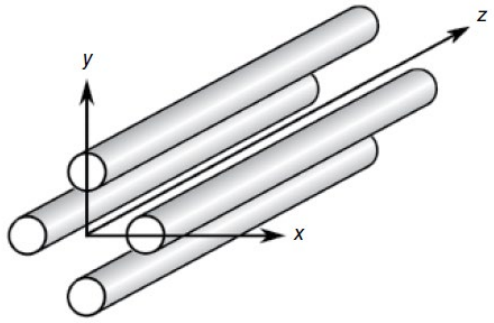
1.5.7.2 MS Mass Analysis.

Following the generation of gaseous ions in the ionisation sources the mass analysers are responsible for the filtering and separation of the ions [103]. Furthermore, some mass analyser designs allow for trapping or further fragmentation of selected ions [103]. There are various types of common mass analysers including time-of-flight, magnetic sector, quadrupole, ion trap, Fourier transform – ion cyclotron resonance, and the most recent type of mass analyser, the Orbitrap [105]. These common mass

analysers have differing principles and designs that provide variability in: their measurable mass-to-charge ratio range, mass resolution and accuracy [103].

1.5.7.2.1 Quadrupole.

The quadrupole mass analyser is comprised of four hyperbolically or cylindrically shaped metal rod electrodes; they are arranged in two pairs, set parallel to each other and the ion beam trajectory in the z-direction and mounted in a square configuration along an xy plane (see Figure 1.5.7.2.1 below) [104, 105]. The quadrupole mass analyser works by generating an electrical field through the application of radiofrequency (RF) and direct current (DC) across pairs of opposing rods [103, 104]. By varying the DC and RF signals the quadrupole mass analyser changes its selectivity for the m/z of ions that will have a stable enough trajectory to pass through to the detector [104]. These scans can be performed rapidly as it only requires manipulation of the electric field, which is an important advantage of quadrupole mass analysers and why they are often paired with continuous ionisation sources and coupled with chromatographic separation techniques [104].



10) **Figure 1.5.7.2.1** Schematic of a cylindrical quadrupole mass analyser. Showing the arrangement of the four rods mounted along an xy plane set parallel in the z-direction. Reproduced from Smoluch *et al.* [104].

One disadvantage of quadrupoles is their comparatively low resolution, operating at unit resolution; however, their ability to rapidly switch between selected m/z values along with their relatively low price and ruggedness make them one of the most common mass analysers [104, 105]. When the DC signal in quadrupoles is reduced to zero the entire ion beam can be guided/focussed through the analyser; a feature that is used to transfer ions and allows for combination with other mass analysers [104]. This focussing/guiding is particularly important in ion guides, often found between the ion source and mass analyser, and in collision cells in triple quadrupole instruments (discussed further in tandem MS below) [103, 105].

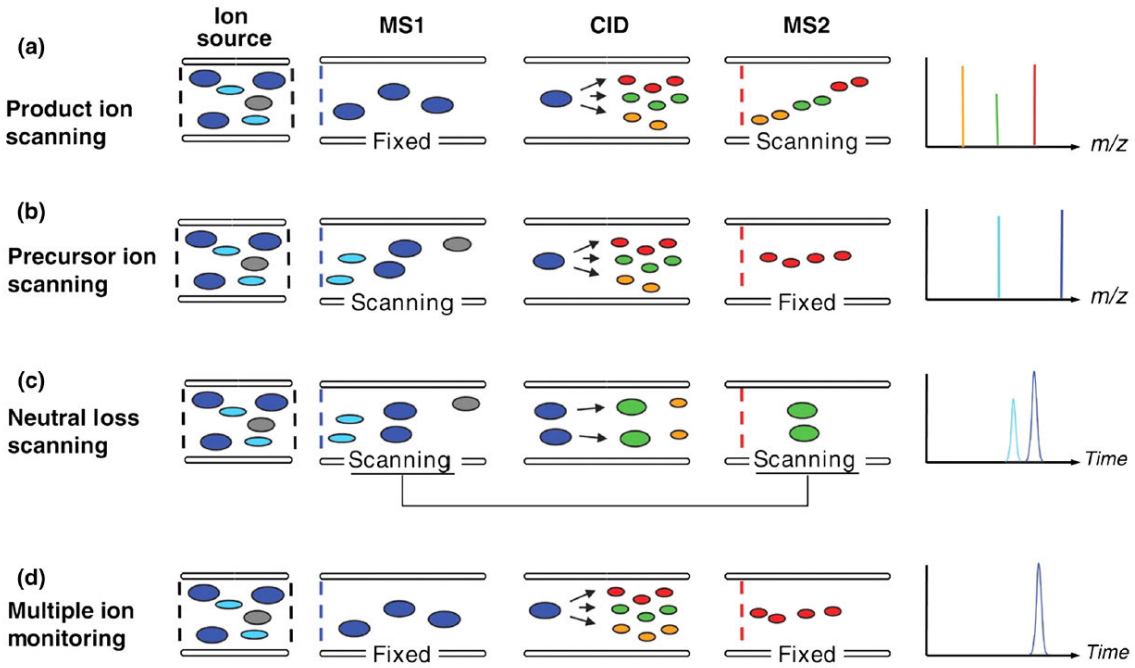
1.5.7.3 Tandem MS.

Tandem mass spectrometry can be referred to as tandem MS, mass spectrometry/mass spectrometry (MS/MS or MS^2), or in the case of multiple consecutive stages of fragmentation (MS^3, MS^4… MS^n) [104, 105]. The advent of tandem mass spectrometry was driven by the lack of structural information attained when utilising 'soft' ionisation techniques, due to their characteristic lack of spontaneous fragmentation [105]. The basic goal of tandem MS is to select and isolate a particular ion known as a precursor ion, then induce fragmentation by breaking some of the precursor ion's covalent bonds and subject the resulting fragment ions known as product ions to further mass spectrometric analysis [104]. The two strategies employed to achieve separation of the precursor ion from the remaining ions prior to fragmentation are known as: tandem-in-time, and tandem-in-space [105]. Tandem-in-time utilises the capabilities of some mass analysers that can store ions of interest, including the various ion trap mass analysers [104]. In these mass analysers all ions except for the target precursor ion can be removed, then fragmentation can be performed before re-analysing the resultant product ions all within the same trapping space; making it possible to repeat the process in generations of fragmentations sequentially in time [104]. Tandem-in-space involves the combination of multiple mass analysers within a single mass spectrometer; most often a combination to two mass analysers mounted either side of a collision chamber in which the fragmentation occurs [104, 105]. The mass analysers commonly found in tandem-in-space instruments include combinations of quadrupole and time-of-flight mass analysers; with the prototypical tandem-in-space depiction being a triple quadrupole tandem mass spectrometer [103, 105].

1.5.7.3.1 Triple Quadrupole Tandem MS.

Triple quadrupole mass spectrometers (QqQ) are the most prevalent example of tandem-in-space mass spectrometers; furthermore, they are particularly powerful when coupling tandem mass spectrometry with chromatographic techniques: GC-MS/MS and LC-MS/MS [103, 105]. Early triple quadrupole mass spectrometers took advantage of the RF-only quadrupoles (q) ion-guiding potential and mounted these between two quadrupole mass analysers; however, it is more common for modern triple quadrupole instruments to utilise a hexapole (h) or octapole (o) in the place of the RF-only quadrupole, as they have improved ion-guiding capabilities [104, 105]. It is in these hexapole/octapole collision cells where fragmentation occurs; whilst there are many methods of inducing fragmentation the most commonly employed in triple quadrupole instruments is collision-induced dissociation (CID) [105]. Collision-induced dissociation is generally realised by accelerating the precursor ions into a collision cell containing a neutral inert gas such as nitrogen; with fragmentation occurring as a result of collisions with the inert gas [103, 105]. Mounted either side of the collision cell are the quadrupole mass analysers used for mass analysis (scanning m/z range) and/or ion selection (fixed m/z isolation); operation of

these quadrupoles in different combinations of their scanning/fixed modes gives rise to the four different operating modes of a triple quadrupole mass spectrometer (shown in Figure 1.5.7.3.1 below) [103, 106].



11) **Figure 1.5.7.3.1** The four operating modes of a triple quadrupole mass spectrometer dependent on the Q1 and Q3 mass analysers' operation modes: (a) Product ion scanning, (b) Precursor ion scanning, (c) Neutral loss scanning, and (d) Multiple ion monitoring. Reproduced from Maher *et al.* [106].

In product scanning mode the first quadrupole (Q1) is set to a value that selects for the known precursor ion; the selected ion enters the collision cell where it undergoes fragmentation by CID, then the fragments or product ions are analysed in the third quadrupole (Q3) operating in m/z scanning mode [103, 106]. In precursor ion scanning Q1 and Q3 operation modes are flipped; the focus is on a specific product ion in Q3 while Q1 scans the m/z range looking for precursor ions that share/generate a particular product ion [104]. Neutral loss scanning has Q1 and Q3 both operating in synchronous scanning modes, focussing on the detection of a specific mass difference between precursor and product ions; helping to provide identification of molecules having specific structural features or conjugational complexes [104, 106]. The last of the four operating modes of triple quadrupole mass spectrometry is multiple ion monitoring (MIM) also commonly referred to as multiple reaction monitoring (MRM) [105, 106]. In this final mode of operation both Q1 and Q3 are selecting for predetermined m/z values: the precursor ion in Q1, and one of its product ions in Q3 [104]. A single reaction (precursor → product ion fragmentation specific to a molecule) can be monitored in a mode

known as single reaction monitoring (SRM), or thanks to the fast scanning speed of modern instruments multiple reactions for many different target analytes can be monitored in a single analytical run in MRM mode [104].

1.5.7.4 MS Detection.

Following the introduction of scanning mass spectrometers, electron multipliers became predominant due to their cost efficiency, sensitivity, and reliability [104, 105]. Electron multipliers consist of several electrodes called dynodes, upon contact with the first dynode an ion's kinetic energy is sufficient to dislodge a few electrons which creates an electron cascade travelling through the dynodes, generating a measurable current [104]. There are other ion-counting detectors that work using similar principles as the electron multiplier, these include the microchannel, photomultiplier, and channel multiplier [104, 105].

These detectors quantitatively transform the ion current into an electrical current, the data from which is presented as a mass spectrum: a two-dimensional representation of the signal intensity (y-axis) as a function of the m/z (x axis) [104, 105]. When observing a mass spectrum the most intense signal/peak is commonly known as the base peak, and it is standard practice to normalise/scale the mass spectrum such that the base peak is 100 % intensity making visual comparisons easier [105]. Other important peaks found in a mass spectrum are: the molecular ion ($M^{+\bullet}$) – the intact ionised molecule found in 'hard' ionisation methods; the pseudomolecular ion ($[M+H]^+$ or $[M-H]^-$) – protonated or deprotonated intact ionised molecule found in 'soft' ionisation methods; fragment ions – several peaks at lower m/z caused by fragmentation of the molecular ion, referred to as product ions in tandem mass spectra; cluster ions (e.g. $[2M-H]^-$) – formations due to two or more of the same ionised molecule complexing; and lastly, adducts (e.g. $[M+Na]^+$ or $[M+NH_4]^+$) – complexes formed between the ionised molecule and metal ions, anions or cations [105].

1.5.8 LC-MS Data Analysis.

When mass spectrometers are employed as chromatographic detectors, a third dimensions of information is gained; a major contributor to the success of the LC-MS technique [102, 105]. The sum of all ion intensities of each successive spectra when plotted as a function of time is known as the total ion chromatogram (TIC), from which the signal intensity of a specific m/z or m/z range can be extracted in a 'reconstructed' or 'extracted' ion chromatogram (RIC and EIC), respectively [105]. Whilst TICs offer an abundance of data for identification of the components of a sample, when quantitation is the goal ion chromatograms can be collected with the mass analyser operating in a more targeted

mode either for a specific m/z in selected ion monitoring mode (SIM) or multiple m/z in multiple ion detection mode (MID) [105]. Lastly, as discussed in the Triple quadrupole tandem mass spectrometry section LC-MS analyses when using tandem MS have the potential to add a fourth dimension to the analysis; in which there are four main operating modes for data collection depending on the application [104, 105].

1.5.9 Method Development, Validation and Verification.

Many analytical methods involve a large number of parameters that require optimisation in order to achieve the desired performance, particularly instrumental methods such as those employing LC-MS systems [102]. Throughout method development the performance of an analytical method is checked and monitored to ensure reliable results; however, more extensive activities such as method validation and verification are indispensable for providing objective evidence that a method is truly fit for purpose [102, 107]. Typically, a more complex method necessitates a more extensive validation and analytical methods employing LC-MS are notorious for their complexity; not just because of the instrument complexity but also because LC-MS is often utilised on the most complex of samples [102]. Further to the inherent necessity for validation, it has become increasingly more important due to regulations affecting laboratories requiring validation of methods, particularly for accreditation purposes, as well as scientific journals requiring validation data for publication of analytical research [102]. This had led to various international organisations and conferences issuing validation guidance documentation for laboratories, both universal and sector-specific [102]. Harmonised validation guidelines between the European Union (EU), Japan and the United States were developed in the 90s within the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [102]. Additionally, validation guidelines have been published by groups such as the Association of Official Analytical Chemists (AOAC), the International Union of Pure and Applied Chemists (IUPAC), and the United States Food and Drug Administration (FDA) [102]. In Australia the National Association of Testing Authorities (NATA) is the recognised national accreditation authority for analytical laboratories and as such their technical note entitled "General Accreditation Guidance – Validation and verification of quantitative and qualitative test methods" is considered the gold standard [107]. Whilst the analytical community is in agreement of the importance of method validation as well as the various performance parameters that require evaluation, there is diversity in the methodology employed for validation and the acceptance criteria [102, 107].

1.5.9.1 Method Validation – Performance Characteristics

Method validation involves the investigation and evaluation of several key method performance characteristics, listed in Table 1.5.9.1 below and adapted from the NATA technical note [107]. The depth of evaluation during the validation is determined by the status of the method under consideration and the requirements of its intended application [107]. A balance between costs, risks and technical capabilities, method validation in most cases involves the investigation of only a handful of the performance characteristics [102, 107]. Most guidelines provide only a general discussion on the performance characteristics including their definition, calculation and interpretation (as seen in Table 1.5.9.1); therefore, the analyst(s) are often tasked with: identifying which specific characteristics require evaluation, designing the method for evaluation, and determining the acceptance criteria [102].

Performance Characteristic	**Procedure(s) for determination/evaluation**
Limit of detection and quantitation	Replicate analysis at multiple concentrations including a concentration close to zero, or replicate analysis at a concentration estimated to be equal to twice the LOQ. Use blanks and a range of standards or samples containing low concentrations of analytes. Separate determinations may be required for different matrices.
Sensitivity	Analysis of spiked or artificially contaminated samples or standards prepared in sample extract solutions. Initial check for satisfactory gradient for plot of response vs concentration.
Selectivity	Analysis of reagent and matrix blanks, standards and matrix samples spiked with standards to which known concentrations of suspected interfering molecules have been added.
Linearity of calibration	Duplicate measurements of standards evenly spaced over expected concentration range of samples.
Measuring interval	Evaluation of bias and possibly LOQ determinations.
Matrix Effects	Analysis of matrix blanks or matrix spiked with standards.
Trueness; bias	Analysis of replicates. Reference samples should be matrix and concentration matched with samples.
Precision (Repeatability and Reproducibility) / Accuracy	Replicate analysis for each sample matrix type under stipulated conditions. For comparing precision of two methods, the F-test is recommended. For accuracy, compare each mixture's true value vs. the measured result.
Ruggedness	Introduce appropriate limits to method parameters likely to impact results if not carefully controlled. Investigated if necessary: single variable tests, and multi variable tests.
Measurement Uncertainty	Calculate a reasonable, fit-for-purpose estimate of MU. Ensure estimates are aligned with the concentration(s) most relevant to the users of the results.

2) **Table 1.5.9.1** A summary of the method validation performance characteristics and notes on how to determine/evaluate them. Adapted from [107]

1.5.9.2 Method Validation – LCMS Specific Aspects Including Ionisation Suppression by Matrix Effects.

There are practical aspects for LC-MS method development and validation that require consideration, particularly when comparing to other LC detectors [102, 108]. These include but are not limited to:

- There is a large number of LC-MS parameters that can be optimised that impact performance
- MS as a detector generally has poorer repeatability when compared to other LC detectors such as spectrophotometric detectors (UV and Fluorescence); this has implications for evaluating trueness, precision and accuracy (often requiring a larger number of replicates for their evaluation)
- MS detectors can be strongly influenced by the sample matrix and matrix variations, causing ionisation suppression or enhancement which has implications for a methods trueness [102, 108]

For the MS detectors that utilise the 'soft' API methods, especially ESI, the influence of the matrix on ionisation efficiency has come to be the most important source of error; with matrix effects being dubbed the 'Achilles heel' of HPLC-ESI-MS/MS and related methods [102, 107, 109]. Generally in analytical chemistry matrix effects are "the combined effects of all components of the sample other than the analyte on the measurement of the quantity"; furthermore any "specific component that can be identified as causing an effect is referred to as an interference" [102, 110]. In LC-MS this most commonly occurs in the form of coeluting compounds that either reduce (ionisation suppression) or increase (ionisation enhancement) the analyte signal [102, 111]. Whilst the exact mechanisms of matrix effects are unknown, various hypotheses explaining the complex mechanisms have been published in reviews; Cappiello *et al* list multiple hypotheses across their several reviews of matrix effects including for example, competition for available charges and access to the droplet surface in ESI [109, 111-113]. The evaluation of matrix effects is crucial for developing and validating LC-MS methods; initially there were two main strategies of evaluating matrix effects: post-extraction addition and post-column infusion [109, 110, 113]. Post-column infusion provides only qualitative assessment of matrix effects, while the post-extraction method provides a quantitative assessment; a modified post-extraction method known as the Slope Ratio Analysis was devised that overcame the need for a matrix blank and provided semi-quantitative assessment of matrix effects across a range of concentrations [110]. Where matrix effects have been assessed and confirmed they should be eliminated or significantly reduced; however, completely eliminating matrix effects may not be possible or practical and in such a case methods for compensating for them are required [102, 114]. There is no universal approach for reducing matrix effects and instead there are many strategies that fall into three main categories: sample preparation modifications (such as sample dilution or more extensive sample clean-up), chromatographic modifications (improved separation or varying stationary/mobile phases for additional selectivity), and

mass spectrometric modifications (using a different ionisation source, polarity or mode) [110, 114, 115]. If the matrix effects cannot be significantly reduced then efforts should be focussed on compensating for them by using an appropriate calibration methodology [110, 114]. The convenient and most widely accepted calibration method for matrix effects compensation is dependent on the availability of a blank matrix, known as the matrix-matched calibration; when a blank matrix is not available the two commonly employed methods for compensation are internal standard calibration and standard addition calibration [110, 114, 115].

1.6 CHEMICAL ANALYSIS OF PHENOLICS IN ALGAE AND TERRESTRIAL PLANTS

There is a growing interest in the study of seaweeds and their bioactive phenolic compounds with applications in a range of industries such as: pharmaceutical, food, cosmetic, and agricultural [116, 117]. However, much of the investigation into phenolics in macroalgae has been focussed on the phenolic compounds unique to or abundant in algae; for example phlorotannins and marine bromophenols [25, 118-120]. Additionally, like other phytochemicals such as phytohormones, much of the methodology for their analysis was developed in terrestrial plants and may not be directly applicable to algae [41]. Therefore, the development of innovative strategies for their analysis in seaweeds, along with the adaption/conversion of established methods from terrestrial plants to seaweeds is a growing area of research [41, 116].

The analysis of phenolics in seaweeds is a multi-step process involving various extraction techniques (described in Section 1.5.5) and analytical methods for their separation, identification, characterisation, and quantification [121]. Typically, phenolic compounds are separated using liquid chromatography, before detection with either spectrophotometric methods such as UV/Vis, or mass spectrometric methods such as ESI-MS [100]. A review of HPLC methods using spectrophotometric and spectrometric detection employed to analyse phenolic compounds in algae based samples is presented in Table 1.6a and b, respectively. As previously discussed, there is a larger body of research pertaining to the analysis of phenolics in terrestrial plants which often serve as the foundations to the development of methods in seaweeds and algae; therefore a similar review of the literature for HPLC methods used for the analysis of phenolics in terrestrial plants is presented in Table 1.6c. The literature review revealed common approaches used for the chromatographic analysis of phenolics in algae/plant samples. Following the Table 1.6a-c a summary of chromatographic, spectrophotometric and spectrometric approaches is shown below.

Algae Species	Phenolics	Analytical Instrumentation and Methodology	Reference(s)
Acanthaphora spicifera, Graciliaria edulis, Padina gymnospora, Ulva fasciata, and *Enteromorpha flexuosa*	Caffeic acid, *p*-coumaric acid, gallic acid, chlorogenic acid, 3,4-dihydroxybenzoic acid	HPLC, No column information provided, kept at 35ºC Gradient elution, Solvent A: 50mM sodium phosphate and 10 % (v/v) methanol, Solvent B: 70 % (v/v) methanol, delivered at 1.0 mL/min, 100 min run time, 20 µL injection	Abirami and Kowsalya, 2017 [122]
Chondrus crispus	Gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, Gentisic acid, catechin, *p*-coumaric acid, cinnamic acid	HPLC-DAD, Symmetry C18 250 x 4.6 5 µm column, Gradient elution, Solvent A: acetonitrile, Solvent B: 0.1 % (v/v) phosphoric acid in water, delivered at 1.0 mL/min, 60 minute run time, 10 µL injection DAD set at 280, 320 and 360 nm	Alkhalaf, 2021 [123]
Turbinaria conoides and *Turbinaria ornate*	Chlorogenic acid, caffeic acid, 2,5-dihydroxybenzoic acid, coumaric acid, ferulic acid, salicylic acid, gallic acid, syringic acid, + more	HPLC-DAD, Phenomenex C18 Luna 150 x 4.6 mm 5 µm column, fitted with guard column Gradient elution, Solvent A: methanol, Solvent B: HPLC water with 0.2 % (v/v) acetic acid, delivered at 0.6 mL/min, 60 min run time, 50 µL injection DAD set to 374 and 277 nm	Chakraborty and Joseph, 2016 [124]
Laminaria and *Ascophyllum nodosum*	Gallic acid, protocatechuic acid, vanillic acid, caffeic acid, *p*-coumaric acid, syringic acid and *p*-hydroxybenzoic acid	HPLC-UV/Vis, TM-LC 18 Supelcosil fitted with pre-column Isocratic elution, mobile phase: water/n-butanol/acetic acid (80.5:18:1.5 v/v/v), delivered at 1.2 mL/min, 20 µL injection UV/Vis set to 275 nm	Ertani et al., 2018 [125]
26 seaweed species (11 brown, 5 green, and 10 red)	Gallic acid, protocatechuic acid, catechin, caffeic acid, vanillic acid, syringic acid, salicylic acid, p-coumaric acid, + more	UHPLC-DAD, Zorbax SB-C18 250 x 4.6 mm 5 µm column, kept at 40ºC Gradient elution, Solvent A: phosphoric acid in water, Solvent B: methanol/acetonitrile 50:50 (v/v), delivered at 0.8 mL/min, 5 µL injection DAD detection at 280, 235, 255, 210, 320, 340, 360, and 520	Farvin et al., 2019 [126]
Cystosira myrica, Padina gymnospora, Sargassum aspirofolium, Sargassum latifolium, Sargassum muticum, Turbinaria sp.	Coumarin, Ellagic acid, kaempherol, naphthalene, phenanthrene, resorcinol acid, ferulic acid	HPLC-DAD, C18 column, Solvent A: methanol, Solvent B: 2 % (v/v) acetic acid in water, delivered at 1.0 mL/min, 15 min run time DAD set to 240, 245 and 250 nm	Fouda et al., 2019 [127]

Dictyota dichotoma and *Padina pavonica*	Protocatechuic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, *t*-ferulic acid, *o*-coumaric acid	HPLC-UV/Vis, UltraAqueous C18 250 x 4.6 mm 5 µm column Gradient elution, Solvent A: 0.2 % (v/v) phosphoric acid in water, Solvent B: methanol/acetonitrile 50:50 (v/v), delivered at 0.8 mL/min UV detection at 280 nm	Generalić Mekinić et al., 2021 [128]
Cladophora glomerata and mixture of baltic seaweeds	Phytohormones including auxins (indoleacetic acid) and cytokinins (kinetin) and abscisic acid	HPLC-PDA, Phenomenex Luna C18 150 x 4.6 mm 5 µm column, kept at 25ºC Gradient elution, Solvent A: acetonitrile, Solvent B: water with formic acid, delivered at 1 mL/min, 30 min run time, 20 µL injection PDA detector set to 214 nm	Górka and Wieczorek, 2017 [117]
Ulva lactuca, Caulerpa racemosa, Acanthophora spicifera, Sargassum dentifolium	Hydroxybenzoic acid, gallic acid, resorcinol, chlorogenic acid, vanillic acid, coumaric acid, salicylic acid, ferulic acid, cinammic acid	HPLC-UV/Vis, Kromasil 150 x 4.6 mm C18 column Isocratic elution, mobile phase: methanol/water/tetrahydrofuran/acetic acid (23:75:1:1 v/v/v/v), delivered at 1.0 mL/min UV detection at 280 nm	Hamed and Messiha, 2018 [129]
Ankistrodesmus sp., Spirogyra sp., Euglena cantabrica, Caespitella pascheri	Gallic acid, epicatechin, chlorogenic acid, protocatechuic acid, syringic acid, catechin	HPLC-DAD, Pursuit XRs C18 250 x 4.6 mm 5 µm column fitted with guard column Gradient elution, Solvent A: water with 0.1 % (v/v) formic acid, Solvent B: methanol, delivered at 1.0 mL/min, 21 min run time, 20 µL injection DAD detection set to 270 and 324 nm	Jerez-Martel et al., 2017 [130]
Cladophora glomerata	Gallic acid, syringic acid, 3,4-dihydroxybenzoic acid, vanillic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid	HPLC-PDA, Phenomenex C18 250 x 4.6 mm 5 µm column, Elution and mobile phase details not provided, run times vary up to 60 min PDA detection at 280 nm	Korzeniowska et al., 2020 [131]
Dunaliella tertiolecta *Phaeodactylum tricornutum*	Various phenolics including: gallic acid, protocatechuic acid, vanillic acid, syringic acid, Gentisic acid	HPLC-DAD, Pursuit XRs C18 250 x 4.6 mm 5 µm column with guard column fitted, kept at 27ºC Gradient elution, Solvent A: Milli-Q water with 0.1 % (v/v) formic acid, Solvent B: methanol, delivered at 1.0 mL/min, 60 µL injection DAD set to 270, 324 and 373 nm	López et al., 2015 [132] Rico et al., 2013 [133]

Edible algal products from: 5 brown, 2 red and 1 green seaweed, As well as 1 cyanobacterium	Vairous flavanols and the phenolic acids: gallic acid and 4-hydroxybenzoic acid	HPLC-DAD, C18 Kinetex 150 x 4.6 mm 2.6 µm column, kept at 23ºC Gradient elution, Solvent A: water with 1 % (v/v) acetic acid, Solvent B: water/acetonitrile/acetic acid (67:32:1 v/v/v), delivered at 1 mL/min, 35 min run time DAD set to 275 nm	Machu et al., 2015 [134]
Saccharina japonica (both studies)	Gallic acid, chlorogenic acid, Gentisic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid and syringic acid	HPLC-UV/Vis, Nucleosil 100-5 C8 250 x 4.6 mm 5 µm column Gradient elution, Solvent A: water with 0.1 % (v/v) glacial acetic acid, Solvent B: acetonitrile with 0.1 % (v/v) glacial acetic acid, delivered at 0.8 mL/min, 30 min run time UV/Vis detection at 280 nm	Nkurunziza et al., 2021 [135] Vo Dinh et al., 2018 [136]
Caulerpa racemosa and *Ulva lactuca*	Gallic acid, chlorogenic acid, gentisic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid	HPLC-UV, Nucleosil C8 Column Gradient elution, Solvent A: water with 0.1 % (v/v) acetic acid, Solvent B: acetonitrile with 0.1 % (v/v) acetic acid, delivered at 1.0 mL/min, UV detection at 280 nm	Pangestuti et al., 2021[137]
16 seaweed species including 8 brown, 2 green and 6 red *Fucus serratus* and *Polysiphonia fucoides*	Gallic acid, protocatechuic acid, Gentisic acid, p-hydroxybenzoic acid, chlorogenic acid, vanillic acid, syringic acid, caffeic acid, salicylic acid, coumaric acid, and ferulic acid (same in both studies)	HPLC-DAD, Zorbax Eclipse XDB C8 150 x 4.6 mm 5 µm column Isocratic elution, mobile phase: methanol/water with 10 mM ammonium acetate buffer (12:88 v/v) pH 5.4, delivered at 1.0 mL/min DAD set to 280 nm	Sabeena Farvin and Jacobsen, 2013 [138] Sabeena Farvin and Jacobsen, 2015 [139]
Nannochloropsis salina, Nannochloropsis limnetica, Desmodesmus sp., Chlorella sorokiniana, Phaeodactylum tricornutom and *Dunaliella salina*	Gallic acid, 2,5-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, chlorogenic acid, 4-hydroxybenzoic acid, syringic acid, salicylic acid, + more	HPLC-DAD, Progidy ODS-3 250 x 4.6 mm 5 µm Gradient elution, Solvent A: de-ionised water with phosphoric acid pH 3, Solvent B: acetonitrile, delivered at 0.9 mL/min, 70 min run time, 20 µL injection DAD set to 280 nm	Safafar et al., 2015 [140]

Sargassum virgatum	Gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, gentisic acid, vanillic acid, syringic acid, cinnamic acid, + more	HPLC-UV/Vis, Kromasil C18 150 x 4.6 mm 3.5 µm column, kept at 25ºC Gradient elution, Solvent A: water with 1 % (v/v) acetic acid, Solvent B: water/acetonitrile/acetic acid (67:32:1 v/v/v), delivered at 1 mL/min, 20 µL injection UV detection set to 280 nm	Semaida et al., 2022 [141]
Ulva lactuca, Enteromorpha intestinales and *Cladophora vagabunda*	Gallic acid, protocatechuic acid, Gentisic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, salicylic acid, benzoic acid, ferulic acid, elagic acid and p-coumaric acid	HPLC-DAD, Zorbax Eclipse XDB C8 150 x 4.6 mm 5 µm column Isocratic elution, mobile phase: methanol/water with 10 mM ammonium acetate buffer (12:88 v/v) pH 5.4, delivered at 1.0 mL/min DAD set to 280 nm	Sirbu et al., 2019 [142]
Gracilaria dura	Gallic acid, vanillic acid, sinapic acid, p-coumaric acid, hydroxybenzoic acid, Phloroglucinol, catechol and cinnamic acid	HPLC-PDA, Luna5-C18 250 x 4.6 mm, kept at 27ºC Isocratic elution, mobile phase: water with potassium hydrogen phosphate/acetonitrile (75:25 v/v)), delivered at 1.0 mL/min UV/Vis detection at 254 nm	Sumayya et al., 2020 [143]
Sargassum cinereum, Sargassum ilicifolium, Sargassum tenerrimum and *Sargassum wightii*	Tannic acid, gallic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid and ferulic acid	HPLC-PDA, Nova-Pak C-18 4 µm 4.6 x 250 mm column, kept at 24ºC Isocratic elution, mobile phase: water/methanol/acetonitrile (5:3:2 v/v/v) with 0.2 % (v/v) triethylamine pH 3.3, delivered at 1.0 mL/min, 15 min run time PDA detection at 280 nm	Waghmode and Khilare, 2018 [144]
Undaria pinnatifida and *Laminaria digitata*	Gallic acid, 2,5-dihydroxybenzoic acid, epicatechin and epigallocatechin	HPLC-UV, Phenomenex C18 150 x 4.6 mm 3µm column, kept at 23ºC Gradient elution, Solvent A: water with 1 % (v/v) acetic acid, Solvent B: water/acetonitrile/acetic acid (67:32:1 v/v/v), delivered at 1.0 mL/min UV detection at 275 nm	Zaharudin et al, 2018 [145]

3) **Table 1.6a** A summary of various methods of analysis of phenolics in algae by HPLC with spectrophotometric detection. Including the algae species, the target phenolics, the analytical instrumentation and methodology, and the reference.

Algae Species	Phenolics	Analytical Instrumentation and Methodology	Reference
Ascophyllum nodosum, Bifurcaria bifurcate, and *Fucus vesiculoses*	Phlorotannins, phenolic acids, and flavonoids	LC-DAD-ESI-MS/MS, Zorbax SB C18 150 x 3.0 mm 3.5 µm column, kept at 25ºC Gradient elution, Solvent A: 2.5 % (v/v) acetic acid in water, Solvent B: 2.5 % (v/v) acetic acid in methanol, delivered at 1.0 mL/min, 75 min run time DAD data collected at 240 and 370 nm Triple quadrupole, ESI source, negative ion mode, mass range of m/z 100 – 1600 Da	Agregán et al., 2017 [146]
Ascophyllum nodosum	Phlorotannins	LC-MS^n, Synergi Hydro C18 2.0 x 150 mm 4 µm column Gradient elution, Solvent A: HPLC water with 0.1 % (v/v) formic acid, Solvent B: acetonitrile with 0.1 % (v/v) formic acid, delivered at 0.3 mL/min, 35 min run time DAD collected in 200-600 nm, and 280, 365, 520 nm Ion trap, ESI source, negative and positive ion modes, mass range of m/z 80-2000, then data-dependent analysis MS/MS for three most intense ions Second MS analysis: Orbitrap, full scan accurate mass, data-dependent analysis at MS^2 and MS^3	Allwood et al., 2020 [147]
Phaeodactylum tricornutum, Diacronema lutheri, Porphyridium purpureum, Haematococcus pluvalis, Chlorella vulgaris, Tetraselmis suecica	Phloroglucinol, *p*-coumaric acid, ferulic acid, caffeic acid, catechin, quercetin, kaempferol, apigenin, luteolin, genistein, + more	UHPLC-MS/MS, Waters Acquity ethylene bridged hybrid BEH-shield RP18 3.0 x 150 mm 1.7 µm and BEH phenyl 2.1 x 100 mm 1.7 µm columns, kept at 40ºC Gradient elution, Solvent A: UHPLC water with 0.1 % (v/v) formic acid, Solvent B: acetonitrile with 0.1 % (v/v) formic acid, delivered at 0.5 mL/min, 23 min run time Triple quadrupole, ESI source, negative ion mode, MRM with analyte specific transitions and collision energies	Goiris et al., 2014 [148]
Bifurcaria bifurcate, Cystoseira humilis, Cystoseira stricta, Fucus spiralis and *Gelidium sesquipedale*	Quinic acid, malic acid, fumaric acid, gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, hesperidin	LC-MS/MS, C18 revered-phase Inertsil ODS-4 150 x 4.6 mm 3 µm C18 column, kept at 40ºC Gradient elution, Solvent A: water with 5 mM ammonium formate and 0.1 % (v/v) formic acid, Solvent B: methanol with 5 mM ammonium formate and 0.1 % (v/v) formic acid, delivered at 0.5 mL/min, 29 min run time, 4 µL injection Triple quadrupole, ESI source, negative ion mode, MRM with analyte specific transitions and collision energies	Grina et al., 2020 [149]

Monostroma oxyspermum, Ulva fasciata, Ulva lactuca, Ulva linza, Ulvareticulata and *Ulva taeniata*	Plant growth regulators including gibberellic acid, Abscisic acid, indole-3-acetic acid, salicylic acid, + more	HPLC-DAD & ESI-MS, Phenomenex Luna-C18 5 µm 4.6 x 250 mm column, kept at 35ºC Isocratic elution, mobile phase: methanol/water (60:40 v/v) with 0.6 % (v/v) acetic acid, delivered at 1.0 mL/min, 50 µL injection UV detection at 208, 254 and 265 nm ESI-MS identification, Quadrupole-TOF, ESI source, negative and positive ion modes, MS/MS mass fragmentation pattern analysis	Gupta et al., 2011 [150]
Spongiochloris spongiosa and cyanobacteria species	Hydroxybenzaldehydes, cinamic acid derivatives, *p*-hydroxybenzoic acid, protocatechuic acid, gallic acid, vanillic acid, and syringic acid	HPLC-ESI-MS, Zorbax SB-C18 75 x 4.6 mm 3.5 µm column, kept at 30ºC Gradient elution, Solvent A: water with 0.2 % (v/v) acetic acid, Solvent B: acetonitrile, delivered at 1.1 mL/min, 15 min run time, 0.5 – 20 µL injections Single quadrupole, ESI source, negative ion mode, pseudomolecular ion monitoring, structural identification using in-source collision induced dissociation at 100 V	Klejdus et al., 2009 [151]
Cystoseira abies-marina, Undaria pinnatifida, Sargassum muticum, Chrondrus crispus	Hydroxybenzaldehydes, cinamic acid derivatives, *p*-hydroxybenzoic acid, protocatechuic acid, gallic acid, salicylic acid, vanillic acid, and syringic acid	Rapid Resolution (RR) LC-MS/MS, Zorbax SB-C18 2.1 x 50 mm 1.8 µm column, kept at 26ºC Gradient elution, Solvent A: water with 0.2 % (v/v) acetic acid, Solvent B: acetonitrile, delivered at 0.8 mL/min, 4.5 min run time, 0.2 – 5 µL injections Triple quadrupole, ESI source, negative ion mode, MRM with analyte specific transitions at 20 eV collision energy	Klejdus et al., 2017 [92]
Jania rubens	Phenolic acids syringic acid and a potential syringic acid sulfate	UPLC-ESI-MS & UPLC-ESI-MS^n, HSS T3 100 x 1.0 mm 1.8 µm column Gradient elution, Solvent A: water with 0.1 % (v/v) formic acid, Solvent B: acetonitrile with 0.1 % (v/v) formic acid, delivered at 0.15 mL/min, 3.1 µL injection MS analysis with quadrupole TOF MS, ESI source, negative ion mode, mass range of m/z 100 - 1000 MS^n analysis with ion trap, ESI source, positive and negative mode, 20 eV collision energy	Maghraby et al., 2022 [152]
Halimeda sp, Spyridia hypnoides, Valoniopsis pachynema, Gracilaria fergusonii, Amphiroa anceps	Non-targetted screening identifying phenoic acids such as: 4-hydroxybenzoic acid and 4-coumaric acid	UHPLC-ESI-MS/MS, Thermo Accucore C18 100 x 2 mm 2.6 µm column, kept at 25ºC Gradient elution, Solvent A: water with 0.1 % (v/v) formic acid, Solvent B: methanol with 0.1 % (v/v) formic acid, delivered at 0.2 mL/min, 70 min run time Orbitrap MS, ESI source, positive and negative ion mode, mass range of m/z 100 - 1500	Mahomoodally et al., 2020 [153]

Pyropia yezoensis, Bangia fuscopurpurea	Salicylic acid	LC-ESI-MS/MS, Zorbax Eclipse XDB-C18 Gradient elution, Solvent A: water with 0.1 % (v/v) formic acid, Solvent B: acetonitrile with 0.1 % (v/v) formic acid Triple quadrupole, ESI source, negative ion mode, MRM transitions at 12 eV collision energy	Mikami et al., 2016 [93]
18 seaweed species including 7 red, 2 green and 9 brown	Identified compounds include: benzoic acid, *p*-hydroxybenzoic acid, vanillic acid 3-sulfate	Untargeted LC-MS/MS, Fortis C18 100 x 2.1 mm 1.7 µm column, kept at 30ºC Gradient elution, Solvent A: water with 0.1 % (v/v) formic acid, Solvent B: acetonitrile with 0.1 % (v/v) formic acid, delivered at 0.4 mL/min, 25 min run time, 5 µL injection Quadrupole TOF MS, ESI source, negative ion mode, full mass scan with range m/z 50 – 1000, for MS/MS analyses collision energy was between 10 and 40 eV	Nørskov et al., 2021 [154]
Gracilaria beckeri, Ecklonia maxima, Ulva rigida and *Gelidium pristoides*	Phlorotannins, flavonoids, and phenolic acids including: vanillic acid, syringic acid	UHPLC-ESI-QTOF-MS, Acclaim RSLC C18 2.1 x 100 mm 2.2 µm Gradient elution, Solvent A: water with 0.1 % (v/v) formic acid, Solvent B: acetonitrile with 0.1 % (v/v) formic acid, delivered at 0.3 mL/min, 40 min run time Quadrupole-TOF, ESI source, positive ion mode, collision energy of 40 eV	Olasehinde et al., 2019 [155]
Spongiochloris spongiosa, Anabaena doliolum, Porphyra tenera and *Undaria pinnatifida*	Hydroxybenzaldehydes, cinamic acid derivatives, and the hydroxybenzoic acids derivatives: *p*-hydroxybenzoic acid, protocatechuic acid, gallic acid, salicylic acid, vanillic acid, and syringic acid	HPLC-ESI-MS, Zorbax SB-C18 150 x 4.6 mm 3.5 µm column, kept at 30ºC Gradient elution, Solvent A: water with 0.2 % (v/v) acetic acid, Solvent B: acetonitrile, delivered at 1.1 mL/min, 15 min run time, injections range from 0.5 – 25 µL Single quadrupole, ESI source, negative ion mode, pseudomolecular ion monitoring, additional structural information detected using in-source CID	Onofrejová et al., 2010 [156]
Himanthalia elongata	Phloroglucinol, gallic acid, chlorogenic acid, caffeic acid, ferulic acid, + more	HPLC-DAD-ESI-MS, Atlantis C-18 250 x 4.6 mm 5 µm column fitted with guard column, kept at 25ºC Gradient elution, Solvent A: water with 0.25 % (v/v) acetic acid, Solvent B: acetonitrile/water (80:20 v/v) with 0.25 % (v/v) acetic acid, delivered at 1.0 mL/min DAD set to 254, 280 and 320 nm Triple quadrupole, ESI source, negative ion mode, mass range of m/z 100 - 1000	Rajauria, 2018 [91]

Himanthalia elongata	*m*-hydroxybenzaldehyde, *p*-hydroxybenzaldehyde, Phloroglucinol, gallic acid, gallic acid 4-*O*-glucoside, + more	HPLC-ESI-MS, Atlantis C-18 250 x 4.6 mm 5 µm column fitted with guard column, kept at 25ºC Gradient elution, Solvent A: water with 0.25 % (v/v) acetic acid, Solvent B: acetonitrile/water (80:20 v/v) with 0.25 % (v/v) acetic acid, delivered at 1.0 mL/min Triple quadrupole, ESI source, negative ion mode, mass range of m/z 100 - 1000	Rajauria et al., 2016 [157]
Palmaria spp., Porphyra spp., Himanthalia elongata, Laminaria ochroleuca and *undaria pinnatifida*	Catechin, epicatechin, gallic acid, catechin gallate, epicatechin gallate, epigallocatechin and epigallocatechin gallate	HPLC-UV and LC-MS, Teknokroma Mediterranean sea-18 150 x 4 mm 3 µm column Gradient elution, Solvent A: water with 1 % (v/v) acetic acid, Solvent B: water/acetonitrile/acetic acid (67:32:1 v/v/v), delivered at 1.2 - 1 mL/min for UV analysis, and 0.5 mL/min for MS, 20 µL injection UV detection set to 280 nm LC-MS, ESI source, negative ion mode, pseudomolecular ion monitoring	Rodríguez-Bernaldo de Quirós et al., 2010 [158]
Laminaria japonica, Undaria pinnatifida, Sargassum fusiforme and *Ascophyllum nodosum*	Flavonoids, Phlorotannins and the phenolic acids: 4-hydroxybenzoic acid, vanillic acid, gallic acid, caffeic acid and ferulic acid	UPLC-ESI-MS/MS, Acquity BEH HILIC 2.1 x 150 mm 1.7 µm column, kept at 65ºC Gradient elution, Solvent A: water with 0.1 % (v/v) formic acid, Solvent B: acetonitrile/water (95:5 v/v) with 0.1 % (v/v) formic acid, delivered at 0.4 mL/min Quadrupole Orbitrap, ESI source, positive ion mode, mass spectra over the range m/z 60 – 900, Q-Exactive HF-X secondary mass spectral analysis	Shen et al., 2021 [159]
Gracilaria birdiae and *Gracilaria cornea*	Gallic acid and apigenin	LC-MS/MS, Supelcosil LC-18 C18 250 x 5 mm Isocratic elution, Solvent A: methanol, Solvent B; water with 0.1 % (w/v) formic acid, mobile phase: (A:B 50:50 v/v), delivered at 0.4 mL/min MS/MS, ESI source, negative and positive ion modes, MS/MS collision energy 15 eV	Souza et al., 2011 [160]
Chlorella minutussima	*p*-hydroxybenzoic acid, salicylic acid and protocatechuic acid	UPLC-PDA-MS/MS, BEH C8 1.7 µm 2.1 x 150 mm column, kept at 30ºC Gradient elution: Solvent A: water with 7.5 mM formic acid, Solvent B: acetonitrile, delivered at 0.25 mL/min, 12 min run time PDA scanning from 210 – 699 nm Triple quadrupole, ESI source, negative ion mode, MRM using analyte ion transitions at 16 eV collision energy	Stirk et al., 2019 [161]

18 tropical seaweeds including 7 green, 4 brown and 7 red	Gallic acid, catechin hydrate, protocatechuic acid, vanillic acid, epicatechin, syringic acid, + more	HPLC-DAD and LC-MS, Kinetex Evo C18 5 µm 100 Å 250 x 4.6 mm column, kept at 28ºC Gradient elution, Solvent A: water with 1 % (v/v) acetic acid, Solvent B: acetonitrile, delivered at 0.7 mL/min, 20 µL injection UV detection set to 272, 280 and 310 nm Quadrupole TOF for identification by mass fragmentation analysis	Tanna et al., 2019 [162]
Ecklonia bicyclis, Laminaria japonica, Sargassum fusiforne, Undaria pinnatifida	17 polyphenols including several phenolic acids: gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, caffeic acid and p-coumaric acid	LC-MS/MS, Zorbax Eclipse XDB-C18 50 x 4.6 mm 1.8 µm column, kept at 50ºC Gradient elution, Solvent A: water with 0.05 % (v/v) formic acid, Solvent B: methanol, delivered at 1.0 mL/min Triple quadrupole, ESI source, negative ion mode, dynamic MRM mode, compound specific parameters	Vlaisavljević et al., 2021 [163]
Ulva intestinalis	Various phenolics including the phenolic acids: gallic acid, vanillic acid, coumaric acid, veratric acid, luteic acid, valoneic acid, + more	HPLC-LRMS, Zorbax SB-C18 50 x 2.1 mm 1.8 µm column Gradient elution, Solvent A: distilled water with 0.5 % (v/v) formic acid, Solvent B: acetonitrile with 0.5 % (v/v) formic acid, delivered at 0.3 mL/min Triple quadrupole, ESI source, positive and negative ion modes, mass range of m/z 100 – 800	Wekre et al., 2019 [164]
8 seaweed species including 3 green, 3 red and 2 brown	22 phenolic acids, 17 flavonoids, 11 other polyphenols and 4 lignans	LC-ESI-QTOF-MS/MS and HPLC-PDA, Synergi Hydro-RP 80 Å 250 x 4.6 mm 4 µm column Gradient elution, Solvent A: water with 2 % (v/v) acetic acid, Solvent B: acetonitrile/water/acetic acid (50:49.5:0.5 v/v/v), delivered at 0.8 mL/min, 90 min run time PDA detection at 280, 320 and 370 nm Quadrupole TOF, ESI source, positive and negative ion modes, mass scan range from m/z 50 – 1300, MS/MS automatic analysis with collision energies of 10, 15 and 30 eV	Zhong et al., 2020 [165]

4) **Table 1.6b** A summary of various methods of analysis of phenolics in algae by HPLC with mass spectrometric detection. Including the algae species, the target phenolics, the analytical instrumentation and methodology, and the reference.

Plant Species	Phenolics	Analytical Instrumentation and Methodology	Reference
Eucomis autumnalis	Hydroxybenzoic acids, hydroxycinnamic acids, flavonoids	UHPLC-MS/MS Analysis, BEH C8 1.7 µm 2.1 x 150 mm column, kept at 30ºC Gradient elution, Solvent A: 7.5 mM formic acid in water, Solvent B: acetonitrile, delivered ay 0.25 mL/min, 12 min run time PDA detection at range of 210 – 600 nm Triple quadrupole, ESI source, negative ion mode, individual MRM transitions, collision energy 16 eV	Aremu et al., 2015 [166]
Miscanthus sacchariflorus	22 phenolic compounds including: veratric acid, protocatechuic acid, gallic acid, *p*-hydroxybenzoic acid, vanillic acid, Gentisic acid, 2,4-dihydroxybenzoic acid, salicylic acid, + more	LC-MS/MS, C18 4.6 x 250 mm 5 µm column, kept at 25ºC Gradient elution, Solvent A: water with 0.1 % (v/v) formic acid, Solvent B: acetonitrile in water, delivered at 0.7 mL/min, 30 min run time, 10 µL injection Triple quadrupole, ESI source, negative ion mode, MRM analysis mode with analyte specific transitions and collision energies	Ghimire et al., 2020 [167]
Trifolium pratense, Glycine max, Pisum sativum and *Ononis spinosa*	Isoflavones, cinamic acid derivatives, gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid	UHPLC-PDA, Zorbax SB CN 50 x 2.1 mm 1.8 µm column, kept at 58ºC Gradient elution, Solvent A: water with 0.3 % (v/v) acetic acid, Solvent B: methanol, delivered at 0.9 mL/min, 1 µL injection PDA detection at 270 nm	Klejdus et al., 2008 [168]
Tabebuia avellanedae	Veratric acid	LC-MS/MS, Kinetex C18 100 Å 150 x 2.1 mm 2.6 µm column, kept at 40ºC Isocratic elution, mobile phase: methanol:water (50:50 v/v) with 0.1 % (v/v) formic acid, delivered at 0.2 mL/min Triple quadrupole, ESI source, positive ion mode, MRM transitions at collision energies of 15, 17, 30 eV	Naveen et al., 2020 [169]
Rosa rugosa	25 phenolic acids	LC-ESI-MS/MS, Eclipse XDB-C18 4.6 x 150 mm 5 µm column, kept at 20ºC Gradient elution, Solvent A: water with 0.1 % (v/v) formic acid, Solvent B: acetonitrile with 0.1 % (v/v) formic acid, delivered at 0.3 mL/min, 22 min run time Triple quadrupole – linear ion trap, ESI source, negative ion mode, MRM analysis mode with analyte specific transitions and conditions	Olech et al., 2020 [170]

Olea europea	16 phenolic acids and aldehydes	LC-ESI/ACPI-QTOF MS, Intensity Solo C18 2.1 x 100 mm 1.8 µm column Gradient elution, Solvent A: water with 0.5 % (v/v) acetic acid, Solvent B: acetonitrile with 0.5 % (v/v) acetic acid, delivered at 0.4 mL/min until 10 min then at 0.6 mL/min thereafter ESI-QTOF, positive and negative ion modes, 6 eV collision energy, full scan spectra in range m/z 30 – 1000, auto MS/MS for fragmentation information APCI-QTOF, positive and negative ion modes, full scan spectra in range m/z 30 – 1000, auto MS/MS for fragmentation information	Olmo-García et al., 2018 [171]
Brassica oleracea L. var. *sabellica*	13 phenolic acids	LC-ESI-MS/MS, Zorbax SB-C18 2.1 x 50 mm 1.8 µm column, kept at 25ºC Gradient elution, Solvent A: water with 0.1 % (v/v) formic acid, Solvent B: methanol with 0.1 % (v/v) formic acid, delivered at 0.4 mL/min, 15 min run time Triple quadrupole ion trap MS, ESI source, negative ion mode, compound specific MRM conditions	Oniszczuk and Olech, 2016 [85]
Vaccinium macrocarpon (Commercial cranberry products)	47 phenolic acids	UPLC-DAD-ESI-TQ MS, Water BEH C18 2.1 x 100 mm 1.7 µm column, kept at 40ºC Gradient elution, Solvent A: water/acetic acid (98:2 v/v), Solvent B: acetonitrile/acetic acid (98:2 v/v), delivered at 0.5 mL/min, 18 min run time DAD detection set to a range from 250 – 420 nm Tandem quadrupole MS/MS, ESI source, negative ion mode, specific MRM conditions for each compound	Sánchez-Patán et al., 2012 [172]

5) **Table 1.6c** A summary of various methods of analysis of phenolics in terrestrial plants by HPLC with spectrophotometric or mass spectrometric detection. Including the plant species, the target phenolics, the analytical instrumentation and methodology, and the reference.

Common Liquid Chromatography Conditions

- The overwhelming majority of methods employed reversed-phase C18 stationary phases, with the some methods using the less hydrophobic C8 stationary phase
- Various column lengths were employed with particles sizes between 3 – 5 μm for most analyses, with some UHPLC methods using smaller particle sizes ≤ 2 μm
- Columns were often kept at around 25 ºC, presumably to prevent temperature fluctuations however, some columns were kept at ≥ 50 ºC for some analyses
- The mobile phase in the majority of cases was a mixture of water with methanol or acetonitrile, often modified with acetic or formic acid between 0.1 – 1.0 % (v/v)
- Most LC methods employed gradient elution, however there were methods that used isocratic elution with great success
- Flow rates are column and detector dependent, but were commonly between 0.2 – 1.0 mL/min
- Run times being were as short as 4.5 min and as long as 100 min

Common Spectrophotometric Detection Conditions

- UV, UV/Vis, PDA were most often set at wavelengths between 270 – 280 nm for the analysis of phenolic acids, with the PDA detectors performing wavelength range scans between 220 – 420 nm

Common Mass Spectrometric Conditions

- The majority of methods employing MS detection used an electrospray ionisation source, most often operating in negative ionisation mode but occasionally both positive and negative ionisation modes were employed
- Full scan mode was regularly used for screening purposes within a range of m/z 0 – 1000
- MS^2 or MS/MS spectra were commonly used for positive identification following fragmentation, which was most commonly achieved through collision induced dissociation
- Collision energies varied but were commonly between 10 – 20 eV, and sometimes up to 40 eV
- Many MS methods employed MRM for qualitative and quantitative analyses, with the pseudomolecular ion serving as the dominant precursor ion

1.7 THE USE OF HYDROXYBENZOIC ACIDS IN AGRICULTURE

Due to the paradigm shift in agricultural strategies towards more sustainable practices to improve productivity, there is a considerable amount of research into natural substances that show potential for improving crop growth, productivity, and quality whilst assisting to mitigate stress-induced limitations due to abiotic and biotic stresses [13, 20, 28]. Additional to the research on biostimulants that was previously discussed in Section 1.2 and 1.3, there is also an extensive body of work focussed on identifying and characterising countless endogenous phytochemicals and their biological activities in plants [13, 28]. Consequently, it is imperative to exploit this abundance of information on endogenous phytochemicals and their role within important plant cellular interactions and processes thereby assisting in elucidating the effects of their exogenous application [14, 61]. The wealth of knowledge on endogenous phytochemicals when combined with the characterisation of the chemical composition of the natural biostimulants, provides grounds to resolve the biological mechanisms underpinning the benefits of biostimulant application; this information can be used in bioassays and field trials investigating the exogenous application potentially uncoupling the relationship between cause and effect [1, 14].

The studies investigating the exogenous application of important phytochemicals directly to seeds, plants, the soil/rhizosphere or the food products pre-/post-harvest tend to focus on the most well characterised phytochemicals, such as plant growth regulators or 'phytohormones' [28, 61]. One potent phytohormone that has received a significant amount of attention due to its well characterised role in regulating a variety of important physiological processes in plants is salicylic acid [17, 57, 173]. The effects of exogenous application of salicylic acid have been tested for a range of benefits including improved growth and development, particularly under abiotic stress conditions which are only increasing in prevalence and severity due to anthropogenic activities [58, 61, 174, 175]. Given salicylic acid's role in plant defence, exogenous application has also been investigated for its potential in increasing resistance to biotic stresses, as well as influencing the plant-microorganism symbiosis in the rhizosphere [10, 57, 173]. Table 1.7 below lists a range of studies investigating the exogenous application of salicylic acid aimed at naturally improving crop growth, quality, and stress tolerance to improve agricultural productivity. Also included in Table 1.7, are studies investigating the exogenous application of other hydroxybenzoic acids through bioassays and field studies that share the same goal of improved agricultural productivity.

Plant(s)	Compound(s)	Dosage and Application	Effect and Agricultural Outcome	Reference(s)
Wheat (S-24 and MH-97 cultivars)	Salicylic acid	0, 0.25, 0.50, 0.75 or 1.00 mM treatment through rooting medium for 7 days	Promotion of growth and yield, and counteracted salt stress-induced growth inhibition. Improved photosynthetic capacity	Arfan et al., 2007 [176]
Barley (*Hordeum vulgare cv* Gerbel)	Salicylic acid	1mM grain soaking presowing	Induced a preadaptive response to salt stress which led to increased photosynthetic pigments and helped maintain membrane integrity resulting in improved plant growth	El-Tayeb, 2005 [177]
Pomegranate (Mollar de Elche cultivar)	Salicylic acid, acetylsalicylic acid and methyl salicylate	1, 5 or 10 mM pre-harvest foliar spray treatment	Higher crop yield, higher quality parameters (firmness, aril colour and individual sugars) and higher concentrations of phenolics, anthocyanins, and ascorbic acid	García-Pastor et al., 2020 [178]
Sweet Cherry (Sweet Heart and Sweet Late cultivars)	Salicylic acid and acetylsalicylic acid	0.5 1.0 or 2.0 mM pre-harvest foliar spray treatment	Increased fruit weight and improved quality attributes, higher concentration of total phenolics and anthocyanins, higher antioxidant activity Improved cherry quality and health benefits for consumers	Giménez et al., 2014 [179]
Wheat (*Tirticum aestivum* L. cv. Raj-3077)	Salicylic acid	0, 10^{-5}, 10^{-4} or 10^{-3} grain soaking for 3, 6 or 9 h	Higher leaf number, increased fresh and dry plant mass, and higher nitrate reductase and carbonic anhydrase activities 10^{-3} treatment had detrimental effects across all parameters	Hayat et al., 2005 [180]
Wheat (*Triticum aestivum* L. cvs. Cheyenne and Chinese Spring)	Salicylic acid and 4-hydroxybenzoic acid	0.5 mM hydroponic growth medium treatment for 1 day	4-hydroxybenzoic acid improved the freezing tolerance of the Chinese Spring and the drought tolerance of the Cheyenne Salicylic acid decreased the drought tolerance of Chinese Spring and decreased the freezing tolerance of Cheyenne Increased abiotic stress tolerance	Horváth et al., 2007 [181]
Cucumber (*Cucumis sativus* L.)	p-hydroxybenzoic acid	0.5 mM hydroponic growth medium treatment	Significant inhibition of root growth observed Characterisation of allelopathic properties of the autotoxin p-hydroxybenzoic acid to improve repeated cultivation outcomes and prevent poor growth and crop yield decline	Huang et al., 2020 [70]

Cocksfoot (*Dactylis glomerata* L.)	p-hydroxybenzoic acid	0.1, 0.5, 1.0 or 1.5 mM irrigation treatment 3 times	Reduction of fresh and dry leaf biomass, shoot and root length, relative water content and leaf osmotic potential. p-hydroxybenzoic acid demonstrated allelopathic potential, for controlling noxious weeds/use as a natural bioherbicide	Hussain et al., 2015 [182]
Arabidopsis thaliana (mutants with altered immune systems)	Salicylic acid	0.5 mM spray on leaves and soil	Salicylic acid modulates rot microbiome composition and in its absence the core root bacterial community composition is substantially altered	Lebeis et al., 2015 [10]
Grapes (*Vitis Vinifera* L. Superior seedless)	Salicylic acid	0, 1, 2 or 4 mM pre-harvest foliar spray	Significantly reduced weight loss, berry shatter, rachis browning index; and preserved berry firmness, separation force, total phenol content and colour hue angle Delayed cluster ripening and maintaining cluster quality during shelf-life at room temperature for four days	Lo'ay, 2017 [183]
Wheat (*AtNPR-1* expressing transgenic Bobwhite)	Salicylic acid	200 µM soil drench	Increased content of wheat spikes, enhancing resistance to Fusarium head blight caused by *Fusarium graminearum*	Makandar et al., 2012 [184]
Tobacco (*Nicotiana tabacum* L.) cell and tissue cultures	3,4-dihydroxybenzoic acid (protocatechuic acid)	0.1, 10 or 1000 µM treatment through supplemented growth media	The highest concentration strongly inhibited leaf tissue proliferation, callus growth, shoot regeneration and root growth The lowest concentration showed auxin-like activity by stimulating cell dedifferentiation, callus induction and rooting of leaf tissues	Mucciarelli et al., 2000 [185]
Rice (*Oryza sativa* L. drought tolerant Q8 and drought susceptible Q2 cultivars)	Vanillic acid and p-hydroxybenzoic acid	25 or 50 µM foliar spray treatment	Both compounds promoted total contents of phenolics, flavonoids, pigments, and DPPH scavenging; resulting in drought tolerance improvement Variable effects that were dose-dependent	Quan and Xuan, 2018 [186]
Spinach (*Spinacia oleracea* L. Reflect)	Salicylic acid	0.5 or 1.0 mM nutrient solution treatment	Salicylic acid did not hamper leaf growth and significantly improved freezing tolerance as observed by reduced ion-leakage and alleviated oxidative stress	Shin et al., 2018 [187]

Lemon (*Citrus limon* L. Burm. F.)	Salicylic acid and methyl jasmonate	2 mM salicylic acid and/or 10 µM methyl jasmonate soak treatment	Treatment was effective in enhancing chilling tolerance of lemon fruit by significantly reducing chilling-induced membrane permeability and membrane lipid peroxidation, total phenolics was also increased	Siboza et al., 2014 [188]
Wheat (*Triticum aestivum* L. cv. HD-2329)	Salicylic acid	1, 2 or 3 mM foliar spray treatment	Treated plants showed a higher moisture content, dry mass, enzymatic activity (Rubisco and superoxide dismutase) and total chlorophyll Salicylic acid plays a role in regulating drought response of plants, improving plant growth under water stress	Singh and Usha, 2003 [189]
Tomato (*Lycopersicon esculentum* Mill. cv. *Roma*)	Salicylic acid	0.1 mM root drenching treatment	Treated plants had greater survival and relative shoot growth rates compared to untreated plants when exposed to salt stress Increased photosynthetic, transpiration and stomatal conductance rates we observed Salicylic acid in appropriate concentrations alleviates salinity stress	Stevens et al., 2006 [190]
Peach (*Prunus persica* L. Batsch. cv. 'Flordaking')	Salicylic acid	0.5, 1.0, 1.5, or 2.0 mM fruit dipping treatment immediately after harvest	Treated fruit showed increased activity for enzymatic antioxidants and radical scavenging, increased fruit firmness, and decreased pH during storage and weight loss Improved fruit keeping quality during postharvest storage	Tareen et al., 2012 [191]
Navel Orange (*Citrus sinensis* Osbeck)	Salicylic acid	0.25 mM treatment solution shoot soak and foliar spray	Upon exposure to citrus canker (*Xanthomonas axonopodis* pv. *citri*) lower disease incidence rate and small lesion sites were observed in treated samples Results suggest salicylic acid evoked a cascade of events that confer resistance to citrus canker	Wang and Liu, 2012 [192]
Apricot (*Prunus armeniaca* L. cv. Dahuang)	Salicylic acid	1.0 or 2.0 mM vacuum-infiltration treatment of fruit post-harvest	Treated fruit were investigated for quality attribute especially related to antioxidant activity Treatment retarded the ripening progress and quality loss, increased phenolic and flavonoid content improving post-harvest quality particularly during storage	Wang et al., 2015 [193]

Cucumber (*Cucumis sativus* L. cv. Jinlv 3)	Syringic acid	0.1 µmol/g of treatment applied to 150 g soil containing one cucumber seedling	Treated soil had decreased bacterial community diversity and increased fungal community richness and diversity, which may exert negative effects on seedling growth through inhibiting plant-beneficial microorganisms Autotoxicity investigation of syringic acid allelopathy revealed important role in the complex interactions between plant and soil microorganisms	Wang et al., 2018 [194]
Chickpea (*Cicer arietinum* L.)	Salicylic acid	1.0, 1.5 or 2.0 mM foliar spray treatment	Plants treated with 1.5 mM showed induction of peroxidase and polyphenol oxidase activities, major components of induced plant defence Plants treated with 2 mM showed phytotoxic symptoms	War et al., 2011 [195]
King Protea (*Protea cynaroides*) explants	3,4-dihydroxybenzoic acid	1, 5, 25, 50, 75, 100 or 500 mg/L *in vitro* treatment incorporated into growth medium	Explant response was strongly dosage dependent, with non-observable effects obtained from 1 – 50 mg/L 75 and 100 mg/L showed an observable increase in root growth, particularly 100 mg/L which was significantly longer and had higher mean root mass The 500 mg/L treatment however appear toxic with severe browning far shorter roots and lower mean root mass	Wu et al., 2007 [196]
Cucumber (*Cucumis sativus* cv. Jinchun no.4)	p-hydroxybenzoic acid	0.25, 0.5 or 0.75 mM treatment incorporated into watering nutrient solution	Pre-treatment enhanced antioxidant enzyme activities under heat stress, enhancing heat tolerance Thus protecting cucumber seedlings from heat stress	Zhang et al., 2012 [197]
Cucumber (*Cucumis sativus* L. cv. Jinlv 3)	p-hydroxybenzoic acid	0.1, 0.25, 0.5 or 1.0 µmol/g of treatment applied to 150 g soil containing one cucumber seedling	Treatment inhibited seedling growth and stimulated an increase in rhizosphere bacterial and fungal communities Results indicate that p-hydroxybenzoic acid plays a role in the chemical plant-microorganism interactions Autotoxic allelopathy of p-hydroxybenzoic acid suggested as one cause for soil sickness from continuous monocropping	Zhou et al., 2012 [198]

6) **Table 1.7** A summary of studies investigating the effects of the exogenous application of hydroxybenzoic acids on plants for improved agricultural outcomes .

1.7.1 Summary of Key Observations from Exogenous Investigations

The studies provided in Table 1.7 are only a small fraction of the research investigating the exogenous application of important plant phytochemicals with aims to improve agricultural productivity; however they provide an overview of the various plant species, compounds, and dosages being investigated, along with the methods of application being employed, and the biological effects and agricultural benefits observed. When reviewing the research involving the exogenous application of important phytochemicals there are a few key observations that warrant further discussion. These key observations are: that the biological mechanisms appear to be general to plants and not species specific, that dosage is critical, and that the biological mechanisms are complex with one phytochemical often eliciting a pleiotropic effect.

The biological mechanisms that underpin the observed benefits appear in many cases to be common amongst plants rather than species specific. By demonstrating that the mechanisms are mostly shared across a wide range of crops it further validates the use of natural substances as a means for increasing agricultural productivity sustainably. For example, the studies conducted by García-Pastor *et al.* and Giménez *et al.* investigated the effects of pre-harvest foliar application of salicylic acid on pomegranate and sweet cherries; finding that fruit quality was improved in treated crops, along with increased antioxidant activity and nutritional content including phenolics and anthocyanins [178, 179]. Since local and systemic acquired resistance in plants are common pathways for pathogenic resistance, phytohormones and elicitor compounds that can induce or regulate these pathways serve as a more natural and sustainable way to improve plant resistance to biotic stresses [173]. Some examples are provided for increased resistance to biotic stresses induced by the exogenous application of a phytochemical; as in the study conducted by Wang and Liu where salicylic acid treatment reduced the disease incident rate of citrus canker in navel oranges [192].

The effects of the application of phytochemicals whether individually or as part of more complex biostimulants are almost always dosage dependent. This is particularly important for phytohormones with which even small fluctuations in endogenous concentrations can have drastic impacts, as well as allelochemicals many of which have hormetic effects where at low doses they exhibit stimulatory effects but show inhibitory effects at high doses [72, 199]. Multiple studies presented showed varying effects due to dosage including the study by Hayat *et al.* where wheat grains were soaked in salicylic acid solutions of various concentrations prior to sowing; the highest concentration having detrimental effects for all measured parameters whilst the lower concentrations improved growth and enzymatic activities [180]. Another study presented investigated the allelochemical, 3,4-dihydroxybenzoic acid (protocatechuic acid), and its effects on root formation in the King Protea (*Protea cynaroides*); they found that plant response was strongly dose dependent and that the highest concentration tested appeared to be toxic [196].

Finally, the studies investigating phytochemicals often conclude by expressing the need for further research into elucidating/unravelling/dissecting the biological mechanisms underpinning the observed benefits [200, 201]. Whilst a great deal of research has been conducted in an attempt to elucidate some biological mechanisms with varying degrees of success, due to their complexity they remain poorly understood or incompletely characterised. For phytochemicals such as the phytohormones their incomplete characterisation can be due to their pleiotropic nature; where often a single phytohormone is involved in regulating many different physiological processes, which makes uncoupling the relationship between cause and effect a difficult task for a specific phenotypical response [57, 174]. Moreover, plants have developed complex phytohormone networks in order to survive environmental stresses, involving highly sophisticated 'crosstalk' between the different phytohormones in order to fine-tune a balanced response between growth/development and defence/resistance [173, 200]. This plant growth/immunity balance gets more complex with the addition of the extra dimension of plant-microorganism interactions, for which there has been a wealth of research in recent years investigating the role of phytochemicals in the establishment/modulation of the rhizosphere [10, 200]. Ultimately, the application of natural substances to crops as safe, effective, and environmentally friendly plant growth regulators and plant protectors is an already well established sustainable agricultural practice. The continued sharing of knowledge and multidisciplinary research effort into these natural substances will increase their contribution to improving agricultural productivity in order to meet the growing food demands. Of particular importance is the potential for seaweed biostimulants to provide a means for optimising the plants growth/immunity balance therefore increasing yield and stress tolerance which would have a profound impact on agriculture globally [200].

1.8 AIMS AND OBJECTIVES

The use of natural biostimulants is a sustainable agricultural practice that has been shown to improve crop productivity whilst remaining adaptable to changing climate patterns, widespread pests, and evolving pathogens. Research on biostimulants has shown that they help to regulate plant growth and development, and assist in the mitigation of environmental stresses. Current research on biostimulants involves a multidisciplinary effort to further identify the functional constituents and elucidate the biological mechanisms underpinning the observed benefits on crop productivity. An important plant phytohormone that has been shown to promote plant growth and development, which is also critical to plant defence is salicylic acid. It is hypothesized then that salicylic acid and structurally related derivatives are present in the commercial seaweed biostimulants and contribute to the biostimulants' observed benefits.

The aims of this research were three-fold and focussed firstly on the identification of hydroxybenzoic acids and related derivatives in commercial seaweed biostimulants; secondly on the quantitative determination of salicylic acid and its isomers in a commercial seaweed biostimulant; and thirdly the investigation into the role of salicylic acid and its isomers in the biological activity of seaweed biostimulants.

The first aim is the identification of hydroxybenzoic acids in a commercial seaweed biostimulant using modern analytical techniques, with the following objectives:

1. The preliminary identification of monohydroxybenzoic acids in a commercial seaweed biostimulant using HPLC-ESI-MS/MS
2. The development of qualitative HPLC-ESI-MS/MS methods to investigate hydroxybenzoic acids and related derivatives in a commercial seaweed biostimulant
3. The investigation into the less explored reversed phase biphenyl stationary phases for the separation of hydroxybenzoic acids and related derivatives
4. The development and partial validation of a quantitative method using mixed-mode SPE sample preparation followed by the HPLC-ESI-MS/MS analysis of the monohydroxybenzoic acids in a commercial seaweed biostimulant

The second aim is the investigation into the role of salicylic acid and its isomers in the biological activity of seaweed biostimulants using a plant growth bioassay, with the following objectives:

1. The investigation of any individual and/or synergistic bioefficacy of aqueous monohydroxybenzoic acids solutions on tomato seedling plant growth
2. The investigation of any synergistic bioefficacy from fortification of the commercial seaweed biostimulant with the monohydroxybenzoic acids on tomato seedling plant growth

1.9 THESIS OUTLINE

There are six chapters in this thesis as follows:

Chapter 1 (Literature Review) presents an introduction into agricultural practices and the shift to sustainable agricultural practices, introduces biostimulants as one of those practices and details algal based biostimulants and their many benefits. It discusses phytochemicals focusing on phenolics acids and hydroxybenzoic acids. It then lays out the analytical methodology for the chemical analysis of complex samples such as biostimulants before discussing the various analytical techniques and instrumentation. Next the methods of analysis for phenolic acids in various algae and plants are compiled and compared to provide the foundations for the method development in this research. The exogenous application of important phytochemicals is discussed with an emphasis on hydroxybenzoic acids and their application to plants for improved agricultural productivity and their role in the bioactivity of the seaweed biostimulants. Lastly, the aims and objectives of the research project are outlined.

Chapter 2 (Materials and Methods) presents details on the samples, materials, reagents, methods of sample preparation and instrumental chemical analysis. Also included are the methods for the root growth bioassay. All described in such a manner that the experiments conducted in this research can be repeated.

Chapter 3 (Results and Discussion I) presents the results for the development and optimisation of qualitative HPLC-ESI-MS/MS methods for the identification of hydroxybenzoic acids and related derivatives in a commercial seaweed biostimulant.

Chapter 4 (Results and Discussion II) presents the results for the development and optimisation of a HPLC-ESI-MS/MS method for the identification and quantification of monohydroxybenzoic acids in a commercial seaweed biostimulant.

Chapter 5 (Results and Discussion III) presents the results for the investigation of the role of the salicylic acid and its isomers in the bioactivity of seaweed biostimulants, investigated through a plant growth bioassay.

Chapter 6 (Conclusions and Future Directions) presents a summary of the discoveries made, discusses the conclusions that can be drawn from this research and proposes further research in this area.

CHAPTER 2: METHODS AND MATERIALS

2.1 INTRODUCTION

This chapter outlines the materials, instrumentation and methods used throughout this research project. Important information regarding the sample is provided before a comprehensive list of all the reagents, chemicals, and materials. Following that, the methodology for the solution and sample preparation, and the instrumental and data analysis are described. This includes significant methods used during investigatory experiments as well as final optimised methods used for qualitative and quantitative purposes. The final part of this chapter outlines the bioassay methodology used in this research project as adapted from a method developed by Seasol R&D.

2.2 COMMERCIAL SEAWEED BIOSTIMULANT SAMPLES

Samples were provided in kind by Seasol International, Bayswater, Australia. The commercial seaweed biostimulant provided is a liquid seaweed extract marketed as "Seasol Commercial"; which is an alkaline hydrolysis product made from two seaweed species – *Durvillaea potatorum* (sourced from King Island and the west coast of Tasmania) and *Ascophyllum nodosum* (sustainably harvested from managed kelp beds in the Northern Hemisphere) [1, 202]. The Seasol Commercial Seaweed Concentrate is a natural product and as stated in the product information, there are small variations in the concentrations of the individual components. A standard chemical technical analysis taken from an average of samples across different batches is available and is duplicated in Table 2.2 [203]. The product analysis in Table 2.2 is for the undiluted concentrate, which requires dilution prior to use at levels ranging from approximately 1 in 200 to 1 in 500. The provided samples were stored in accordance with the guidelines outlined in the MSDS [203].

Seasol Commercial Seaweed Concentrate

Composition	Amount	Element	Amount
pH	11.5 – 12.5	Calcium (Ca)	458 mg/L
Total Solids	17 % w/v	Cobalt (Co)	<0.5 mg/L
Total Organic Matter	8 %	Copper (Cu)	<0.5 mg/L
Specific Gravity	1.1	Iron (Fe)	115 mg/L
Appearance	Dark Brown Liquid, Seaside Odour	Magnesium (Mg)	972 mg/L
Filtration	150 µm	Manganese (Mn)	2 mg/L
		Molybdenum (Mo)	<0.5 mg/L
Element	**Amount**	Selenium (Se)	<0.5 mg/L
Nitrogen (N)	0.2 % w/v	Silicon (Si)	56 mg/L
Phosphorus (P)	0.02 % w/v	Sodium (Na)	6820 mg/L
Potassium (K)	3.7 % w/v	Sulphur (S)	2574 mg/L
Boron (B)	15 mg/L	Zinc (Zn)	5 mg/L

7) **Table 2.2** The product analysis of the undiluted Seasol Commercial Seaweed Concentrate, sourced from Seasol at https://www.seasol.com.au/wp-content/uploads/2017/03/Product-Analysis-Seasol-Commercial-Seaweed-Concentrate-April-2017.pdf [203]

2.3 CHEMICALS, MATERIALS AND REAGENTS

A comprehensive list of all chemicals, materials and reagents used throughout this research project are collated along with their source in Table 2.3.

Chemical/Material/Reagent	Source
LCMS Grade Methanol (Optima®)	Fisher Chemical A456-4
LCMS Grade Water (Optima®)	Fisher Chemical W6-4
LCMS Hypergrade Acetonitrile (LiChrosolv®)	Merck 1.00029.2500
LCMS Grade Formic Acid (Optima®)	Fisher Chemical A117-50
LC Grade Methanol (LiChrosolv®)	Merck 1.06018.4000
LC Grade Acetonitrile (LiChrosolv®)	Merck 1.00030.4000
HPLC Grade 2-propanol (Chromasolv®)	Sigma-Aldrich 34863-4L
Formic Acid, Reagent Grade, ≥95 %	Sigma-Aldrich F0507-500ML
Hydrochloric Acid, AR Grade, 34 %	Merck 6.10307.2511
Ammonium Hydroxide, 25 – 30 % NH_3 basis	Sigma Aldrich 05003
Ultrapure Water (18 MΩ resistivity)	Purite Select HP Water Purification System
LC-Pak® Ultrapure Water – Trace Organics Filter	Merck LCPAK00A1
Salicylic acid, ≥99.0 %	Sigma-Aldrich S5922-100G
3-hydroxybenzoic acid, ≥99 %	Honeywell Fluka 54610-50G
Sodium 4-hydroxybenzoate, ≥96 %	Sigma-Aldrich H3766-25G
2,3-dihydroxybenzoic acid, 99 %	Sigma-Aldrich 126209-5G
2,4-dihydroxybenzoic acid, 97 %	Sigma-Aldrich D109401-5G
Gentisic acid, 98 %	Sigma-Aldrich G-5254
2,6-dihydroxybenzoic acid, 98 %	Sigma-Aldrich D109606-25G
3,4-dihydroxybenzoic acid, ≥97 %	Sigma-Aldrich 37580
3,5-dihydroxybenzoic acid, 97 %	Sigma-Aldrich D110000-100G
2,3,4-trihydroxybenzoic acid, 97 %	Sigma-Aldrich 253847-5G
2,4,6-trihydroxybenzoic acid monohydrate, 90 %	Sigma-Aldrich 367346-25G
Gallic acid, 97.5 – 102.5 %	Sigma-Aldrich G-7384-1KG
Vanillic Acid, ≥97 %	Sigma-Aldrich 94770-10G
Syringic Acid, ≥95 %	Sigma-Aldrich S6881-5G
3,4-dimethoxybenzoic acid, ≥99 %	Sigma-Aldrich D131806-100G
3,4,5-trimethoxybenzoic acid, 99 %	Sigma-Aldrich T69000-100G
Anthranilic Acid, 98+ %	Sigma-Aldrich A89855-500G
EVOLUTE EXPRESS AX SPE Columns 60 mg/ 3 mL (Biotage)	Biotage 613-0006-BXG
0.45 µm PTFE 25 mm Syringe Filters (PHENEX)	Phenomenex AF0-0512
Amber Verex HPLC Vials 2 mL (Phenomenex)	Phenomenex AR0-3801-13
Verex HPLC Vial Screw Caps 8 mm (Phenomenex)	Phenomenex AR0-8857-13-B
Verex HPLC Vial Inserts 175 µL w/ bottom spring (Phenomenex)	Phenomenex AR0-4521-12
Consumable items below are from the university bulk stock	
5000 µL Clear Pipette Tips	Centrifuge tubes 5, 15, 20 and 50 mL
1000 µL Blue Pipette Tips	Disposable Luer Lock Syringes 1, 3, 5 and 10 mL
200 µL Yellow Pipette Tips	pH test strips
10 µL Clear Pipette Tips	

8) **Table 2.3** A comprehensive list of all chemicals, materials and reagents used throughout this research project.

2.4 PREPARATION OF HYDROXYBENZOIC ACID STANDARD SOLUTIONS

This section details the preparation of various hydroxybenzoic acid and related derivatives standard solutions used in the qualitative and quantitative investigations described in Chapters 3 and 4, respectively.

2.4.1 Hydroxybenzoic Acid Standard Solutions used in Qualitative Investigation

2.4.1.1 Primary Stock Standard Solutions.

The primary stock standard solutions were made to a concentration of 4000 mg/L. This involved accurately weighing out the equivalent of 20 mg of each hydroxybenzoic acid using an analytical balance before volumetrically pipetting 5 mL of HPLC grade methanol with 0.1 % (v/v) formic acid. These stock solutions were mixed until complete dissolution by inversion and then vortexed for 1 minute.

2.4.1.2 Secondary Stock Standard Solutions.

Secondary stock solutions of each hydroxybenzoic acid were prepared to a concentration of 200 mg/L by direct dilution. A 100 μL aliquot of the primary stock standard solution was diluted to 2 mL with LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid.

2.4.1.3 Individual Working Standard Solutions.

Individual working standard solutions of each hydroxybenzoic acid were prepared to a concentration of 0.5 mg/L by direct dilution. A 5 μL aliquot of the secondary stock standard solution was diluted to 2 mL in a HPLC vial with LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid.

2.4.1.4 Mixed Working Standard Solutions.

Mixed working standard solutions were prepared for the monohydroxybenzoic acids, dihydroxybenzoic acids, and trihydroxybenzoic acids for the LC investigation and optimisation experiments. The concentration of the mixed working standard solutions used for the LC investigation and optimisation experiments catered for the different instrument response observed for the various hydroxybenzoic acids and related derivatives. Further dilution of the 200 mg/L secondary stock solutions allowed for the preparation of the following mixed standard solutions in LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid.

a) The monohydroxybenzoic acid mixed standard solution was prepared via direct dilution to the concentrations of 25, 500 and 200 μg/L for 2-, 3- and 4-hydroxybenzoic acid, respectively.
b) The dihydroxybenzoic acid mixed standard solution was prepared via direct dilution to the concentrations of 63, 250, 167, 25, 250 and 500 μg/L for 2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-dihydroxybenzoic acid, respectively.
c) The trihydroxybenzoic acid mixed standard solution was prepared via direct dilution to the concentrations of 2000, 500 and 500 μg/L for 2,3,4-, 2,4,6- and 3,4,5-trihydroxybenzoic acid, respectively.

2.4.2 Preparation of Standard Solutions for Validation Study and Quantitation of Monohydroxybenzoic Acids.

Various mixed standard solutions were required for the validation study as well as the quantitative analysis of the monohydroxybenzoic acids in Chapter 4. Using the same direct dilution methodology discussed in Section 2.4.1 the following mixed standard solutions were prepared in LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid.

2.4.2.1 Standard Solution for Instrument Repeatability.

A mixed standard solution was prepared by further dilution of the secondary stock solutions in LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid to a concentration of 25, 250, 100 μg/L of 2-, 3- and 4-hydroxybenzoic acid.

2.4.2.2 Standard Solutions for Linearity.

By further dilution of the secondary stock solutions a tertiary stock solution was prepared in LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid. The concentrations of 2-, 3-, and 4-hydroxybenzoic acid in the tertiary stock solution were 0.5, 5 and 5 mg/L, respectively. This tertiary stock solution was then used to prepare six mixed working standards in a linear calibration range with the following concentrations: 2-hydroxybenzoic acid – 10, 20, 30, 40, 50, 60 μg/L; 3-hydroxybenzoic acid – 100, 200, 300, 400, 500, 600 μg/L; 4-hydroxybenzoic acid – 100, 200, 300, 400, 500, 600 μg/L.

2.4.2.3 Standard Spike Solution for Recovery.

A mixed standard recovery spike solution was required to spike the sample prior to preparation, extraction and instrumental analysis for the recovery study. It was prepared from the 200 mg/L

secondary stock solutions by direct dilution in LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid to a concentration of 5, 50 and 50 mg/L of 2-, 3-, and 4-hydroxybenzoic acid, respectively. For the recovery study one sample was spiked with a blank spike of 100 μL of LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid, and three samples were individually spiked with 100 μL of the mixed standard recovery spike solution. The final volume of sample + spike was 5 mL, therefore the spike added the equivalent of 0.1, 1 and 1 ppm for 2-, 3-, and 4-hydroxybenzoic acid, respectively.

2.4.2.4 Standard Additions Solutions.

The standard additions procedure required three standard additions solutions of varying concentrations. The three standard additions solutions were prepared by direct dilution in LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid. By further dilution of the secondary stock solution a tertiary stock solution was prepared in LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid. The concentrations of 2-, 3-, and 4-hydroxybenzoic acid in the tertiary stock solution were 1, 10 and 10 mg/L, respectively. This tertiary stock solution was then used to prepare three standard additions solutions in the following concentrations: 2-hydroxybenzoic acid – 0.2, 0.4, 0.6 mg/L; 3-hydroxybenzoic acid – 2, 4, 6 mg/L; 4-hydroxybenzoic acid – 2, 4, 6 mg/L.

2.4.3 Preparation of Standard Solutions for the Qualitative Analysis of Hydroxybenzoic Acids and Related Derivatives.

Various standard and mixed standard solutions were required for the qualitative analysis of the hydroxybenzoic acids and related derivatives in Chapter 3. Using the same direct dilution methodology discussed in Section 2.4.1 the following standard solutions were prepared in LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid.

2.4.3.1 Individual Standard Solutions for Qualitative Analysis of Benzoic Acid Derivatives.

Individual standard solutions were prepared for vanillic, syringic, anthranilic, veratric and eudesmic acids by direct dilution using LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid. Aliquots of the secondary stock solutions from Section 2.4.1.2 were diluted into a final volume of 2 mL in HPLC sample vials for each derivative. The concentrations of vanillic, syringic, anthranilic, veratric and eudesmic acid was 1, 1, 0.5, 0.5 and 0.5 mg/L, respectively.

2.4.3.2 Mixed Standard Solutions for Qualitative Analysis of Benzoic Acid Derivatives.

Mixed standard solutions were prepared for the qualitative analysis of the dihydroxybenzoic acids and trihydroxybenzoic acids. Further dilution of the secondary stock solutions from Section 2.4.1.2 were used to prepare the mixed working standard solutions via direct dilution using LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid as follows:

a) The dihydroxybenzoic acid mixed standard solution was prepared via direct dilution to the concentrations of 250, 250, 170, 100, 250 and 500 μg/L for 2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-dihydroxybenzoic acid, respectively.
b) The trihydroxybenzoic acid mixed standard solution was prepared via direct dilution to the concentrations of 1.5, 1.5 and 0.1 mg/L for 2,4,6-, 2,3,4- and 3,4,5-trihydroxybenzoic acid, respectively.

2.5 SAMPLE EXTRACTION AND PREPARATION PROCEDURES

Various sample extraction and preparation procedures were used for the qualitative and quantitative analyses of the hydroxybenzoic and benzoic acid derivatives in a commercial seaweed biostimulant. These include: acetonitrile partitioning, mixed-mode solid-phase extraction, sample identification spiking and standard additions calibration. The following section will describe the final optimised processes for these analyses in the commercial seaweed biostimulant. Lastly, an example workflow used for sample extraction and preparation in the quantitative analysis of the monohydroxybenzoic acids is provided as a demonstration of the systematic approach employed to minimise the variability and ensure a suitably repeatable method of analysis.

2.5.1 Partitioning.

A 5 mL aliquot of the commercial seaweed biostimulant sample was pipetted into a 15 mL conical bottom centrifuge tube. The pH was adjusted to approximately 1 with the addition of 800 μL of concentrated hydrochloric acid before 5 mL of HPLC grade acetonitrile was pipetted into the centrifuge tube. This was then shaken for 10 seconds and vortexed for 15 seconds before adding approximately 3 g of magnesium sulfate heptahydrate and repeating the shake/vortex protocol three more times. The sample tube was then centrifuged in an MSE Super Minor Centrifuge (fixed with an unmarked swinging bucket rotor with a radius equal to 17 cm, equivalent to ~4750 *g* of force) for 5 minutes at approximately 5000 rpm, twice in succession. The sample tube was then shaken for 20 seconds before two to four more successive spins in the centrifuge. Following the centrifugation there were two separate protocols:

a) Qualitative Analysis Protocol - an aliquot of the top layer was taken, evaporated under a stream of nitrogen, reconstituted in LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid, filtered through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter and transferred into a HPLC vial.
b) Quantitative Analysis - an aliquot of the top layer was taken and diluted with 0.1 % (v/v) formic acid in water in preparation for loading onto a solid-phase extraction cartridge.

2.5.2 Sample Identification Spiking.

The sample extract from the Qualitative Analysis Protocol mentioned above in Section 2.5.1a was used for the identification of the hydroxybenzoic acids and related derivatives in Chapter 3. To further support the identification of the various derivatives this sample extract was spiked with small amounts of each derivative. Aliquots of the secondary stock solutions prepared in Section 2.4.1.2 were added to the sample extract in a HPLC vial.

2.5.3 Solid-Phase Extraction.

The solid-phase extraction was performed using Biotage EVOLUTE® EXPRESS AX Advanced Polymeric SPE mixed-mode non-polar and strong anion exchange cartridges; developed for the extraction of acidic analytes from biological fluids and other aqueous samples. The specifications of the cartridges used are as follows: 3 mL cartridge volume, 60 mg sorbent bedmass, 50 μm particle size, and 54 Å mean pore size. The solid-phase extraction protocol began with conditioning with one cartridge volume (3mL) of HPLC grade methanol then equilibrating with 3 mL of 0.1 % (v/v) formic acid in water. Then 1 mL of load solution (prepared in Section 2.5.1b) was loaded before entering the four wash steps: 3 mL of 0.1 % (v/v) formic acid in water, 3 mL of ultrapure water, 3 mL of 2 mM ammonia in water, then lastly 2 mM ammonia in acetonitrile. The solid-phase cartridge was then dried under vacuum for 1 minute before the elution step. The elution step involved four 1.5 mL extractions using 1 % (v/v) formic acid in acetonitrile into a HPLC vial. After each single elution the HPLC vial was placed in a Bio-Dynamics Inc. I400 Dry Well Incubator and evaporated under a stream of nitrogen. The final step was reconstituting with the mobile phase: LCMS grade methanol/water 30:70 (v/v) with 0.1 % (v/v) formic acid. All steps in solid-phase extraction protocol were approximately conducted at the recommended flowrate of 3 mL/min. The filtration of the sample with a 0.45 μm PTFE syringe filter was integrated into the solid-phase extraction protocol; the conditioning, equilibrating, loading and first two wash steps were passed through the syringe filter (explained in Section 4.2.3). The reconstituted solid-phase extraction sample could then be analysed using HPLC-ESI-MS/MS or go through standard additions preparation for quantitation.

2.5.3.1 Preparation of Solid-Phase Extraction Solvents.

- The conditioning solvent was HPLC grade methanol.
- The equilibration solvent was the same as the first wash solvent, as well as the dilution solvent post partitioning; this was prepared by adding 0.1 % (v/v) reagent grade (≥95 %) formic acid to the ultrapure water (~18 MΩ resistivity) prepared using a Purite Select HP water purification system with an Merck LC-Pak® Polisher to remove trace organics.
- The second wash was with the same ultrapure water.
- The third wash solution was 2 mM ammonia in water prepared by adding 0.2 mL 1 M ammonium hydroxide solution to 100 mL of ultrapure water.
- The fourth wash solution was 2 mM ammonia acetonitrile solution prepared by adding 0.2 mL 1 M ammonium hydroxide to LC grade acetonitrile.
- The final solvent was the 1 % (v/v) formic acid in acetonitrile elution solvent, which was prepared using the reagent grade formic acid and LC grade acetonitrile previously mentioned.

2.5.4 Standard Additions.

For the standard additions four 95 μL aliquots of the solid-phase extraction extract were individually transferred to separate HPLC vials with vial inserts before being spiked with 5 μL of the standard additions solutions described in Section 2.4.2.4. Then finally a quick vortex to ensure homogenization and inspection for the formation of air bubbles before HPLC-ESI-MS/MS analysis.

2.5.5 Sample Extraction Workflow for Validation Study and Quantitation.

A consistent protocol was used for the extraction and preparation of the seaweed biostimulant sample for the quantitative analysis of the monohydroxybenzoic acids. This was done to minimise the variability of the extraction and preparation procedures and therefore ensure repeatability. A workflow schematic was generated which would be printed and secured into the laboratory logbook and filled out during the sample extraction and preparation (an example is shown below in Figure 2.5.5).

Example Workflow

Date: / /

Partitioning

5 mL Sample [identification]
800 µL HCl
5 mL MeCN
Shake virgorously 10 sec + Vortex 15 sec

3 g MgSO4.7H2O
3 x Shake virgorously 10 sec + Vortex 15 sec

2 x Centrifuge 5 min @ 5000 rpm

Load Solution Preparation (1 in 20 Dilution)

1900 µL 0.1 % Formic H2O
100 µL Top Layer from Partitioning

Solid-Phase Extraction

Condition	MeOH	3 mL	*
Equilibrate	0.1 % Formic H2O	3 mL	*
Load	Load Solution	1 mL	*
Wash	0.1 % Formic H2O	3 mL	*
Wash	H2O	3 mL	*
Wash	2 mM NH3 H2O	3 mL	
Wash	2 mM NH3 MeCN	3 mL	
Dry	vacuum	1 min	
Elute	1% Formic MeCN	4 x 1.5 mL	
(Evaporate w N2 after each elution, in same vial)			
Reconstitute	Mobile Phase	750 µL	

* indicates solvents passed through 0.45 µm PTFE Syringe Filter prior to S.P.E cartridge

Standard Addition for Quantitiation

Four even aliquots of S.P.E Extract (95 µL) + (5 µL) of Standard Additions Spike

Stnd Addtn 1	Blank (Mobile Phase)
Stnd Addtn 2	Standard Additions Spike 1
Stnd Addtn 3	Standard Additions Spike 2
Stnd Addtn 4	Standard Additions Spike 3

Vortex each HPLC vial to ensure thorough mixing for 10 sec

Identification of sample vials:

Stnd Addtn 1	[labelling incl. date]
Stnd Addtn 2	[labelling incl. date]
Stnd Addtn 3	[labelling incl. date]
Stnd Addtn 4	[labelling incl. date]

12) **Figure 2.5.5** An example workflow schematic for sample extraction and preparation for the method for the quantitative analysis of monohydroxybenzoic acids in a commercial seaweed biostimulant.

2.6 ANALYTICAL INSTRUMENTATION, MATERIALS AND METHODS

This section details the instrumentation and materials used, as well as the different method parameters for the investigation, separation, identification, and quantitation work performed throughout this research project.

2.6.1 Analytical Instrumentation and Materials.

All HPLC-ESI-MS/MS experiments were performed on a Shimadzu Nexera X2 Ultra High Performance Liquid Chromatograph System equipped with a DGU-20A_{5R} Degassing Unit, twin LC-30AD Solvent Delivery Modules, MR 20 μL Small Capacity Gradient Mixer, SIL-30AC Autosampler, CTO-20A Column oven, CBM-20A Communications Bus Module, and a LCMS-8045 Mass Spectrometer. The CBM-20A allowed for remote operations and data collection on a Desktop PC using the Shimadzu LabSolutions v5.96 software package. Routine preventative maintenance of the HPLC system was performed weekly by the researcher including flushes with various solvents.

The LCMS-8045 Liquid Chromatograph Mass Spectrometer used Argon as the Collision-Induced Dissociation Gas and Nitrogen (N_2) as the Drying, Nebulizer and Heating gases. The source for the N_2 was liquid nitrogen that was filtered by dual Shimadzu High Flow Moisture/Hydrocarbon Combi Filters to ensure high purity. The LCMS-8045 was tuned using a Shimadzu Standard Sample (P/N S225-14122-04), which is a mixture of polyethylene glycol 200, 600, 1000, 2000, polypropylene glycol and raffinose. Similar to the HPLC system, the researcher performed routine preventative maintenance on the LCMS-8045 including weekly inspection and cleaning of the ESI unit, Desolvation Line (DL), Sampling Cone and Heated Block.

The ESI-MS/MS standard operating conditions were as follows: interface voltage of -3.00 kV in negative ionisation mode, interface voltage of 4.00 kV in positive ionisation mode, detector voltage of -1.88 kV, interface temperature of 300 °C, DL temperature of 250 °C, heat block temperature of 400 °C, and nebulizing, heating and drying gas flows of 3, 10 and 10 L/min, respectively.

Three HPLC columns were available for this research project, they are as follows:

- Agilent Polaris 3 μm C18-A 150 × 2.0 mm
- Restek Raptor 1.8 μm C18 150 × 2.1 mm
- Restek Raptor 2.7 μm Biphenyl 150 × 2.1 mm

When injecting samples each column was fitted with a matching guard column, they are as follows: Agilent Polaris C18-A MetaGuard 10 × 2.0 mm, and Restek EXP®Direct Connect Holder fitted with either a Raptor C18 UHPLC 5 × 2.1 mm cartridge or a Raptor 2.7μm Biphenyl 5 × 2.1 mm cartridge. Column preventative maintenance involved routine flushing, equilibration prior to every

batch analysis, and programming a column flush as part of each HPLC run for each sample injection.

The HPLC systems twin delivery modules (pumps) and mixing chamber were always used in accordance to an in-house standard operating procedure. The two pumps, termed mobile phase pump A and pump B, were set up so pump A was the polar/aqueous solvent line and pump B was the non-polar/organic solvent line. Three solvents were used in the HPLC analyses, water was always the aqueous solvent and the organic solvent was either acetonitrile or methanol. Formic acid was often used as a mobile phase acid modifier and was prepared in both solvent lines in low concentrations. To reduce contamination and ensure consistent chromatographic results the preparation of the mobile phase was done following standard operating procedures which included degassing via sonication.

2.6.2 Analytical Method Development.

There were various HPLC-ESI-MS/MS methods utilised throughout Chapters 3 and 4, from simple investigatory methods to the final optimised quantitative and/or qualitative methods. This section details the instrument parameters both HPLC and ESI-MS/MS that are significant and specific to the methods employed throughout Chapters 3 and 4 for the analysis of the monohydroxybenzoic acids, dihydroxybenzoic acids, trihydroxybenzoic acids, and other related derivatives.

2.6.2.1 Method for MRM Voltage Optimisation.

The instrument software used in this research (see Section 2.6.1) employs an automatic MRM optimisation protocol that allows for the optimisation of the collision energy (C.E) and quadrupole Q1 and Q3 "Pre-rod Bias". The instrument optimisation protocol directly injects the standard solutions whilst making incremental changes to each of the conditions. The first was the CE which was performed in increments of 5.0 V before being re-optimised in finer detail at increments of 1.0 V. Then finally the Q1 and Q3 pre-rod bias were changed in 1.0 V increments.

2.6.2.2 Methods for MS Optimisation

Mobile Phase: Solvent A comprised of LCMS grade water with 0.1 % (v/v) formic acid and Solvent B comprised of LCMS grade methanol with 0.1 % (v/v) formic acid

Elution: Isocratic elution of 70:30 (v/v) for pumps A/B, respectively

Flow Rate: 0.4 mL/min

Column: No column

Injection Volume: 1 – 5 μL

LC Time Program: the HPLC system was configured for direct injection to the MS

ESI-MS/MS Conditions: The ESI-MS was operated in negative mode.

MS Analysis Mode: Various MS Analysis modes used successively as follows:

- Q3 scan mode with a range of m/z 100 – 220 at a scan speed of 7500 u/sec
- Product Ion (PI) scan mode using two events in a single injection with a scan speed between 1071 - 5000 u/sec, a scan range of m/z 40 – 240, discrete collision energies of 10 and 20 V, and the pseudomolecular ion for each derivative as the precursor ion.
- MRM Voltage Optimisation mode with final parameters as follows:

Analyte(s)	MS/MS Transition (m/z)	Dwell Time (ms)	Collision Energy (V)	Q1 Pre-bias (V)	Q3 Pre-bias (V)
Monohydroxybenzoic acids	137 → 93	100	15	14	15
Dihydroxybenzoic acids	153 → 109	100	15	17	18
Trihydroxybenzoic acid	169 → 125	100	17	18	21
Vanillic acid	167 → 152	100	16	20	28
Syringic acid	197 → 182	100	13	11	17
Veratric acid	181 → 137	100	12	21	23
Eudesmic acid	211 → 167	100	12	11	16
Anthranilic acid	136 → 92	100	16	15	30

2.6.2.3 Methods for LC Phase Investigation and Optimisation

Mobile Phase: Two mobile phase configurations:

1. Solvent A comprised of LCMS grade water with 0.1 % (v/v) formic acid and Solvent B comprised of LCMS grade methanol with 0.1 % (v/v) formic acid
2. Solvent A comprised of LCMS grade water with 0.1 % (v/v) formic acid and Solvent B comprised of LCMS grade acetonitrile with 0.1 % (v/v) formic acid

Elution: Isocratic elution of 70:30 (v/v) for pumps A/B, respectively

Flow Rate: 0.4 mL/min

Column: Three separate columns all maintained at 30ºC as follows:

1. Agilent Polaris 3 μm C18-A 150 × 2.0 mm
2. Restek Raptor 1.8 μm C18 150 × 2.1 mm
3. Restek Raptor 2.7 μm Biphenyl 150 × 2.1 mm

Injection Volume: 1 – 5 μL

LC Time Program: Isocratic elution for required run time, between 5 – 25 min

ESI-MS/MS Conditions: The ESI-MS was operated in negative mode.

MS Analysis Mode: MRM analysis mode with the following parameters:

Analyte(s)	MS/MS Transition (m/z)	Dwell Time (ms)	Collision Energy (V)	Q1 Pre-bias (V)	Q3 Pre-bias (V)
Monohydroxybenzoic acids	137 → 93	100	15	14	15
Dihydroxybenzoic acids	153 → 109	100	15	17	18
Trihydroxybenzoic acid	169 → 125	100	17	18	21
Vanillic acid	167 → 152	100	16	20	28
Syringic acid	197 → 182	100	13	11	17
Veratric acid	181 → 137	100	12	21	23
Eudesmic acid	211 → 167	100	12	11	16
Anthranilic acid	136 → 92	100	16	15	30

2.6.2.4 Methods for Positive Ion Mode MS Optimisation

Mobile Phase: Solvent A comprised of LCMS grade water with 0.1 % (v/v) formic acid and Solvent B comprised of LCMS grade methanol with 0.1 % (v/v) formic acid

Elution: Isocratic elution of 70:30 (v/v) for pumps A/B, respectively

Flow Rate: 0.4 mL/min

Column: No column

Injection Volume: 5 μL

LC Time Program: the HPLC system was configured for direct injection to the MS

ESI-MS/MS Conditions: The ESI-MS was operated in positive mode.

MS Analysis Mode: Various MS Analysis modes used successively as follows:

- Q3 scan mode with a range of m/z 100 – 220 at a scan speed of 7500 u/sec
- Product Ion (PI) scan mode using two events in a single injection with a scan speed of 1071 u/sec, a scan range of m/z 40 – 240, discrete collision energies of 10 and 20 V, and the pseudomolecular ion for each derivative as the precursor ion.
- MRM Voltage Optimisation mode with final parameters as follows:

Analyte(s)	ESI Polarity	MS/MS Transition (m/z)	Dwell Time (ms)	Collision Energy (V)	Q1 Pre-bias (V)	Q3 Pre-bias (V)
Veratric acid	+	183 → 139	100	-13	-11	-29
Eudesmic acid	+	213 → 154	100	-14	-11	-29

2.6.2.5 A HPLC-ESI-MS/MS Method for the Qualitative and Quantitative Analysis of Monohydroxybenzoic Acids in a Commercial Seaweed Biostimulant.

Mobile Phase: Solvent A comprised of LCMS grade water with 0.1 % (v/v) formic acid and Solvent B comprised of LCMS grade methanol with 0.1 % (v/v) formic acid

Elution: Isocratic elution of 70:30 (v/v) for pumps A/B, respectively

Flow Rate: 0.4 mL/min

Column: A Restek Raptor 2.7 μm Biphenyl 150 × 2.1 mm column with matching guard column was housed in the column oven maintained at 30 °C

Injection Volume: 1 μL

LC Time Program (including pre- and post-column flush): diversion post-column to waste from 0 – 1.5 min; diversion to detector at 1.5 min; standard isocratic elution from 1.5 – 10 min; diversion back to waste at 10 min; increase from 30 % (v/v) to 80 % (v/v) B from 10 – 11 min; 80 % (v/v) B flush from 11 – 14 min; decrease back to 30 % (v/v) B from 14 – 15 min; re-equilibration at 30 % (v/v) B from 15 – 20 min.

ESI-MS/MS Conditions: The ESI-MS was operated in negative mode and was set to acquire data from 1.5 – 10 min.

MS Analysis Mode: MRM analysis mode with the following parameters:

Analyte(s)	MS/MS Transition (m/z)	Dwell Time (ms)	Collision Energy (V)	Q1 Pre-bias (V)	Q3 Pre-bias (V)
Monohydroxybenzoic acids	137 → 93	100	15	14	15

2.6.2.6 A HPLC-ESI-MS/MS Method for the Qualitative Analysis of Dihydroxybenzoic Acids in a Commercial Seaweed Biostimulant.

Mobile Phase: Solvent A comprised of LCMS grade water with 0.1 % (v/v) formic acid and Solvent B comprised of LCMS grade methanol with 0.1 % (v/v) formic acid

Elution: Isocratic elution of 80:20 (v/v) for pumps A/B, respectively

Flow Rate: 0.4 mL/min

Column: A Restek Raptor 2.7 μm Biphenyl 150 × 2.1 mm column with matching guard column was housed in the column oven maintained at 30 °C

Injection Volume: 1 μL for standards and 1 – 10 μL for samples

LC Time Program (including pre- and post-column flush): diversion post-column to waste from 0 – 1.0 min; diversion to detector at 1.0 min; standard isocratic elution from 1.0 – 10 min; diversion back to waste at 10 min; increase from 20 % (v/v) to 80 % (v/v) B from 10 – 11 min; 80 % (v/v) B flush from 11 – 14 min; decrease back to 20 % (v/v) B from 14 – 15 min; re-equilibration at 20 % (v/v) B from 15 – 20 min.

ESI-MS/MS Conditions: The ESI-MS was operated in negative mode and was set to acquire data from 1 – 10 min.

MS Analysis Mode: MRM analysis mode with the following parameters:

Analyte(s)	MS/MS Transition (m/z)	Dwell Time (ms)	Collision Energy (V)	Q1 Pre-bias (V)	Q3 Pre-bias (V)
Dihydroxybenzoic acids	153 → 109	100	15	17	18

2.6.2.7 A HPLC-ESI-MS/MS Method for the Qualitative Analysis of Trihydroxybenzoic Acids in a Commercial Seaweed Biostimulant.

Mobile Phase: Solvent A comprised of LCMS grade water with 0.1 % (v/v) formic acid and Solvent B comprised of LCMS grade methanol with 0.1 % (v/v) formic acid

Elution: Isocratic elution of 85:15 (v/v) for pumps A/B, respectively

Flow Rate: 0.4 mL/min

Column: A Restek Raptor 2.7 μm Biphenyl 150 × 2.1 mm column with matching guard column was housed in the column oven maintained at 30 °C

Injection Volume: 2 μL for standards and 2 – 10 μL for samples

LC Time Program (including pre- and post-column flush): diversion post-column to waste from 0 – 1.0 min; diversion to detector at 1.0 min; standard isocratic elution from 1.0 – 10 min; diversion back to waste at 10 min; increase from 15 % (v/v) to 80 % (v/v) B from 10 – 11 min; 80 % (v/v) B flush from 11 – 14 min; decrease back to 15 % (v/v) B from 14 – 15 min; re-equilibration at 15 % (v/v) B from 15 – 20 min.

ESI-MS/MS Conditions: The ESI-MS was operated in negative mode and was set to acquire data from 1 – 10 min.

MS Analysis Mode: MRM analysis mode with the following parameters:

Analyte(s)	MS/MS Transition (m/z)	Dwell Time (ms)	Collision Energy (V)	Q1 Pre-bias (V)	Q3 Pre-bias (V)
Trihydroxybenzoic acids	169 → 125	100	17	18	21

2.6.2.8 HPLC-ESI-MS/MS Methods for the Qualitative Analysis of Vanillic, Syringic, and Anthranilic Acids in a Commercial Seaweed Biostimulant.

Mobile Phase: Solvent A comprised of LCMS grade water with 0.1 % (v/v) formic acid and Solvent B comprised of LCMS grade methanol with 0.1 % (v/v) formic acid

Elution: Isocratic elution of 70:30 (v/v) for pumps A/B, respectively

Flow Rate: 0.4 mL/min

Column: A Restek Raptor 2.7 μm Biphenyl 150 × 2.1 mm column with matching guard column was housed in the column oven maintained at 30 °C

Injection Volume: 5 μL for standards and 5 – 10 μL for samples

LC Time Program (including pre- and post-column flush): diversion post-column to waste from 0 – 1.0 min; diversion to detector at 1.0 min; standard isocratic elution from 1.0 – 10 min; diversion back to waste at 10 min; increase from 30 % (v/v) to 80 % (v/v) B from 10 – 11 min; 80 % (v/v) B flush from 11 – 14 min; decrease back to 30 % (v/v) B from 14 – 15 min; re-equilibration at 30 % (v/v) B from 15 – 20 min.

ESI-MS/MS Conditions: The ESI-MS was operated in negative mode and was set to acquire data from 1 – 10 min.

MS Analysis Mode: MRM analysis mode with the following parameters:

Analyte(s)	MS/MS Transition (m/z)	Dwell Time (ms)	Collision Energy (V)	Q1 Pre-bias (V)	Q3 Pre-bias (V)
Vanillic acid	167 → 152	100	16	20	28
Syringic acid	197 → 182	100	13	11	17
Anthranilic acid	136 → 92	100	16	15	30

2.6.2.9 HPLC-ESI-MS/MS Methods for the Qualitative Analysis of Veratric and Eudesmic Acids in a Commercial Seaweed Biostimulant.

Mobile Phase: Solvent A comprised of LCMS grade water with 0.1 % (v/v) formic acid and Solvent B comprised of LCMS grade methanol with 0.1 % (v/v) formic acid

Elution: Isocratic elution of 50:50 (v/v) for pumps A/B, respectively

Flow Rate: 0.4 mL/min

Column: A Restek Raptor 2.7 μm Biphenyl 150 × 2.1 mm column with matching guard column was housed in the column oven maintained at 30 °C

Injection Volume: 5 μL for standards and 5 – 10 μL for samples

LC Time Program (including pre- and post-column flush): diversion post-column to waste from 0 – 1.0 min; diversion to detector at 1.0 min; standard isocratic elution from 1.0 – 10 min; diversion back to waste at 10 min; increase from 50 % (v/v) to 80 % (v/v) B from 10 – 11 min; 80 % (v/v) B flush from 11 – 14 min; decrease back to 50 % (v/v) B from 14 – 15 min; re-equilibration at 50 % (v/v) B from 15 – 20 min.

ESI-MS/MS Conditions: The ESI-MS was operated in positive mode and was set to acquire data from 1 – 10 min.

MS Analysis Mode: MRM analysis mode with the following parameters:

Analyte(s)	MS/MS Transition (m/z)	Dwell Time (ms)	Collision Energy (V)	Q1 Pre-bias (V)	Q3 Pre-bias (V)
Veratric acid	183 → 139	100	-13	-13	-29
Eudesmic acid	213 → 154	100	-14	-11	-29

2.7 PLANT GROWTH BIOASSAYS – GREENHOUSE EXPERIMENTS

The plant growth bioassays were conducted at Seasol facilities using an in-house assay developed at Seasol R&D laboratories.

2.7.1 Plant Preparation.

Tomato (*Lycopersicon esculentum*) seeds were germinated in a seedling tray filled with commercially purchased seed raising mix (Seasol Seed Raising and Cutting Potting Mix) [204]. The tomato seedlings were grown in a temperature-controlled greenhouse at 24 ºC and a relative humidity no higher than 60 %. They were grown for four weeks exposed to local ambient spring and summer lighting conditions before being prepared for the plant growth assay.

Tomato seedlings were selected for uniformity before being removed from their tray. The roots were cleaned by carefully removing as much soil as possible by gentle agitation before being washed under running tap water until the roots appeared clear. Once cleaned, the roots could be trimmed carefully with sharp pointed scissors until a trimmed tap root was all that remained. Finally, each prepared tomato seedling was placed into jars filled with the treatment or control solutions.

2.7.2 Preparation of Treatment Solutions.

All solutions were made using ultrapure water (~18 MΩ resistivity) prepared using a Purite Select HP water purification system. The total volume of each solution was 2000 mL, except for where 5 mL Commercial Seasol Concentrate was to be added in order to prepare 2000 mL of 1:400 dilution, then 1995 mL was provided.

To prepare aqueous solutions of the individual and combination monohydroxybenzoic acids stock solutions were made by dissolving the appropriate mass in the ultrapure water. From which the 2000 mL solutions were prepared via direct dilution. The fortified Seasol solutions were also prepared using the same stock solutions; however, the direct dilution method took into account the addition of 5 mL Seasol and compensated such that the final 2000 mL fortified Seasol would have been spiked with the same concentration as employed in the aqueous solutions.

2.7.3 Experiment 1 – Pilot Bioassay.

The first bioassay experiment involved five treatment solutions and two control solutions. There were six tomato seedlings per treatment/control, for a total of 42 tomato seedlings. The first root growth bioassay experiment began 6/10/2021 in the same greenhouse, under the same conditions the seedlings were raised (see Section 2.7.1).

Treatment Solution	Description
1) Treatment Control (Seasol)	1 in 400 dilution of concentrate
2) Water Control	18 MΩ deionised water
3) 2-hydroxybenzoic acid	2 nM aqueous solution
4) 3-hydroxybenzoic acid	62 nM aqueous solution
5) 4-hydroxybenzoic acid	32 nM aqueous solution
6) Combination of monohydroxybenzoic acids	2 nM 2-hydroxybenzoic acid, 62 nM 3-hydroxybenzoic acid and 32 nM 4-hydroxybenzoic acid aqueous solution
7) Seasol fortified with Combination	2 nM 2-hydroxybenzoic acid, 62 nM 3-hydroxybenzoic acid and 32 nM 4-hydroxybenzoic acid spiked into 1 in 400 dilution of concentrate

13) **Figure 2.7.3** A representative photo of the tomato seedling plant growth assay set up, as per Experiment 1 with seven treatment/control solutions each with 6 replicate plants, commenced 6/10/21.

2.7.4 Experiment 2 – Repeatability Bioassay

The second bioassay experiment was a repeat of Experiment 1 and involved five treatment solutions and two control solutions. There were six tomato seedlings per treatment/control, for a total of 42 tomato seedlings. The second root growth bioassay began on the 9/11/2021 in the same greenhouse, under the same conditions the seedlings were raised (see Section 2.7.1).

Treatment Solution	Description
1) Treatment Control (Seasol)	1 in 400 dilution of concentrate
2) Water Control	18 MΩ deionised water
3) 2-hydroxybenzoic acid	2 nM aqueous solution
4) 3-hydroxybenzoic acid	62 nM aqueous solution
5) 4-hydroxybenzoic acid	32 nM aqueous solution
6) Combination of monohydroxybenzoic acids	2 nM 2-hydroxybenzoic acid, 62 nM 3-hydroxybenzoic acid and 32 nM 4-hydroxybenzoic acid aqueous solution
7) Seasol fortified with Combination	2 nM 2-hydroxybenzoic acid, 62 nM 3-hydroxybenzoic acid and 32 nM 4-hydroxybenzoic acid spiked into 1 in 400 dilution of concentrate

2.7.5 Quantitative Parameters

Five key parameters were measured at the conclusion of the assay (harvest) to assess the efficacy/bioactivity of the various treatments: longest root length, fresh and dry root weight, and fresh and dry shoot weight.

2.7.5.1 Longest Root Length

The longest root length for each plant was measured using the methodology described by Doerner *et al.* [205]. The roots were carefully washed under running tap water before the longest root was measured from the hypocotyl region to the tip.

2.7.5.2 Fresh and Dry Root Weight

The dry root weight was measured using the methodology described by Huang *et al.*, with small modifications [206]. The roots were carefully washed under running tap water before being measured using a digital weighing scale. Following which roots were dried at 70 ºC in a drying oven before dry weights were measured using a digital weighing scale.

2.7.5.3 Fresh and Dry Shoot Weight

The dry shoot weight was measured using the methodology described by Huang *et al.*, with small modifications [206]. The shoots were carefully washed under running tap water before being measured using a digital weighing scale. Following which shoots were dried at 70 ºC in a drying oven before dry weights were measured using a digital weighing scale.

2.7.6 Data Analysis

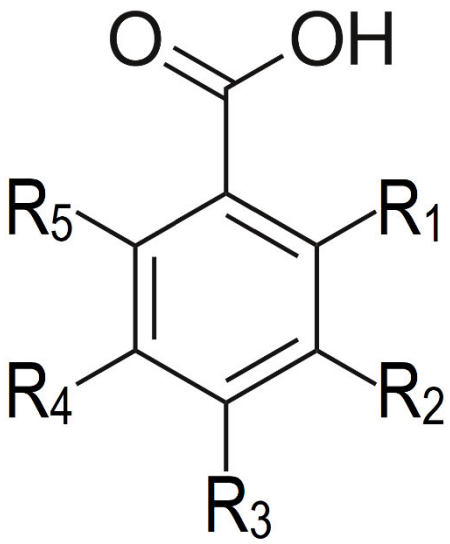
To assess data for outliers both z-score and interquartile range outlier tests were confirmed, when there was a consensus between the two tests for the presence of an outlier that data point was omitted. Data were then subjected to one-way ANOVA at 5 % level of significance to assess treatment effects. Significant differences between means were determined using the Fisher Least Significant Difference (LSD).

CHAPTER 3: METHOD DEVELOPMENT FOR THE TARGETED METABOLOMIC HPLC-ESI-MS/MS QUALITATIVE ANALYSIS OF HYDROXYBENZOIC ACIDS AND RELATED DERIVATIVES IN A COMMERCIAL SEAWEED BIOSTIMULANT

3.1 INTRODUCTION

There are a number of phytochemicals commonly grouped in the subgroup of phenolic acids known as hydroxybenzoic acids that are well characterised and involved in the same biochemical pathways (see Section 1.4.6). This includes mono-, di- and tri-hydroxybenzoic acids as well as methoxylated hydroxybenzoic acids, and methoxylated and amino substituted benzoic acids that are products of the same biosynthetic pathways (presented below in Table 3.1). Some of their biological activities, particularly the responses of the exogenous application of these compounds to plants, bear comparison to the benefits observed from the application of some seaweed biostimulants (see Sections 1.3 and 1.7) [1, 57]. In order to further investigate the mode-of-action for the observed benefits from the seaweed biostimulants, sophisticated analytical methods are required for the detection, identification and quantification of target analytes in complex samples (see Sections 1.5 and 1.6). The development of targeted metabolomic methods is therefore crucial for the advancement of biostimulants as a sustainable agricultural practice that can help to meet the growing global food demands. The aim of this part of the work was to develop qualitative HPLC-ESI-MS/MS methods for the identification of hydroxybenzoic acids and related derivatives in a commercial seaweed biostimulant. Additionally, the chromatographic behaviour of these compounds on the less explored reversed phase phenyl based stationary phases will be investigated and compared to the more common C18 phases.

Table 3.1 shows the derivatives selected for this investigation and their molecular weight. The table also includes their structural information relative to the C_6-C_1 backbone. The selection of these hydroxybenzoic acids and related derivatives took into consideration their prevalence in the literature and their structural idiosyncrasies; however, the availability of pure standard materials was also a major deciding factor.



Hydroxybenzoic acids and related derivatives	M.W	R_1	R_2	R_3	R_4	R_5
2-hydroxybenzoic acid (Salicylic)	138.12 g/mol	OH	H	H	H	H
3-hydroxybenzoic acid	138.12 g/mol	H	OH	H	H	H
4-hydroxybenzoic acid	138.12 g/mol	H	H	OH	H	H
2,3-dihydroxybenzoic acid (Pyrocatechuic)	154.12 g/mol	OH	OH	H	H	H
2,4-dihydroxybenzoic acid (β-Resorcyclic)	154.12 g/mol	OH	H	OH	H	H
2,5-dihydroxybenzoic acid (Gentisic)	154.12 g/mol	OH	H	H	OH	H
2,6-dihydroxybenzoic acid	154.12 g/mol	OH	H	H	H	OH
3,4-dihydroxybenzoic acid (Protocatechuic)	154.12 g/mol	H	OH	OH	H	H
3,5-dihydroxybenzoic acid (α-Resorcyclic)	154.12 g/mol	H	OH	H	OH	H
2,3,4-trihydroxybenzoic acid	170.12 g/mol	OH	OH	OH	H	H
2,4,6-trihydroxybenzoic acid	170.12 g/mol	OH	H	OH	H	OH
3,4,5-trihydroxybenzoic acid (Gallic)	170.12 g/mol	H	OH	OH	OH	H
4-hydroxy-3-methoxybenzoic acid (Vanillic)	168.15 g/mol	H	OCH_3	OH	H	H
4-hydroxy-3,5-dimethoxybenzoic acid (Syringic)	198.17 g/mol	H	OCH_3	OH	OCH_3	H
3,4-dimethoxybenzoic acid (Veratric)	182.17 g/mol	H	OCH_3	OCH_3	H	H
3,4,5-trimethoxybenzoic acid (Eudesmic)	212.20 g/mol	H	OCH_3	OCH_3	OCH_3	H
2-aminobenzoic acid (Anthranilic)	137.18 g/mol	NH_2	H	H	H	H

9) **Table 3.1** List of hydroxybenzoic acids and related derivatives investigated in this study. Including structural information relative to their C6-C1 backbone.

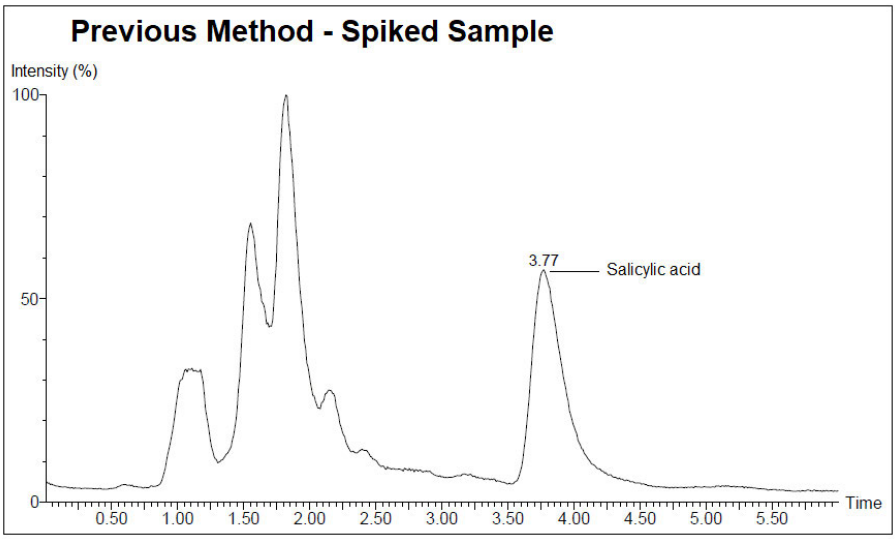
3.1.1 Chapter 3 Outline.

The results in this chapter are divided into the following sections.

- Preliminary qualitative investigations of salicylic acid and its isomers (Section 3.2) - This section describes the preliminary HPLC-ESI-MS-MS qualitative investigation of monohydroxybenzoic acids in a commercial seaweed biostimulant.
- The development of qualitative methods for the analysis of hydroxybenzoic acids and related derivatives in a commercial seaweed biostimulant - HPLC-ESI-MS/MS Method Development (Section 3.3) - This section describes the optimisation of the instrumental analysis including the mass spectrometric and liquid chromatographic conditions
    - Investigation of the selectivity of phenyl phases vs C18 for the separation of these aromatic compounds
- The qualitative analysis of the hydroxybenzoic acids and related derivatives in a commercial seaweed biostimulant using the newly developed HPLC-ESI-MS/MS methods (Section 3.4) - This section describes the application of the newly developed methods for the identification of the dihydroxybenzoic acids, trihydroxybenzoic acids, vanillic acid, syringic acid, veratric acid, eudesmic acid, and anthranilic acid in a commercial seaweed biostimulant.

3.2 PRELIMINARY QUALITATIVE INVESTIGATION OF SALICYLIC ACID AND ITS ISOMERS

An in-house LC-MS method for the analysis of salicylic acid in a commercial seaweed biostimulant was previously developed in our laboratories [207]. The method employed an octadecylsilane-bonded silica (ODS) reversed-phase HPLC column with an acetonitrile/water mobile phase modified with formic acid. The method of detection was electrospray ionisation (ESI) mass spectrometry (MS) in negative ionisation mode. To produce a sample suitably clean for instrumental analysis, and allow preconcentration of analytes, this method employed a sample preparatory procedure involving acetonitrile partitioning followed by solid-phase extraction. Figure 3.2.1 shows the LC-MS chromatogram of a sample analysed by the aforementioned method in Selected Ion Monitoring (SIM) mass spectrometry mode at 137 m/z; the peak with a retention time (t_R) of 3.77 min. was identified as salicylic acid. The cluster of peaks preceding the salicylic acid peak suggested the presence of salicylic acid's isomers, with a similar molecular ion. This observation sparked the interest for this current study.

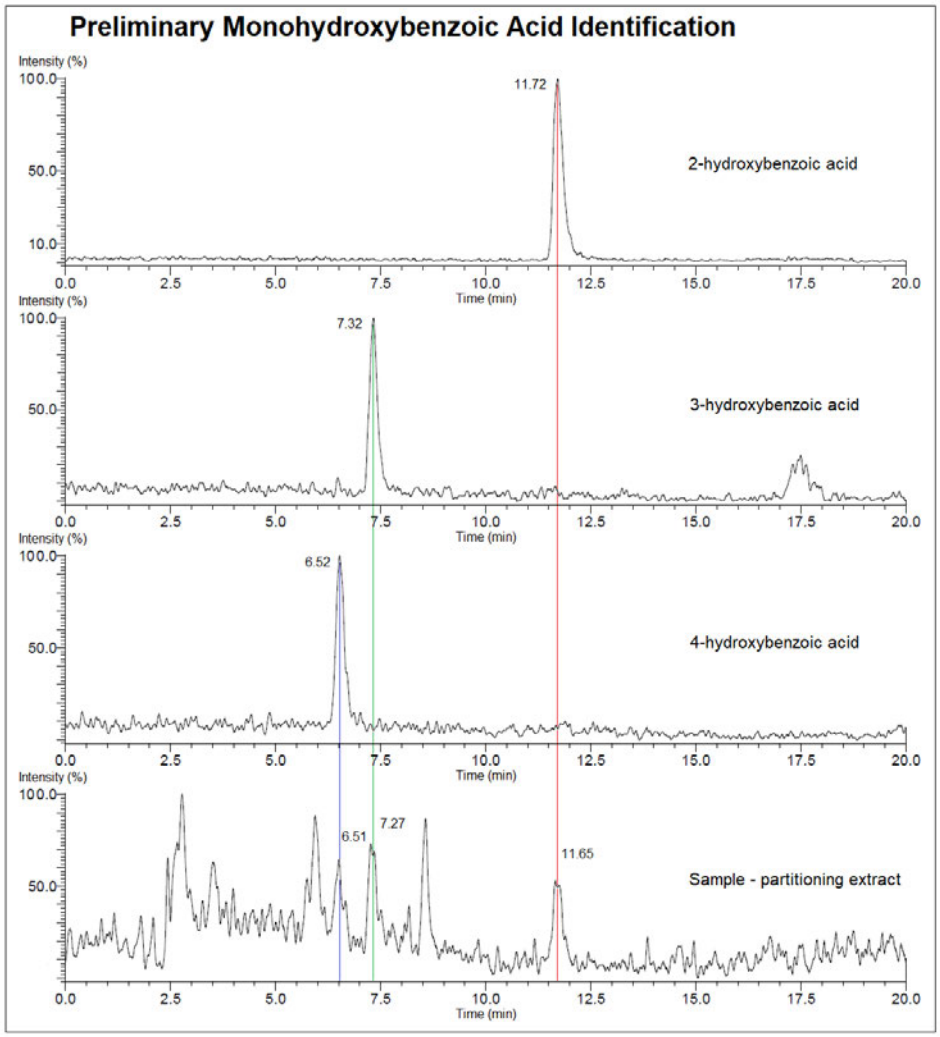


14) **Figure 3.2.1** Chromatogram showing the separation and identification of salicylic acid in a spiked sample using an octadecylsilane (C18) reverse-phase HPLC column with an acetonitrile/water mobile phase modified with formic acid. The LC-MS chromatogram was run in SIM mode at 137 m/z [207].

3.2.1 Preliminary Qualitative Investigation of Monohydroxybenzoic Acids using HPLC-ESI-MS/MS.

The initial qualitative investigation began by transposing the HPLC-ESI-MS method described above to HPLC-ESI-MS/MS (see Section 2.6.1 for instrument details) with the goal to achieve enough separation of the preceding peaks observed in the chromatogram shown in Figure 3.2.1 to allow for a preliminary identification of compounds present. It was hypothesised that the cluster of peaks observed in Figure 3.2.1 included the presence of the other monohydroxybenzoic acids. For this preliminary work an Agilent Polaris 3 μm C18-A 150 × 2.0 mm with an Agilent Polaris C18-A MetaGuard 10 × 2.0 mm column was used along with aqueous acetonitrile mobile phases modified with formic acid. Gradient elution chromatography was used and mobile phase conditions along with other instrumental conditions are listed in figure legends.

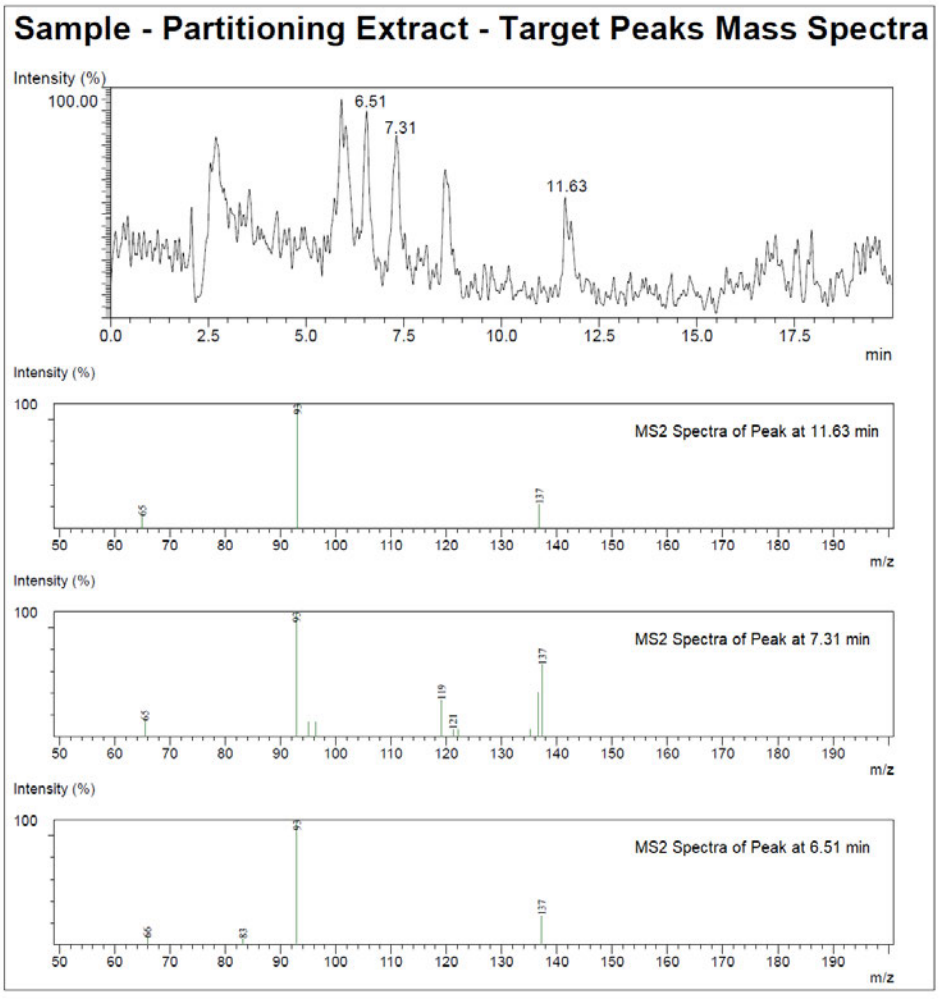
To begin the qualitative investigation a crude sample preparatory extraction was performed on the seaweed biostimulant following the basic partitioning steps from the previous method [207]. An aliquot of the biostimulant sample was placed into a centrifuge tube and the pH was dropped to approximately 1 using concentrated HCl. An equal aliquot of acetonitrile was added to the tube which was then vigorously shaken before being centrifuged. A 1 mL aliquot of the top organic layer was then diluted with 9 mL of 30 % (v/v) aqueous acetonitrile with 0.1 % (v/v) formic acid and then filtered through a 0.45 μm PTFE syringe filter prior to instrumental analysis using the mass spectrometer in full scan mode (m/z 100 – 400). Standard solutions of the three monohydroxybenzoic acids were prepared via direct dilution in the same solvent as the sample extract and chromatographed under the same conditions. Figure 3.2.2a shows the extracted ion chromatograms using m/z 137 (the pseudomolecular ion or $[M-H]^-$ of monohydroxybenzoic acids) of 2-, 3- and 4-hydroxybenzoic acid as well as the sample extract. As can be seen via the coloured lines retention time matching of the standard monohydroxybenzoic acids with peaks present in the sample supports the hypothesis that the isomers of salicylic acid are present in the biostimulant sample. This could be further supported with mass spectral evidence of the fragmentation of the suspected peaks in the sample using Product Ion scanning mode (PI scan) with the MS/MS. By selecting the pseudomolecular ions of the three suspected peaks and fragmenting them using collision-induced dissociation the fragment peaks could be compared to that of the pure standards under the same conditions as well as literature values.



15) **Figure 3.2.2a** Extracted ion chromatograms (m/z 137) of each monohydroxybenzoic acid standard solution and sample extract. The mobile phase consisted of: Solvent A - water modified with 0.1 % (v/v) formic acid, Solvent B - acetonitrile. The gradient LC time program used was: 10 % (v/v) B from 0 – 1 min, increase from 10 % to 40 % (v/v) B from 1 – 10 min, increase from 40 % to 90 % (v/v) B from 10 – 14 min, hold at 90 % (v/v) B from 14 – 15.5 min. The MS was configured in full scan mode scan mode with a scan range of 100 – 400 m/z.

Using the same LC conditions the Product Ion scanning experiment was conducted by selecting for the pseudomolecular ion of m/z 137, fragmenting with a collision energy of 20 V and then scanning for product ions between the mass range of m/z 50 – 200. Figure 3.2.2b shows the chromatogram of the sample extract along with three mass spectra, one for each of the peaks previously tentatively identified as the monohydroxybenzoic acids. All three spectra contained the pseudomolecular ion of m/z 137 as well as the base peak of m/z 93, which constitutes a loss of CO_2 or m/z 44. This loss is commonly described for monohydroxybenzoic acids such as salicylic acid [85]. The MS^2 spectra for the peaks in the sample are similar to those for pure standards, and when combined with retention time

matching provide suitable evidence for the presence and positive identification of the three monohydroxybenzoic acids. This is a significant finding as it is the first known report of monohydroxybenzoic acids in a commercial seaweed biostimulant. This finding will allow for further investigation into the biological mechanisms underpinning the reported benefits of commercial seaweed biostimulants. The significance of this finding is further discussed in Chapter 4 where the monohydroxybenzoic acids are quantitatively determined in the commercial seaweed biostimulant, as well as Chapter 5 where their role in seaweed extracts is investigated using plant growth bioassays. Furthermore, these results demonstrate that a suitable approach for the qualitative investigation of hydroxybenzoic acids in commercial seaweed biostimulants is the development of HPLC-ESI-MS/MS methods which combine retention time and MS/MS data (as described in Section 1.5).



16) **Figure 3.2.2b** A product ion chromatogram of the sample extract. The three target peaks of interest are labelled with their respective retention times and their MS^2 spectra is displayed below the chromatogram. LC conditions similar to Figure 3.2.2a.

3.3 HPLC-ESI-MS/MS METHOD DEVELOPMENT FOR THE QUALITATIVE ANALYSIS OF HYDROXYBENZOIC ACIDS AND RELATED DERIVATIVES IN A COMMERCIAL SEAWEED BIOSTIMULANT

This section describes the optimisation of mass spectrometric and liquid chromatographic conditions in the development of fit-for-purpose HPLC-ESI-MS/MS methods for the identification of hydroxybenzoic acids and related derivatives in a commercial seaweed biostimulant sample. The approach adopted in this part of the study was to optimise the MS/MS detection conditions before investigating and optimising the liquid chromatographic conditions. Mass spectrometric optimisation was conducted first in order to establish detection polarity and pseudomolecular and fragment ions for the specific and selective detection of the analytes via MRM. Alongside the optimisation of LC conditions, it was of interest to explore the liquid chromatographic behaviour of the various hydroxybenzoic acids and related derivatives on reversed-phase stationary phases including C18 and the less explored phenyl based stationary phases. This section will discuss MS and LC investigations of standard solutions and mixed standard solutions of the various hydroxybenzoic acids and related derivatives. The standard solutions were prepared via direct dilution following the steps outlined in Section 2.4.1.

3.3.1 Investigation and Optimisation of MS Conditions.

The optimisation of MS/MS conditions followed a standardised approach that began with the determination of the pseudomolecular ion ($[M-H]^-$) for each of the analytes; followed by fragmentation studies to determine the characteristic MS/MS transition for each analyte before finally optimising the collision conditions. The results from the determination of the pseudomolecular ion, fragment ions, and optimal MS/MS transitions were compared with literature values and databases. For MS investigation experiments the HPLC-ESI-MS/MS instrument was configured with the operating parameters outlined in Section 2.6.2.2.

3.3.1.1 Determination of the Pseudomolecular Ion and Fragment Ions.

Dilute solutions of each of the hydroxybenzoic acids and related derivatives were directly injected into the MS/MS operating in full scan mode with a mass range of m/z 100 – 220. All analytes showed the characteristic pseudomolecular ion, $[M-H]^-$ in negative mode; listed in Table 3.3.1.1 below. As can be seen in negative mode all the derivatives displayed a pseudomolecular ion that was m/z 1 lower than the molecular weight typical of the deprotonation of the carboxylic acid moiety. After the molecular ion has been established fragment ions can be determined using MS/MS in Product Ion (PI) scanning

mode. The two collision energies used in this experiment were 10 and 20 eV; these are considered low and moderate levels of collision energy, which for small molecules such as the hydroxybenzoic acids and related derivatives are suitable. Table 3.3.1.1 shows the key observed fragment ions for each of the analytes collated from all of the product ion scans at both collision energies; spectral data in Appendix A.1.

10) **Table 3.3.1.1** Determination of the Pseudomolecular and Fragment Ions

Compound	$[M-H]^-$ (m/z)	Observed MS/MS Fragments (m/z)
2-hydroxybenzoic acid (Salicylic)	137	137, 93, 65
3-hydroxybenzoic acid	137	137, 93, 63, 45
4-hydroxybenzoic acid	137	137, 93, 45
2,3-dihydroxybenzoic acid (Pyrocatechuic)	153	109, 91, 53
2,4-dihydroxybenzoic acid (β-Resorcyclic)	153	109, 67, 65, 41
2,5-dihydroxybenzoic acid (Gentisic)	153	109, 108
2,6-dihydroxybenzoic acid	153	135, 109, 91, 67, 65, 41
3,4-dihydroxybenzoic acid (Protocatechuic)	153	109, 91, 81, 65, 53
3,5-dihydroxybenzoic acid (α-Resorcyclic)	153	109, 80, 67, 65, 45
2,3,4-trihydroxybenzoic acid	169	151, 125, 110, 95, 81
2,4,6-trihydroxybenzoic acid	169	151, 125, 107, 83, 65, 41
3,4,5-trihydroxybenzoic acid (Gallic)	169	151, 125, 97, 79, 69, 53
4-hydroxy-3-methoxybenzoic acid (Vanillic)	167	152, 149, 123, 108, 99, 79
4-hydroxy-3,5-dimethoxybenzoic acid (Syringic)	197	182, 167, 151, 138, 123, 107
3,4-dimethoxybenzoic acid (Veratric)	181	137, 113, 93, 69, 45
3,4,5-trimethoxybenzoic acid (Eudesmic)	211	193, 167, 165, 147, 137, 121
2-aminobenzoic acid (Anthranilic)	136	92

3.3.1.2 Determination of the Characteristic MS/MS Transition.

As discussed in Section 1.5.7.3, the MS/MS transition allows for more selective analysis and often provides increased sensitivity. It is considered another method of filtering out background noise to select for only the precursor to product ion reaction of the target analytes. Depending on the application MS/MS transitions can be selected that are either unique to the compound offering the highest level of selectivity; or conversely, a MS/MS transition can be specific to a group of compounds such as isomers that share the same molecular ion and a fragment ion which can allow for the targeted analysis of a group of compounds. Both approaches will be employed to target the benzoic acids derivatives being investigated in this chapter. To determine the MS/MS transition for each derivative

the spectra from the product ion scanning will be compared to literature and online databases; including the Human Metabolome Database (HMDB) which has an extensive list of compounds for which there are both experimental and predicted LC-MS/MS spectra [208].

3.3.1.2.1 MS/MS Transition of Monohydroxybenzoic Acids

All three of the monohydroxybenzoic acids are isomers and therefore share the same pseudomolecular ion; furthermore they all share the same benzoic acid backbone that has a characteristic fragmentation pattern, the loss of CO_2 (loss of m/z 44). The transition from the pseudomolecular ion to that specific fragment ion can be selected for analysis; allowing for detection of molecules that only exhibit this same m/z 137 → 93. This MS/MS transition has been used to analyse salicylic acid and other benzoic acid derivatives in multiple studies [82, 209, 210]. While m/z 137 → 93 is the optimal transition for the monohydroxybenzoic acids in pure standard solutions, it is worth noting that interferences can often occur as a result of the sample matrix. For example, this was the case in a study conducted by Segarra *et al.* where the chosen MS/MS transition for jasmonic acid showed to be suboptimal due to coeluting peaks [82]. Therefore, the m/z 137 → 93 MS/MS transition is selected for the monohydroxybenzoic acids with the assumption that any coeluting peaks will be able to be chromatographically separated with suitable resolution in order to distinguish between analytes.

3.3.1.2.2 MS/MS Transition of Dihydroxybenzoic Acids

The dihydroxybenzoic acids all shared the fragment of m/z 109; which is consistent with experimental and predicted spectra that can be found on HMDB [208, 211, 212]. Therefore, the transition of m/z 153 → 109, again due to the loss of CO_2, could be used to detect for all six of the dihydroxybenzoic acids simultaneously, under the condition that they can be chromatographically separated. In examining the literature for methods that aim to separate and identify dihydroxybenzoic acids by LCMS/MS, the vast majority of methods employed the m/z 153 → 109 MS/MS transition. Gentisic acid (2,5-dihydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), and 3,5-dihydroxybenzoic acid were analysed by LCMS/MS using the m/z 153 → 109 transition across various studies with great success; therefore this was the MS/MS transition employed in this study for the dihydroxybenzoic acids [92, 151, 209, 210].

3.3.1.2.3 MS/MS Transition of Trihydroxybenzoic Acids

The trihydroxybenzoic acids all shared two fragment ions that could serve as the product ion in the MS/MS transition: m/z 151 and 125. The m/z 125 is the fragment ion that is the result of the loss of m/z 44 due to the loss of the carboxylic acid moiety, as previously described for the mono- and dihydroxybenzoic acids. When investigating spectra for trihydroxybenzoic acids in the HMDB, both the predicted and experimental spectra were in agreement with the observations made in the product ion scans from this study as listed in Table 3.3.1.1 above. [208]. At the lower to moderate collision energies of 10 and 20 eV, the m/z 151 and 125 fragment ions were the most abundant [211, 212]. The literature also reports that the most common trihydroxybenzoic acid: gallic acid, is often analysed and quantified using the MS/MS transition of m/z 169 →125 [157, 209, 210, 213]. Therefore, for this study the m/z 169 → 125 was the chosen MS/MS transition for the analysis of trihydroxybenzoic acids.

3.3.1.2.4 MS/MS Transition of Methoxylated Hydroxybenzoic Acids

The two methoxylated hydroxybenzoic acids: vanillic and syringic acid, both had product ions that showed a loss of m/z 15 from the pseudomolecular ion. This loss of m/z 15 is due to the loss of a methyl group which is characteristic of the derivatives with aromatic methoxy groups [209]. The characteristic methyl dissociation served as the primary candidate for an MS/MS transition for both methoxylated derivatives: m/z 167 → 152 and 197 → 182 for vanillic and syringic acid, respectively. These transitions were used in a study isolating phenolics from sea algae by Klejdus *et al.* with great success, therefore these were the transitions employed for further analysis of vanillic and syringic acid in this study [92].

3.3.1.2.5 MS/MS Transition of Methoxylated Benzoic Acids

The methoxylated benzoic acid derivatives, veratric and eudesmic acid both have only a few experimental mass spectra on the HMDB, with the bulk being predicted spectra [208]. Of the product ions detected for veratric acid in negative ionisation mode, it was the m/z 137 ion that was clearly present in both the experimental and predicted spectra on HMDB; this was the product ion used in two studies that analysed veratric acid via ESI-MS/MS with the following MS/MS transition: m/z 181 → 137 [167, 170, 212]. As for eudesmic acid, even though there is no experimental negative ionisation mode spectra on HMDB, of the ions detected in this study the m/z 167 and 137 ions matched the predicted mass spectra [208, 212]. The MS/MS transition of 211 → 167 m/z was employed in three separate studies that analysed eudesmic acid by ESI-MS/MS in negative ionisation mode [172, 214, 215]. Therefore, for the

chromatographic investigation and optimisation study the m/z 181 → 137 and 211 → 167 negative ion transitions, representing the loss of CO_2, were the chosen MS/MS transitions for the analysis of veratric and eudesmic acids, respectively. It should be noted that these transitions were eventually not deemed suitable for the analysis of veratric and eudesmic acid in the seaweed biostimulant sample as discussed in Section 3.3.6.

3.3.1.2.6 MS/MS Transition of Aminobenzoic Acids

In the product ion analysis conducted in this study in negative ionisation mode anthranilic acid had one very dominant product ion of m/z 92, this was also observed in all of the experimental and predicted mass spectra on HMDB [208]. The literature suggests that anthranilic acid is most commonly analysed in positive mode, for example two studies use both m/z 138 → 120 and 138 → 92 MS/MS transitions for the analysis of anthranilic acid [216, 217]. However, as will be shown in Sections 3.3.6 and 3.4.7 anthranilic acid displayed suitable sensitivity when analysed in negative mode in this study, therefore the negative ionisation mode transition of m/z 136 → 92 was employed for this study.

3.3.1.3 Optimisation of the MS/MS Collision Conditions.

Once a characteristic MS/MS transition is selected it is important to optimise the collision conditions. Even analytes that share the same characteristic transitions will often have differences in the optimal amount of energy to initiate the desired fragmentation [218]. The most important and thus the most widely reported parameter is the Collision Energy (CE); often reported in volts (V) or electronvolt (eV). There are other variables that are occasionally optimised simultaneously, these are reported under various names that are instrument manufacturer specific that include: collision cell entrance/exit potentials, declustering potential, fragmentor voltage, and quadrupole prebias potentials [92, 210, 219]. These collision conditions can all be optimised by manually changing them individually and monitoring the instrumental response, however many instrument manufacturers have software protocols that will automatically optimise these conditions. The instrument used in this research (see Section 2.6.1) employs an MRM optimisation protocol that allows for the optimisation the CE and quadrupole (Q_1 and Q_3) "Pre-rod Bias" (see Section 2.6.2.1). The precursor and product ions for each analyte were manually determined in this study as described above to yield MS/MS transitions, for each the three remaining conditions were optimised. The MRM optimisation protocol was run and the optimised collision conditions are reported below in Table 3.3.1.3.

11) **Table 3.3.1.3** Optimisation of the MS/MS Collision Conditions

Compound	E.S.I Polarity	MS/MS Transition (m/z)	Q1 PreBias (V)	Collision Energy (V)	Q3 PreBias (V)
2-hydroxybenzoic acid (Salicylic)	-	137 → 93	15	16	15
3-hydroxybenzoic acid	-	137 → 93	14	15	15
4-hydroxybenzoic acid	-	137 → 93	15	16	15
2,3-dihydroxybenzoic acid (Pyrocatechuic)	-	153 → 109	17	16	18
2,4-dihydroxybenzoic acid (β-Resorcyclic)	-	153 → 109	16	14	18
2,5-dihydroxybenzoic acid (Gentisic)	-	153 → 109	17	15	10
2,6-dihydroxybenzoic acid	-	153 → 109	17	17	17
3,4-dihydroxybenzoic acid (Protocatechuic)	-	153 → 109	17	16	17
3,5-dihydroxybenzoic acid (α-Resorcyclic)	-	153 → 109	17	13	18
2,3,4-trihydroxybenzoic acid	-	169 → 125	21	17	21
2,4,6-trihydroxybenzoic acid	-	169 → 125	19	17	22
3,4,5-trihydroxybenzoic acid (Gallic)	-	169 → 125	18	16	22
4-hydroxy-3-methoxybenzoic acid (Vanillic)	-	167 → 152	20	16	28
4-hydroxy-3,5-dimethoxybenzoic acid (Syringic)	-	197 → 182	11	13	17
3,4-dimethoxybenzoic acid (Veratric)	-	181 → 137	21	12	23
3,4,5-trimethoxybenzoic acid (Eudesmic)	-	211 → 167	11	12	16
2-aminobenzoic acid (Anthranilic)	-	136 → 92	15	16	30

3.3.1.4 Final Optimised MS/MS Methods for Simultaneous Analysis.

For the hydroxybenzoic acids that could share a single MS/MS transition (monos, dis and tris) the collision conditions for each derivative can be collated and used to find one optimal set of conditions per MS/MS transition. Then a single optimised method would allow for the identification of all derivatives that share that same transition. As can be seen in Table 3.3.1.3 there is little variation in voltages amongst analytes that share a MS/MS transition. The final conditions chosen shown in Table 3.3.1.4 allowed for the best sensitivity for all isomers in a group.

12) **Table 3.3.1.4** Final Optimised MS/MS Methods for Simultaneous Analysis

Compound	Polarity	MS/MS Transition (m/z)	Q1 PreBias (V)	Collision Energy (V)	Q3 PreBias (V)
All three monohydroxybenzoic acids	-	137 → 93	14	15	15
All six dihydroxybenzoic acids	-	153 → 109	16	15	18
All three trihydroxybenzoic acid	-	169 → 125	18	17	21

3.3.2 Investigation and Optimisation of LC Phases and Conditions.

Following on from the establishment of MS/MS detection conditions the next part of the study focussed on the investigation of suitable liquid chromatographic conditions for the separation of the target analytes listed in Table 3.1. Three LC columns with different stationary phases were available for this work and were investigated for their applicability for the separation of the target analytes in the seaweed biostimulant sample. Typically the main goal in chromatography is "the best resolution in the shortest possible time", however the literature alludes to highly complex seaweed biostimulant matrices; therefore the complexity of these matrices necessitates longer analysis times in order to ensure specificity as well as minimise common MS detector restraints such as ionisation suppression. The three columns investigated were: Agilent Polaris 3 μm C18-A 150 × 2.0 mm, Restek Raptor 1.8 μm C18 150 × 2.1 mm, and Restek Raptor 2.7 μm Biphenyl 150 × 2.1 mm. The Polaris column uses a fully porous polar modified 3 μm C18 stationary phase suitable for polar organic compounds and helps to avoid poor peak shape and retention issues in low organic conditions, and has been used successfully in our laboratories for the HPLC separation of the three monohydroxybenzoic acids (see Section 3.2) [220]. The Raptor C18 column uses 1.8 μm superficially porous particles (core-shell) and this general purpose C18 column offers fast and efficient UHPLC method development [221]. The Raptor Biphenyl bonded silica column uses 2.7 μm superficially porous particles and these columns offer increased retention of hydrophilic aromatic compounds and enhanced selectivity with methanolic (protic) mobile phases [222]. The two mobile phases investigated with the three columns include acetonitrile/water and methanol/water, both with formic acid modifier, as these are typical mobile phases for reversed phase LC-MS. Initially all the derivatives were analysed in negative ionisation mode using their respective MS/MS transitions determined in Section 3.3.1.

A standard set of operating conditions was used to allow for a simple and direct comparison of columns and mobile phase combinations. The standard solutions of the target analytes used throughout this study were prepared as described in Section 2.4.1.3 for individual standard solutions and Section 2.4.1.4 for mixed standard solutions. For direct comparison of separations on three columns isocratic elution using 30 % (v/v) organic modifier was used and the LC-MS operating conditions are listed in Section 2.6.2.3.

The results in the sections presented below show chromatograms of the target analytes using the three columns and two mobile phases. These chromatograms are normalised to 100 % intensity for the most intense chromatographic peak.

3.3.3 Chromatographic Investigation of Monohydroxybenzoic Acids.

The first group of target analytes investigated were the monohydroxybenzoic acids. Figures 3.3.3a and 3.3.3b show the six chromatograms for the monohydroxybenzoic acids on the three columns and two mobile phases. Peak assignments were confirmed by retention time matching using individual standard solutions of the three compounds. The elution order was the same on all three columns and is listed below in Table 3.3.3.

13) **Table 3.3.3** Peak assignments for monohydroxybenzoic acids

Peak	Analyte
1	4-hydroxybenzoic acid
2	3-hydroxybenzoic acid
3	2-hydroxybenzoic acid

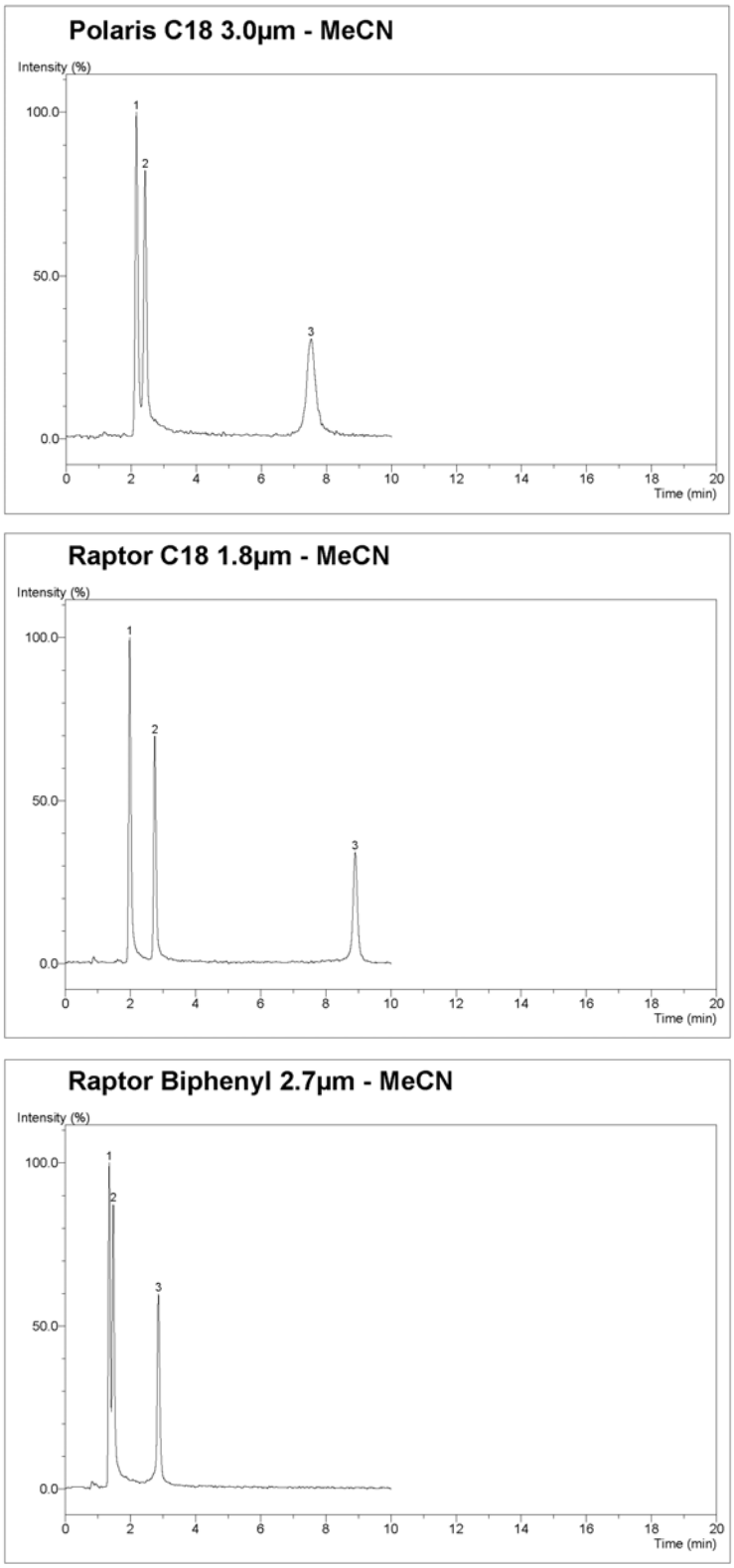
Using acetonitrile separation of all three analytes was achieved in under 9 minutes on the Raptor C18 column, which provided the best resolution of all three analytes with sharp and symmetrical peaks. For the same mobile phase conditions the Polaris C18 also separated all three analytes but with less resolution between peaks 1 and 2, and a broader peak shape for peak 3. Much less retention was observed on the Biphenyl with all three analytes eluting in under 3 minutes. This result is perhaps not unexpected using an acetonitrile based mobile phase given that phenyl stationary phases prefer protic mobile phases, such as methanol for optimum performance [223]. Using methanol all three columns separated the three analytes with adequate resolution, in particular peaks 1 and 2. Both Raptor columns achieved good separation of all three analytes with sharp and symmetrical peaks in under 10 minutes. Whereas the Polaris showed greater retention of all three analytes resulting in broader peak shapes, in particular peak 3 which eluted at around 16 minutes.

The observations made from the chromatography of the monohydroxybenzoic acids show that for isocratic elution and similar operating parameters all three columns show potential for the separation of all three analytes. For the Polaris column which uses 3.0 μm porous particles it is obvious that gradient elution would be needed to improve overall chromatographic performance; such as more retention for early eluting compounds and less retention for the more strongly retained compounds. The Raptor C18 is a UHPLC column with core-shell technology and 1.8 μm particles and these columns offers faster analysis times and superior selectivity to their porous particle equivalents. The separating power of these small particle solid-core columns was definitely on display here and with

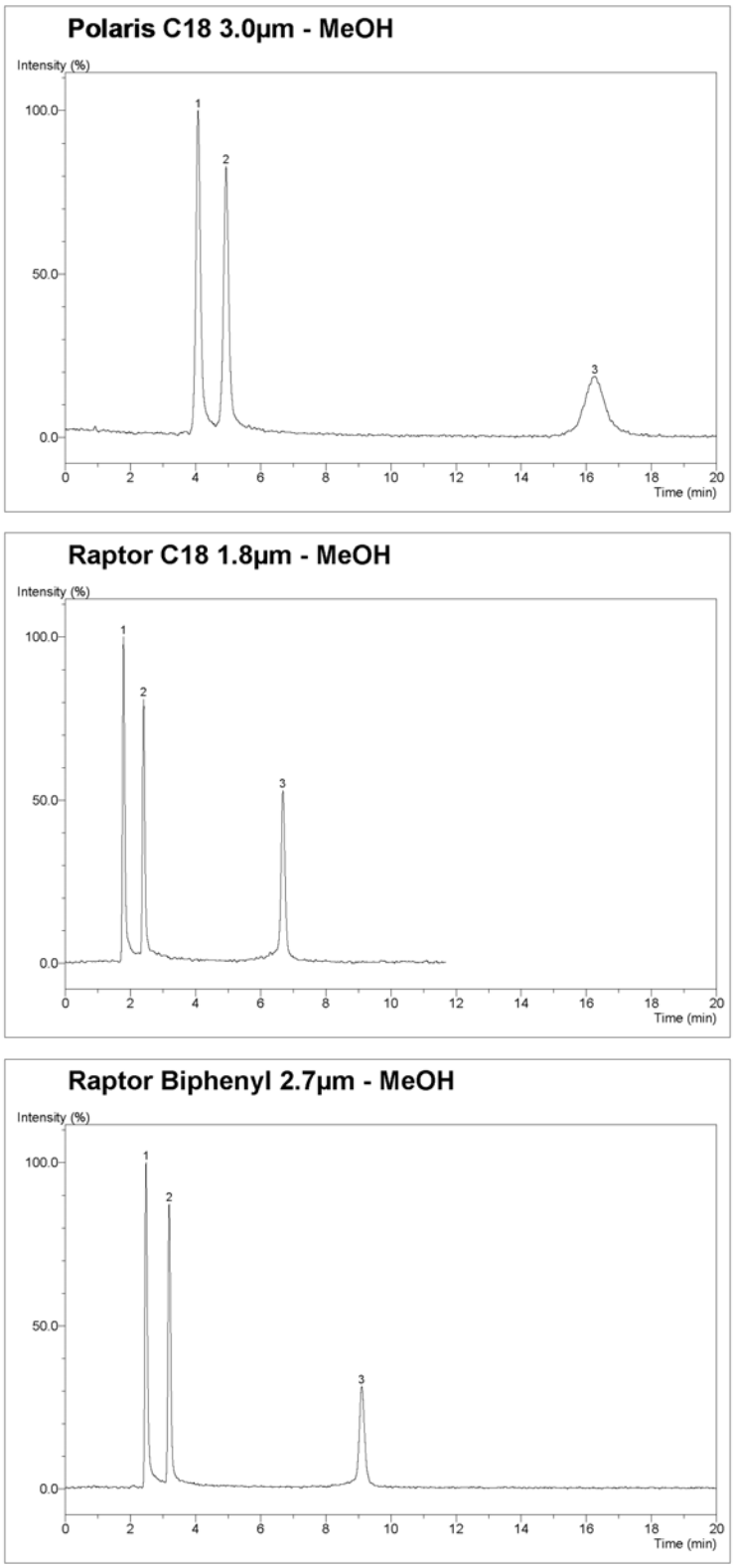
both methanol and acetonitrile based mobile phases all three structurally similar compounds were separated in under 10 minutes with adequate resolution. The Raptor Biphenyl column which also uses core shell technology and has larger 2.7 μm particles offered similar high performance to the Raptor C18 column.

The two most commonly analysed monohydroxybenzoic acids, 2- and 4-hydroxybenzoic acid, have been separated many times in algae and terrestrial plants using C18 stationary phases with acetonitrile/water and methanol/water mobile phases [85, 92, 156, 167]. In these studies 4-hydroxybenzoic acid was always eluted before 2-hydroxybenzoic acid, which was strongly retained. A study that employed a C18 column for the separation of phenolic compounds in freshwater algae, including 3- and 4-hydroxybenzoic acid, showed similar selectivity for 3- and 4-hydroxybenzoic acid, where the 3-hydroxybenzoic acid eluted just after 4-hydroxybenzoic acid. Two studies that were able to achieve separation of all three monohydroxybenzoic acids used C18 stationary phases with acetonitrile/water mobile phases, where all three were separated in under 15 minutes with the elution order: 4-, 3- then 2-hydroxybenzoic acid [170, 172].

Not surprisingly the results from this study are in agreement with other studies separating monohydroxybenzoic acids using C18 stationary phases. The C18 columns used in this study showed similar selectivity for 3- and 4-hydroxybenzoic acid and strong retention for 2-hydroxybenzoic acid, and both these columns would be suitable for the separation of these compounds. The choice to investigate the less explored biphenyl stationary phase as a novel approach for the separation of the monohydroxybenzoic acids proved successful. This is believed to be the first report of the separation of all three monohydroxybenzoic acids on a biphenyl stationary phase. The Biphenyl column has different retention mechanisms, π-π interactions with aromatic analytes, and as the results show offers great potential for the separation of these aromatic compounds when using methanol based mobile phases [224-226].



17) **Figure 3.3.3a** Chromatograms showing the separation of the three monohydroxybenzoic acids on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with an acetonitrile/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).



18) **Figure 3.3.3b** Chromatograms showing the separation of the three monohydroxybenzoic acids on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with a methanol/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).

3.3.4 Chromatographic Investigation of Dihydroxybenzoic Acids.

The second group of target analytes investigated were the dihydroxybenzoic acids. Figures 3.3.4a and 3.3.4b show the six chromatograms for the dihydroxybenzoic acids on the three columns and two mobile phases. Peak assignments shown in Table 3.3.4 below were confirmed by retention time matching using individual standard solutions of the six compounds.

14) **Table 3.3.4** Peak assignments for dihydroxybenzoic acids

Peak	Analyte
1	3,5-dihydroxybenzoic acid
2	3,4-dihydroxybenzoic acid
3	2,5-dihydroxybenzoic acid
4	2,4-dihydroxybenzoic acid
5	2,3-dihydroxybenzoic acid
6	2,6-dihydroxybenzoic acid

All six dihydroxybenzoic acids were separated in under 4 min using the Raptor C18 column and the elution order of the six dihydroxybenzoic acids is shown in Table 3.3.4. For the same mobile phase conditions the Polaris C18 column showed much less selectivity where both 3,5- and 3,4-dihydroxybenzoic acids coeluted as did 2,4- and 2,3-dihydroxybenzoic acid. The most striking difference observed with the Polaris column was the strong retention of 2,6-dihydroxbenzoic acid which appeared as a very broad mound at t_R = 17 min. When compared to the C18 columns the Raptor Biphenyl column showed extremely poor retention resulting in all six compounds eluting under 2 minutes with resulting poor resolution. As was stated with the separation of the monohydroxybenzoic acids, these biphenyl columns offer enhanced selectivity when used with methanolic mobile phases.

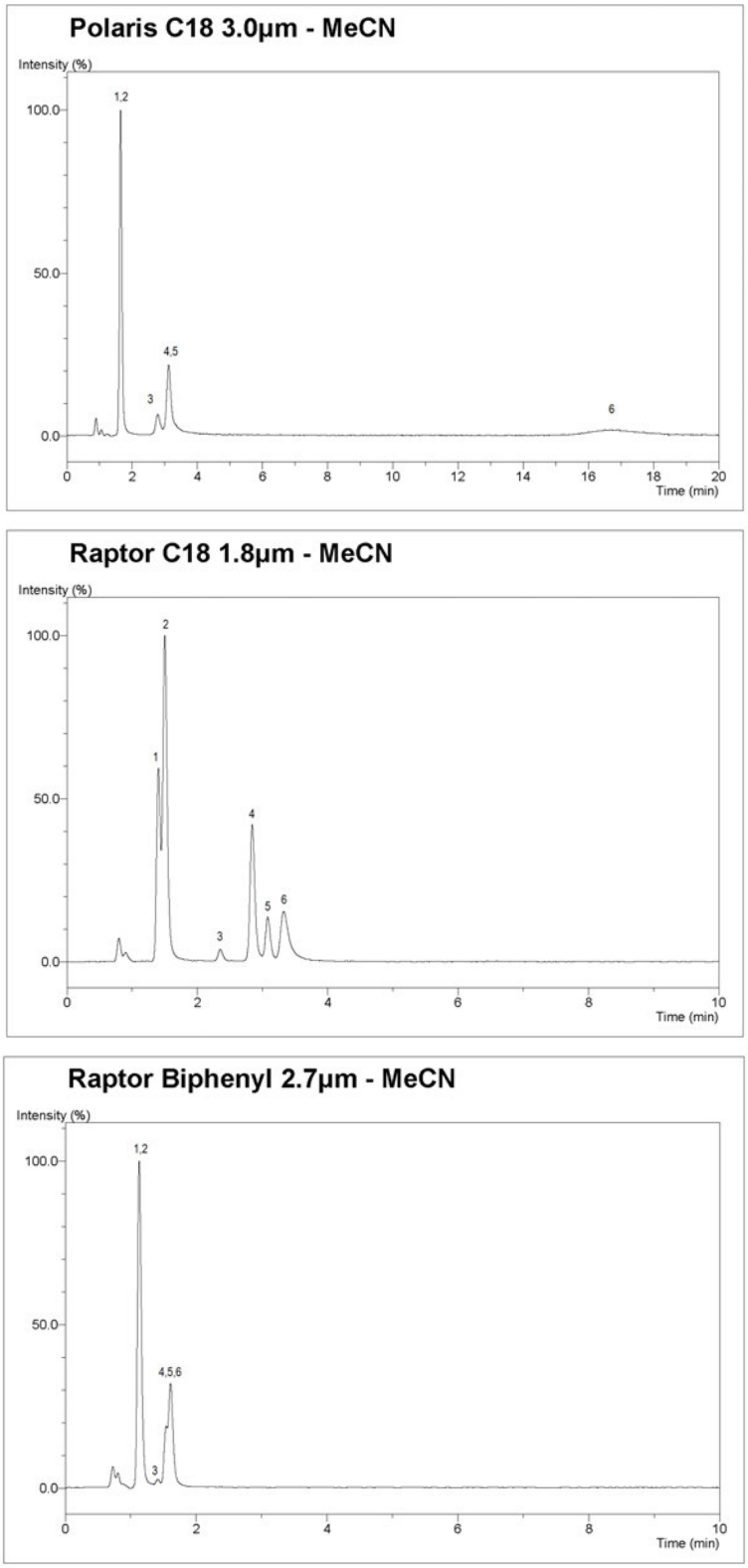
Similar to acetonitrile when using the methanol mobile phase the Polaris C18 column again displayed poor chromatography in terms of increased retention and less selectivity; with similar coelution. The 2,6-dihydroxybenzoic acid again was very strongly retained and appeared as a mound at t_R = 37 minutes. When using methanol all six dihydroxybenzoic acids were separated in under 4 min using the Raptor Biphenyl column. Interestingly the elution order of the last three compounds differed to that observed on the Raptor C18 with acetonitrile; being 2,6- 2,4- and 2,3-dihydroxybenzoic acid. The Raptor C18 column using methanol showed potential to separate all six compounds in under 3 min. The main difference between the Raptor C18 and Biphenyl being the decreased resolution of the 3,5-

and 3,4-dihydroxybenzoic acids and the elution order which showed a reversal for the 2,3- and 2,4-dihydroxybenzoic acids.

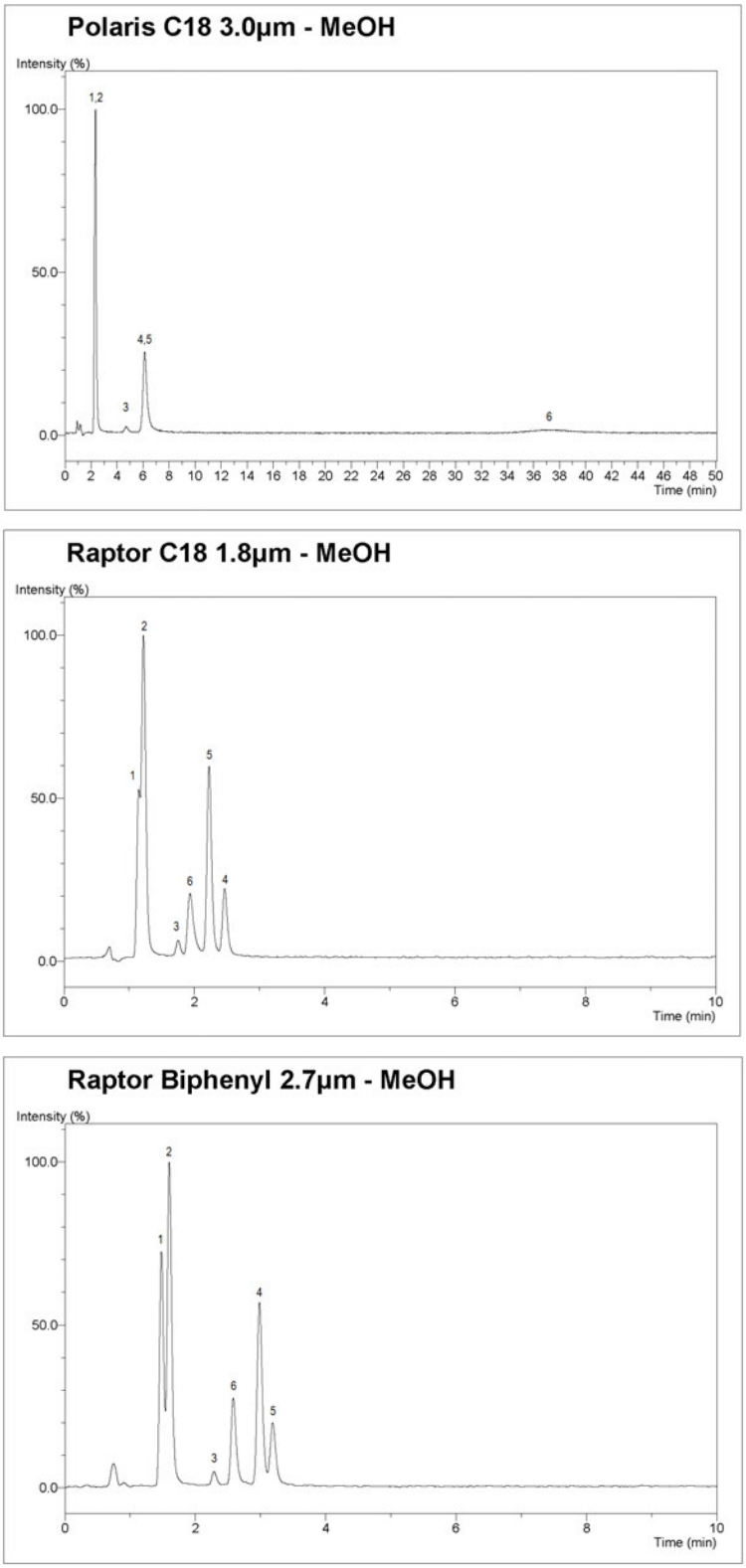
The observations made from the chromatography of the dihydroxybenzoic acids show that for isocratic elution and similar operating parameters all three columns show potential for the separation of all six compounds. For the Polaris column it is obvious that gradient elution would be needed to offer more retention for early eluting compounds and less retention for the 2,6-dihydroxbenzoic acid. Again excellent chromatography was observed with the Raptor C18 column and for both methanol and acetonitrile based mobile phases all six structurally similar compounds were separated except for the early eluting 3,5- and 3,4-dihydroxybenzoic acids. Obviously adjustments to mobile phase solvent strength would allow separation of the 3,5- and 3,4-dihydroxybenzoic acids. Interestingly the 2,6-dihydroxbenzoic acid was not strongly retained on the Raptor C18 column and when using methanol as the polar modifier it eluted before the 2,3- and 2,4-dihydroxbenzoic acids. This suggests there may be different retention mechanisms occurring for this compound on this particular solid-core C18 stationary phase. The Raptor Biphenyl column offers a point of difference to the two C18 columns because it has different retention mechanisms, which provided similar excellent chromatography.

When reviewing the literature for the separation of dihydroxybenzoic acids (see Section 1.6), no studies reported the separation of all six dihydroxybenzoic acids. The successful separation of all six isomers, as shown below on the Raptor C18 and Biphenyl columns, may potentially be the first reported separation of all six isomers in a single liquid chromatographic run. Multiple studies investigating a range of phenolic acids found that the dihydroxybenzoic acid studied was often weakly retained and eluted early, when compared to the other phenolic acids [85, 92, 131, 149, 168, 172]. Studies that investigated multiple dihydroxybenzoic acids and achieved separation using C18 showed similar selectivity for two groups, the 3,4- and 3,5-dihydroxybenzoic acid isomers were always less retained than the group consisting of 2,3-, 2,4-, 2,5- and 2,6-dihydroxybenzoic acid isomers [156, 163, 167, 170, 171]. One study analysed five of the six dihydroxybenzoic acids using a C18 column, achieving separation of the five in under 10 minutes with an elution order of 3,5-, 3,4-, 2,5-, 2,6- and 2,4-dihydroxybenzoic acid [170]. Interestingly, the elution order on the Raptor C18 column used in this study was similar to the above study, except for the reversal in the elution order of 2,4- and 2,6-dihydroxybenzoic acid.

As expected the results from this study are in agreement with other studies separating dihydroxybenzoic acids using C18 stationary phases. The C18 columns used in this study showed similar selectivity for the groups of dihydroxybenzoic acids mentioned above, and both these columns would be suitable for the separation of these compounds. The results for the Biphenyl column showed excellent separation performance with a methanol/water mobile phase. These biphenyl-bonded phases are advantageous in terms of offering better selectivity and increased retention for compounds that elute early on C18 and offer greater aromatic selectivity than other phenyl phases [222, 223, 227]. These advantages, and without comparing columns with similar sized solid-core particles, it is postulated, allowed the Raptor Biphenyl column with the larger 2.7 μm particles and with a methanol/water mobile phase to offer similar high performance to the Raptor C18 column with smaller 1.8 μm particles.

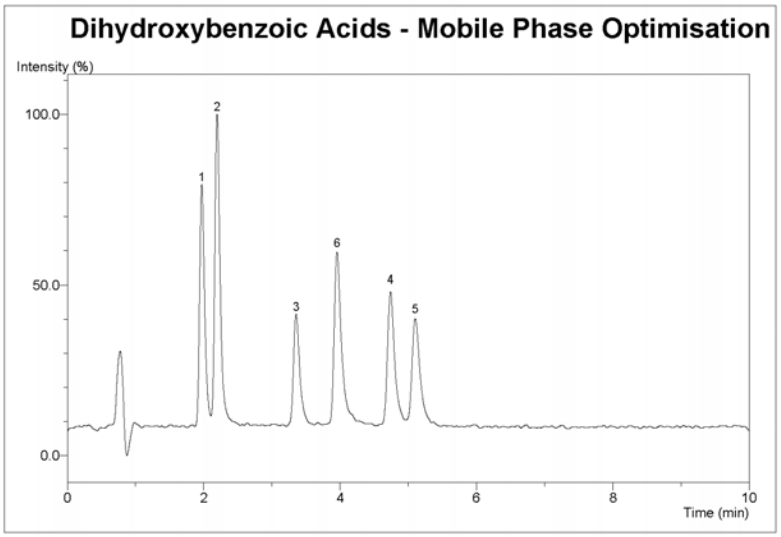


19) **Figure 3.3.4a** Chromatograms showing the separation of the six dihydroxybenzoic acids on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with an acetonitrile/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).



20) **Figure 3.3.4b** Chromatograms showing the separation of the six dihydroxybenzoic acids on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with a methanol/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).

The chromatographic conditions required optimisation in order to achieve separation of the six dihydroxybenzoic acids with better resolution between the first two peaks on the Raptor Biphenyl column with a methanol/water/formic mobile phase. In reversed phase chromatography the solvent strength governs retention, where a reduction in the solvent strength by decreasing the methanol content in the mobile phase is expected to provide an increase in retention and which may aid in the separation of closely eluting analytes. Various mobile phase compositions were investigated and it was found that methanol/water at 20:80 (v/v) provided adequate resolution of the six dihydroxybenzoic acids (as shown below in Figure 3.3.4c). Using this mobile phase allowed for the separation of all six dihydroxybenzoic acids in under 6 minutes with greater resolution of peaks 1 and 2 (3,4- and 3,5-dihydroxybenzoic acids) on the Raptor Biphenyl column. Similar to the monohydroxybenzoic acids, the Biphenyl column offers great potential in terms of simple and fast separation for the dihydroxybenzoic acids. The choice to investigate the biphenyl stationary phase as a novel approach for the separation of the dihydroxybenzoic acids proved extremely successful, with the separation of all six dihydroxybenzoic acids. This is a significant finding as it is also believed to be the first report of their successful separation on any reversed phase stationary phase and further highlights the potential of the biphenyl reversed phase system for aromatic phytochemicals.



21) **Figure 3.3.4c** Chromatogram showing the separation of the six dihydroxybenzoic acids on the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification, after altering the mobile phase composition from 30 % to 20 % (v/v) methanol. Run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.6).

3.3.5 Chromatographic Investigation of Trihydroxybenzoic Acids.

The third group of target analytes investigated were the trihydroxybenzoic acids. Figures 3.3.5a and 3.3.5b show the six chromatograms for the trihydroxybenzoic acids on the three columns and two mobile phases. Peak assignments shown in Table 3.3.5 below were confirmed by retention time matching using individual standard solutions of the three compounds.

15) **Table 3.3.5** Peak assignments for trihydroxybenzoic acids

Peak	Analyte
1	3,4,5-trihydroxybenzoic acid
2	2,4,6-trihydroxybenzoic acid
3	2,3,4,-trihydroxybenzoic acid

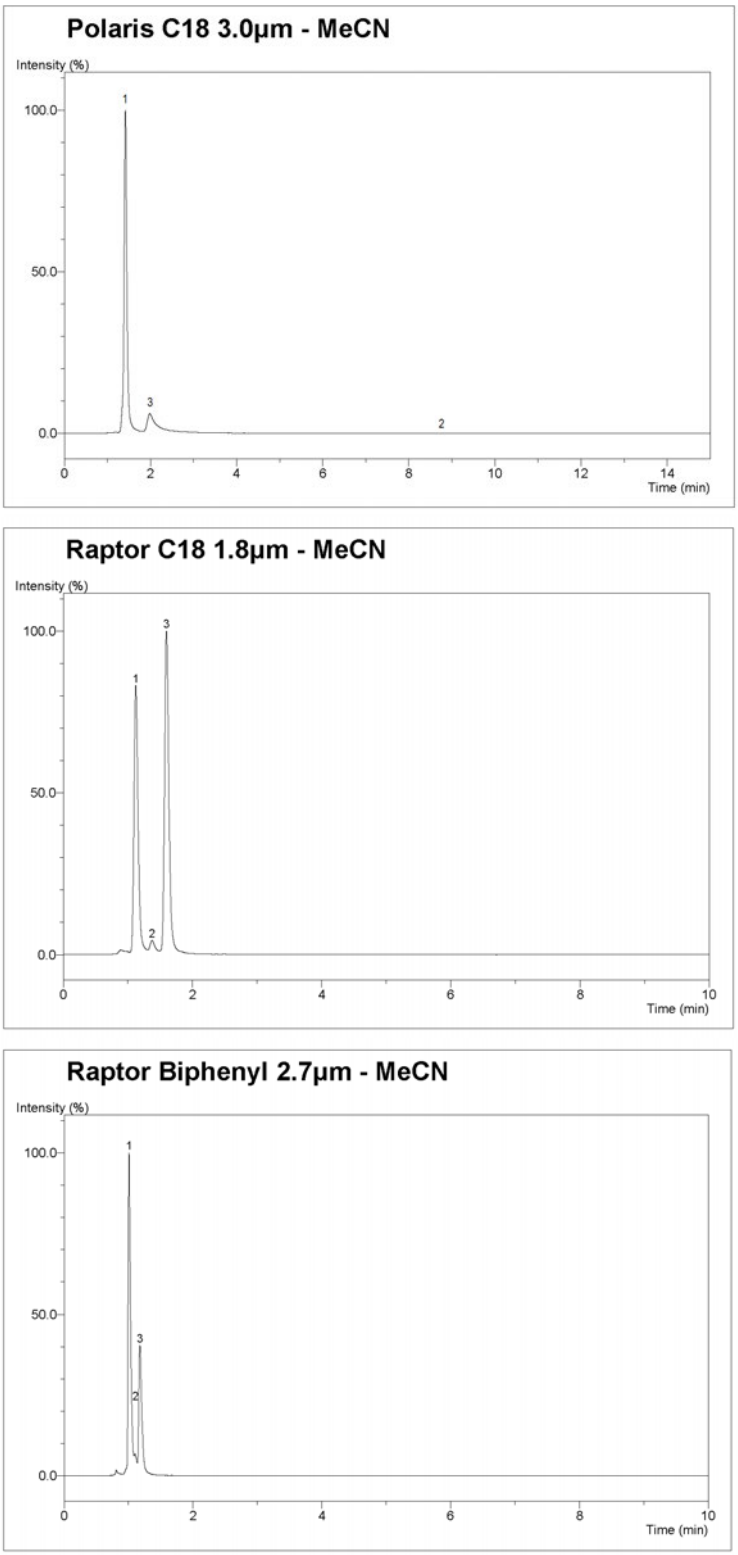
Interestingly the behaviour of trihydroxybenzoic acids on the three columns using the same mobile phase conditions was very similar to that of the dihydroxybenzoic acids. All three trihydroxybenzoic acids were separated under 2 min using the Raptor C18 column and the elution order of the three trihydroxybenzoic acids was 3,4,5-, 2,4,6 and 2,3,4 trihydroxybenzoic acid, as shown in Table 3.3.5. For the Polaris C18 column 3,4,5- and 2,3,4 trihydroxybenzoic acid were separated in under 2.5 min with the later displaying significant tailing. Based on the retention time of individually chromatographed standard solutions the 2,4,6-trihydroxybenzoic acid eluted at around 9 minutes but was not observed due to the normalisation of the chromatogram. As mentioned previously phenyl stationary phases prefer protic mobile phases for optimum performance so perhaps the loss of retention and hence selectivity with the Raptor Biphenyl column when compared to the Raptor C18 column was not unexpected.

Similar to the separations using acetonitrile when using the methanol on the Polaris C18 the 3,4,5- and 2,3,4 trihydroxybenzoic acids were easily separated early in under 4 minutes but again the latter displayed significant tailing. Based on the retention time of individually chromatographed standard solutions the 2,4,6-trihydroxybenzoic acid eluted at around 17.5 minutes but was not observed due to the normalisation of the chromatograms. The Raptor C18 column showed a decrease in retention and hence selectivity when using methanol which resulted in the 3,4,5- and 2,4,6-trihydroxybenzoic acids coeluting early followed by the elution of the 2,3,4-trihydroxybenzoic acid. When using the methanol mobile phase all three trihydroxybenzoic acids were separated in under 2 minutes on the Raptor Biphenyl column with the same elution order as shown in Table 3.3.5.

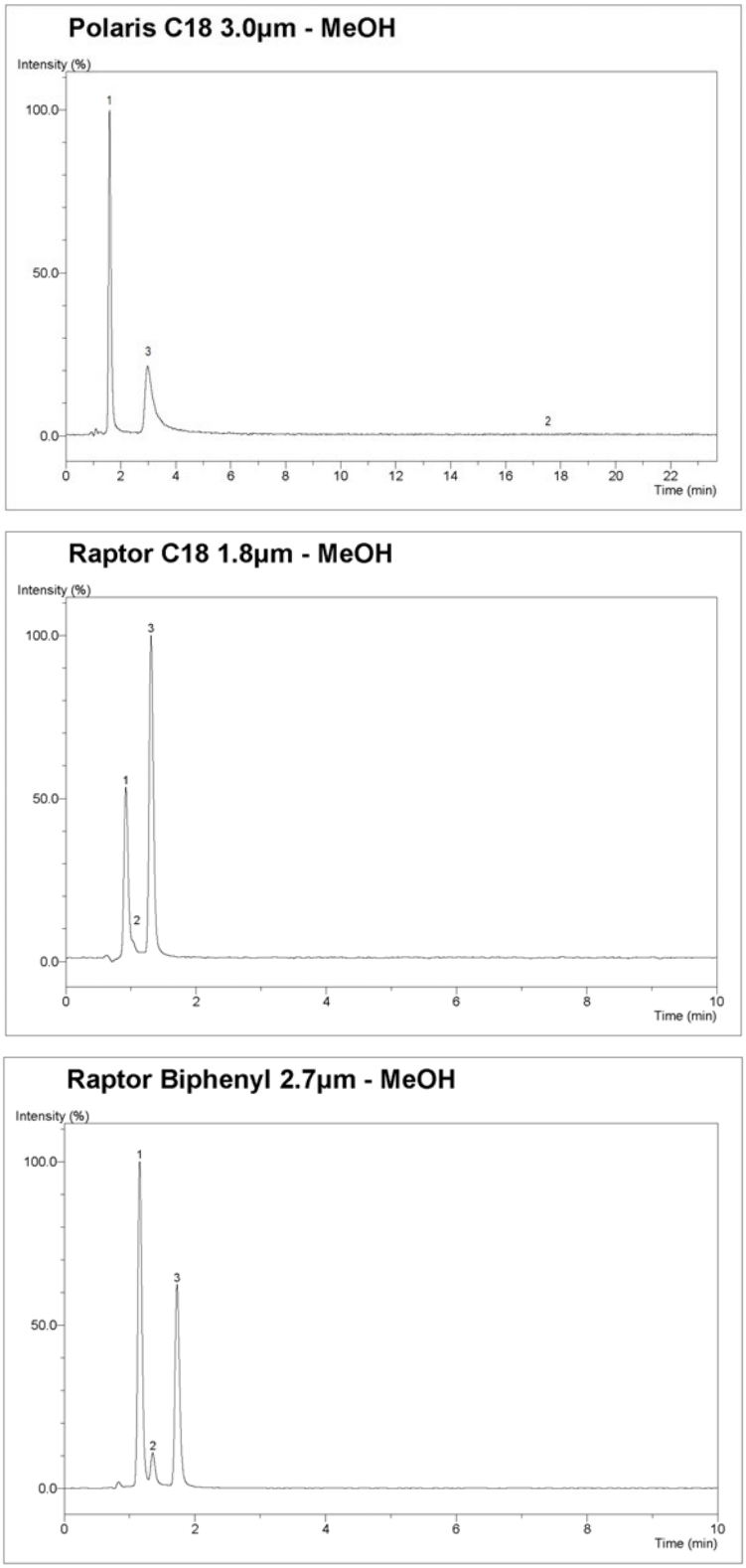
The observations made from the chromatography of the trihydroxybenzoic acids show that for isocratic elution and similar operating parameters the Raptor columns offer greater potential than the Polaris column for the separation of the three trihydroxybenzoic acids. The potential of the Polaris column may be hindered by tailing of the 2,3,4-trihydroxybenzoic acid and strong retention of the 2,4,6-trihydroxybenzoic acid probably caused by interaction with free silanol groups on the stationary phase. Obviously the solvent strength needs to be adjusted for the separation of the compounds when using the Raptor C18 column with methanol and likewise the Raptor Biphenyl column with acetonitrile. As discussed and shown below for the separation of dihydroxybenzoic acids, the advantages of the Raptor solid-core C18 column with 1.8 μm particles and that of the Raptor solid-core Biphenyl column with 2.7 μm particles also offered fast and efficient separation of the trihydroxybenzoic acids.

Many studies analysing phenolic acids include the analysis of 3,4,5-trihydroxybenzoic acid, better known as gallic acid (as seen in Table 1.6) [92, 131, 149, 163-165, 167, 168, 170-172]. However, there were no studies that included any of the other trihydroxybenzoic acids. The studies that analysed gallic acid using C18 used acetonitrile/water and methanol/water mobile phases and found that gallic acid was often the first compound to elute. With Klejdus *et al.* stating that it is expected to have the lowest retention of the commonly analysed hydroxybenzoic acids due to "its aromatic ring containing three polar hydroxyl groups in 3', 4' and 5' positions" [168]. Accordingly, all three trihydroxybenzoic acids are predicted to have weak retention in reversed phase chromatography.

When using Raptor C18 column with the same LC conditions the trihydroxybenzoic acids have the lowest retention, eluting between 1 and 2 minutes. The dihydroxybenzoic acids have greater retention than the trihydroxybenzoic acids, eluting between 1.5 and 4 minutes; followed by the monohydroxybenzoic acids which have the greatest retention, eluting between 2 and 10 minutes. These general observations are in accordance with Klejdus *et al.*, the addition of polar hydroxyl groups to the aromatic ring results in lower retention [168]. Similar to the separation of mono- and dihydroxybenzoic acids, the previously discussed advantages of the Biphenyl column including better selectivity and increased retention for compounds that elute early on C18, offers great potential for the separation of trihydroxybenzoic acids [222, 223, 227].

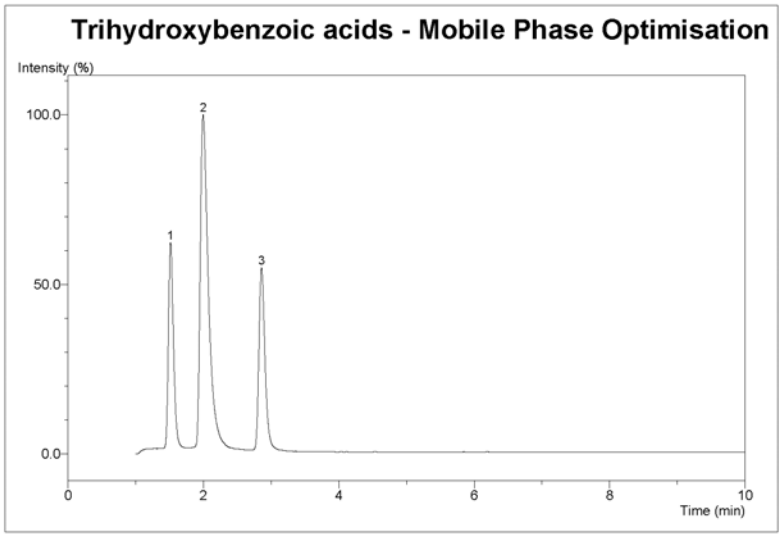


22) **Figure 3.3.5a** Chromatograms showing the separation of the three trihydroxybenzoic acids on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with an acetonitrile/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).



23) **Figure 3.3.5b** Chromatograms showing the separation of the three trihydroxybenzoic acids on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with a methanol/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).

The chromatographic conditions required optimisation in order to achieve separation of the three trihydroxybenzoic acids with better resolution on the Raptor Biphenyl with a methanol/water/formic mobile phase. Similarly to the dihydroxybenzoic acids chromatographic optimisation, a reduction in the solvent strength by decreasing the methanol content in the mobile phase was expected to provide extra retention and consequently better resolution. Various mobile phase compositions were investigated and it was found that methanol/water at 15:85 (v/v) provided adequate resolution of the three trihydroxybenzoic acids (as shown below in Figure 3.3.5c). The improvement in resolution by altering the composition of the mobile phase facilitates a higher chance of positive identification of the trihydroxybenzoic acids in a seaweed biostimulant sample. The choice to investigate the biphenyl stationary phase as a novel approach for the separation of the trihydroxybenzoic acids proved to be successful, with the separation of 3,4,5-, 2,4,6- and 2,3,4-trihydroxybenzoic acid in under 3 minutes (as shown below in Figure 3.3.5c). This is a significant finding as it is believe to be the first report of the successful separation on a biphenyl stationary phase, further demonstrating the applicability of the biphenyl reversed phase system for polar aromatic phytochemicals.



24) **Figure 3.3.5c** Chromatogram showing the separation of the three trihydroxybenzoic acids on the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification, after altering the mobile phase composition from 30 % to 15 % (v/v) methanol. Run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.7).

3.3.6 Chromatographic Investigation of Hydroxybenzoic Acid Related Derivatives.

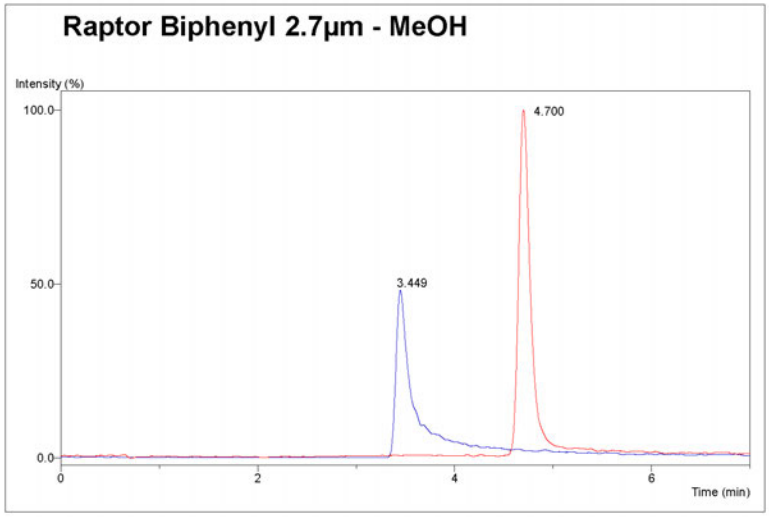
The final group of target analytes investigated were the hydroxybenzoic acid related derivatives: vanillic, syringic, veratric, eudesmic and anthranilic acids. Appendix A.2 shows the chromatograms obtained for the five derivatives using the three columns with both the acetonitrile and methanol mobile phases. To better observe the retention differences between structurally similar compounds, that is similar aromatic substitution, the individual chromatograms for vanillic and syringic acids were overlayed, as were the chromatograms for veratric and eudesmic acid. A visual inspection of the chromatograms shows very similar results to the chromatography observed for the mono, di and trihydroxybenzoic acids, with these five derivatives chromatographing reasonably well on all three columns.

These various hydroxybenzoic acid related derivatives have been successfully separated using C18 stationary phases [85, 92, 131, 156, 163, 164, 167, 168, 170-172, 216, 228]. The studies investigating the hydroxybenzoic acids with methoxy substitution, vanillic acid (4-hydoxy-3-methoxybenzoic acid) and syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), observed similar selectivity for these two compounds on C18 columns. The retention of these methoxy-substituted hydroxybenzoic acids increased when compared to their di- and tri-hydroxy counterparts, as the methoxy group ($-OCH_3$) is less polar than the hydroxyl group (-OH) [168]. In all of these studies vanillic acid elutes shortly before syringic acid, as predicted given the extra methoxy group in syringic acid. The increased retention following methoxy substitution is also observed in studies that investigated the methoxylated benzoic acid derivatives, veratric acid (3,4-methoxybenzoic acid) and eudesmic acid (3,4,5-methoxybenzoic acid); where the former elutes before the latter [164, 167, 170-172]. There are many studies that demonstrate the applicability of reversed phase chromatography using C18 for the separation of anthranilic acid (2-aminobenzoic acid) [216, 228-232]. In a study that separated 4-aminobenzoic acid and 4-hydroxybenzoic acid using C18, it was observed that the hydroxy derivative was more strongly retained [231].

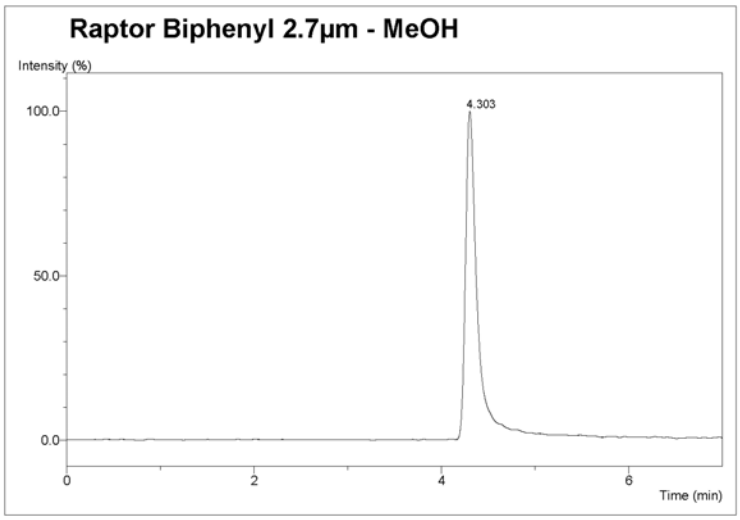
As anticipated the results from this study are in agreement with other studies separating these hydroxybenzoic acid related derivatives using C18 stationary phases. The C18 columns used in this study showed similar selectivity for vanillic and syringic acid, as well as the increased retention that is expected with further aromatic methoxy-substitution. In accordance with the general observations made for aminobenzoic acids by Deming *et al.*, 2-aminobenzoic acid always eluted well before 2-hydroxybenzoic acid counterpart, when separated under the same LC conditions. The different retention mechanisms of the Biphenyl column, π-π interactions with aromatic analytes, are suitable for

the separation of these types of aromatic compounds [101, 222, 223, 227]. The Raptor Biphenyl column with a methanol mobile phase showed great potential in terms of selectivity between the derivatives when considering individual retention times, that is, a mixture of all five compounds could be separated. For this reason including the advantages already mentioned for the mono, di and trihydroxybenzoic acids, this column was chosen for the qualitative analysis of these derivatives in the seaweed biostimulant sample.

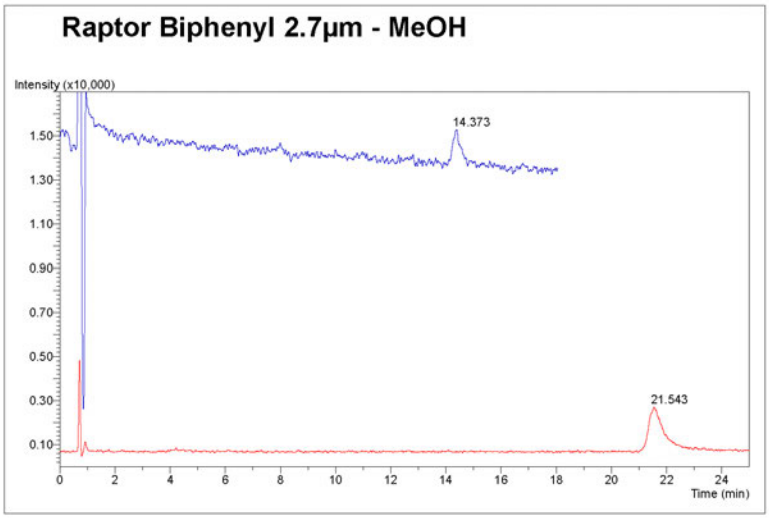
Figure 3.3.6a shows the separation of vanillic and syringic acids, Figure 3.3.6b shows the separation of veratric and eudesmic acids, and Figure 3.3.6c shows the separation of anthranilic acid all on the Biphenyl column with a methanol mobile phase. The discussion below will focus on the optimisation of LC conditions of the Raptor Biphenyl column with methanol mobile phase to be used for the qualitative analysis of veratric and eudesmic acids, which required optimisation to improve analysis time.



25) **Figure 3.3.6a** Chromatograms (overlayed) of vanillic (blue) and syringic (red) acids on the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).



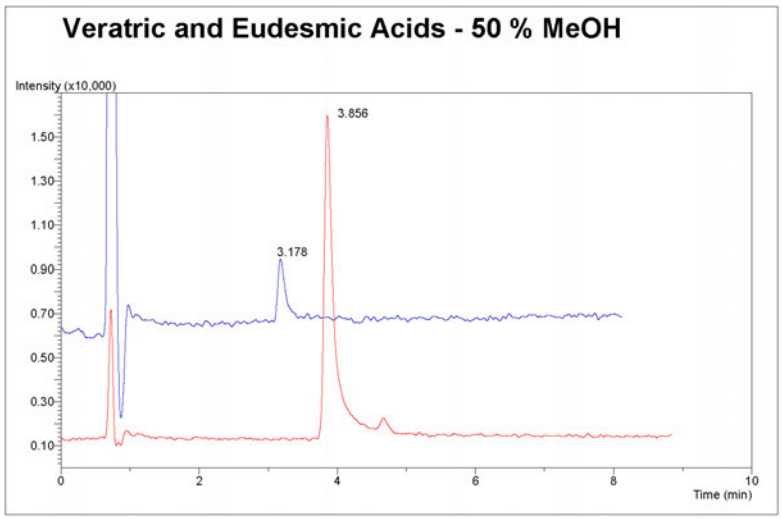
26) **Figure 3.3.6b** Chromatogram of anthranilic acid on the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).



27) **Figure 3.3.6c** Chromatograms (overlayed) of veratric (blue) and eudesmic (red) acids on the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).

As can be seen in Figure 3.3.6c veratric and eudesmic acid are strongly retained under the standard conditions. Similar to the di- and trihydroxybenzoic acids, the chromatography of veratric and eudesmic acid on the Raptor Biphenyl with a methanol/water/formic mobile phase required optimisation. Veratric and eudesmic acid retained too long and needed to be eluted more rapidly and this was achieved by increasing the solvent strength. Various mobile phase compositions were

investigated and 50:50 methanol/water (v/v) allowed suitable rapid separation. As can be seen in Figure 3.3.6d an increase in solvent strength from 30:70 (v/v) methanol/water to 50:50 (v/v) methanol/water allowed the separation of veratric and eudesmic acids in under 4 min on the Raptor Biphenyl column.



28) **Figure 3.3.6d** Chromatograms (overlayed) of veratric (blue) and eudesmic (red) acids on the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification, after altering the mobile phase composition from 30 % to 50 % (v/v) methanol, respectively. Run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.9).

The decrease in retention time improved the signal-to-noise ratio, however this was still considerably lower than the other analytes investigated. To improve the sensitivity for these two compounds it was decided to investigate their behaviour under positive ionisation mode conditions. According to the literature and experimental mass spectra from the HMDB both these compounds have been analysed in positive ion mode [169, 208, 233]. The product ion scanning analysis discussed in Section 3.3.1.1 was repeated in positive ion mode for these analytes. Table 3.3.6a shows the molecular ion and key observed fragment ions for each of the benzoic acid derivatives collated from the positive mode product ion scan; with full spectral data in Appendix A.3.

16) **Table 3.3.6a** Determination of pseudomolecular and fragment ions of veratric and eudesmic acid in positive ion mode

Compound	$[M+H]^+$ (m/z)	Observed MS/MS Fragments (m/z)
3,4-dimethoxybenzoic acid (Veratric)	183	165, 151, 139, 124, 108, 96
3,4,5-trimethoxybenzoic acid (Eudesmic)	213	195, 181, 169, 154, 139, 123

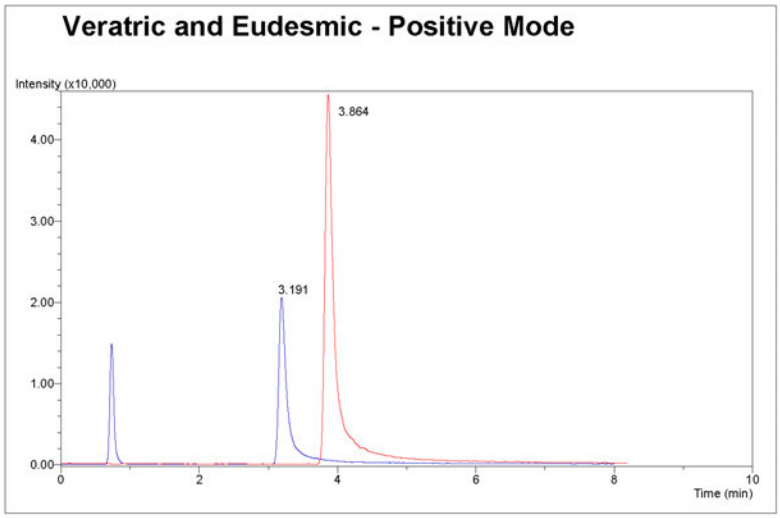
The analysis of veratric acid in positive ionisation mode in this study yielded product ions that were seen in predicted mass spectra on HMDB; including the m/z 165 and 139 ions [208]. Two studies that successfully analysed veratric acid in positive ion mode also utilised the 139 product ion with the MS/MS transition: 183 → 139 m/z [169, 233]. Therefore the positive ionisation mode transition m/z 183 → 139 which is due to the loss of CO_2, will be tested for improved sensitivity. The experimental mass spectra for eudesmic acid in positive ionisation mode and the product ion data from this study share the m/z 195, 169, 154, 139 and 123 ions [208]. Although the HMDB only contains experimental mass spectra for eudesmic acid collected in positive mode, no reliable source for a positive mode MS/MS transition could be found in the literature. Interestingly, the product ion that was the base peak in the MS^2 spectra was the m/z 154 ion, which is a loss of m/z 59. Although no studies could be found that employed a m/z 213 → 154 transition, two studies were found that employed a m/z 211 → 152 transition in negative mode; which is the same loss of m/z 59 [234, 235]. This loss of m/z 59 is believed to be due to the loss of CO_2 and CH_3, also postulated by Qin *et al.* [235]. Based on these findings the m/z 213 → 154 positive ion MS/MS transition will be tested for improved sensitivity. The new positive ion MS/MS transitions for veratric and eudesmic acid underwent the same MRM optimisation protocol outlined in Section 3.3.1.3, the results are listed in Table 3.3.6b below.

17) **Table 3.3.6b** Optimised MS/MS collision conditions for veratric and eudesmic acids in positive ion mode

Compound	E.S.I Polarity	MS/MS Transition (m/z)	Q1 PreBias (V)	Collision Energy (V)	Q3 PreBias (V)
3,4-dimethoxybenzoic acid (Veratric)	+	183 → 139	-13	-13	-26
3,4,5-trimethoxybenzoic acid (Eudesmic)	+	213 → 154	-11	-14	-29

Figure 3.3.6e shows the overlayed chromatograms for veratric and eudesmic acids using the positive mode MRM conditions in Table 3.3.6.b. As can be seen there was a significant increase in signal-to-noise which provided more confidence in the methods ability to identify the analytes amongst the complex sample matrix of the seaweed biostimulant. The successful application of the unique positive

ionisation mode MRM transition of m/z 213 → 154 offers a novel approach for the analysis of eudesmic acid.



29) **Figure 3.3.6e** Chromatograms (overlayed) of veratric (blue) and eudesmic (red) acids on the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification, using their positive ionisation mode MS/MS transitions. Run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.9).

3.3.7 Additional LC Considerations Prior to Sample Analysis

The impacts of the sample matrix on the LC and the MS were considered and necessary alterations were made to the methodology in order to have a proactive and preventative response to any fouling, contamination, and other matrix effects. The two key additions to the methods were a time delay on the MS detector data acquisition where the first portion of the chromatographic run was directed to waste, and a column flush to elute any strongly retained compounds from the column which was also directed to waste.

Poorly retained compounds can be a problem and may linger in the electrospray ionisation unit, leading to fouling and poor ionisation efficiency. This can be avoided by directing the flow to waste at the beginning of the chromatographic run, then at a set time redirecting the flow to the detector for the analysis of the target analytes [110]. An adequate amount of time to expel the poorly retained compounds without impacting on the first eluting target analyte was added to the beginning of the chromatographic methods.

A column flush can be programmed into the LC time program to quickly remove compounds that retain strongly and may lead to carry-over between chromatographic runs. This approach does extend the individual analysis run time and requires more solvent, however this proactive approach to preventative care and maintenance is important in managing the impacts of more complex sample matrices on the instrument. The main considerations for a column flush are the concentration of the organic component of the mobile phase and the duration of the flush. It was decided that 80 % (v/v) methanol for a minimum of three column volumes would be sufficient for a column flush programmed into the analysis, with the same re-equilibration volume. Therefore, the methods included a column flush programmed into the LC analysis as follows: flow is directed to waste at 10 minutes; methanol concentration increased to 80 % (v/v) from 10 – 11 minutes; column flush from 11 – 14 minutes; methanol concentration decreased back to the starting concentration from 14 – 15 minutes; re-equilibration at the starting concentration of methanol from 15 – 20 minutes where the analysis ends.

3.4 PRELIMINARY QUALITATIVE INVESTIGATION OF HYDROXYBENZOIC ACIDS AND RELATED DERIVATIVES IN A COMMERCIAL SEAWEED BIOSTIMULANT

Given that the monohydroxybenzoic acids were already positively identified in Section 3.2, the method developed in Section 3.3 will be used for the development of a quantitative method for the analysis of monohydroxybenzoic acids in a commercial seaweed biostimulant, which is the focus of Chapter 4. The results presented in this section are for the di- and trihydroxybenzoic acids and related derivatives only. The specificity and selectivity of the MRM analysis modes optimised in Section 3.3 will be used for the tentative identification, supplemented by retention time matching with standard solutions. Furthermore, following the qualitative analysis of the sample, a sample spiked with standard benzoic acid derivatives was analysed for further evidence to support the findings with retention time matching. The spiked sample additionally allowed for an investigation of detection limitations brought about by matrix effects, and if they are suppressing the ionisation/detection of the target derivatives.

The seaweed biostimulant sample used for the qualitative analysis was prepared using acetonitrile partitioning, before evaporation under nitrogen, reconstituting, and filtering as outlined in Section 2.5.1a. An aliquot of the sample was transferred into an LC vial for analysis; whilst a second aliquot was spiked with standard stock solutions of the hydroxybenzoic acids and related derivatives for spiked sample analysis as described in Section 2.5.2. The standard solutions used for the qualitative analysis were prepared as described in Section 2.4.3.

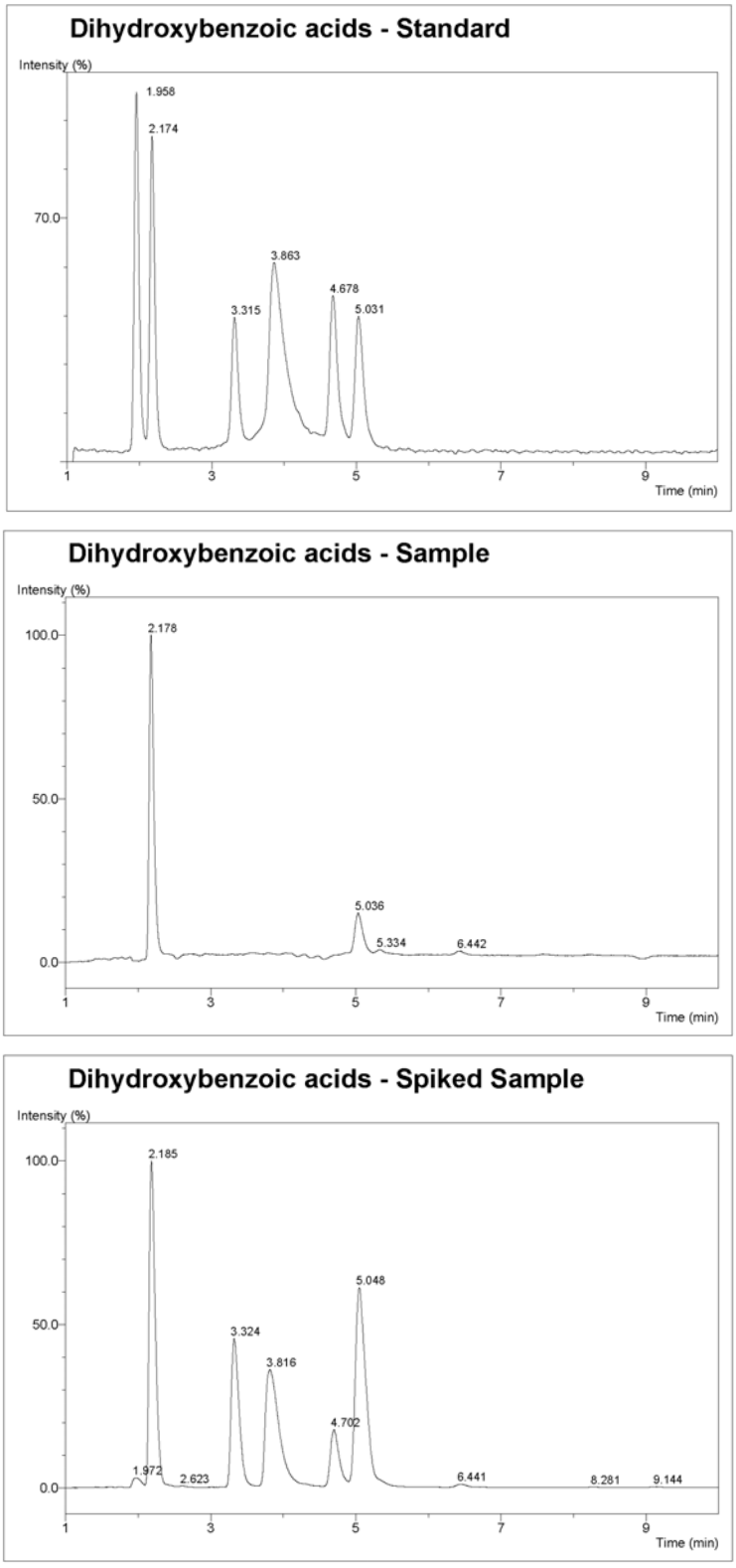
The findings of the preliminary qualitative analysis of the hydroxybenzoic acids and related derivatives in a commercial seaweed biostimulant are discussed below in Section 3.4.

3.4.1 Identification of Dihydroxybenzoic Acids in a Commercial Seaweed Biostimulant

The HPLC-ESI-MS/MS conditions specific to the qualitative method for the analysis of dihydroxybenzoic acids (DHBA) in a commercial seaweed biostimulant are described in Section 2.6.2.6. Figure 3.4.1 shows the three chromatograms for a mixed standard solution of the dihydroxybenzoic acids, a seaweed biostimulant sample, and a spiked sample.

The first chromatogram shows six peaks corresponding to the six dihydroxybenzoic acid isomers where the elution order based on retention time matching was: 3,5-DHBA, 3,4-DHBA, 2,5-DHBA, 2,6-DHBA, 2,4-DHBA, then 2,3-DHBA. The second chromatogram is of the seaweed biostimulant sample extract; in which there are two observable peaks with retention times of 2.178 and 5.036 minutes which share retention times with 3,4-DHBA and 2,3-DHBA, respectively. These results suggest the presence of both 3,4- and 2,3-DHBA in the biostimulant sample. This is a significant finding as it is believed to be the first report of any dihydroxybenzoic acids in a commercial seaweed biostimulant (further discussed in Section 3.5).

The results for the spiked sample are not immediately as expected: the peak at 1.9 minutes has dramatically decreased, whereas the peak at 2.1 minutes appears to not have increased, and the relative sizes of the next three peaks are not the same as in the standard mixture. Only the last peak displays the expected increase in peak height. These results strongly suggest that matrix effects via ionisation suppression are significant and need to be taken into account in developing qualitative and quantitative methods of analysis for these compounds. In particular increasing retention for early eluting peaks and including extra sample clean-up will help. Interestingly the sample chromatogram does show other smaller peaks at 5.3 and 6.4 minutes indicating the presence of other compounds with similar precursor and product ions.



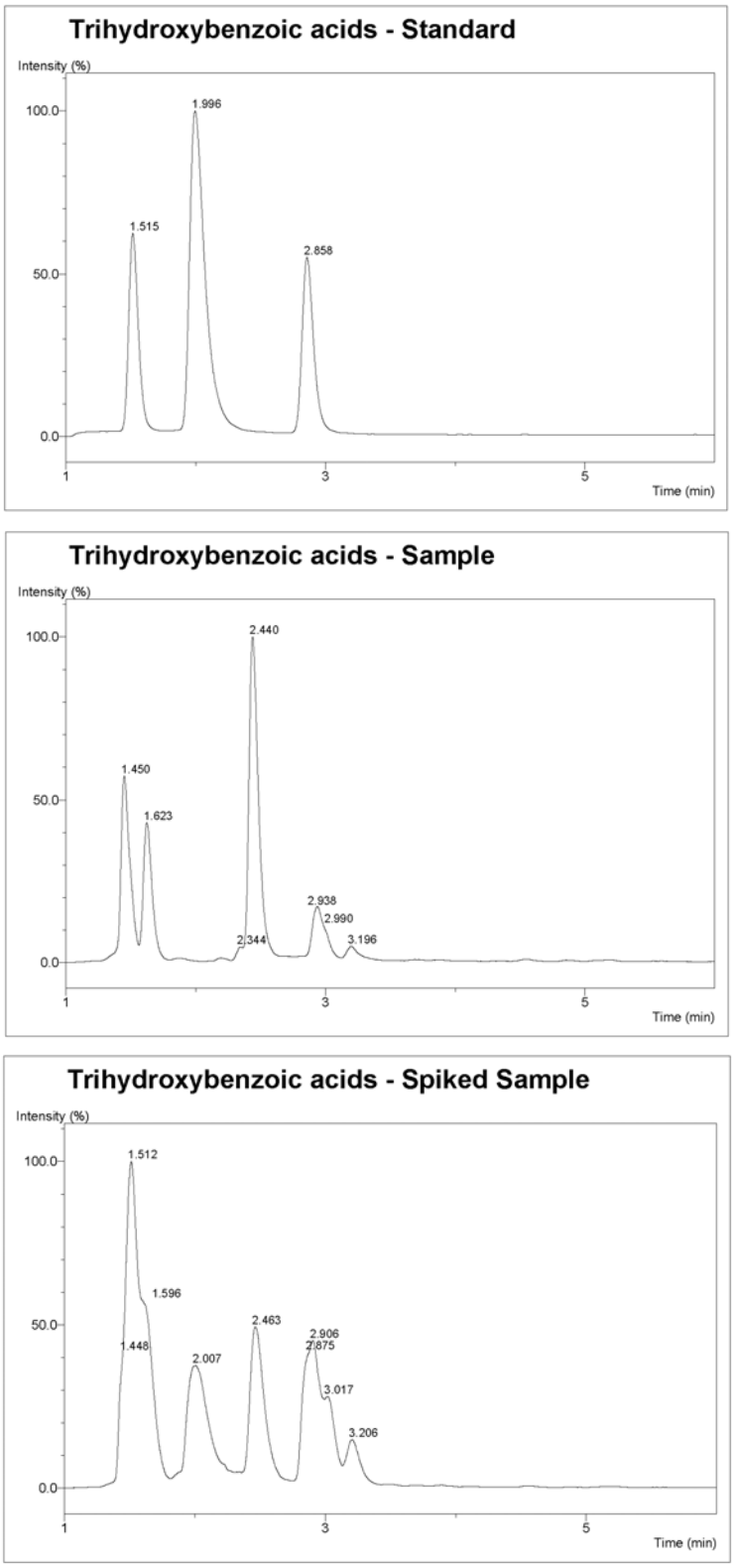
30) **Figure 3.4.1** Chromatograms of a dihydroxybenzoic acid mixed standard solution, a sample, and a spiked sample; each analysed using the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification and the 153 → 109 m/z MRM transition. Full LC-MS/MS conditions outlined in (Chapter 2.6.2.6).

3.4.2 Identification of Trihydroxybenzoic Acids in a Commercial Seaweed Biostimulant

The HPLC-ESI-MS/MS conditions specific to the qualitative method for the analysis of trihydroxybenzoic acids (THBA) in a commercial seaweed biostimulant are described in Section 2.6.2.7. Figure 3.4.2 shows the three chromatograms for a mixed standard solution of the trihydroxybenzoic acids, a seaweed biostimulant sample, and a spiked sample.

The first chromatogram is of the mixed standard solution, in which three peaks are observable with retention times of 1.515, 1.996, and 2.858 minutes. These three peaks have previously been identified through retention time matching as 3,4,5-THBA, 2,4,6-THBA, and 2,3,4-THBA, respectively. The second chromatogram is the analysis of the seaweed biostimulant sample extract; several peaks are present between approximately 1 – 3.5 minutes and on first inspection it appears that 3,4,5- and 2,3,4-THBA are present in the sample. The third chromatogram is of the spiked sample and this result suggests that 3,4,5- and 2,3,4-THBA are not present in the sample with the 3,4,5-THBA eluting between the sample peaks with retention times of 1.450 and 1.623 minutes; and the 2,3,4-THBA eluting just before the sample peak of 2.938 minutes. Surprisingly it appears that none of the three THBA were present in the sample and in particular gallic acid which is prevalent in the literature.

Perhaps what was not found is not the most surprising aspect of this THBA study but what was found, that being the presence of several other compounds with similar precursor and product ions? It is obvious that further work on THBA and these other compounds in the seaweed biostimulant sample requires adjustment of chromatographic conditions to obtain improved resolution, which due to time restraints will have to be addressed in future research.



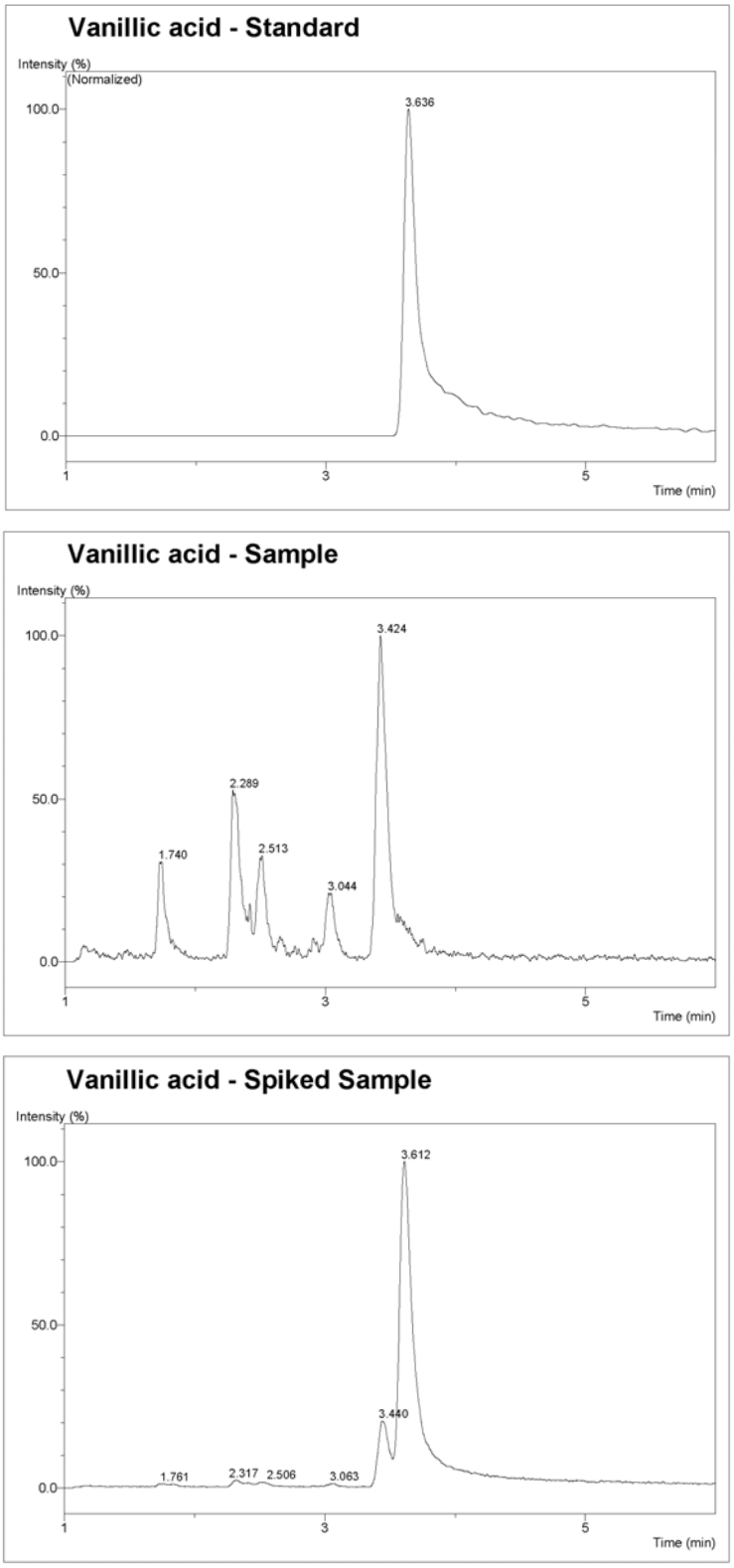
31) **Figure 3.4.2** Chromatograms of a trihydroxybenzoic acid mixed standard solution, a sample, and a spiked sample; each analysed using the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification and the 169 → 125 m/z MRM transition. Full LC-MS/MS conditions outlined in (Chapter 2.6.2.7).

3.4.3 Identification of Vanillic Acid in a Commercial Seaweed Biostimulant

The HPLC-ESI-MS/MS conditions specific to the qualitative method for the analysis of vanillic acid in a commercial seaweed biostimulant are described in Section 2.6.2.8. Figure 3.4.3 shows the three chromatograms for a standard solution of vanillic acid, a seaweed biostimulant sample, and a spiked sample.

The first chromatogram is of the vanillic acid standard solution and as can be seen the vanillic acid peak shows considerable tailing under the chromatographic conditions used. Further work would be required to improve the chromatography here but the current result was considered adequate for this qualitative investigation. The second chromatogram is of the seaweed biostimulant sample extract where several peaks are observed, however none of these peaks match the retention time of the standard vanillic acid of 3.636 minutes. The third chromatogram is of the spiked sample and shows the peak due to the vanillic acid spike (t_R = 3.612 min) elutes after the last peak in the sample (t_R = 3.440 min); and this result suggests that under the conditions used vanillic acid was unable to be detected in the seaweed biostimulant sample.

Similar to the study of DHBA and THBA, the sample chromatogram showed several other peaks indicating the presence of other compounds with similar precursor and product ions. Due to the chromatographic relationship often observed between isomers and the specificity of the MRM transition it is probable that one of these peaks, in particular the one at t_R = 3.424 min, may be isovanillic acid. Unfortunately an isovanillic acid reference standard was not available for this study.



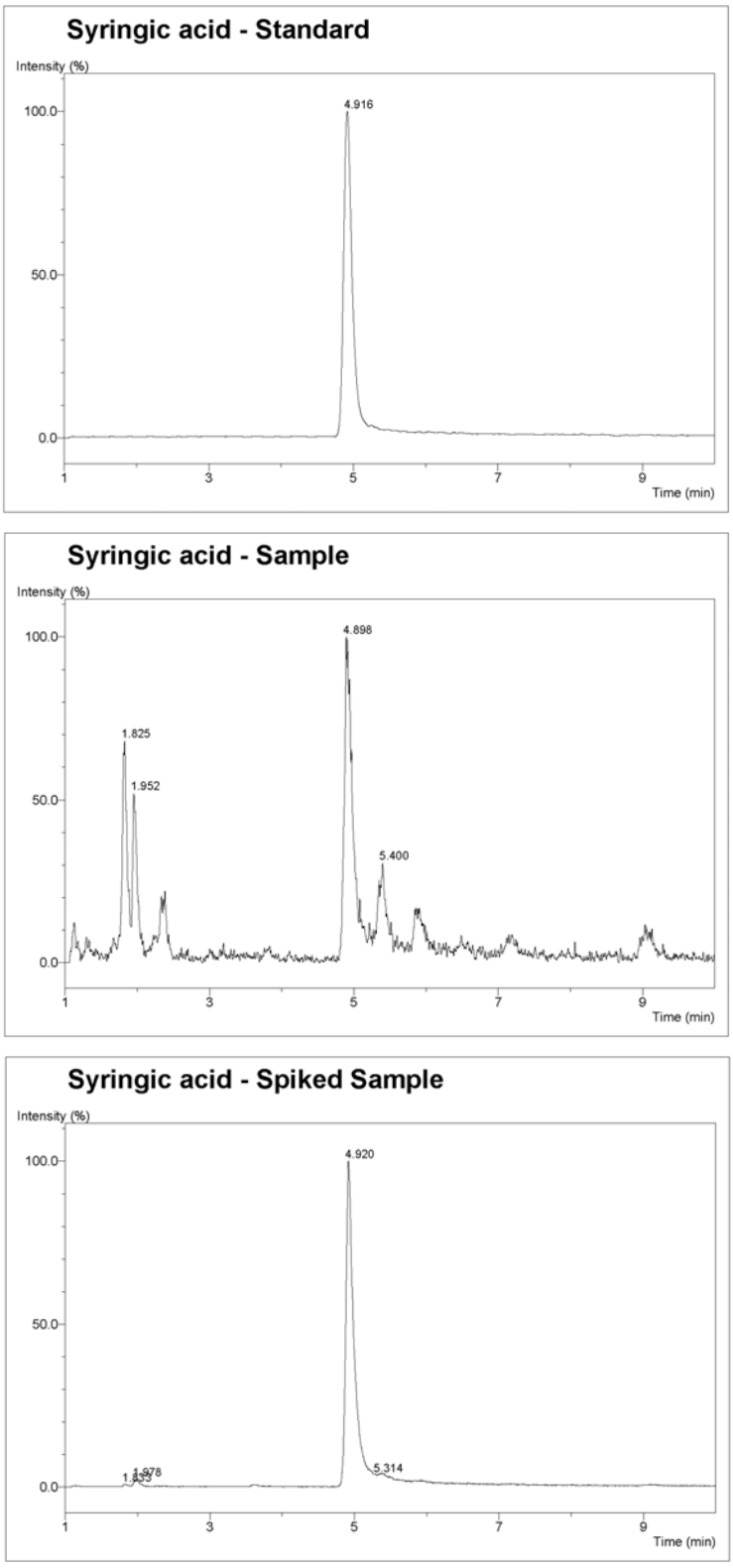
32) **Figure 3.4.3** Chromatograms of a vanillic acid standard solution, a sample, and a spiked sample; each analysed using the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification and the 167 → 152 m/z MRM transition. Full LC-MS/MS conditions outlined in (Chapter 2.6.2.8).

3.4.4 Identification of Syringic Acid in a Commercial Seaweed Biostimulant

The HPLC-ESI-MS/MS conditions specific to the qualitative method for the analysis of syringic acid in a commercial seaweed biostimulant are described in Section 2.6.2.8. Figure 3.4.4 shows the three chromatograms for a standard solution of syringic acid, a seaweed biostimulant sample, and a spiked sample.

The first chromatogram is of a syringic acid standard solution and as can be seen under the conditions used syringic acid displays good chromatography with a single symmetric and sharp peak eluting at t_R = 4.916 min. The second chromatogram is of the seaweed biostimulant sample extract which contains several peaks, most notably a peak with a retention time of 4.898 minutes which matches that of the peak for syringic acid in the chromatogram for the standard solution The third chromatogram is of the sample spiked with syringic acid and as expected the sample peak thought to be syringic acid has grown considerably in intensity and this result strongly suggests that syringic acid is present in the seaweed biostimulant sample. This is a significant finding as it is believed to be the first report of syringic acid in a commercial seaweed biostimulant (further discussed in Section 3.5).

Again a number of other peaks present in the sample chromatogram suggests the presence of other similar compounds with the same precursor and product ions as syringic acid.



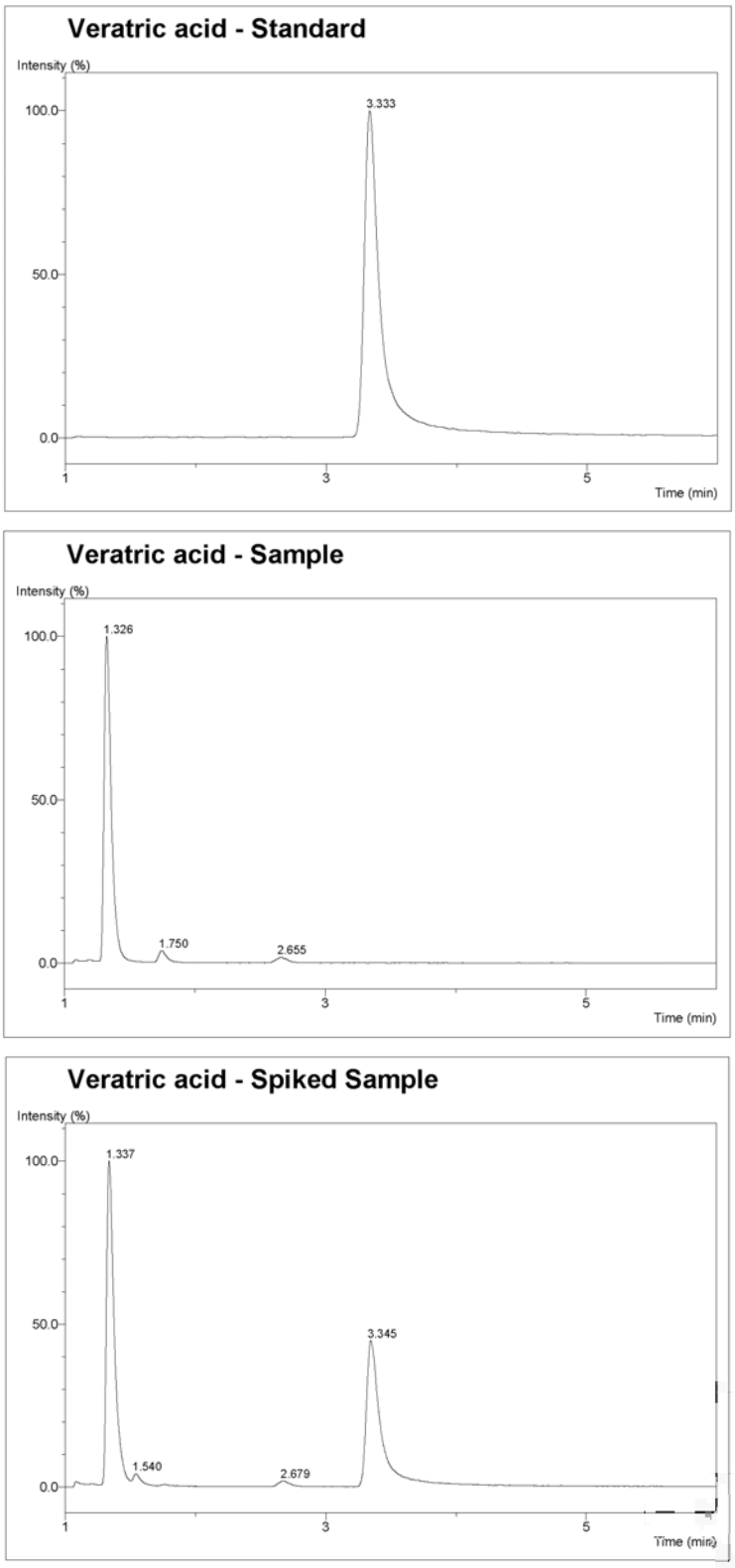
33) **Figure 3.4.4** Chromatograms of a syringic acid standard solution, a sample, and a spiked sample; each analysed using the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification and the 197 → 182 m/z MRM transition. Full LC-MS/MS conditions outlined in (Chapter 2.6.2.8).

3.4.5 Identification of Veratric Acid in a Commercial Seaweed Biostimulant

The HPLC-ESI-MS/MS conditions specific to the qualitative method for the analysis of veratric acid in a commercial seaweed biostimulant are described in Section 2.6.2.9. Figure 3.4.5 shows the three chromatograms for a standard solution of veratric acid, a seaweed biostimulant sample, and a spiked sample.

In the first chromatogram of the veratric acid standard solution, a single peak with some tailing is observed at a retention time of 3.333 minutes. The second chromatogram of the seaweed biostimulant sample extract shows three peaks are present but none matching the retention time of the veratric acid peak in the standard chromatogram. This result suggests that under the conditions used veratric acid is unable to be detected in the seaweed biostimulant sample. This result is further supported by the third chromatogram of the sample spiked with veratric acid which also shows the peak due to the veratric acid spike (t_R = 3.345 min) elutes after the last peak in the sample (t_R = 2.655 in).

Again a number of other peaks present in the sample chromatogram suggests the presence of other similar compounds with the same precursor and product ion as veratric acid.



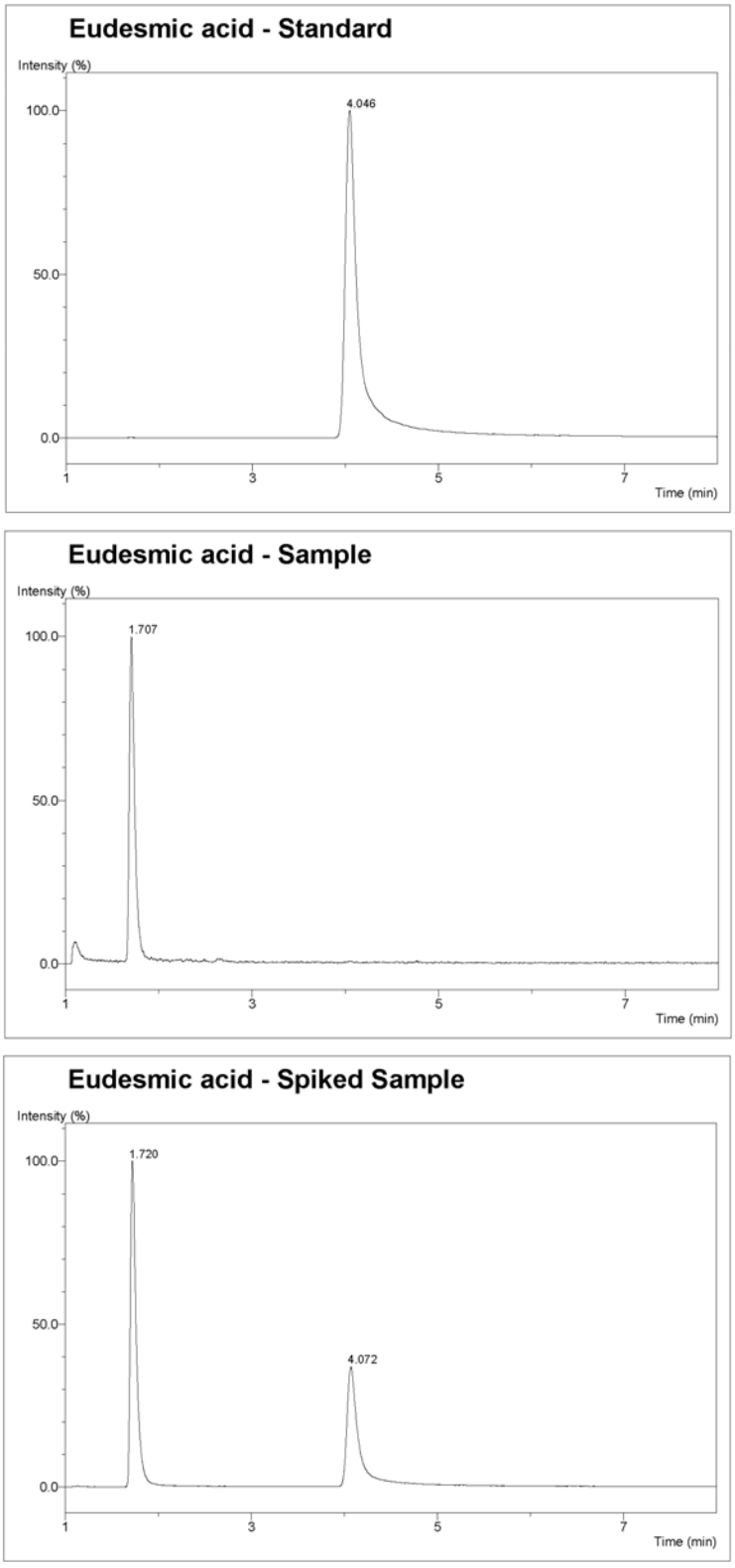
34) **Figure 3.4.5** Chromatograms of a veratric acid standard solution, a sample, and a spiked sample; each analysed using the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification and the 183 → 139 m/z MRM transition. Full LC-MS/MS conditions outlined in (Chapter 2.6.2.9).

3.4.6 Identification of Eudesmic Acid in a Commercial Seaweed Biostimulant

The HPLC-ESI-MS/MS conditions specific to the qualitative method for the analysis of eudesmic acid in a commercial seaweed biostimulant are described in Section 2.6.2.9. Figure 3.4.6 shows the three chromatograms for a standard solution of eudesmic acid, a seaweed biostimulant sample, and a spiked sample.

The first chromatogram is of a eudesmic acid standard solution, in which a single, symmetrical and sharp peak is observed with a retention time of 4.046 minutes. The second chromatogram is of the seaweed biostimulant sample extract and it also contains a single peak at a retention time of 1.707 minutes that does not match with the retention time of eudesmic acid in the standard solution. This result suggests that under the conditions used eudesmic acid is unable to be detected in the seaweed biostimulant sample. The third chromatogram is of the sample spiked with eudesmic acid and as expected shows a combination of the first and second chromatograms and further supports that eudesmic acid may not be present in the sample.

The sample peak at t_R = 1.707 min suggests the presence of a similar compound to eudesmic acid with the same precursor and product ions.



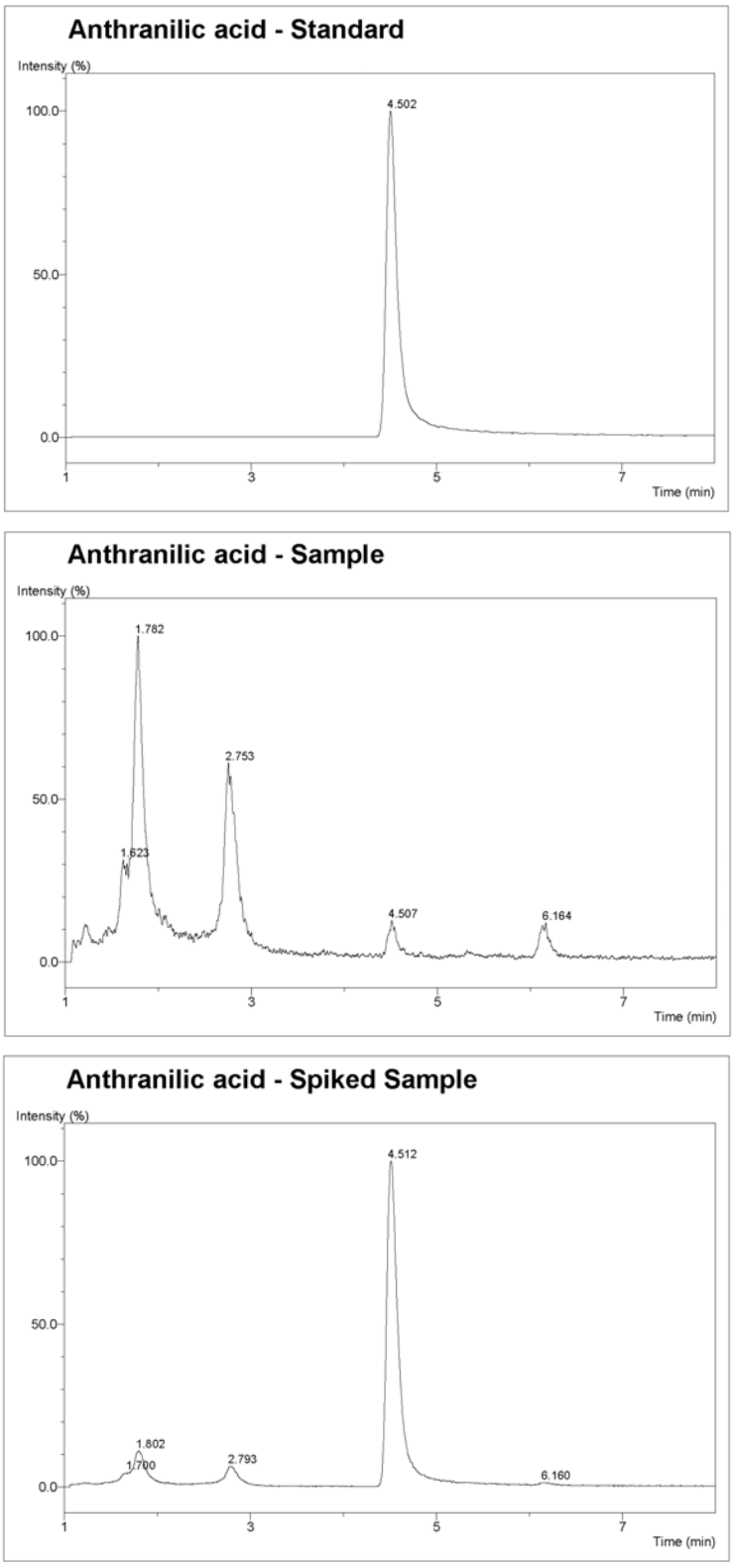
35) **Figure 3.4.6** Chromatograms of a eudesmic acid standard solution, a sample, and a spiked sample; each analysed using the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification and the 213 → 154 m/z MRM transition. Full LC-MS/MS conditions outlined in (Chapter 2.6.2.9).

3.4.7 Identification of Anthranilic Acid in a Commercial Seaweed Biostimulant

The HPLC-ESI-MS/MS conditions specific to the qualitative method for the analysis of anthranilic acid in a commercial seaweed biostimulant are described in Section 2.6.2.8. Figure 3.4.7 shows the three chromatograms for a standard solution of anthranilic acid, a seaweed biostimulant sample, and a spiked sample.

The first chromatogram is of an anthranilic acid standard solution and shows a single peak with good symmetry and minor tailing at a retention time of 4.502 minutes. The second chromatogram is of the seaweed biostimulant sample extract in which there several peaks, with the peak at t_R = 4.507 minutes matching the retention time of anthranilic acid the standard chromatogram. This suggests the presence of anthranilic acid in the seaweed biostimulant sample. The third chromatogram is of the sample spiked with anthranilic acid and the increase in intensity of the peak at t_R = 4.512 min further supports the presence of anthranilic acid in the sample. This is a significant finding as it is believed to be the first report of anthranilic acid in a commercial seaweed biostimulant (further discussed in Section 3.5).

The apparently low intensity of the anthranilic acid in the sample may not be reflective of its concentration in the sample but rather on the extraction method used given that anthranilic acid is amphoteric. Depending on the pH of aqueous solutions anthranilic acid can exist in four different microforms: cation, zwitterion, neutral species, and anion [236]. According to Zapala *et al* at a pH of 1 (the pH used for extraction of compounds from the sample, see Section 2.5.1a) anthranilic acid is mostly in its amino-protonated form skewing its distribution in the organic/aqueous partition strongly in favour of the aqueous phase. Further work on the qualitative and quantitative study of anthranilic acid in the seaweed biostimulant sample would require optimisation of the extraction method used. Again a number of other peaks present in the sample chromatogram suggests the presence of other similar compounds with the same precursor and product ions as anthranilic acid.



36) **Figure 3.4.7** Chromatograms of an anthranilic acid standard solution, a sample, and a spiked sample; each analysed using the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification and the 136 → 92 m/z MRM transition. Full LC-MS/MS conditions outlined in (Chapter 2.6.2.8).

3.5 SUMMARY OF THE FINDINGS OF THE QUALITATIVE STUDY

Of the derivative investigated in this study, only four had sufficient evidence to suggest that they are present in the seaweed biostimulant. These four compounds are 2,3-dihydroxybenzoic (Pyrocatechuic) acid, 3,4-dihydroxybenzoic (Protocatechuic) acid, 4-hydroxy-3,5-dimethoxybenzoic (Syringic) acid, and 2-aminobenzoic (Anthranilic) acid. The identification of these phenolic acids in the seaweed biostimulant is significant from two perspectives.

(i) Firstly, whether these compounds have been identified in one or both of the species from which the seaweed extract is prepared

(ii) Secondly, whether for the reported benefits of commercial seaweed biostimulants to crops can be explained by the presence of these compounds

3.5.1 Identification of Hydroxybenzoic Acids and Related Derivatives in a Seaweed Extract

The commercial seaweed biostimulant sample investigated in this study is an alkaline hydrolysis product made from two seaweed species: *Durvillaea potatorum* and *Ascophyllum nodosum* [1]. After reviewing the literature for both *D.potatorum* and *A.nodosum*, no records of the detection or identification of pyrocatechuic acid, protocatechuic acid, syringic acid and anthranilic acid were found for *D.potatorum*. However, protocatechuic acid and syringic acid have been detected in extracts of *A.nodosum* [125]. When the literature search was extended to include other species of seaweeds and algae these derivatives were identified in various species. Two review articles authored by Fernando *et al.* and Sudhakar *et al.* both feature a comprehensive list of phenolic acids identified in various algal species [237, 238]. Included in those lists of phenolic acids are three of the four derivatives identified in this study: pyrocatechuic acid, protocatechuic acid, and syringic acid. Of these three derivatives, pyrocatechuic acid shows up in only a single study. This study, conducted by Onofrejová *et al.*, identified and quantified pyrocatechuic acid in the two freshwater algae species *Spongiochloris spongiosa* and *Anabaena doliolum*, as well as two food products made from the marine algal species *Porphyra tenera* and *Undaria pinnatifida* [156]. Multiple studies conducted by the same research team of Klejdus *et al.,* have also identified protocatechuic acid and syringic acid in various algal species [92, 151, 156, 168]. Further studies that have identified protocatechuic acid and/or syringic acid in algae samples include: isolation of protocatechuic acid and syringic acid amongst other phenolic acids in the freshwater algae *Cladophora glomerata* by Korzeniowska *et al.*, screening of protocatechuic acid and syringic acid amongst other phenolic acids in seaweeds from the Danish coast by Sabeena Farvin *et al.*, identification of syringic acid and other phenolic acids in two brown seaweed species by Chakraborty *et al.*, and the extraction of protocatechuic acid, syringic acid and other phenolic acids from the brown seaweed *Saccharina japonica* [124, 131, 136, 239]. The fourth derivative

identified in this study is a benzoic acid derivative but is not considered a phenolic acid, that is anthranilic acid (2-aminobenzoic acid). There were no studies that had identified anthranilic acid in either of the two species of seaweed the extract in this study is prepared from and furthermore, no literature could be found that investigated anthranilic acid in seaweeds or other algae. Instead, much of the literature surrounding anthranilic acid in marine life focuses on marine fungi, and some marine bacteria [240]. While this study points to the presence of these compounds in the seaweed biostimulant sample, it does not provide any indication of whether they are endogenously present in the seaweed species from which the biostimulant is prepared, or if they are degradation products. The manufacturing processes for this commercial biostimulant involve alkaline hydrolysis at elevated temperature and pressure, which has the potential to generate a large number of degradation products [241]. Therefore, further work investigating the raw seaweed samples would serve to supplement this work and investigate the phenolic acid profile within the seaweeds; for which the methodology developed throughout this study would provide an excellent foundation. Developing methods to investigate the phenolic acid profile of the commercial seaweed biostimulant helps to elucidate its complex composition. Furthermore, the methods developed in this study have shown other compounds are present that share similar chromatographic behaviour with the specificity of the mass spectrometric detection, suggesting the possibility of discovering new metabolites through untargeted analysis and structural elucidation using MS/MS or even MS^n.

3.5.2 Identification of Hydroxybenzoic Acids and Related Derivatives in a Commercial Seaweed Biostimulant

The literature on seaweed biostimulants strongly suggests that it's the phytochemicals that are responsible for their benefits to crops which include improved plant growth and development as well as increased resistance to stressors [1, 23]. Therefore, elucidation of the phytochemical composition of the commercial seaweed biostimulant can help to better understand and characterise the complex modes of action underpinning the observed benefits of the application of biostimulants. The identification of specific phytochemicals allows for comparison of the observed benefits of biostimulants with the endogenous and exogenous bioactivity of phytochemicals previously characterised, and paves the way for new experiments to investigate the impacts of their application to plants/crops. Many phytochemicals have had their endogenous biosynthesis and bioactivity characterised, with some of the more important phytochemicals having also had their exogenous application investigated. Here a short summary of important characteristics of the four identified derivatives will be discussed.

Pyrocatechuic acid or 2,3-dihydroxybenzoic acid occurs in many plant species, as well as algae, bacteria and fungi [242]. Pyrocatechuic acid like most phenolic acids, is an antioxidant and radical scavenger [242]. It is also a known metal-ion chelator, where in microorganisms pyrocatechuic acid along with salicylic acid serve as precursors for siderophores; the uptake of Fe^{3+} being essential for the survival of microorganisms, and also the virulence in some bacteria such as *Escherichia coli* [53-55]. The role of pyrocatechuic acid in plants is not fully characterised, initially thought as a product of metabolic inactivation of salicylic acid by the additional hydroxylation of the aromatic ring [53]. However, studies have found that pyrocatechuic acid is involved in plant defence; for example the study investigating the accumulation of pyrocatechuic acid following elicitation of *Catharanthus roseus* cell cultures with an extract from the common soil borne plant pathogen *Pythium aphanidermatum* [53]. Furthermore, the glycosylation of pyrocatechuic acid has been suggested to play an important role in regulating the activity of important signal molecules such as salicylic acid, impacting the plants immune response to biotic stress [243].

Protocatechuic acid or 3,4-dihydroxybenzoic acid occurs widely throughout the plant kingdom and is also found in algae, bacteria and fungi [242, 244]. It has several bioactivities including antioxidant, free radical scavenger, antibacterial, antifungal, and metal-ion chelator; similarly to pyrocatechuic acid, serving as the binding ligand of an iron chelating siderophore in some bacteria [54, 244]. Protocatechuic acid has been investigated for various pharmacological activities such as anticancer, antiulcer, antidiabetic, antiaging, analgesic, and neuroprotective activities [242, 244]. In plants though, protocatechuic acid is best known as an allelochemical; exhibiting beneficial and detrimental allelopathy depending on the concentration [245, 246]. One study that demonstrates the allelopathic potential of protocatechuic acid was conducted by Mucciarelli *et al.* on tobacco cell and tissue cultures [185]. They tested three concentrations orders of magnitude apart and found the highest concentration strongly inhibited growth, while the lowest concentration showed stimulatory activity [185]. Another in vitro study, on *Protea cynaroides*, found that exogenous application of protocatechuic acid significantly increased the root mass at one concentration, then showed inhibitory effects at higher concentrations [196]. Furthermore, propagation cuttings showed a considerable increase in protocatechuic acid from initial planting to root formation, indicating that it may play an important role in root formation in *P.cynaroides* [196].

Syringic acid or 4-hydroxy-3,5-dimethoxybenzoic is widely abundant in plants, fungi and algae [242, 247]. Syringic acid has antioxidant, antimicrobial and antifungal properties; furthermore it has been investigated for pharmacological properties such as anti-inflammation, anticancer, antidiabetic, and protective properties for the heart, liver and brain [247]. It is a key component in lignin (plant cell wall component) key structural material in plants critical to the rigidity of bark and wood [247]. Syringic acid is considered to be an allelochemical, being found in the leachates of various *Eucalyptus* species;

furthermore, bioassay results found it significantly inhibited the germination of black lentils (*Phaseolus mungo L.*) [246]. It has been found to have significant fungitoxicity; with a study investigating the Oil palm basal stem rot caused by the *Ganoderma boninense* fungus finding syringic acid as a key compound found in oil palm roots [248]. Chong *et al.* tested syringic acid *in vitro* and found that even at the lowest concentration tested it was very fungitoxic to *G.boninense*, inhibiting the pathogen [248]. Its antimicrobial and antifungal properties have led to syringic acid being investigated for interactions with rhizosphere microbial communities [194]. The responses of cucumber rhizosphere bacterial and fungal communities to treatment with syringic acid were analysed by Wang *et al.*; they found that syringic acid changed the cucumber rhizosphere microbial communities, potentially exerting detrimental effects on the cucumber seedling through inhibiting plant-beneficial microbes [194].

Anthranilic acid or 2-aminobenzoic acid is a compound that is important for the physiology of plants, bacteria, fungi and algae [228, 236]. Whilst anthranilic acid is not a phenolic acid, it is sometimes considered a derivative due to the C_6-C_1 benzoic acid backbone and that it is a product of the shikimate pathway [249]. Most importantly, anthranilic acid is the precursor to many different alkaloids, a different group of phytochemicals involved in biological processes of plants, animals and microorganisms [249, 250]. A Particularly important group of alkaloids that anthranilic acid is the precursor for is auxins, which play a pivotal role in the growth and development of plants; most notably indole-3-acetic acid (IAA) [251]. Anthranilic acid is not only the precursor to the main plant auxin IAA, it has also been shown to exhibit auxin-like properties; moreover, it is suspected to have a distinct role in regulating root elongation separate from its conversion to IAA [251, 252]. Lastly, anthranilic acid plays a role in plant defence; anthranilic acid was found to be a key determinant identified in *Bacillus* bacterium secretions that exhibit induced systemic resistance activity against the soft-rot disease in tobacco [253].

Each of the four derivatives identified in the commercial seaweed biostimulant have been investigated for their roles in plant growth and development, and resistance to stressors. The identification of these compounds within the biostimulant now provides the opportunity to develop experiments to test if any of these derivative are involved in the benefits observed from the biostimulants application. It is worth noting that without quantitative data it is difficult to construct experiments that could compare to the commercial seaweed biostimulant directly; however, these qualitative methods provide an ideal foundation for the development of quantitative methods for these derivatives.

3.6 CONCLUSION

The primary aim of this part of the study was to develop qualitative HPLC-ESI-MS/MS methods for the identification of hydroxybenzoic acids and related derivatives in a commercial seaweed biostimulant. This was successfully achieved whereby 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid and 2-aminobenzoic acid were all identified in a commercial seaweed biostimulant using the various methods developed.

The secondary aim of this part of the study was the investigation into the chromatographic behaviour of the hydroxybenzoic acids and related derivatives on the less explored reversed phase phenyl based stationary phases when compared to the more common C18 phases. This was successfully achieved as the results demonstrated the applicability of the biphenyl stationary phase for the separation of these aromatic compounds. This was the first report of the separation of the monohydroxybenzoic acids, the dihydroxybenzoic acids and the trihydroxybenzoic acids using a biphenyl stationary phase.

This chapter's key observations, significant findings and future research prospects are all further discussed in Chapter 6.

CHAPTER 4: METHOD OPTIMISATION AND VALIDATION FOR THE TARGETED METABOLOMIC HPLC-ESI-MS/MS QUANTITATIVE ANALYSIS OF MONOHYDROXYBENZOIC ACIDS IN A COMMERCIAL SEAWEED BIOSTIMULANT

4.1 INTRODUCTION

The three monohydroxybenzoic acids: 2-hydroxybenzoic acid (salicylic acid), 3-hydroxybenzoic acid, and 4-hydroxybenzoic acid are all important phytochemicals that have been reported in various plant, fungal and algal samples [17, 47, 67, 69]. Each of the three isomers differs only in the position of the hydroxyl group, and this difference results in variability in their biological activities that has been reported in scientific literature [65, 68, 70]. Salicylic acid is an important phytohormone that has be shown to be a safe and efficacious plants protector and growth regulator [173]. The improved plant growth and immunity reported for the exogenous application of salicylic acid bear comparison to the benefits observed from the application of some seaweed biostimulants (see Section 1.7) [1, 57]. The presence of salicylic acid and its isomers in a commercial seaweed biostimulant was confirmed in the qualitative investigation conducted in Chapter 3. In order to assess whether salicylic acid and its isomers play a role in the benefits observed from the application of seaweed biostimulants, quantitative data is required to develop proof-of-concept bioassays. The HPLC-ESI-MS/MS method developed and optimised in Chapter 3 for the qualitative investigation of the monohydroxybenzoic acids in a seaweed biostimulant is a fit-for-purpose method that could be used for quantification following partial validation to ensure statistical confidence for the quantitative data generated. The aim of this study was to successfully validate then apply a quantitative HPLC-ESI-MS/MS method for the analysis of salicylic acid and its isomers in a commercial seaweed biostimulant. Any quantitative method needs to take into account the effects of the sample matrix on instrumental analysis and therefore this study begins with further sample purification and extraction (Section 4.2) followed by the partial validation of the fit-for-purpose quantitative method (Section 4.3), then its application to the commercial seaweed biostimulant sample (Section 4.4).

4.1.1 Chapter Outline

The results in this chapter are divided into the following sections.

- Optimisation of Sample Preparatory Procedures (Section 4.2) - This section describes the optimisation of sample preparation procedures.
- Partial Validation of New Quantitative Method (Section 4.3) - This section describes the partial validation of the newly developed quantitative method following the National Association of Testing Authority (NATA) protocol.
- Simultaneous Quantitative Analysis of 2-, 3- and 4-Hydroxybenzoic Acid in a Commercial Seaweed Biostimulant using HPLC-ESI-MS/MS (Section 4.4) - This section describes the application of the newly developed and partially validated method for the quantitative analysis of monohydroxybenzoic acids in a commercial seaweed biostimulant.

4.2 OPTIMISATION OF SAMPLE PREPARATORY PROCEDURES

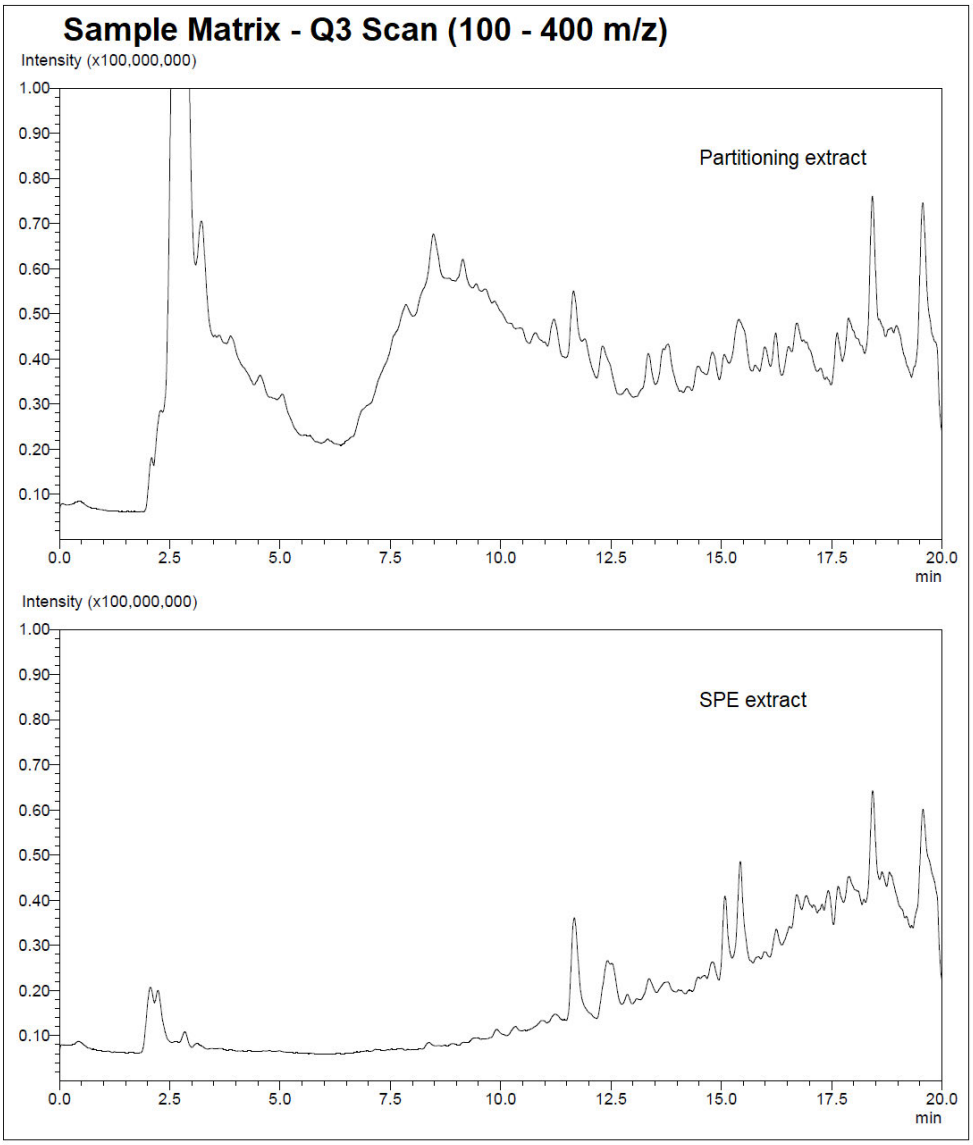
Previous work in these laboratories (Collins, 2016) used a sample preparatory procedure that employed partitioning of the seaweed biostimulant sample with acetonitrile and SPE to selectively target and preconcentrate salicylic acid in the biostimulant sample. This section will highlight how the previous sample preparation procedure was further optimised for the repeatable and robust quantitative investigation of all three hydroxybenzoic acids in the seaweed biostimulant sample. This section first highlights the complexity of the sample matrix, then outlines and discusses the optimisation of the two major components (partitioning and SPE) of the sample preparatory procedure. For this sample preparatory investigation the same analytical methods employed for the preliminary qualitative investigation of monohydroxybenzoic acids were used (see Section 3.2). This method employed an Agilent Polaris 3 μm C18-A 150 × 2.0 mm with an Agilent Polaris C18-A MetaGuard 10 × 2.0 mm column was used along with aqueous acetonitrile mobile phases modified with formic acid. Gradient elution chromatography was used and mobile phase conditions along with other instrumental conditions are listed in figure legends.

4.2.1 Sample Matrix Complexity.

The seaweed biostimulant sample is aqueous with a naturally high salt content as described in Chapter 2 and the high salt content allows partitioning with water-soluble organic solvents, such as acetonitrile. This is advantageous in that the subsequent acetonitrile extracts are amenable with LCMS reversed-phase stationary phases and mobile phases. The biostimulant sample is quite complex and for qualitative purposes partitioning into acetonitrile is a simple means of preparing the sample for instrumental analysis that is adequate for identification work using retention time matching and mass spectral structure elucidation, as demonstrated in Chapter 3. The sample matrix however is still quite complex and consequently, this places greater demands on the chromatographic/mass spectrometric system to separate sample matrix interferences. The extra complexity of the matrix means more complex chromatography is required, such as the use of gradient elution. Furthermore, the increased number of interfering compounds means a high likelihood of ionisation suppression which is colloquially referred to as the 'Achilles Heel' of HPLC-ESI-MS/MS, along with more frequent instrument maintenance being required due to fouling [109].

Figure 4.2.1 shows the full scan chromatograms (mass range m/z 100 – 400) of seaweed biostimulant sample at two stages of the sample preparation: the acetonitrile partitioning extract prior to SPE and following SPE (SPE conditions as per [207]). The chromatograms are set to the same scale for comparison

and as can be seen the SPE extract shows considerably less background, particularly in the early- to mid-range of the chromatogram which is where the monohydroxybenzoic acids elute. This reduction in background is why a sample preparatory procedure that employs SPE was chosen for the development of a quantitative method for the analysis of monohydroxybenzoic acids in the commercial biostimulant sample.



37) **Figure 4.2.1** Chromatograms showing the difference between a partitioning extract and an SPE extract of a sample. The chromatograms are in Q3 Scan analysis mode with a mass range of m/z 100 - 400. The mobile phase consisted of: Solvent A - water modified with 0.1 % (v/v) formic acid, Solvent B - acetonitrile. The gradient LC time program used was: 10 % (v/v) B from 0 – 2.5 min, increase from 10 % to 90 % (v/v) B from 2.5 – 15 min, increase from 40 % to 90 % (v/v) B, hold at 90 % (v/v) B from 15 – 17 min.

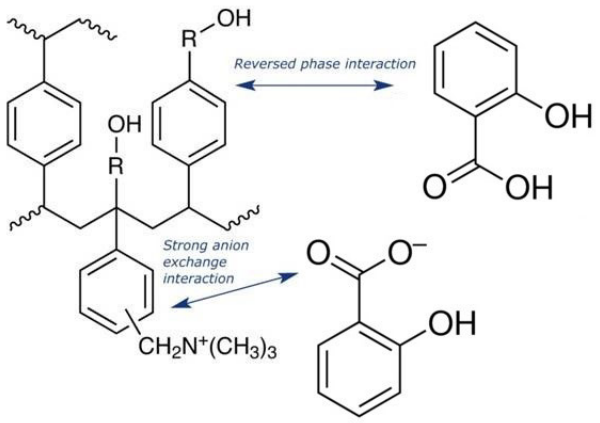
4.2.2 Partitioning Using Quechers Methodology.

In order to ensure the monohydroxybenzoic acids are in the correct form to partition into the acetonitrile layer, the sample's pH needs to be dropped from approximately 11 down to 1. The acid dissociation constants (pKa) of 2-hydroxy, 3-hydroxy- and 4-hydroxybenzoic acids are 2.98, 4.06 and 4.38 respectively, and at a pH of 1 (greater than 2 pH units below the pKa of all three hydroxybenzoic acids) all three will be un-ionised and transfer into the acetonitrile layer. The pH is dropped using concentrated HCl, then acetonitrile is added to the sample, and it is shaken vigorously and centrifuged. This results in two distinct layers with the aqueous seaweed biostimulant sample layer on the bottom and an acetonitrile layer on top. The previous method that served as the starting point for the development of this method centrifuged the sample only once, from which the full volume of acetonitrile was not recovered. It was found that the variance in the acetonitrile layer volume post-centrifugation meant that this procedure was not repeatable nor quantitative. Initially it was thought that further centrifugation would improve the recovery of the acetonitrile layer; however it was found that even after centrifuging another 10 times no further improvement in acetonitrile recovery was observed. As previously mentioned this sample is amenable to acetonitrile partitioning due to its naturally high salt content and it was thought that increasing the salt content would aid in the recovery of the acetonitrile layer. This approach is commonly used in Quechers methodologies, that also employed acetonitrile partitioning with the addition of various salts to the sample [94]. The first step in Quechers methodologies is partitioning with acetonitrile, this requires the addition of magnesium sulfate ($MgSO_4$) which provides the most complete liquid-liquid phase separation [94]. Typically the Quechers method uses a 1:1 ratio of sample to acetonitrile, as well as 2:1 ratio of sample to salt [94]. To test the applicability of $MgSO_4$ for improving the recovery of the acetonitrile layer, varying amounts of $MgSO_4$.7H2O were added to 5 mL of sample. After repeating the extraction process with varying amounts of $MgSO_4$.7H2O it was found that partitioning with the addition of 3 g of MgSO4.7H2O provided full recovery of the 5 mL acetonitrile, making the partitioning step suitable for quantitative analysis. The final partitioning methodology employed for this study is believed to be the first report of the use of Quechers methodologies for sample extraction of a commercial seaweed biostimulant and its protocol is described in Section 2.5.1.

4.2.3 Solid-Phase Extraction of Monohydroxybenzoic Acids.

Mixed-mode SPE utilising sorbents developed for acidic compounds was deemed ideally suited for the present study in order to produce targeted sample clean-up of the complex acetonitrile seaweed biostimulant extracts. The SPE protocol developed for this study used a sorbent comprising a quaternary amine modified polystyrene-divinyl benzene polymer incorporating non-ionisable hydroxyl

groups [254]. As described in the sorbent technical information the sorbet allows for non-polar interactions between sorbent and analyte aromatic groups and anion exchange interactions between the cationic quaternary amino group of the sorbent and anionic carboxylate group of the hydroxybenzoic acids, as seen in Figure 4.2.3 [254].



38) **Figure 4.2.3** Interaction of salicylic acid and the salicylate ion with the mixed-mode sorbent. Image adapted from Biotage SPE technical information [254].

The mixed-mode SPE procedure takes advantage of the acid-base chemistry of the hydroxybenzoic acids and allows weakly retained non-polar and polar analytes to be washed away. The procedure initially involves retention of analytes via the non-polar mechanisms followed by 'flipping' to allow retention of analytes via anion exchange. The 'flipping' is achieved using various wash solutions beginning with acidic and finishing with slightly alkaline. This unique approach to mixed-mode SPE of 'flipping' the target analytes from one retention mechanism to the other provide extra opportunity to clean the sample using various washes. Additionally, this offers extra selectivity for the target analytes and is predicted to help reduce matrix effects such as ionisation suppression.

In developing the SPE protocol three key considerations were;

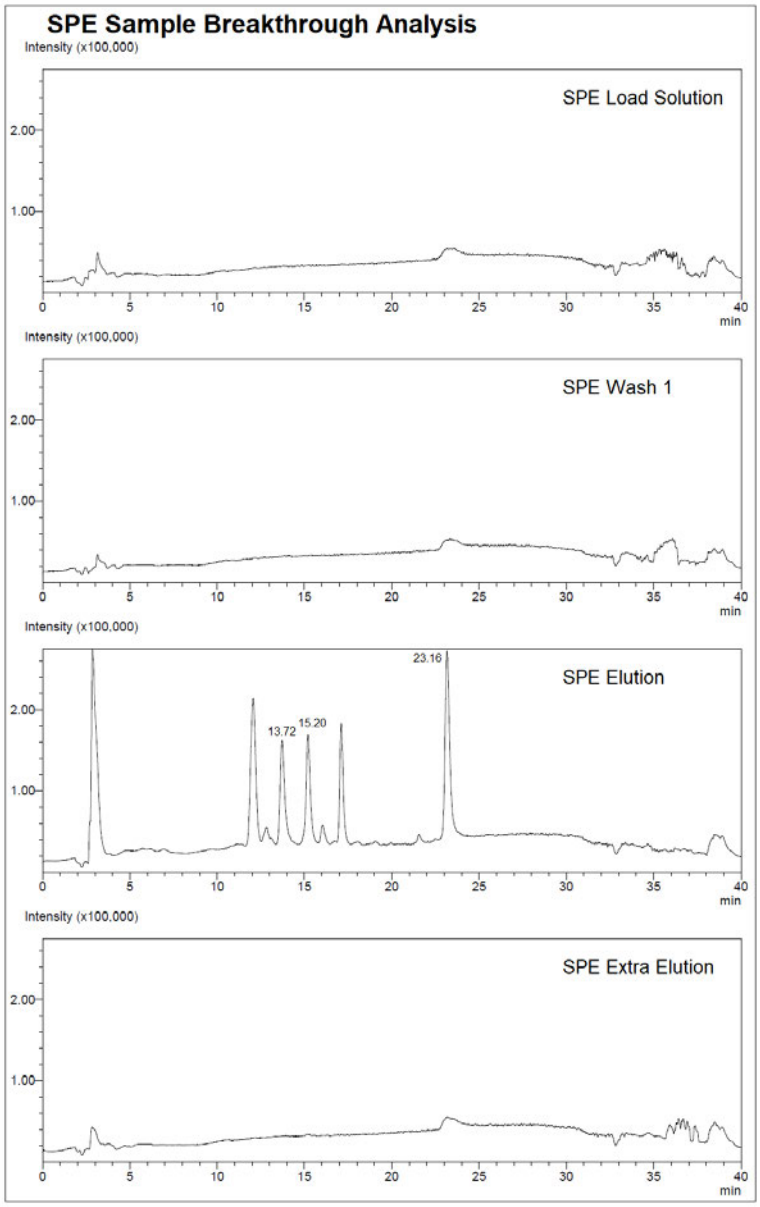
- filtering of the acetonitrile partitioning extract
- non-selective elution or breakthrough of the target analytes during sample loading or washing, as well as ensuring selective and complete elution of the target analytes when desired
- final elution of target analytes, evaporation and reconstitution in a solvent compatible with the LCMS conditions

Beginning with the filtering step, which was done prior to loading the sample onto the SPE cartridge. The choice of syringe filter was polytetrafluoroethylene due to its use across a wide pH range. The PTFE syringe filters require pre-wetting before use with aqueous samples, a step that was integrated into the SPE methodology. The same solvent could be used to pre-wet the PTFE syringe filter and condition the SPE column, therefore the filter was placed inline before the SPE cartridge and the conditioning and equilibrating steps were performed for both the filter and the SPE cartridge.

Following the conditioning and equilibration of the SPE cartridge, the next step is to load the sample. The sample loading solution is one common source of breakthrough: if the loading solution has a high solvent strength then the sample may not selectively retain and some of the target analytes may breakthrough at this step. Careful consideration must be made when preparing the load solution for SPE and even still it is good practice to collect the eluate when loading the sample onto the SPE cartridge for analysis of breakthrough. To reduce the chance of breakthrough upon loading, the sample (acetonitrile partition aliquot) is diluted in water with 0.1 % (v/v) formic acid, as this was believed to keep the solvent strength low enough to avoid breakthrough of the monohydroxybenzoic acids. That same solvent was also used as the first wash solution to elute polar interfering molecules but continue to retain the monohydroxybenzoic acids via the non-polar retention mechanisms. In order to assess any possible breakthrough the eluate from the SPE cartridge during the loading and first wash were collected and analysed for traces of the monohydroxybenzoic acids (as shown below in Figure 4.2.3). The chromatograms in Figure 4.2.3 show that there were no traces of the monohydroxybenzoic acids in the load or first wash eluates when comparing to the SPE elution chromatogram (peaks with t_R of 13.72, 15.20, and 23.16 minutes identified through retention time matching). This indicates that there was no breakthrough upon loading the sample onto the SPE cartridge, and furthermore that the choice of solvent for sample dilution and first wash successfully allowed for the selective retention of the monohydroxybenzoic acids. The same type of analysis was performed on all wash solutions to ensure they are not eluting the monohydroxybenzoic acids non-selectively. Finally, after the final elution of monohydroxybenzoic acids an extra elution was performed to confirm complete recovery of the monohydroxybenzoic acids, and as seen in Figure 4.2.3 it was evident that all of the monohydroxybenzoic acids were selectively eluted in the elution step as required.

Acetonitrile with 1% (v/v) formic acid was chosen as the final elution solvent, which selectively eluted the monohydroxybenzoic acids as shown in Figure 4.2.3 and was also volatile enough to provide the opportunity for evaporation and reconstitution. This had two benefits: evaporating and reconstituting in a smaller volume effectively concentrating the monohydroxybenzoic acids in the SPE elution, and reconstituting in a solution that matches the mobile phase conditions in the LC minimising solvent mixing effects.

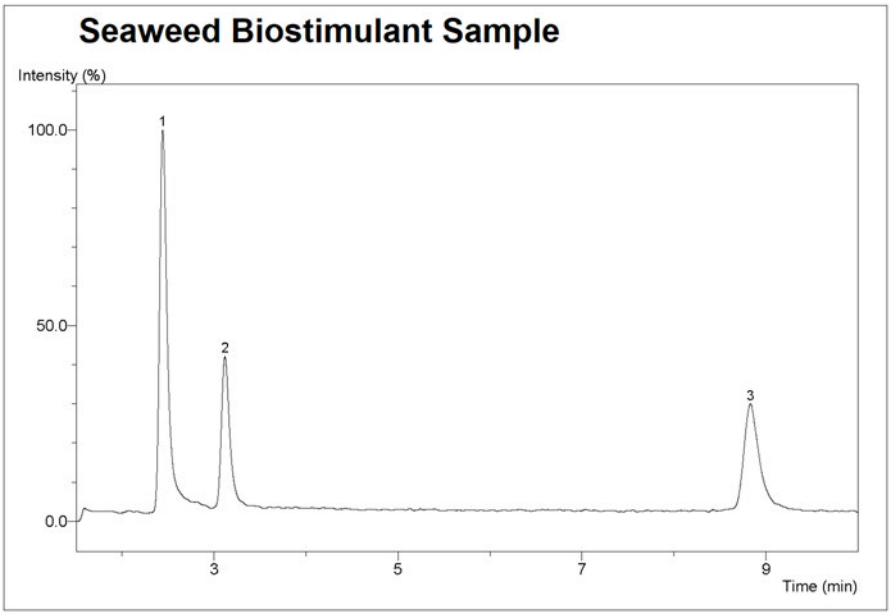
The culmination of these investigations resulted in a SPE protocol that allowed for the highly selective extraction of the monohydroxybenzoic acids from the seaweed biostimulant sample. This protocol also significantly reduced the background of the complex biostimulant matrix and this would aid in reducing instrumental matrix effects. The full protocol is described in Section 2.5.3.



39) **Figure 4.2.3** Chromatograms investigating the presence of the monohydroxybenzoic acids in various SPE solutions to determine any breakthrough and ensure complete selective elution. The chromatograms are in SIM analysis mode at 137 m/z. The mobile phase consisted of: Solvent A - water modified with 0.1 % (v/v) formic acid, Solvent B - acetonitrile. The gradient LC time program used was: 5 % (v/v) B from 0 – 5 min, increase from 5 % to 50 % (v/v) B from 5 – 30 min, and increase from 50 % to 90 % (v/v) B from 30 – 35 min.

4.3 PARTIAL VALIDATION OF THE NEW METHOD

In this section a quantitative method employing the sample preparatory procedure developed in Section 4.2 and the HPLC-ESI-MS/MS method developed in Section 3.3 was partially validated for the determination of monohydroxybenzoic acids in a commercial seaweed biostimulant. This HPLC-ESI-MS/MS method (described in Section 2.6.2.5) successfully separates 2-, 3-, and 4-hydroxybenzoic acid on the Raptor Biphenyl column with enough chromatographic resolution in order to positively identify these monohydroxybenzoic acids in complex samples. Furthermore it also has preventative measures built into the LC time program that aim to mitigate contamination of the instrument and degradation of the LC column by addressing any poorly retained or strongly retained compounds. An example chromatogram of a sample analysed using this method is shown in Figure 4.3.1. The partial validation involves an evaluation of some significant quantitative analysis parameters to ensure a high level of confidence in the final quantitative results. The method validation was conducted in accordance with the National Association of Testing Authorities (NATA) publication "Validation and verification of quantitative and qualitative test methods".



40) **Figure 4.3.1** Chromatograms showing the separation of the three monohydroxybenzoic acids in the seaweed biostimulant sample prepared using the methodology described in Section 2.5.5. Chromatographed on the Raptor Biphenyl column with a methanol/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.5).

4.3.2 Evaluation of Method Validation Parameters.

The validation and verification of an analytical method is most often performed for the purposes of accreditation to International Standards; however, technical notes such as the NATA technical note for validation and verification offer guidance for providing objective evidence on the statistical confidence of data generated by a newly developed analytical method. The extent of validation required depends on many factors; nevertheless, the intended application is the key consideration when deciding which aspects of the method will be investigated and evaluated. For the purposes of this research project the partial method validation aims to provide confidence in the quantitative analysis that will be performed in-house and isn't seeking official standardisation or accreditation. Therefore, the rigour of method validation testing required is only a portion of what is outlined in the technical note and includes the following parameters: linearity, instrument repeatability, method repeatability, instrument limit-of-detection (IDL), instrument limit-of-quantitation (IQL), matrix effects and method recovery. The evaluation of each validation parameter was performed for each of the three monohydroxybenzoic acids separately because each compound may differ in their instrument response and matrix effects.

4.3.2.1 Linearity.

Linearity is considered to be the most important performance parameter of an instrumental analytical method; furthermore, when performing an analysis using HPLC-ESI-MS/MS a linear response is assumed in theory [255]. The linearity study aims to evaluate the ability for the instrument to elicit a response that is directly proportional to the concentration of the target analyte [107]. Determination of linearity for instrumental analyses is often performed using a calibration range of standards that: includes a blank, are evenly spaced over the range, encompass 0-150 % of the expected concentration to be encountered in the sample, and are run in at least duplicate or preferably triplicate [107]. A plot of the data is constructed with the instrument response on the y-axis (ordinate) and analyte concentration on the x-axis (abscissa). Visual inspection of the plot will give an initial indication of the linearity but for statistical validity further graphical and numerical approaches are required. A least squares regression method to establish the relationship between the instrument response and analyte concentration is the most commonly implemented method that results in a linear model of algebraic form $y = a + bx$, where a is the y-intercept and b is the slope of the regression line. From the equation for the linear model the most common tool to assess linearity is the correlation coefficient (r) and moreover, the coefficient of determination (R^2); which are derived from the regression analysis. In more recent years these have been found to be misleading and many scientists are recommending not relying on these parameters alone [255]. Instead, the r and R^2 values are used alongside other tools such as the residuals examination, standard error of the regression, and response factor plots. A residuals

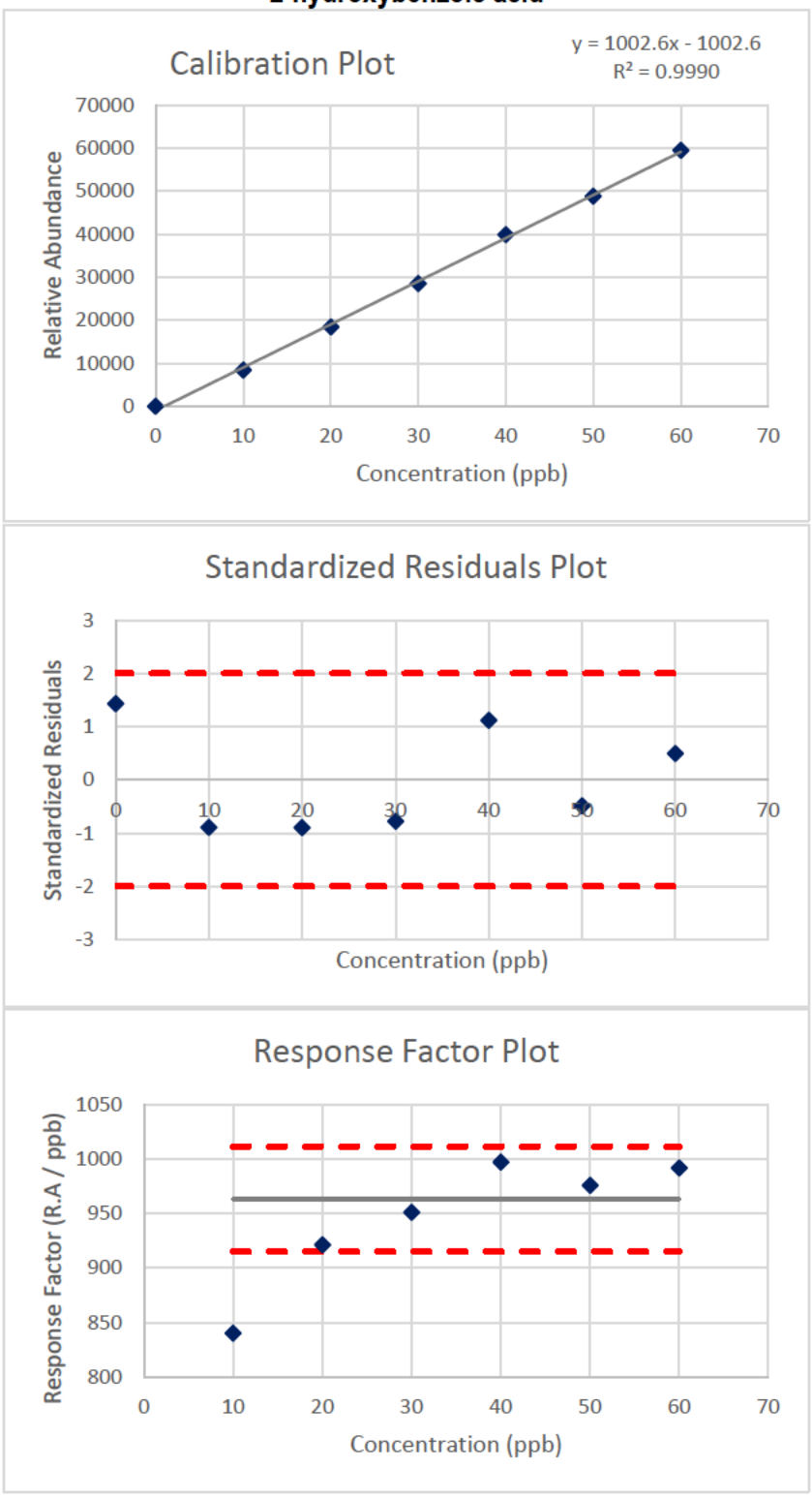
plot of the standardized residuals that is obtained using the standard error of the regression and residual leverage scores gives a standardized plot that can be used to assess goodness-of-fit, homoscedasticity, influence points and outliers [255]. The final plot used to assess linearity that is common in chromatographic method development is a response factor plot. The response factor plot is obtained by plotting the signal-to-concentration ratio (response factor) against the analyte concentration; from which the median response factor and the 5 % confidence lines are traced, and any deviations outside the confidence limits can be evaluated.

The linearity study was conducted using 1 blank and 6 mixed standard solutions that varied in concentration to make a suitable calibration range. The preparation of these solutions is described in Section 2.4.2.2. The blank and each of the mixed standard solutions were injected in triplicate and the tabulated calibration data is shown in Appendix B.1. Calibration data is shown in Table 4.3.2.1 and the calibration plots are shown in Figures 4.3.2.1a – 4.3.2.1c. Also included alongside the calibration plots is the standardized residuals plot and response factor plot for each monohydroxybenzoic acid, data for which is available in Appendices B.2 and B.3, respectively.

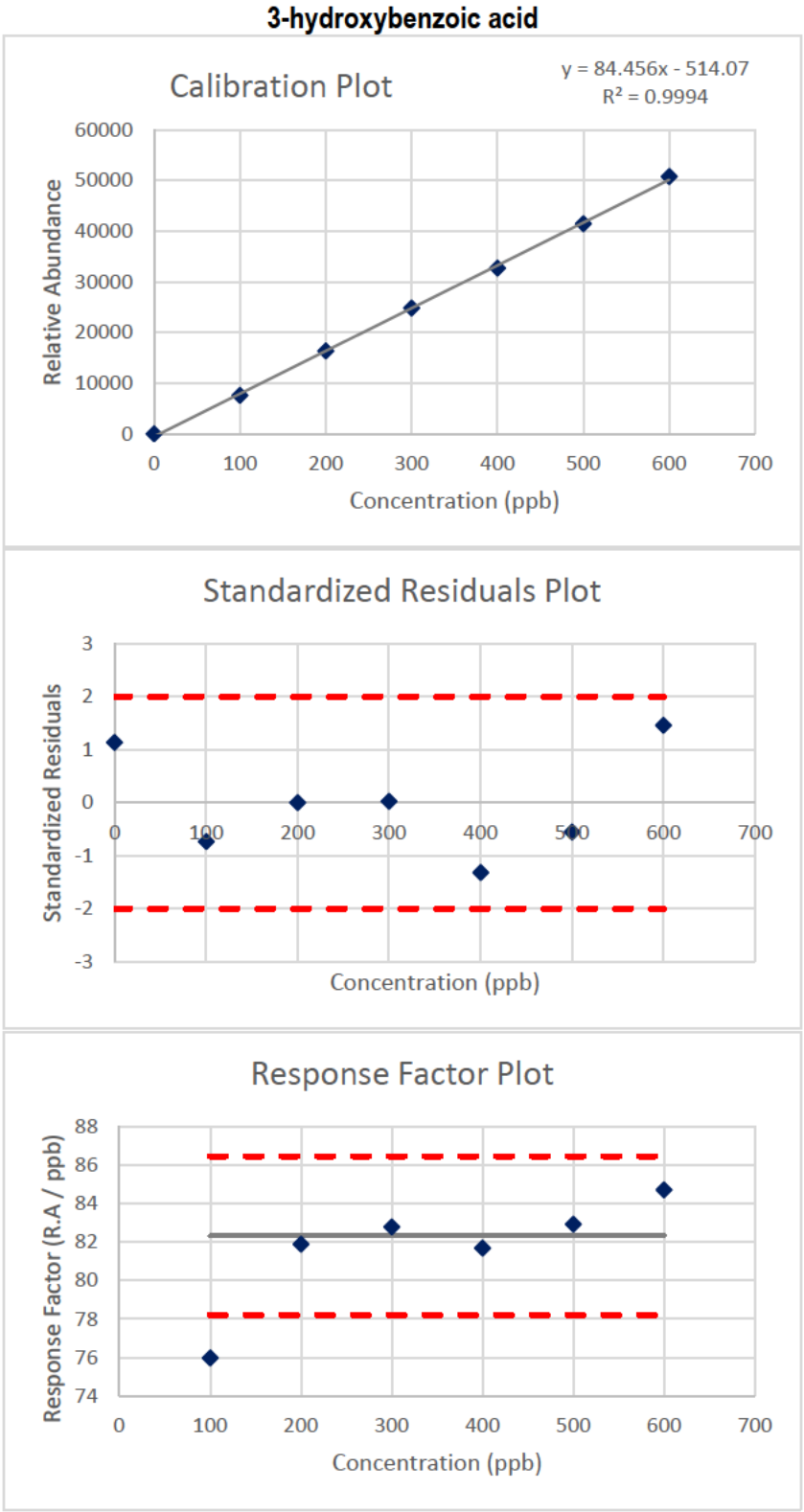
18) **Table 4.3.2.1** Linear Calibration Data for Monohydroxybenzoic Acids

Standard	Concentration Range (ppb)	Correlation Coefficient (r)	Coefficient of Determination (R^2)
2-hydroxybenzoic acid	0, 10, 20, 30, 40, 50, 60	0.9995	0.9990
3-hydroxybenzoic acid	0, 100, 200, 300, 400, 500, 600	0.9997	0.9994
4-hydroxybenzoic acid	0, 100, 200, 300, 400, 500, 600	0.9996	0.9992

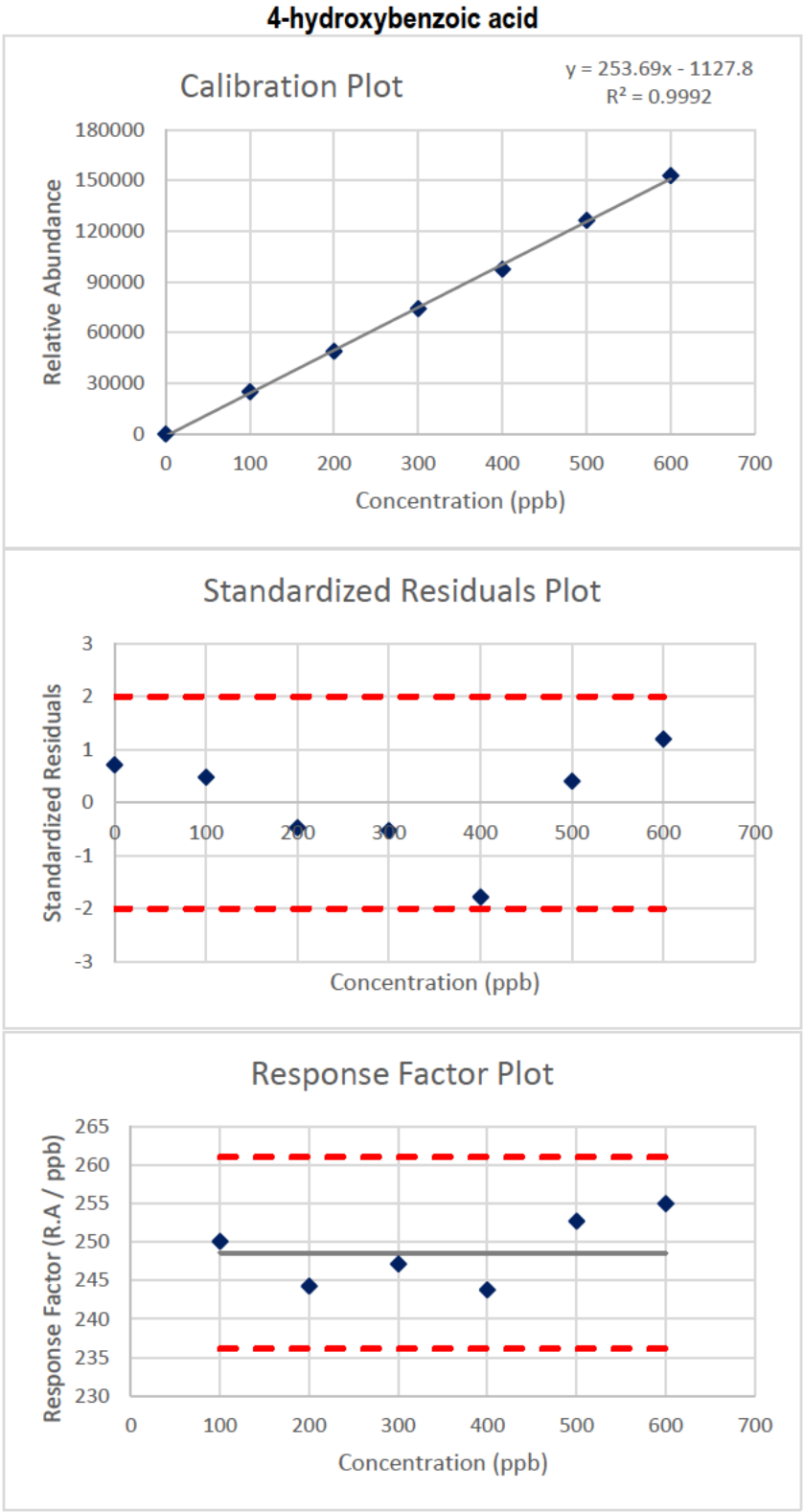
2-hydroxybenzoic acid



41) **Figure 4.3.2.1a** Linearity study plots for 2-hydroxybenzoic acid consisting of a standard calibration plot, a standardized residuals plot, and a response factor plot.



42) **Figure 4.3.2.1b** Linearity study plots for 3-hydroxybenzoic acid consisting of a standard calibration plot, a standardized residuals plot, and a response factor plot.



43) **Figure 4.3.2.1c** Linearity study plots for 4-hydroxybenzoic acid consisting of a standard calibration plot, a standardized residuals plot, and a response factor plot.

Visual inspection of each calibration plot and its line of best-fit shows there is a strong positive linear relationship between the instrument response and analyte concentration. This is further confirmed by the correlation coefficient showing a very strong positive linear relationship. The coefficient of determination for each calibration plot being above 0.999 indicates that no less than 99.9 % of the variability in instrument response is explainable by the model of the relationship between instrument response and analyte concentration. As previously outlined the standardized residuals plot and response factor plot can also be examined for each monohydroxybenzoic acid to assess linearity in the calibration data. The standardized residuals plots for all three monohydroxybenzoic acids show the residuals lie between ± 1.96 standard deviations of the mean of residuals over the whole calibration range, which indicates each model is acceptably linear and that there are no outliers. The distribution of the residuals appears to be random and there are no signs of homoscedasticity, which would be evident if the variance increased or decreased proportionately with concentration. The response factor plots show that all of the response factors lie within the ± 5 % limits (5 % of the mean response factor show as black line in Figures 4.3.2.1a – 4.3.2.1c), except for the first response factor for both 2- and 3-hydroxybenzoic acid. This deviation from linearity at the low end of the calibration curve is not uncommon and may be investigated in the limits of detection and quantitation study. The need to evaluate the IDL and IQL particularly for 2- and 3-hydroxybenzoic acid is highlighted in the response factor plots, however the linearity of the calibration has been determined to be fit-for-purpose for quantitative analysis of each analyte.

4.3.2.2 Instrument Repeatability.

Instrument repeatability is an estimation of the precision in measurements performed under the most constant conditions possible; one operator acquiring data on the same instrument, in the same facility, in a short time interval, with experimental conditions such as temperature held as constant as possible. For chromatographic analyses, successive injections of standard solutions are made in a single batch to provide an indication of the short-term variation in instrumental response. The two chromatographic parameters assessed are retention time and peak area. The data is collated and from it the mean, standard deviation and percent relative standard deviation (coefficient of variation) can be calculated. The coefficient of variation (CV) is used to express the instrument repeatability as it shows the variability in relation to the mean. To ensure statistical significance it is recommended that the measurements be performed with at least 6 degrees of freedom, with 7 or more replicates for a single parameter providing adequate degrees of freedom. A mixed standard solution was prepared following the standard solutions preparation procedure outlined in Section 2.4.2.1. The concentration for ortho-, meta-, and para-hydroxybenzoic acid were: 25, 250 and 100 ppb, respectively. In order to satisfy the

required amount of degrees of freedom, 10 successive injections were made. The full dataset is tabulated in Appendix B.4 and a summary of the data is provided below in Table 4.3.2.2. The HPLC-ESI-MS/MS method shows little short-term variation in both the retention time and peak area data.

19) **Table 4.3.2.2** Instrument Repeatability Data for Monohydroxybenzoic Acids

Chromatographic Parameter / Standard	Retention Time (% CV)	Peak Area (% CV)
2-hydroxybenzoic acid	0.21	2.1
3-hydroxybenzoic acid	0.16	3.7
4-hydroxybenzoic acid	0.11	3.0

4.3.2.3 Method Repeatability.

The instrument repeatability provides only a portion of the short-term variability in the quantitative data generated. It is important to additionally evaluate the variability in the total method including the sample preparation. The method repeatability can be determined by spiking samples to a level suitable for recovery analysis, each of these samples is then prepared and analysed in the shortest time-frame possible under the most constant conditions attainable. The chromatographic analysis is then performed and from the data acquired only the peak area data is evaluated. Similarly to the instrument repeatability the data is collated and the mean, standard deviation and CV is calculated. The method repeatability studies were aligned with the method recovery studies discussed later in this chapter, where the lowest spiked standard addition sample analysis was used to determine the method repeatability (sample preparation described in Section 2.5.5). The triplicate data from the lowest standard addition calibration level across each of the three samples was collated and used to determine the percent CV as a measure of the variation in the full method, including sample preparation. Below in Table 4.3.2.3 is a summary of the full dataset which can be found in Appendix B.6. The full method including the sample preparation and HPLC-ESI-MS/MS shows very little short-term variation and is considered to be fit-for-purpose for further quantitative analysis.

20) **Table 4.3.2.3** Method Repeatability Data for Monohydroxybenzoic Acids

Standard / Chromatographic Parameter	Peak Area (% CV)
2-hydroxybenzoic acid	2.3
3-hydroxybenzoic acid	4.0
4-hydroxybenzoic acid	3.8

4.3.2.4 Instrument Detection and Quantitation Limits.

The instrument detection and quantitation limits (IDL and IQL) were evaluated based on signal-to-noise (S/N). This determination involved successively injecting a mixed standard solution of low concentration 10 times and calculating the mean signal-to-noise ratio of the peaks corresponding to each monohydroxybenzoic acid. The signal-to-noise data is calculated by the instrument software by comparing peak height to baseline noise. From the mean signal-to-noise ratio for each monohydroxybenzoic acid the two limits can be calculated for each as follows: S/N of 3:1 for instrument detection limit and S/N 9:1 for instrument quantitation limit (see equations below in Figure 4.3.2.4). This data was collected for the mixed standard solution used in Section 4.3.2.2 for instrument repeatability where the concentration for ortho-, meta-, and para-hydroxybenzoic acid were: 25, 250 and 100 ppb, respectively. The full tabulated data is presented in Appendix B.5. A summary of the IDL and IQL data is shown in Table 4.3.2.4.

$$IDL = \frac{Conc.}{S/N} \times 3 \qquad IQL = \frac{Conc.}{S/N} \times 9$$

44) **Figure 4.3.2.4** The equations used to calculate the instrument detection limit (IDL) and instrument quantitation limit (IQL).

21) **Table 4.3.2.4** Instrument Detection and Quantitation Limits for Monohydroxybenzoic Acids

Standard / Validation Parameter	IDL (ppb)	IQL (ppb)
2-hydroxybenzoic acid	5.2	15.6
3-hydroxybenzoic acid	37.2	111.5
4-hydroxybenzoic acid	12.3	36.8

4.3.2.5 Matrix Effects.

The complexity of the sample matrix is not to be understated for seaweed biostimulants. It is therefore necessary to evaluate the impacts that the matrix has on the analysis of the monohydroxybenzoic acids. The other components found in the sample matrix have the potential to impact the results in different ways; for example, in HPLC-ESI-MS/MS analyses matrix effects are notorious for altering the ionization efficiency of the electrospray which often leads to suppression of the instrument response [109]. Due to the frequency of the presence of matrix effects in HPLC-ESI-MS/MS it was determined that they should be investigated. The NATA technical note suggests making standard additions to a typical sample extract at a minimum of 3 concentrations, each measured in at least duplicate. Then the matrix effects on the instrument response can be determined by comparing the slope for the standard additions calibration plot vs the slope for the direct calibration plot for the mixed standard solutions. Any differences greater than 10 % are expected to be compensated for or further investigated, where the most cost-effective and simple method for compensating for matrix effects is the use of standard additions for quantitation [107, 256]. A standard addition calibration plot was constructed for each of four samples (one spiked with blank; and three spiked with the recovery spike solution) that underwent the sample preparation procedure including SPE that is outlined in Section 2.5.5. The mean slope for each of the monohydroxybenzoic acid standard addition plots was determined and compared to the slope for the direct calibration of standard solutions used for the linearity studies in Section 4.3.2.1. The standard addition calibration plots and equation for the linear regression models is presented in Appendix B.7, and a summary of this data is shown below in Table 4.3.2.5.

22) **Table 4.3.2.5** Matrix Effects Data for Monohydroxybenzoic Acids

Standard	Mean Slope for Standard Addition Calibrations	Slope for Direct Calibration	Matrix Effects |%|
2-hydroxybenzoic acid	1505	1002	50.2
3-hydroxybenzoic acid	83	84	1.2
4-hydroxybenzoic acid	250.5	254	1.4

The matrix effects values provided in Table 4.3.2.5 show the percentage difference between the mean slope for the standard addition calibrations data and the slope for the direct calibration plots. There appears to be no significant difference in slopes for 3- and 4-hydroxybenzoic acid, with 1.2 and 1.4 % matrix effects respectively. For 2-hydroxybenzoic acid the 50 % difference in slopes indicates that

matrix effects are present, therefore direct calibration would be inadequate in providing accurate quantitative determinations.

Strategies for reducing matrix effects include diluting samples to lower background noise, improving MS parameters to be more selective, optimising LC conditions to reduce co-elution and more efficient and selective sample extraction methods [109, 110]. All of these strategies have been considered and employed in this study: the sample is heavily diluted and the extraction methodology involves multiple selective steps to reduce the matrix effects, particularly in the SPE protocol which involves the unique 'flipping' to allow for extra washes. Furthermore, the HPLC conditions provide excellent separation (as seen above in Figure 4.3.1), the injection volume is kept deliberately low to prevent overloading, and finally the MS/MS methodology uses a MRM that is specific to the monohydroxybenzoic acids.

4.3.2.6 Method Recovery.

A recovery study where no matrix blanks or any certified reference materials are available for comparison can be conducted by spiking (fortifying) the original sample prior to the sample preparation procedure, then following analysis the method recovery is calculated from the difference between the measured concentrations of the unfortified and fortified samples [107]. The recovery study was conducted using triplicate analysis of four samples, one fortified with a blank solutions and three fortified with a mixed stock solution of monohydroxybenzoic acids. Samples were prepared by fortifying with a mixed stock solution before undergoing sample preparation and standard addition as described in Section 2.5.5. The method recovery was calculated (see equation below in Figure 4.3.2.6) from the quantitative data presented in Appendix B.8 and is summarised below in Table 4.3.2.6.

$$\%\, Recovery = \frac{C_1 - C_2}{C_3} \times 100$$

45) **Figure 4.3.2.6** The equation used to calculate the percent recovery where: C_1 = measured concentration of spiked sample, C_2 = measured concentration of unspiked sample, and C_3 = concentration of spike [107].

23) **Table 4.3.2.6** Method Recoveries for Monohydroxybenzoic Acids

Validation Parameter / Standard	Method Recovery %
2-hydroxybenzoic acid	73.5
3-hydroxybenzoic acid	75.0
4-hydroxybenzoic acid	87.3

The results indicate that there is bias in the trueness of the measurements of all three monohydroxybenzoic acid using the developed method. This deviation from trueness indicates systematic errors in the method, laboratory, and/or the operator; in some cases however, even reference methods recognised nationally or internationally have a known bias that is an excepted limitation of the method and is compensated for when analysing quantitative data [107]. Therefore, the recoveries as calculated whilst significant, are not an indication that the method is not fit-for-purpose; but instead, indicate that there is a bias that needs to be compensated for when employing the developed method.

4.3.3 Summary of Partial Method Validation.

The partial method validation conducted in this study was able to provide objective evidence on the statistical confidence of the data generated by the newly developed analytical method. The validation parameters investigated included: linearity, instrument repeatability, method repeatability, instrument limit-of-detection (IDL), instrument limit-of-quantitation (IQL), matrix effects and method recovery. The acceptance criteria for these performance parameters is not very well defined for the analysis of phytochemicals in biostimulants, however the statistical tools used for their assessment have standard criteria for acceptance (e.g. R^2 values > 0.995). To supplement the standard acceptance criteria for these statistical analyses the validation data was compared to studies investigating similar phytochemicals/phytohormones in seaweeds and other algal samples using HPLC methodologies [91, 117, 150, 158].

All three monohydroxybenzoic acids showed good linearity with coefficients of determination all ≥ 0.999. Visual inspection of the standardised residuals and response factor plots provided further evidence for the linearity, as well as the goodness of fit for the linear regression models. The precision was assessed in terms of instrument repeatability and method repeatability; with the percent coefficients of variation not exceeding 5 %, and this is considered a good result when analysing samples of biological origin [117]. The specificity of the method is highlighted by the excellent chromatographic separation and because no other significant peaks are present in the chromatogram the method can be considered highly specific [102, 257]. The sensitivity of the method was assessed by determination of the limits of detection and quantitation, and the method was determined to be suitably sensitive for the detection and quantitation of the monohydroxybenzoic acids in the commercial seaweed biostimulant.

The accuracy of the method in terms of recovery was assessed whilst investigating the single biggest source of error in the accuracy of LCMS analyses: matrix effects [114, 115]. The analysis of standard addition calibrations for the monohydroxybenzoic acids revealed that 3- and 4-hydroxybenzoic acid

exhibited no significant matrix effects, whereas there were significant matrix effects for 2-hydroxybenzoic acid. Using the standard addition calibration method the recovery assessment revealed a bias in the trueness of the measurements of the monohydroxybenzoic acids, recoveries of 73.5, 75.0 and 87.3 % were determined for 2-, 3- and 4-hydroxybenzoic acid, respectively. In conclusion these findings suggest that the HPLC-ESI-MS/MS method for the analysis of the monohydroxybenzoic acids in a commercial seaweed biostimulant is sufficiently fit-for-purpose.

4.4 SIMULTANEOUS QUANTIATIVE ANALYSIS OF 2-, 3-, AND 4-HYDROXYBENZOIC ACID IN A COMMERCIAL SEAWEED BIOSTIMULANT USING HPLC-ESI-MS/MS

The concentrations of 2-, 3-, and 4-hydroxybenzoic acid were determined in a commercial seaweed biostimulant by HPLC-ESI-MS/MS. The commercial seaweed biostimulant sample was prepared for analysis via partitioning, solid-phase extraction and standard additions as described in Section 2.5.5. The analysis utilised the HPLC-ESI-MS/MS method developed in Section 3.3.3 and partially validated above in Section 4.3, the conditions for which are described in Section 2.6.2.5. The sample was analysed in triplicate, data collated and analysed, and the mean concentrations with standard errors summarised in Table 4.4 (see Appendix B.9 for full regression analysis). The mean concentrations and standard errors adjusted to compensate for recoveries are also included in Table 4.4, using the recoveries determined above in Section 4.3.2.6.

24) **Table 4.4** Concentrations of Monohydroxybenzoic Acids in Commercial Seaweed Biostimulant

Standard	Concentration (µg / L)	Compensated Concentration (µg / L)
2-hydroxybenzoic acid	100 ± 11	137 ± 16
3-hydroxybenzoic acid	2557 ± 70	3409 ± 93
4-hydroxybenzoic acid	1526 ± 102	1748 ± 117

From Table 4.4 it can be seen that the levels of 2-, 3- and 4-hydroxybenzoic acid are significantly different in the seaweed biostimulant sample, with salicylic acid being present in the lowest concentration. The identification and quantitative determination of the salicylic acid and its isomers in this work is a significant finding. It should be noted that this is the first reported identification and quantitative determination of salicylic acid and its isomers in a commercial seaweed biostimulant, which now paves the way for biological assessment of these compounds for plant growth biostimulation. Furthermore, the seaweed biostimulant sample is prepared from two seaweed species, in which salicylic acid and 3-hydroxybenzoic acid have never been detected. Further significance and comparisons to relevant literature is provided below.

4.4.1 Identification and Quantitation of 2-, 3- and 4-Hydroxybenzoic Acid in a Seaweed Extract.

The seaweed biostimulant investigated in this study is an alkaline hydrolysis product from two seaweed species: *Durvillaea potatorum* and *Ascophyllum nodosum* [1]. Importantly there is no reported literature for the identification of salicylic acid nor 3-hydroxybenzoic acid in either of the seaweed species or an extract of them, however the presence of 4-hydroxybenzoic acid has been reported in *A.nodosum* but not in *D.potatorum* [125, 159]. Whilst there is little reported for the two algal species mentioned, monohydroxybenzoic acids have been identified in other various seaweeds or seaweed products before. Salicylic acid, being the most well-characterised and prominent in the literature, has been detected in various red, green, and brown seaweeds [41, 124, 150]. The other monohydroxybenzoic acid that is well characterised and represented in the literature, 4-hydroxybenzoic acid, has also been detected in various seaweeds [123, 156, 237]. Significantly less has been reported on 3-monohydroxybenzoic acid and its presence in seaweeds, although it has been identified in red seaweeds [258]. The presence of 3-hydroxybenzoic acid is seemingly far rarer as demonstrated in the study of eight different South African seaweeds conducted by Rengasamy *et al*, it was only detected in one seaweed species whereas, 4-hydroxybenzoic acid was detected in all eight [258]. Interestingly, in the quantitative determinations above 3-hydroxybenzoic acid was the most prominent monohydroxybenzoic acid of the three. The presence of monohydroxybenzoic acids in other seaweed species alludes to the possibility of their presence in the two species from which this biostimulant is produced, but it is not conclusive evidence for their presence in these two seaweed species. The manufacturing processes for this particular biostimulant involve alkaline hydrolysis at elevated temperature and pressure which has the potential to generate a large number of degradation products [241]. Therefore, it is possible that the monohydroxybenzoic acids identified in the biostimulant are a result of those manufacturing processes, indicating that further work is required to determine whether these compounds are endogenous. If the monohydroxybenzoic acids are present in the seaweeds, then it is likely that they are methylated or glycosylated as they are in plants, however it is unlikely that any glycosylated derivatives would be present in the biostimulant following alkaline hydrolysis [55]. It is possible that some common methoxylated hydroxybenzoic acid derivatives may be also be present as shown in the qualitative investigation in Chapter 3 where syringic acid was tentatively identified. Developing methods for the raw seaweeds themselves was beyond the scope of this research project but altering and optimising the qualitative and quantitative methodology developed in this study may help to provide a more comprehensive profile of hydroxybenzoic acids within the seaweeds. This profile could be compared to biosynthetic pathways and could be used to help fill in the gaps of what is present in the seaweeds and how they are synthesised in vivo, providing a more complete picture of their metabolome.

4.4.2 Identification and Quantitation of 2-, 3- and 4-Hydroxybenzoic Acid in a Commercial Seaweed Biostimulant.

The identification of all three monohydroxybenzoic acids in a commercial seaweed biostimulant is an important discovery. Given the current literature surrounding phytochemicals and their importance on plant growth and development as well as resistance to stressors, the identification of these significant phenolic acids facilitates the potential elucidation of the biological modes of action of one or more of the many benefits that the biostimulants offer. Additionally, the quantitative data serves to provide a more comparable reference for the investigation of the efficacy of these monohydroxybenzoic acids in the biostimulants. This also provides a link between previous studies conducted on the exogenous application of any individual monohydroxybenzoic acid on plants and the previous studies that investigate the benefits of biostimulant use. A lot of research has been conducted investigating salicylic acid's role in plants endogenously as well as the effects of exogenous application, including its crucial role in plant growth and development processes and regulating plant defence, namely systemic acquired resistance (SAR) [57, 59, 173]. Likewise, 4-hydroxybenzoic acid is well represented in the literature, where in plants it is known most for being an allelochemical that has exhibited hormesis-like effects [70, 72]. Both of these compounds have been used in plant studies to determine biological efficacy as shown in Table 1.7 in Chapter 1, where some of these studies mirror the investigations into the seaweed biostimulants. The identification of 2- and 4-hydroxybenzoic acid in the biostimulant helps to bridge the gap between these studies such that future studies can now investigate if their presence in the biostimulant helps to explain any of the biostimulant's benefits. Far less is reported about 3-hydroxybenzoic acid with regards to plant studies, however the concentrations determined in the biostimulant warrant the inclusion of 3-hydroxybenzoic acid in future studies to investigate its biological activity. It is worth noting that the levels of monohydroxybenzoic acids in the seaweed biostimulants are subject to the variability that comes from producing an extract from a natural resource such as a living organism. These phytochemicals are often found in small concentrations and are upregulated at times when required by the host organism, particularly salicylic acid whose synthesis is known to be upregulated as a response to stress [259]. Therefore the environmental impacts on the organism at the time of harvest is likely to play a role in the concentration and profile of these monohydroxybenzoic acids. The novelty and significance of the quantitative determination of salicylic acid and its isomers described above will be further highlighted in Chapter 5, where simple but powerful plant bioassays will assess their role in plant growth both individually and as part of complex seaweed biostimulants.

4.5 CONCLUSION

The aim of this part of the study was to successfully validate then apply a quantitative HPLC-ESI-MS/MS method for the analysis of salicylic acid and its isomers in a commercial seaweed biostimulant. This was successfully achieved by firstly developing and optimising a sample preparation procedure involving acetonitrile partitioning and mixed-mode SPE to selectively extract the monohydroxybenzoic acids from the commercial seaweed biostimulant sample. Secondly the HPLC-ESI-MS/MS method for the analysis of the monohydroxybenzoic acids developed and optimised in Chapter 3 was partially validated following the NATA protocol and found to be sufficiently fit-for-purpose. Lastly the partially validated method for the quantitative determination of the monohydroxybenzoic acids was successfully employed, and the concentrations of 2-, 3- and 4-hydroxybenzoic acids in the commercial seaweed biostimulant were determined to be 137 ± 16, 3409 ± 93 and 1748 ± 117 μg/L, respectively.

For the full discussion of this chapter's key observations, significant findings and future research prospects refer to Chapter 6.

CHAPTER 5: THE BIOLOGICAL ROLE OF SALICYLIC ACID AND ITS ISOMERS IN SEAWEED EXTRACTS

5.1 INTRODUCTION

There is a plethora of studies investigating the potential of salicylic acid and other closely related phenolic acids for improving crop productivity through their exogenous application (a review of which is presented in Table 1.7). The exogenous application of these endogenous phytochemicals has been shown to improve plant growth, quality and stress tolerance (as discussed in Section 1.7). In reviewing these studies some important trends were observed: that the biological mechanisms are more often general to plants rather than species specific; that the dosage is vital; that the biological mechanisms are complex; and that compounds such as salicylic acid elicit a response that is commonly pleotropic in nature. Further to these proof-of-concept studies are the field trials that provide the most significant evidence for the potential of phytochemicals like salicylic acid to improve agricultural productivity.

5.1.1 The Use of Salicylic Acid in Agriculture.

The exogenous application of salicylic acid to plants yields many benefits; as such there are a number of review articles reporting on the studies investigating the agricultural application of salicylic acid [17, 57, 61, 173-175]. A recent review article that provides examples to demonstrate salicylic acid's role as a safe plant growth regulator and protector is the one by Koo *et al.* [173]. With a focus on crops of agronomical significance, the review article provides a plethora of examples of the application of salicylic acid enhancing disease resistance as well as abiotic stress resistance [173, 260].

One example provided by Koo *et al.* is a study that investigates the effects of exogenous methyl jasmonate and salicylic acid on rice resistance to *Oebalus pugnax*, the most injurious insect pest of heading rice in the United States [261]. This study is chosen as an example due to its use of both proof-of-concept bioassays coupled with field trials to demonstrate efficacy of salicylic acid in enhancing biotic resistance. In this study Stella de Freitas *et al.* found that treatment with salicylic acid could be used as an elicitor to trigger defence against the rice stink bug *O.pugnax* [261]. A different study investigating salicylic acid's efficacy for increasing abiotic resistance that also employed field trials was conducted by Abreu and Munné-Bosch, in which they investigated salicylic acid's role in regulating drought-induced leaf senescence in field-grown sage (*Salvia officinalis* L.) [262]. They concluded that salicylic acid, possibly along with other phytohormones was involved in the regulation of drought-induced leaf senescence in perennials [262]. These studies offer the most significant evidence for the use

of salicylic acid to improve agricultural outcomes as they provide statistical significance of its effects in different agricultural production settings despite the high level of environmental variation experienced in field trial studies.

5.1.2 Plant Tolerance to Stresses.

Plant growth and development is severely impacted by both abiotic and biotic environmental stresses which present challenging threats to agricultural systems, reducing crop yield and quality and resulting in considerable economic losses [61, 63, 263]. To cope with these challenges plants have developed a number of physiological and molecular defence mechanisms that are largely governed by phytohormones [63, 173, 263]. The network of phytohormones in plants is highly sophisticated, involving crosstalk between the different phytohormones in order to balance responses to environmental stresses with the plants' ability to grow and develop in order to minimise defence-associated fitness costs [173, 200]. This plant growth-immunity equilibrium involves the harmony and/or disharmony of phytohormones in response to specific stimuli to elicit a specific response [173].

5.1.3 The Role of Salicylic Acid in Plant Tolerance to Stresses.

Amongst the list of phytohormones known to play a pivotal role in plant immunity as well as growth is the phenolic acid, salicylic acid [173]. Some of the underlying mechanisms of salicylic acid induced abiotic stress tolerance that have been demonstrated include: accumulation of osmolytes that help to maintain osmotic homeostasis, regulation of mineral nutrition uptake and metabolism which is a basic requirement for proper growth and development, enhanced reactive oxygen species (ROS) scavenging and antioxidant metabolism, enhanced secondary metabolite production, and regulation of other phytohormone pathways through the aforementioned crosstalk [61, 173]. Salicylic acid is most well-known for being a defence hormone, primarily for its role in the regulation of plant resistance to biotic stresses [173]. The biological role of salicylic acid is central to the regulation of local and systemic immunity in plants, playing a critical role in triggering the systemic acquired resistance (SAR) pathway [63, 173]. Pathogenic infection stimulates the production of salicylic acid which induces the generation of mobile signals to trigger systemic acquired resistance [63]. The elevated level of salicylic acid promotes massive transcriptional reprogramming in pathogenesis related (PR) genes, binding directly to receptor transcriptional coactivators such as Non-expresser of PR genes 1 (NPR1) activating defence-related genes/pathways to establish plant immunity [63, 173].

5.1.4 Agricultural Biostimulants.

Given that salicylic acid is such an important phytohormone with exciting potential as a natural product for the sustainable improvement of stress tolerance and growth in crops it was an ideal candidate compound for identification in biostimulants [20, 28, 200]. Most commercial biostimulants are often complex mixtures containing many different bioactive constituents that offer a great diversity of responses in various crops [14, 263]. Most importantly the application of biostimulants has been shown to improve plant growth and development under abiotic and biotic stress conditions [13, 263]. In fact, some studies investigating seaweed biostimulants for improved plant productivity have paired salicylic acid with seaweed extracts to assess their potential in both comparative and synergistic experimental designs [264-266].

If the ultimate goal is to optimise the plant's growth-immunity balance to maximise both yield as well as immune resilience, then investigating natural products such as biostimulants with the potential for influencing this growth-immunity balance is a prospective avenue to assisting in elucidating the mechanisms whilst improving agricultural productivity sustainably [200]. This includes addressing the fact that complex agricultural biostimulants such as the ones prepared from seaweeds are poorly characterised, due to their diversity and molecular complexity [1, 23, 26]. This requires new analytical research using modern techniques and instruments to profile the bioactive constituents that contribute to their efficacy and agricultural benefits [23, 73, 118].

The research in Chapters 3 and 4 is dedicated to addressing this knowledge gap, with Chapter 3 describing new analytical research profiling important phytochemicals in seaweed biostimulants and the research in Chapter 4 leading to the first positive identification and quantitative determination of salicylic acid and its isomers in a commercial seaweed biostimulant. This new research made it possible to design experiments to investigate the biological role of salicylic acid and its isomers in seaweed extracts using concentrations similar to those found in the commercial seaweed biostimulant.

5.1.5 Investigating the Role of Salicylic Acid and its Isomers in Seaweed Extracts.

To investigate the role of salicylic acid and its isomers in seaweed extracts, primary screening assays that focus on growth and development are more achievable than assays for stress tolerance for the identification of molecules that trigger a phenotype of interest [263]. A practical way of testing growth is by testing root growth and development; this is because the plant root system is often directly exposed to the nutrients in the soil or growth medium and a healthy root system is crucial for plant survival [57, 70]. In this chapter the biological roles of salicylic acid and its isomers in seaweed extracts were

investigated using a liquid plant growth bioassay that assesses root and shoot growth. The simple yet effective plant growth bioassay aimed to provide insights into the role of salicylic acid and its isomers in the growth and development benefits to plants observed following the application of the seaweed extracts as an agricultural biostimulant.

5.1.6 Chapter Outline

This chapter is divided into the following sections.

- Bioassay Design (Section 5.2) – This section details the design of the bioassay experiments using the quantitative data generated for the monohydroxybenzoic acids in Chapter 4.
- Plant Growth Bioassay Results (Section 5.3) – This section reports the results from the two independent plant growth bioassays. Additionally photographs of the plants are included for phenotypical analysis.
- Discussion (Section 5.4) – This section includes a detailed discussion interpreting the results and comparing them with the relevant scientific literature.

5.2 BIOASSAY DESIGN – TREATMENT DOSAGES BASED ON DATA FROM CHAPTER 4

The data from Chapter 4 was used in the design of these bioassays to investigate the role of salicylic acid and its isomers in seaweed extracts. The concentrations of 2-, 3- and 4-hydroxybenzoic acid in the Seasol seaweed extract after compensating for their recovery were calculated to be 137, 3409 and 1748 μg/L, respectively. As shown in Table 1.7, studies investigating the exogenous application of important phytochemicals report dosage concentrations in molarity or mol/L. Therefore the aforementioned concentrations correlate to 0.992, 24.7 and 12.7 μM for 2-, 3- and 4-hydroxybenzoic acid in the concentrate, respectively. The positive treatment control in the bioassays was the Seasol seaweed extract (Commercial Seasol Concentrate) used at the recommended 1:400 dilution. As such the concentrations of 2-, 3- and 4-hydroxybenzoic acid in the diluted Seasol seaweed extract are 2, 62 and 32 nM, respectively (when rounded to the nearest integer). These were the concentrations used throughout the bioassays both individually and in combination. Finally, water was used as one of the controls (negative control) to avoid any potential interactions of the monohydroxybenzoic acids with plant nutrients.

5.3 PLANT GROWTH BIOASSAYS RESULTS

The liquid-based plant growth bioassays assessed the impact of salicylic acid and its isomers on plant growth and development, using tomato seedlings grown in a liquid growth system, by measuring five growth parameters: longest root length, root fresh and dry weight, and shoot fresh and dry weight. For the full methodology for all bioassay experiments see Section 2.7. The following section reports the results from the two independent plant growth bioassay experiments conducted in this study.

5.3.1 Experiment 1 – Pilot Bioassay

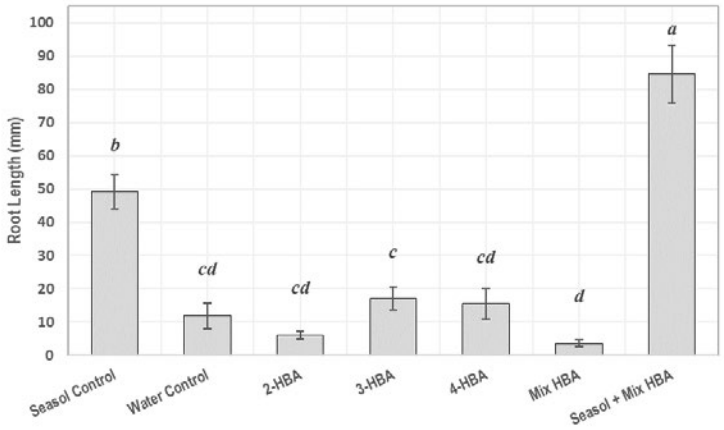
The first plant growth bioassay consisted of 7 treatment solutions, 2 controls treatments and 5 treatments containing monohydroxybenzoic acids as described in Section 2.7.3. After 14 days of treatment the various parameters were measured. Data were subjected to two outlier tests to determine the presence of any outliers before being subjected to one-way ANOVA analysis at 5% significance level to assess treatment effects as described in Section 2.7.6. The Fisher Least Significant Difference (LSD) test was used to determine significant differences between the means. Full data and ANOVA statistical analyses are presented in Appendix C.1. The results reported below are separated into three sections: the impacts of the treatments on the longest root length, the impacts of the treatments on the root weight, and the impacts of the treatments on the shoot weight.

5.3.1.1 Experiment 1 Root Length Results

The longest root length of each tomato plant was measured by the method described in Section 2.7.5.1. Table 5.3.1.1 presents the collated data with the mean longest root length for each treatment along with the standard error of the means (n = 6); means denoted with the same letter do not differ significantly at $P < 0.05$.

25) **Table 5.3.1.1** Experiment 1 Longest Root Length Results

Treatment	Replicates	Mean Longest Root Length (mm)
T1) Seasol Control	6	49.2 ± 5.1^{b}
T2) Water Control	6	12.0 ± 3.8^{cd}
T3) 2-HBA	6	6.2 ± 1.2^{cd}
T4) 3-HBA	6	17.2 ± 3.5^{c}
T5) 4-HBA	6	15.5 ± 4.7^{cd}
T6) HBA Mix	6	3.7 ± 1.0^{d}
T7) Seasol + HBA Mix	6	84.7 ± 8.7^{a}



46) **Figure 5.3.1.1** Tomato longest root length after 14 days treatment with Seasol control, Water control, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, a 2-, 3- & 4-hydroxybenzoic acid mix, and Seasol + 2-, 3- & 4-hydroxybenzoic acid mix. Error bars represent standard error of the means (n = 6). Means denoted with the same letter do not differ significantly at $P < 0.05$.

Treatment 7 composed of Seasol + hydroxybenzoic acid mixture produced the mean longest root length at 84.7 mm which was significantly longer than all other treatments including the positive control Treatment 1 composed of Seasol which had a mean longest root length of 49.2 mm. Treatments 3 – 6 did not significantly differ from the negative treatment control Treatment 2 composed of water.

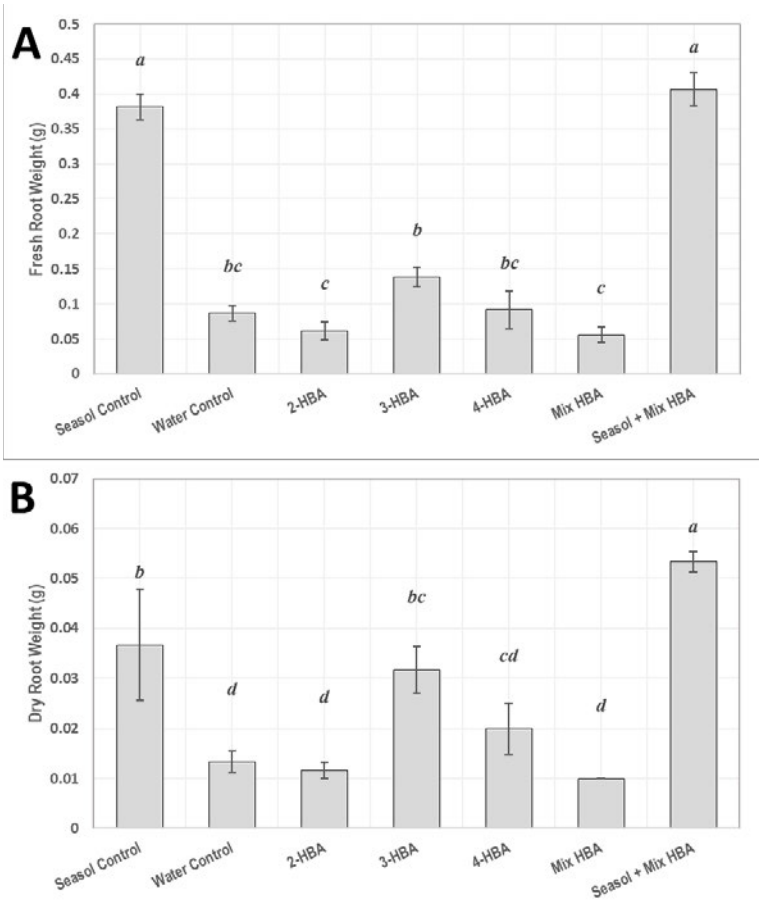
5.3.1.2 Experiment 1 Root Weight Results

The fresh and dry root weight of each tomato plant was measured by the method described in Section 2.7.5.2. Table 5.3.1.2 presents the collated data with the mean fresh and dry root weight for each treatment along with the standard error of the means (n = 6); means denoted with the same letter do not differ significantly at $P < 0.05$.

26) **Table 5.3.1.2** Experiment 1 Root Weight Results

Treatment	Replicates	Mean Root Weight (g)	
		Fresh	Dry
T1) Seasol Control	6	0.38 ± 0.02^{a}	0.04 ± 0.01^{b}
T2) Water Control	6	0.09 ± 0.01^{bc}	0.01 ± 0.00^{d}
T3) 2-HBA	6	0.06 ± 0.01^{c}	0.01 ± 0.00^{d}
T4) 3-HBA	6	0.14 ± 0.01^{b}	0.03 ± 0.00^{bc}
T5) 4-HBA	6	0.09 ± 0.03^{bc}	0.02 ± 0.01^{cd}
T6) HBA Mix	6	0.06 ± 0.01*^{c}	0.01 ± 0.00^{d}
T7) Seasol + HBA Mix	6	0.41 ± 0.02^{a}	0.05 ± 0.00^{a}

* One outlier rejected (n = 5)



47) **Figure 5.3.1.2** Tomato fresh root weight (A) and dry root weight (B) after 14 days treatment with Seasol control, Water control, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, a 2-, 3- & 4-hydroxybenzoic acid mix, and Seasol + 2-, 3- & 4-hydroxybenzoic acid mix. Error bars represent standard error of the means (n = 6). Means denoted with the same letter do not differ significantly at $P < 0.05$.

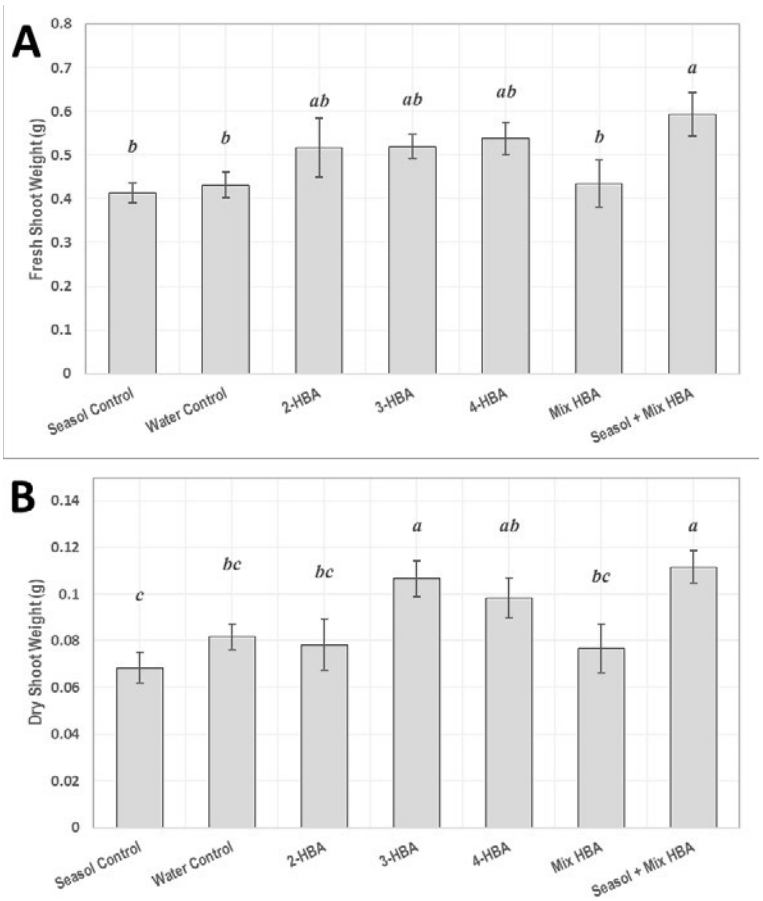
Treatment 7 had the largest mean fresh root weight at 0.41 g, although it did not significantly differ from the positive control Treatment 1 which had a mean fresh root weight of 0.38 g. Both Treatments 1 and 7 were significantly different from the remaining 5 treatments. Treatment 4 composed of 4-hydroxybenzoic acid had the largest mean fresh root weight of the individual hydroxybenzoic acid treatments at 0.14 g, however it was not significantly different from the negative control Treatment 2. Treatments 3 – 6 did not significantly differ from the negative control Treatment 2. Treatment 7 also produced the largest mean dry root weight of 0.05 g which was found to be significantly larger than the positive control Treatment 1 which had a mean dry root weight of 0.04 g. Treatment 1 did not significantly differ from Treatment 4 also, with the 3-hydroxybenzoic acid treatment producing a mean dry root weight of 0.03 g which was statistically comparable to the positive control. Treatments 3, 5 and 6 all did not significantly differ from the negative control Treatment 2.

5.3.1.3 Experiment 1 Shoot Weight Results

The fresh and dry shoot weight of each tomato plant was measured by the method described in Section 2.7.5.3. Table 5.3.1.3 presents the collated data with the mean fresh and dry shoot weight for each treatment along with the standard error of the means (n = 6); means denoted with the same letter do not differ significantly at $P < 0.05$.

27) **Table 5.3.1.3** Experiment 1 Shoot Weight Results

Treatment	Replicates	Mean Shoot Weight (g)	
		Fresh	Dry
T1) Seasol Control	6	0.41 ± 0.02^{b}	0.07 ± 0.01^{c}
T2) Water Control	6	0.43 ± 0.03^{b}	0.08 ± 0.01^{bc}
T3) 2-HBA	6	0.52 ± 0.07^{ab}	0.08 ± 0.01^{bc}
T4) 3-HBA	6	0.52 ± 0.03^{ab}	0.11 ± 0.01^{a}
T5) 4-HBA	6	0.54 ± 0.04^{ab}	0.10 ± 0.01^{ab}
T6) HBA Mix	6	0.44 ± 0.05^{b}	0.08 ± 0.01^{bc}
T7) Seasol + HBA Mix	6	0.59 ± 0.05^{a}	0.11 ± 0.01^{a}



48) **Figure 5.3.1.3** Tomato fresh shoot weight (A) and dry shoot weight (B) after 14 days treatment with Seasol control, Water control, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, a 2-, 3- & 4-hydroxybenzoic acid mix, and Seasol + 2-, 3- & 4-hydroxybenzoic acid mix. Error bars represent standard error of the means (n = 6). Means denoted with the same letter do not differ significantly at $P < 0.05$.

The largest fresh shoot weight was produced by Treatment 7 with 0.59 g, which was statistically comparable to Treatments 3, 4 and 5. The mean fresh shoot weight for Treatments 1 – 6 did not significantly differ. Treatment 7 also produced the largest mean dry shoot weight with 0.11 g which was statistically comparable to Treatments 4 and 5 which had mean dry shoot weights of 0.11 and 0.10 g, respectively. Treatments 1 -3, and 6 all did not significantly differ with mean dry shoot weights between 0.7 and 0.8 g.

5.3.2 Experiment 2 – Repeatability Bioassay

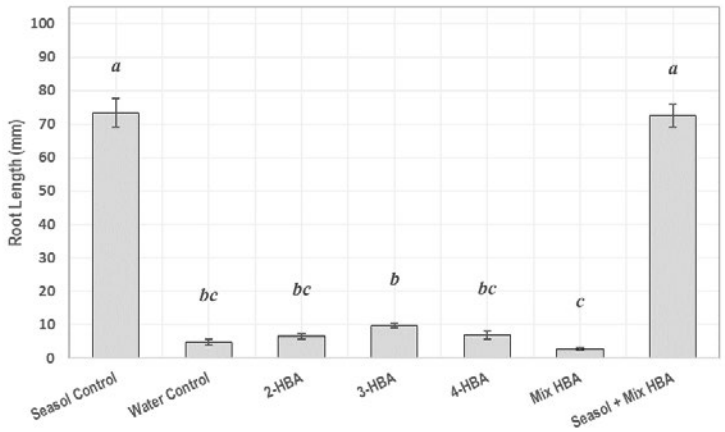
The second plant growth bioassay consisted of 7 treatment solutions, 2 controls treatments and 5 treatments containing monohydroxybenzoic acids as described in Section 2.7.3. After 14 days of treatment the various parameters were measured. Data were subjected to two outlier tests to determine the presence of any outliers before being subjected to one-way ANOVA analysis at 5% significance level to assess treatment effects as described in Section 2.7.6. The Fisher Least Significant Difference (LSD) test was used to determine significant differences between the means. Full data and ANOVA statistical analyses are presented in Appendix C.2. The results reported below are separated into three sections: the impacts of the treatments on the longest root length, the impacts of the treatments on the root weight, and the impacts of the treatments on the shoot weight.

5.3.2.1 Experiment 2 Root Length Results

The longest root length of each tomato plant was measured by the method described in Section 2.7.5.1. Table 5.3.2.1 presents the collated data with the mean longest root length for each treatment along with the standard error of the means (n = 6); means denoted with the same letter do not differ significantly at $P < 0.05$.

28) **Table 5.3.2.1** Experiment 2 Longest Root Length Results

Treatment	Replicates	Mean Longest Root Length (mm)
T1) Seasol Control	6	73.3 ± 4.2^{a}
T2) Water Control	6	4.8 ± 0.9^{bc}
T3) 2-HBA	6	6.5 ± 0.8^{bc}
T4) 3-HBA	6	9.7 ± 0.6^{b}
T5) 4-HBA	6	6.8 ± 1.1^{bc}
T6) HBA Mix	6	2.8 ± 0.4^{c}
T7) Seasol + HBA Mix	6	72.5 ± 3.3^{a}



49) **Figure 5.3.2.1** Tomato longest root length after 14 days treatment with Seasol control, Water control, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, a 2-, 3- & 4-hydroxybenzoic acid mix, and Seasol + 2-, 3- & 4-hydroxybenzoic acid mix. Error bars represent standard error of the means (n = 6). Means denoted with the same letter do not differ significantly at $P < 0.05$.

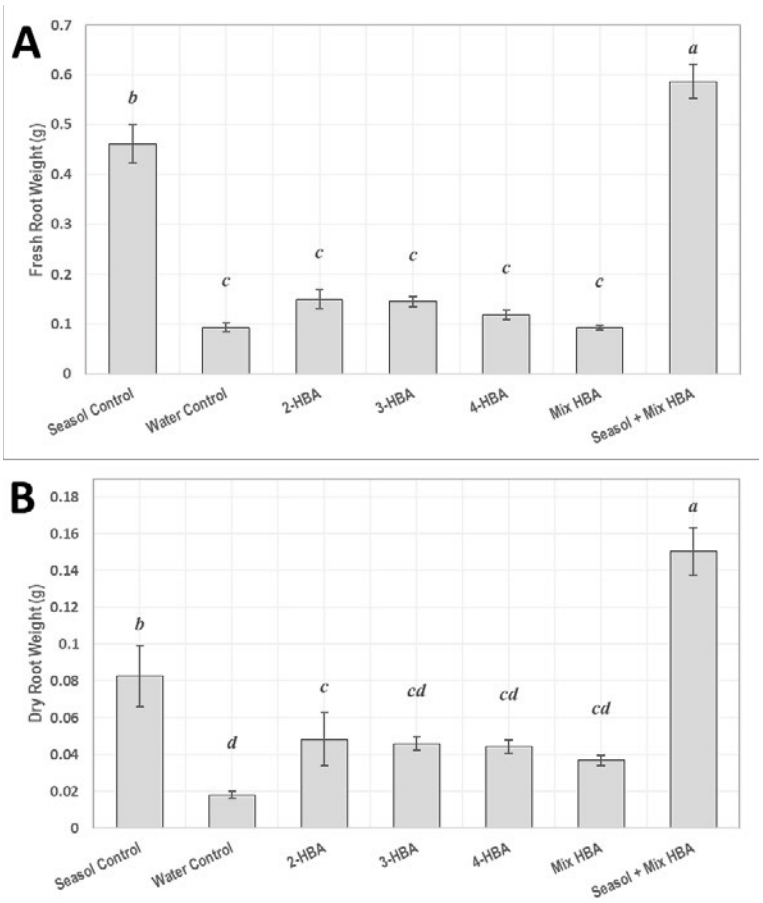
The positive control Treatment 1 produced the mean longest root length at 73.3 mm. Treatment 7 was not significantly different with a mean longest root length of 72.5 mm. Treatments 3 – 6 did not significantly differ from the negative control Treatment 2, with mean longest root lengths ranging from 2.8 – 9.7 mm.

5.3.2.2 Experiment 2 Root Weight Results

The fresh and dry root weight of each tomato plant was measured by the method described in Section 2.7.5.2. Table 5.3.2.2 presents the collated data with the mean fresh and dry root weight for each treatment along with the standard error of the means (n = 6); means denoted with the same letter do not differ significantly at $P < 0.05$.

29) **Table 5.3.2.2** Experiment 2 Root Weight Results

Treatment	Replicates	Mean Root Weight (g)	
		Fresh	Dry
T1) Seasol Control	6	0.46 ± 0.04^{b}	0.08 ± 0.02^{b}
T2) Water Control	6	0.09 ± 0.01^{c}	0.02 ± 0.00^{d}
T3) 2-HBA	6	0.15 ± 0.02^{c}	0.05 ± 0.01^{c}
T4) 3-HBA	6	0.15 ± 0.01^{c}	0.05 ± 0.00^{cd}
T5) 4-HBA	6	0.12 ± 0.01^{c}	0.04 ± 0.00^{cd}
T6) HBA Mix	6	0.09 ± 0.00^{c}	0.04 ± 0.00^{cd}
T7) Seasol + HBA Mix	6	0.59 ± 0.03^{a}	0.15 ± 0.01^{a}



50) **Figure 5.3.2.2** Tomato fresh root weight (A) and dry root weight (B) after 14 days treatment with Seasol control, Water control, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, a 2-, 3- & 4-hydroxybenzoic acid mix, and Seasol + 2-, 3- & 4-hydroxybenzoic acid mix. Error bars represent standard error of the means (n = 6). Means denoted with the same letter do not differ significantly at $P < 0.05$.

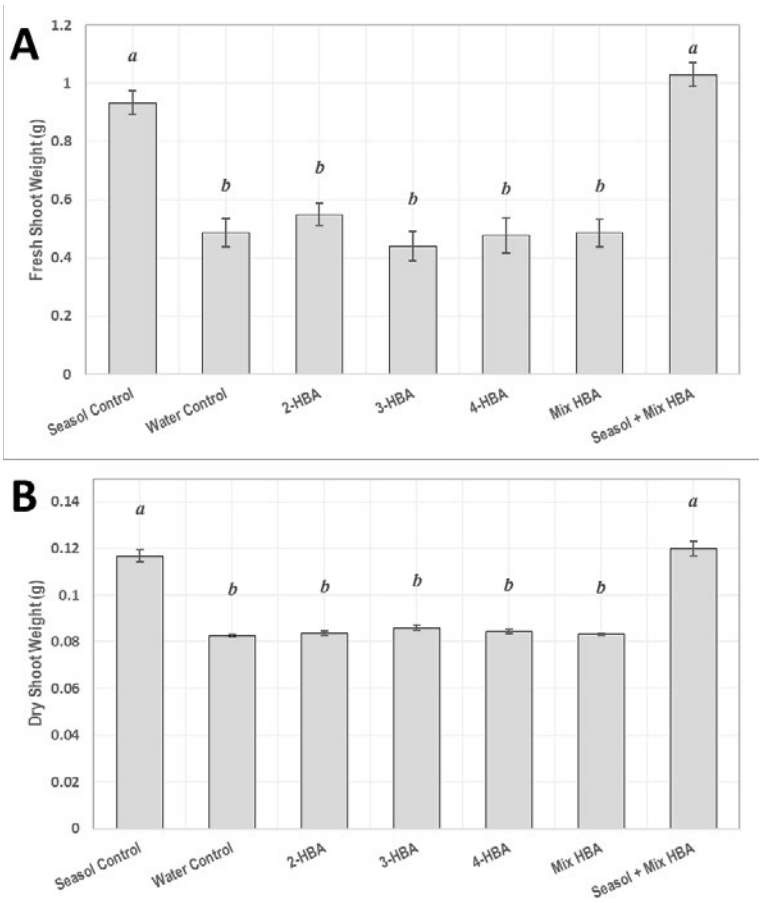
Treatment 7 produced the mean largest fresh root weight at 0.59 g which was statistically larger than the positive control Treatment 1 mean fresh root weight of 0.46 g. Treatment 1 was significantly different form the remaining Treatments 2 – 6, which were all statistically comparable with mean fresh root weights between 0.09 and 0.15 g. The dry root weight results were in reasonable agreement with the fresh root weight results. Treatment 7 again produced the largest mean dry root weight with 0.15 g and was significantly larger than the positive control Treatment 1 which had a mean dry root weight of 0.08 g. Treatment 3 composed of 2-hydroxybenzoic acid was the only remaining treatment that was statistically different from the negative control with a mean dry root weight of 0.05 g.

5.3.2.3 Experiment 2 Shoot Weight Results

The fresh and dry shoot weight of each tomato plant was measured by the method described in Section 2.7.5.3. Table 5.3.2.3 presents the collated data with the mean fresh and dry shoot weight for each treatment along with the standard error of the means (n = 6); means denoted with the same letter do not differ significantly at $P < 0.05$.

30) **Table 5.3.2.3** Experiment 2 Shoot Weight Results

Treatment	Replicates	Mean Shoot Weight (g)	
		Fresh	Dry
T1) Seasol Control	6	0.93 ± 0.04[a]	0.12 ± 0.00[a]
T2) Water Control	6	0.49 ± 0.05[b]	0.08 ± 0.00[b]
T3) 2-HBA	6	0.55 ± 0.04[b]	0.08 ± 0.00[b]
T4) 3-HBA	6	0.44 ± 0.05[b]	0.09 ± 0.00[b]
T5) 4-HBA	6	0.48 ± 0.06[b]	0.08 ± 0.00[b]
T6) HBA Mix	6	0.48 ± 0.05[b]	0.08 ± 0.00[b]
T7) Seasol + HBA Mix	6	1.03 ± 0.04[a]	0.12 ± 0.00[a]

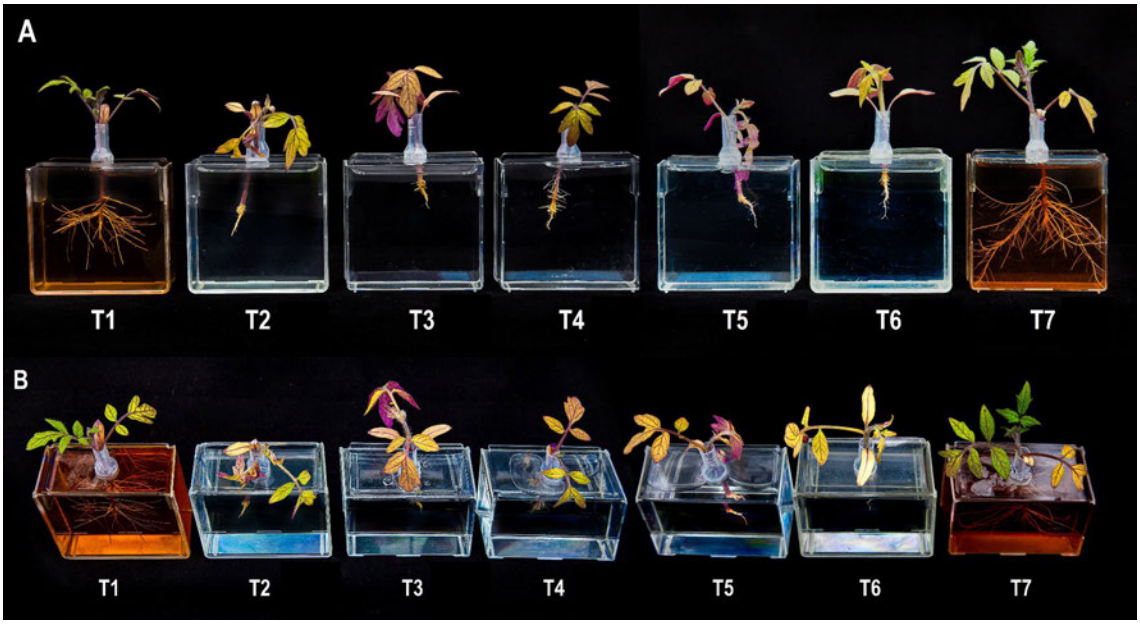


51) **Figure 5.3.2.3** Tomato fresh shoot weight (A) and dry shoot weight (B) after 14 days treatment with Seasol control, Water control, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, a 2-, 3- & 4-hydroxybenzoic acid mix, and Seasol + 2-, 3- & 4-hydroxybenzoic acid mix. Error bars represent standard error of the means (n = 6). Means denoted with the same letter do not differ significantly at $P < 0.05$.

Treatment 7 produced the largest mean fresh shoot weight with 1.03 g but did not differ significantly to the positive control Treatment 1 which produced a mean fresh shoot weight of 0.93 g. Treatments 2 – 6 were all statistically comparable with mean fresh shoot weights ranging from 0.44 – 0.55 g. The results for the dry shoot weight were in agreement with the fresh shoot weight, with Treatments 1 and 7 producing the largest mean dry shoot weights of 0.12 g each which were significantly larger than the other treatments. Treatments 2 – 6 were all statistically comparable with mean dry shoot weights ranging from 0.08 – 0.09 g.

5.3.3 Phenotypical Observations

To further supplement the quantitative data of the five plant growth parameters and to provide qualitative evidence for the effect of the various treatments on plant growth, photos of representative plants from each treatment were captured. These photos were captured on day 14 of Experiment 1 prior to quantitative data collection.



52) **Figure 5.3.3** Representative tomato plants after 14 days treatment with Seasol control (T1), Water control (T2), 2-hydroxybenzoic acid (T3), 3-hydroxybenzoic acid (T4), 4-hydroxybenzoic acid (T5), a 2-, 3- & 4-hydroxybenzoic acid mix (T6), and Seasol + 2-, 3- & 4-hydroxybenzoic acid mix (T7). A) Front-on view for observation of the root growth and, B) Top view for observation of shoot growth and health.

The plants in Figure 5.3.3 show significant differences in root growth as well as visual plant health. Upon observing the seven plants in the front-on view (A) it can be seen that the root growth is most prominent in Treatment 7, followed by Treatment 1, then Treatment 4, with the remaining treatments seemingly having no effect in the promotion of adventitious roots. When observing the plants in the top view (B) the plants in Treatments 2 – 6 all look severely discoloured and unhealthy. Treatments 1 and 7 however haven't discoloured to the same degree, with Treatment 7 showing new growth that appears to be in good health. Suggesting the enhanced development of the root system promotes and supports overall plant health.

5.4 DISCUSSION

The goal of this chapter was to determine the biological effects, on tomato seedlings, of salicylic acid and its two structural isomers (i) alone and (ii) in combination with the Seasol seaweed extract. Fresh and dry root and shoot weight data along with the longest root length data and phenotypical observations were used to evaluate the effects that different treatments had on tomato seedling growth and development. Two identical greenhouse experiments (with 6 replicates per treatment) were conducted to determine the effects of (i) individual aqueous monohydroxybenzoic acid solutions, (ii) an aqueous combination of all three monohydroxybenzoic acids, and (iii) fortifying the Seasol seaweed extract matrix with a combination of all three monohydroxybenzoic acids. The application rates of the monohydroxybenzoic acid treatment solutions (described above in Section 5.2) were based on the concentrations in the Seasol seaweed extract which were determined in Chapter 4.

The research in this study found:

(i) The main biological effect was a significant increase in tomato dry root weight when the monohydroxybenzoic acids were used in combination with the Seasol seaweed extract.

(ii) The treatments using aqueous monohydroxybenzoic acid solutions alone did not demonstrate consistent biological effects at application rates mirroring the respective concentrations in the Seasol seaweed extract.

(iii) Salicylic acid and its isomers in combination with the Seasol seaweed extract enhanced root weight growth, but no consistent effect on root length and shoot growth were observed.

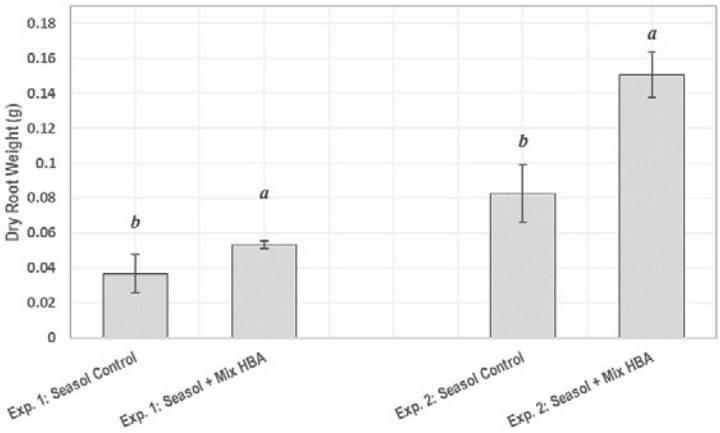
5.4.1 The Plant Growth Parameters

5.4.1.1 Fresh Root Weight

The individual aqueous monohydroxybenzoic acid solutions had no significant effect on fresh root weight in either experiment. In both experiments the only two treatments that differed significantly in fresh root weight from the water control were the Seasol control and the fortified Seasol. In Experiment 1 the Seasol control and the fortified Seasol were not significantly different however, in Experiment 2 the fortified Seasol produced plants with significantly larger fresh root mass. This suggests that fortification of the Seasol seaweed extract with a combination of the monohydroxybenzoic acids significantly enhanced root growth.

5.4.1.2 Dry Root Weight

Significant effects were observed for dry root weight when treated with aqueous hydroxybenzoic acid solutions; with the aqueous 3-hydroxybenzoic acid treatment in Experiment 1, and the 2-hydroxybenzoic acid treatment in Experiment 2 producing plants with a significantly larger dry root weight than the water control. In Experiment 1 the Seasol control, aqueous 3-hydroxybenzoic acid treatment and fortified Seasol produced plants with significantly larger dry root mass. Furthermore, the results of Experiment 1 suggest that fortification of the Seasol seaweed extract with the monohydroxybenzoic acids enhanced root growth when measured assessing dry root weight. Additionally in Experiment 1, the aqueous 3-hydroxybenzoic acid treatment was not significantly different from the Seasol control, demonstrating efficacy for root growth. Experiment 2 saw a repeat of the results observed for the Seasol control and fortified Seasol, whereby the fortified Seasol produced plants with significantly larger dry root mass, as shown below in Figure 5.4.1.2. In Experiment 2 the aqueous 2-hydroxybenzoic acid treatment produced plants that had significantly larger dry root mass than the water control. The dry root weight results across both experiments suggest that the fortification of the Seasol seaweed extract increased its root growth potential, but experimental results were inconsistent for the individual aqueous monohydroxybenzoic acid solutions as each experiment returned differences in the significance of the effects of 2- and 3-hydroxybenzoic acid.



53) **Figure 5.4.1.2** Tomato seedling dry root weight after 14 days treatment with Seasol control and Seasol + 2-, 3- & 4-hydroxybenzoic acid mix in Experiments 1 and 2. Error bars represent standard error of the means (n = 6). Means denoted with the same letter do not differ significantly at $P < 0.05$.

5.4.1.3 Longest Root Length

In both experiments the only two treatments that were statistically different from the water control were Treatments 1 and 7, the Seasol control and fortified Seasol, respectively. In Experiment 1 the Seasol control and the fortified Seasol were statistically different from one another, with the fortified Seasol producing significantly longer roots than the Seasol control. Additionally the longest root length recorded across both experiments was a plant being treated with the fortified Seasol in Experiment 1 with a longest root length of 119 mm. In Experiment 2 the Seasol control and fortified Seasol were not significantly different from one another. Both experiments demonstrate the efficacy of the Seasol seaweed extract for enhancing root growth, with the results from Experiment 1 suggesting that fortification of the Seasol seaweed extract with a mixture of the monohydroxybenzoic acids may significantly increase its root growth promoting effects.

5.4.1.4 Fresh and Dry Shoot Weight

The various treatments did not have a significant effect on the fresh and dry shoot weights. Experiment 1 returned variable results in which there was no significant difference in fresh or dry shoot mass between the Seasol control Treatment 1 and the water control Treatment 2. The largest observed fresh and dry shoot mass in Experiment 1 was the fortified Seasol. Experiment 2 returned results that better reflected the root growth observed, with the Seasol control and fortified Seasol treatments being statistically larger in both fresh and dry shoot mass than the water control Treatment 2. There was no significant difference between the Seasol control Treatment 1 and fortified Seasol Treatment 7 in fresh and dry shoot mass in Experiment 2. The fresh and dry shoot weight results failed to provide any significant evidence to demonstrate efficacy of any of the treatments containing monohydroxybenzoic acids that could be distinguished from the effects of the Seasol seaweed extract.

5.4.2 The Study and the Broader Scientific Literature

When comparing to other studies in the scientific literature the first critical point that needs to be addressed is the dosages employed throughout this study relative to the dosages used by others. The dosages employed in the studies listed in Table 1.7 in Chapter 1 ranged on average from 0.1 – 10 mM, which are far above the dosages employed in this study which were between 2 – 62 nM. Furthermore, in the review article by Koo *et al.* it is stated that doses above 2 mM are considered 'high' and they suggest that efficacy screening be conducted at concentrations in the range of micromolar to low millimolar [173]. The reasoning behind the low concentration range is due to inhibitory effects observed at high concentrations [57, 58, 173]. Phytochemicals like salicylic acid have a range of concentrations where

promoting effects are observed, above which the application can be inhibitory; these hormetic effects are why lower concentrations are recommended. A major consideration of the outcomes from this study is the very low dosages employed and therefore, the treatments where no significant efficacy was observed could still have potential and instead require further assessment of a wider range of concentrations.

5.4.2.1 Plant Efficacy of Monohydroxybenzoic Acids Alone

In this study both 2- and 3-hydrobenzoic acid showed some bioefficacy alone, with increased dry root weight observed for each compound. This was not repeatable though as 2-hydroxybenzoic acid increase dry root weight in Experiment 2 but not in Experiment 1, and vice versa for 3-hydroxybenzoic acid.

There is significant evidence to support salicylic acid's (2-hydroxybenzoic acid) role in plant growth and development, it is does however have promoting or inhibiting effects when applied exogenously depending on its concentration, the plant growth conditions and developmental stages [58, 60, 173]. In Experiment 2 treatment with 2 nM aqueous salicylic acid saw a significant increase in root growth when assessing dry root weight when compared to the water control. Whilst this result was not observed in both experiments, this result does support other studies where the exogenous application of salicylic acid promoted plant growth and development [57, 58, 176, 190, 267-269]. As previously stated the concentration of the treatment containing salicylic acid that elicited a significant root growth promoting response in Experiment 2 is lower than the levels used in most other studies; therefore, further investigation with a wider range of concentrations would be recommended to assess optimal levels for plant growth promotion. Additionally, investigating a wide range of concentrations could lead to the observation of the hormetic effects that are reported in the literature; as there are reports of high levels of salicylic acid being detrimental to plant growth and development [57, 58, 173]. An example of salicylic acid's hormetic effects with respects to root growth is a study conducted by Pasternak *et al.*, in which they demonstrated that salicylic acid influenced *Arabidopsis* root growth in a concentration-dependent manner [270]. Pasternak *et al.* found that treatment with salicylic acid below 50 μM promoted adventitious roots whereas treatments above 50 μM inhibited all growth processes, proposing that low-concentration of salicylic acid plays an important role in root meristem structure [270].

The increase in root growth observed following treatment with 3-hydroxybenzoic acid in Experiment 1 provides significant interest owing to it being the monohydroxybenzoic acid that is least explored [68, 242]. Furthermore the observed increase in dry root weight was not only significantly different from the water control, but was statistically comparable to the Seasol control treatment being used as the experimental positive control. Very little information is available regarding the assessment of bioefficacy of 3-hydroxybenzoic acid in crops of agronomic significance. One study investigating the autotoxicity of root exudates from taro assessed the allelopathic potential for 3-hydroxybenzoic acid and found no significant inhibition of taro plantlet growth [271]. That same research group investigated autotoxic substances in eight leafy vegetables, finding that plants treated with 3-hydroxybenzoic acid exhibited significantly inhibited growth in two out of the eight vegetable species [272].

A study investigating the growth stimulation/inhibition effects of 4-hydroxybenzoic acid on a freshwater green alga also investigated 3-hydroxybenzoic acid, they observed that 3-hydroxybenzoic acid stimulated algal growth weakly but not to a statistically significant extent [72]. This study highlights an important and often underestimated notion for efficacy studies, that the complexity and sophisticated balancing act of the growth and immunity pathways requires synergistic effects [173]. Various phytochemicals working in harmony/disharmony are often required to provide the optimal response to external stimuli. For this reason biological efficacy studies sometimes include treatments that involve a combination of different phytohormones alongside individual treatments [57, 61, 186]. A study investigating the chilling tolerance in cold-stored lemons assessed both methyl jasmonate and salicylic acid individually and in combination for their effects on improving the chilling tolerance, finding that a combination of the two offered the most significant improvements [188]. To investigate salicylic acid's role in root growth it has been paired with both hydrogen peroxide and indoleacetic acid (IAA), finding synergistic effects in regulating adventitious rooting [273].

5.4.2.2 Plant Efficacy of Monohydroxybenzoic Acids in Combination with Seasol Seaweed Extract

The most important outcome from this study is that tomato root growth (measured as root weight) was significantly increased when the Seasol seaweed extract was fortified with a combination of three similarly structured monohydroxybenzoic acids: salicylic acid (2-hydroxybenzoic acid), 3-hydroxybenzoic acid and 4-hydroxybenzoic acid.

Treatment 7 comprised of the 1:400 dilution of Seasol seaweed extract (Seasol Commercial Concentrate) fortified with a combination of all three monohydroxybenzoic acids had the most significant results of all treatments investigated. However, the results produced from treatment with the fortified Seasol for longest root length and fresh root weight were not repeated across both experiments. Consequently, the dry root weight is the recommended assessment for further testing based on concentrations identified in the Seasol seaweed extract. The variability in fresh weight is not uncommon as Huang *et al.* report that fresh weight determination is less reliable and instead recommend dry weight determination be used for plant growth promotion tests [206].

The increase in root biomass is an important plant growth parameter to assess as increased root biomass is associated with enhanced uptake and accumulation of water and important nutrients such as nitrogen and sulphur [15]. This improves plant growth overall, enhances the nutritional value of food crops, and can increase the efficiency of nutrient/water usage in plants which is critical for crops in dealing with the worsening environmental stresses such as drought [1, 15]. Seaweed biostimulants have been shown to increase root biomass both on their own and in combination with other additives such as N-P-K fertilizer and humic acid [15, 24, 30]. The combination of seaweed extracts and salicylic acid has also been explored, with several studies investigating the use of seaweed extracts and salicylic acid individually and in combination finding that a combination had the most significant effects [264-266]. The results in the study conducted by El-Kareem and El-Rahman on the response of Ruby seedless grapevines to foliar application share similarities with the observations made in this study. In that study the combination of a seaweed extract (*A.nodosum*) with salicylic acid and roselle extract stimulated main shoot length, leaf area and percentages of N, P and K in the leaves [265].

The plant growth benefits of seaweed extracts/biostimulants are extensively reported in the scientific literature. It is established that their physiological responses are not simply due to nutrient fertilization, but rather the complex compositions of seaweed extracts which includes a diverse range of biologically active constituents such as phytohormones [1, 15, 23, 24, 26, 27]. The Seasol seaweed extract control is expected to produce significant plant growth, particularly root growth in the bioassay and the hypothesis was that one or potentially all of the monohydroxybenzoic acids may have a role in the observed benefits. Interestingly, there was no available literature investigating the effects of a combination of all three monohydroxybenzoic acid in combination with seaweeds extracts on plant growth, making this proof-of-concept study a significant first. This study set out to investigate the role of monohydroxybenzoic acid's role in the biological effects of seaweed extracts and found that the fortification of the seaweed

extract with a combination of the monohydroxybenzoic acids had a significant effect on root growth when measuring dry root weight. These results point towards a potentially novel mechanism involving the synergy of all three monohydroxybenzoic acids in combination, possibly with further unknown constituents in the seaweed extract matrix. Therefore it is possible to identify and select for specific combinations of synergistic molecules in order to achieve a desired phenotypical outcome.

Trials using fractions of seaweed extracts have reported that no single fraction was able to replicate all of the effects observed when the original seaweed extract was used [274]. The combined effect of a complex seaweed extract is more than the sum of their independent effects [13, 274]. This school of thought is not just prominent within the field of biostimulants and sustainable agricultural practices but also in many other fields including the treatment of human diseases; where single compound antibiotics are becoming problematic due to microbial resistance and investigations into combinations of phytochemicals and their synergistic antimicrobial activity represent a possible source of effective and safe antimicrobial agents [16].

5.4 CONCLUSION

The results from this study indicate that the monohydroxybenzoic acids play a biological role in the effects of seaweed extracts on plant growth, potentially through a novel mechanism. Made possible by the analytical research conducted in Chapters 3 and 4, this plant growth study developed using the quantitative data from Chapter 4 excitingly provides a significant foundation for the further investigation into the benefits of seaweed biostimulants and some of their important biologically active constituents, hydroxybenzoic acids.

The observations of synergistic effects of the monohydroxybenzoic acids in combination with the Seasol seaweed extract significantly enhancing tomato seedling root growth is an important step forward in our understanding of crop management, providing further evidence and insights for the use of seaweed biostimulants as a sustainable agricultural practice. The synergistic effects of seaweed extracts as biostimulants is one of their greatest attributes, given that crops are often faced with multiple/combined abiotic and biotic constraints which are only forecast to increase in severity owing to climate change. Consequently, as Rouphael and Colla state: "research on the potential synergistic effects among plant biostimulants should be at the core of future efforts in addressing global food security, complimented by sustainable and optimised use of nutrients" [275].

It is recommended that the further investigation of the role of monohydroxybenzoic acids in seaweed extracts aim to assess the impacts of fortifying the Seasol seaweed extract with individual monohydroxybenzoic acids, and at different dosages. The further testing could include other seaweed extracts with different combinations of the monohydroxybenzoic acids. Given the research conducted in Chapter 3 alluded to the possibility of a wide variety of similar compounds being present in the seaweed biostimulants it is also recommended that further analytical research be conducted to provide the required quantitative data to develop future plant bioassays. If the ultimate goal is to optimise the plant's growth-immunity balance to maximise both yield and immune resilience, then this research highlights that a combination of plant protector molecules like salicylic acid with seaweed biostimulants could be biologically efficacious. Additionally, synergistic combinations of hydroxybenzoic acids and important plant protector/growth-promoting molecules at concentrations found in seaweed biostimulants could be an emerging concept to improve crop productivity; demonstrating the need for further research.

The chapter's key observations, significant findings and future research prospects are further discussed in Chapter 6.

CHAPTER 6: SUMMARY AND FURTHER WORK

6.1 GENERAL CONCLUSION

The chemical analysis of the commercial seaweed biostimulant investigated in this study confirmed the presence of important hydroxybenzoic acids and other related derivatives. These include the monohydroxybenzoic acids, two dihydroxybenzoic acids, syringic acid and anthranilic acid. The identification of these important phytochemicals in a commercial seaweed biostimulant further characterises biologically active constituents present within the complex seaweed extracts. Quantitative determinations of the monohydroxybenzoic acids provided the data necessary for the investigation into their biological role in seaweed biostimulants. Preliminary results from which revealed that they may synergistically effect plant growth through a potentially novel mechanism. This research provides insights into the applicability of seaweed biostimulants and their biologically active constituents for the optimisation of plant growth and stress tolerance; consequently increasing seaweed biostimulants' contribution to improving global agricultural productivity in order to meet growing food demands. The three major discoveries from this research and to the best of our knowledge reported for the first time are:

- the presence of 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid and 2-aminobenzoic acid in a commercial seaweed biostimulant (Chapter 3)
- the quantitative determination of salicylic acid (2-hydroxybenzoic acid) and its isomers (3- and 4-hydroxybenzoic acid) in a commercial seaweed biostimulant (Chapter 4)
- synergistic effects of salicylic acid and its isomers in combination with a commercial seaweed biostimulant significantly enhancing tomato seedling root growth (Chapter 5)

6.2 CHAPTER OUTCOMES AND KNOWLEDGE CONTRIBUTION

6.2.1 Chapter 3 – Method Development for the Targeted Metabolomic HPLC-ESI-MS/MS Qualitative Analysis of Hydroxybenzoic Acids and Related Derivatives in a Commercial Seaweed Biostimulant.

A preliminary HPLC-ESI-MS/MS method was developed for the qualitative analysis of monohydroxybenzoic acids in a commercial seaweed biostimulant that allowed for separation and identification, using retention time matching and MS^2 spectra matching from product ion scanning data. The method developed demonstrated the power of combining HPLC with MS/MS analysis for qualitative investigations of important phytochemicals in a commercial seaweed biostimulant. The significant finding of this part of the study was:

- All three monohydroxybenzoic acid isomers were successfully characterised in the commercial seaweed biostimulant

The success of the preliminary method for the monohydroxybenzoic acids led to the development and optimisation of HPLC-ESI-MS/MS methods for the analysis of hydroxybenzoic acids and related derivatives in a commercial seaweed biostimulant. This investigation was in two parts (i) the investigation into the optimal MS/MS conditions for the analysis of the various derivatives and (ii) the investigation into the chromatographic behaviour of the various derivatives on both C18 and the less explored biphenyl reversed phase stationary phases. The different retention mechanisms of the biphenyl column showed great potential for the separation of these aromatic compounds when using protic/methanol based mobile phases. This was best highlighted by the successful separation of all six dihydroxybenzoic acid isomers in under 6 minutes with good resolution, believed to be the first reported separation of all six on any reversed phase column. The MS/MS study delivered the necessary MRM data for the various derivatives including:

- A unique positive ionisation mode MRM transitions of m/z 213 → 154 was successfully employed for the analysis of eudesmic acid

The novel reversed phase system employing the biphenyl stationary phase with methanol/water/formic mobile phases produced a number of significant findings, including:

- Separation of all three monohydroxybenzoic acids on a biphenyl stationary phase using a methanol/water/formic mobile phase
- All six dihydroxybenzoic acids were separated in a single chromatographic run

- All six dihydroxybenzoic acids were separated on a biphenyl stationary phase using a methanol/water/formic mobile phase
- Three trihydroxybenzoic acids were separated on a biphenyl stationary phase using a methanol/water/formic mobile phase

The preliminary qualitative investigation of hydroxybenzoic acids and related derivatives in a commercial seaweed biostimulant using the HPLC-ESI-MS/MS methods developed and optimised in this study positively identified pyrocatechuic acid, protocatechuic acid, syringic acid and vanillic acid. Additionally, there were a number of peaks in the sample chromatogram that shared characteristic MRM transitions, indicating the presence of other related and potentially novel derivatives. The sample chromatograms also alluded to the presence of matrix effects via ionisation suppression which require further characterisation if future research hopes to develop qualitative and quantitative methods for the analysis of other important phytochemicals in these matrices. The significant findings of this part of the study were:

- The tentative identification of 2,3-dihydroxybenzoic acid, 3-4,dihydroxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid and 2-aminobenzoic acid in a commercial seaweed biostimulant. This commercial seaweed biostimulant is prepared from *D.potatorum* and *A.nodosum* and therefore:
    - The first reported identification of 2,3-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid and 2-aminobenzoic acid in a seaweed extract prepared from *D.potatorum*
    - The first reported identification of 2,3-dihydroxybenzoic acid and 2-aminobenzoic acid in a seaweed extract prepared from *A.nodosum*

6.2.2 Chapter 4 – Method Optimisation and Validation for the Targeted Metabolomic HPLC-ESI-MS/MS Quantitative Analysis of Monohydroxybenzoic Acids in a Commercial Seaweed Biostimulant.

A novel sample preparation method employing the acidified acetonitrile partitioning step from Quechers methodology along with mixed-mode reversed phase and strong anion-exchange SPE was successfully adapted and optimised for the selective extraction and preconcentration of the monohydroxybenzoic acids. The methodology for the mixed-mode SPE utilised a unique 'flipping' of

the target analytes on column by taking advantage of their acid-base chemistry, achieved by varying the pH of the wash solutions.

The newly developed quantitative method was partially validated following NATA protocols. The following parameters were evaluated for each monohydroxybenzoic acid: linearity, instrument repeatability, method repeatability, instrument limit-of-detection (IDL), instrument limit-of-quantitation (IQL), matrix effects and method recovery. The method was found to be fit-for-purpose with all evaluated parameters being deemed suitable for the analysis of all three monohydroxybenzoic acids, with method recoveries of 73.5, 75.0 and 87.3 % for 2-, 3- and 4-hydroxybenzoic acid, respectively. Significant matrix effects were observed for the analysis of 2-hydroxybenzoic acid which were compensated for through the use of standard addition calibration.

The fit-for-purpose quantitative method was used to determine the concentrations of 2-, 3- and 4-hydroxybenzoic acid in a commercial seaweed biostimulant and these were found to be 137, 3409 and 1748 μg/L, respectively. This is believed to be the first time that all three monohydroxybenzoic acids had been detected and quantitatively determined in a commercial seaweed biostimulant. Furthermore, it is also believed to be the first reporting of salicylic acid and 3-hydroxybenzoic acid in an extract of the seaweeds species *D.potatorum* and *A.nodosum*, and 4-hydroxybenzoic acid in *D.potatorum*. The quantitative data was crucial for the development of the plant growth bioassays conducted in Chapter 5, which used dosages relative to the concentrations determined in the commercial seaweed biostimulant. The significant findings of this part of the study were:

- The detection and quantitative determination of 2-hydroxybenzoic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid in a commercial seaweed biostimulant. This commercial seaweed biostimulant is prepared from *D.potatorum* and *A.nodosum* and therefore:
    - The first reported detection and quantitative determination of 2-hydroxybenzoic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid in a seaweed extract prepared from *D.potatorum*
    - The first reported detection and quantitative determination of 2-hydroxybenzoic acid, and 3-hydroxybenzoic acid in a seaweed extract prepared from *A.nodosum*

6.2.3 Chapter 5 – The Biological Role of Salicylic Acid and its Isomers in Seaweed Extracts

The impact of salicylic acid and its isomers on plant growth was assessed using a liquid-based plant growth bioassay in two independent experiments. The treatments using aqueous monohydroxybenzoic

acid solutions alone and in combination did not demonstrate consistent biological effects at concentrations similar to the seaweed biostimulant. Both 2-hydroxybenzoic acid and 3-hydroxybenzoic acid produced significant increases in dry root weight, however these results were not repeatable across both experiments.

The fortification of the Seasol seaweed extract with a combination of the monohydroxybenzoic acids enhanced root weight growth, however no consistent effect was observed on root length or shoot growth. The statistically significant increase in root growth as measured by the increase in dry root weight from the fortification of the Seasol seaweed extract was the most important outcome from the bioassay experiments. This is the first report of all three monohydroxybenzoic acids being tested for synergistic effects on plant growth, and the results point towards a potentially novel mechanism involving all three monohydroxybenzoic acids, possibly with other unknown constituents of the complex seaweed biostimulant matrix. The significant finding of this part of the study was:

- Fortification of the Seasol seaweed extract with a combination of the monohydroxybenzoic acids significantly enhanced root growth of tomato seedlings
    - The results suggest a potentially novel synergistic mechanism involving the monohydroxybenzoic acids and Seasol seaweed extract matrix for improve plant root growth

6.3 RECOMMENDATIONS FOR FUTURE RESEARCH

With respect to future research, a number of interesting observations and results have been identified as needing further investigation:

- A natural extension to the qualitative study is further mass spectrometric confirmation of the peaks identified through retention time matching. Identification of more characteristic fragments and mass spectral matching of the sample peaks with pure reference compounds would provide more supporting evidence for their presence.
- Additionally, to help with the identification of the unidentified peaks in the sample chromatograms, it would be advantageous to acquire more reference standards of similar derivatives of agricultural importance (for example isovanillic acid).
- Other work could include the characterisation of the unidentified peaks in the preliminary qualitative analysis sample chromatograms using an untargeted metabolomic approach. It is possible that some of those unidentified peaks could be novel compounds so an untargeted analysis approach could help to provide enough structural information for a tentative identification; following which isolation from the sample and supporting confirmative structural elucidation by NMR would be highly valuable.
- Where necessary, for example the trihydroxybenzoic acids, further optimisation of the chromatography in the sample would help with the separation and identification of closely eluting peaks. This would include further investigation and optimisation using the biphenyl stationary phase which was shown to have great potential for these types of compounds.
- It would be advantageous to explore the possibility of examining the seaweed species individually to investigate the presence of these derivatives endogenously, as the compounds identified may be degradation products that result from biostimulant manufacturing processes. The chromatographic methods developed in this study provide as excellent foundation to the analysis of the seaweed species and one would postulate that the sample preparatory procedures would need the most attention to investigate the seaweed materials.
- If any of the identified derivatives were to be investigated for their biological activity and/or role in the seaweed biostimulants bioactivity in improving plant growth and/or stress tolerance then quantitative data would be required. The qualitative methods developed serve as ideal foundations to develop quantitative methods, which could be tested using the same method validation procedure employed in this study.
- The quantitative method for the analysis of the monohydroxybenzoic acids could be used for batch analysis of the biostimulants to assess variability could be a useful tool for identifying

natural ways to increase the concentration of the monohydroxybenzoic acids in the seaweed biostimulants, which may help to optimise their impact on agricultural productivity.

- An obvious aim of further work following on from the plant growth bioassay study is to investigation the impacts of fortifying the seaweed biostimulant with individual monohydroxybenzoic acids. Furthermore, similar experiments should be conducted at various dosages in an attempt to identify an optimal dosage or range.
- Quantitative data for the other identified derivatives would allow for the investigation into their biological role in the seaweed biostimulant using the same bioassay methodology.
- The synergistic effects observed in the bioassay study highlight the need for further research characterising and profiling the biologically active components of the seaweed biostimulants, possibly allowing for the selection of specific combinations of molecules that act synergistically to provide a desirable phenotypical response.

REFERENCES

1. Arioli, T.; Mattner, S. W.; Winberg, P. C., Applications of seaweed extracts in Australian agriculture: past, present and future. *Journal of Applied Phycology* **2015,** *27* (5), 2007-2015.
2. Talukder, B.; Blay-Palmer, A.; vanLoon, G. W.; Hipel, K. W., Towards Complexity of Agricultural Sustainability Assessment: Main Issues and Concerns. *Environmental and Sustainability Indicators* **2020**, 100038.
3. Anderson, R.; Bayer, P. E.; Edwards, D., Climate change and the need for agricultural adaptation. *Current Opinion in Plant Biology* **2020**.
4. Adegbeye, M. J.; Ravi Kanth Reddy, P.; Obaisi, A. I.; Elghandour, M. M. M. Y.; Oyebamiji, K. J.; Salem, A. Z. M.; Morakinyo-Fasipe, O. T.; Cipriano-Salazar, M.; Camacho-Díaz, L. M., Sustainable agriculture options for production, greenhouse gasses and pollution alleviation, and nutrient recycling in emerging and transitional nations - An overview. *Journal of Cleaner Production* **2020,** *242*, 118319.
5. Foguesatto, C. R.; Borges, J. A. R.; Machado, J. A. D., A review and some reflections on farmers' adoption of sustainable agricultural practices worldwide. *Science of The Total Environment* **2020**, 138831.
6. Armanda, D. T.; Guinée, J. B.; Tukker, A., The second green revolution: Innovative urban agriculture's contribution to food security and sustainability – A review. *Global Food Security* **2019,** *22*, 13-24.
7. Kopittke, P. M.; Menzies, N. W.; Wang, P.; McKenna, B. A.; Lombi, E., Soil and the intensification of agriculture for global food security. *Environment International* **2019,** *132*, 105078.
8. Kour, D.; Rana, K. L.; Yadav, A. N.; Yadav, N.; Kumar, M.; Kumar, V.; Vyas, P.; Dhaliwal, H. S.; Saxena, A. K., Microbial biofertilizers: Bioresources and eco-friendly technologies for agricultural and environmental sustainability. *Biocatalysis and Agricultural Biotechnology* **2020,** *23*, 101487.
9. Leong, W.-H.; Teh, S.-Y.; Hossain, M. M.; Nadarajaw, T.; Zabidi-Hussin, Z.; Chin, S.-Y.; Lai, K.-S.; Lim, S.-H. E., Application, monitoring and adverse effects in pesticide use: The importance of reinforcement of Good Agricultural Practices (GAPs). *Journal of Environmental Management* **2020,** *260*, 109987.
10. Lebeis, S. L.; Paredes, S. H.; Lundberg, D. S.; Breakfield, N.; Gehring, J.; McDonald, M.; Malfatti, S.; Glavina del Rio, T.; Jones, C. D.; Tringe, S. G.; Dangl, J. L., PLANT MICROBIOME. Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* **2015,** *349* (6250), 860-4.
11. Bulgari, R.; Franzoni, G.; Ferrante, A., Biostimulants application in horticultural crops under abiotic stress conditions. *Agronomy* **2019,** *9* (6), 306.
12. Lindsey, A. P. J.; Murugan, S.; Renitta, R. E., Microbial disease management in agriculture: Current status and future prospects. *Biocatalysis and Agricultural Biotechnology* **2020,** *23*, 101468.
13. Yakhin, O. I.; Lubyanov, A. A.; Yakhin, I. A.; Brown, P. H., Biostimulants in plant science: a global perspective. *Frontiers in plant science* **2017,** *7*, 2049.
14. du Jardin, P., Plant biostimulants: Definition, concept, main categories and regulation. *Scientia Horticulturae* **2015,** *196*, 3-14.
15. Battacharyya, D.; Babgohari, M. Z.; Rathor, P.; Prithiviraj, B., Seaweed extracts as biostimulants in horticulture. *Scientia Horticulturae* **2015,** *196*, 39-48.
16. Barbieri, R.; Coppo, E.; Marchese, A.; Daglia, M.; Sobarzo-Sánchez, E.; Nabavi, S. F.; Nabavi, S. M., Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity. *Microbiological Research* **2017,** *196*, 44-68.
17. Barman, K.; Sharma, S.; Kumari, P.; Siddiqui, M. W., Salicylic Acid. In *Postharvest Management Approaches for Maintaining Quality of Fresh Produce*, Siddiqui, W. M.; Ayala Zavala, F. J.; Hwang, C.-A., Eds. Springer International Publishing: Cham, 2016; pp 51-68.
18. Kaur, H.; Chahal, S.; Jha, P.; Pandey, D. K.; Arencibia, A. D.; Kumar, V., Chapter 2 - Biostimulants, the cinderella for plant development. In *Biostimulants for Crops from Seed Germination to Plant Development*, Gupta, S.; Van Staden, J., Eds. Academic Press: 2021; pp 61-72.

19. Wong, W.; Tan, S.; Ge, L.; Chen, X.; Yong, J., The importance of phytohormones and microbes in biofertilizers. In *Bacterial metabolites in sustainable agroecosystem*, Springer: 2015; pp 105-158.
20. Del Buono, D., Can biostimulants be used to mitigate the effect of anthropogenic climate change on agriculture? It is time to respond. *Science of The Total Environment* **2021,** *751*, 141763.
21. Shukla, P. S.; Mantin, E. G.; Adil, M.; Bajpai, S.; Critchley, A. T.; Prithiviraj, B., Ascophyllum nodosum-based biostimulants: Sustainable applications in agriculture for the stimulation of plant growth, stress tolerance, and disease management. *Frontiers in plant science* **2019,** *10*, 655.
22. Hamed, S. M.; Abd El-Rhman, A. A.; Abdel-Raouf, N.; Ibraheem, I. B. M., Role of marine macroalgae in plant protection & improvement for sustainable agriculture technology. *Beni-Suef University Journal of Basic and Applied Sciences* **2018,** *7* (1), 104-110.
23. Kapoore, R. V.; Wood, E. E.; Llewellyn, C. A., Algae biostimulants: A critical look at microalgal biostimulants for sustainable agricultural practices. *Biotechnology Advances* **2021,** *49*, 107754.
24. Sangha, J.; Kelloway, S.; Critchley, A.; Prithiviraj, B., *Seaweeds (Macroalgae) and Their Extracts as Contributors of Plant Productivity and Quality: The Current Status of Our Understanding*. 2014.
25. Balboa, E. M.; Conde, E.; Moure, A.; Falqué, E.; Domínguez, H., In vitro antioxidant properties of crude extracts and compounds from brown algae. *Food Chemistry* **2013,** *138* (2–3), 1764-1785.
26. Craigie, J. S., Seaweed extract stimuli in plant science and agriculture. *Journal of Applied Phycology* **2011,** *23* (3), 371-393.
27. Nabti, E.; Jha, B.; Hartmann, A., Impact of seaweeds on agricultural crop production as biofertilizer. *International Journal of Environmental Science and Technology* **2016**, 1-16.
28. Gupta, S.; Kulkarni, M. G.; White, J. F.; Stirk, W. A.; Papenfus, H. B.; Doležal, K.; Ördög, V.; Norrie, J.; Critchley, A. T.; Van Staden, J., Chapter 1 - Categories of various plant biostimulants – mode of application and shelf-life. In *Biostimulants for Crops from Seed Germination to Plant Development*, Gupta, S.; Van Staden, J., Eds. Academic Press: 2021; pp 1-60.
29. Mattner, S. W.; Wite, D.; Riches, D. A.; Porter, I. J.; Arioli, T., The effect of kelp extract on seedling establishment of broccoli on contrasting soil types in southern Victoria, Australia. *Biological Agriculture & Horticulture* **2013,** *29* (4), 258-270.
30. Yusuf, R.; Kristianse, P.; Warwick, N., Effect of Two Seaweed Products and Equivalent Mineral Treatments on Lettuce (Lactuca sativa L.) Growth. *Journal of Agronomy* **2019,** *18*, 100-106.
31. Elansary, H. O.; Skalicka-Woźniak, K.; King, I. W., Enhancing stress growth traits as well as phytochemical and antioxidant contents of Spiraea and Pittosporum under seaweed extract treatments. *Plant Physiology and Biochemistry* **2016**.
32. Jayaraman, J.; Norrie, J.; Punja, Z. K., Commercial extract from the brown seaweed Ascophyllum nodosum reduces fungal diseases in greenhouse cucumber. *Journal of Applied Phycology* **2011,** *23* (3), 353-361.
33. Jayaraman, J.; Wan, A.; Rahman, M.; Punja, Z., Seaweed extract reduces foliar fungal diseases on carrot. *Crop Protection* **2008,** *27* (10), 1360-1366.
34. Saharan, B.; Nehra, V., Plant growth promoting rhizobacteria: a critical review. *Life Sci Med Res* **2011,** *21* (1), 30.
35. Zhai, R., Effects of the brown seaweed, Ascophyllum nodosum, on the nodulation and growth of alfalfa. **2013**.
36. Huang, Y.; Xiao, D.; Burton-Freeman, B. M.; Edirisinghe, I., Chemical Changes of Bioactive Phytochemicals during Thermal Processing. In *Reference Module in Food Science*, Elsevier: 2016.
37. Bayir, A. G.; Kiziltan, H. S.; Kocyigit, A., Chapter 1 - Plant Family, Carvacrol, and Putative Protection in Gastric Cancer. In *Dietary Interventions in Gastrointestinal Diseases*, Watson, R. R.; Preedy, V. R., Eds. Academic Press: 2019; pp 3-18.
38. Patra, B.; Schluttenhofer, C.; Wu, Y.; Pattanaik, S.; Yuan, L., Transcriptional regulation of secondary metabolite biosynthesis in plants. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* **2013,** *1829* (11), 1236-1247.

39. Vieira da Silva, B.; Barreira, J. C. M.; Oliveira, M. B. P. P., Natural phytochemicals and probiotics as bioactive ingredients for functional foods: Extraction, biochemistry and protected-delivery technologies. *Trends in Food Science & Technology* **2016,** *50*, 144-158.
40. Jamwal, K.; Bhattacharya, S.; Puri, S., Plant growth regulator mediated consequences of secondary metabolites in medicinal plants. *Journal of Applied Research on Medicinal and Aromatic Plants* **2018**.
41. Mori, I. C.; Ikeda, Y.; Matsuura, T.; Hirayama, T.; Mikami, K., Phytohormones in red seaweeds: A technical review of methods for analysis and a consideration of genomic data. *Botanica Marina* **2017,** *60* (2), 153-170.
42. Li, Y.; Kong, D.; Fu, Y.; Sussman, M. R.; Wu, H., The effect of developmental and environmental factors on secondary metabolites in medicinal plants. *Plant Physiology and Biochemistry* **2020,** *148*, 80-89.
43. Sanchita; Sharma, A., Chapter 23 - Gene Expression Analysis in Medicinal Plants Under Abiotic Stress Conditions. In *Plant Metabolites and Regulation Under Environmental Stress*, Ahmad, P.; Ahanger, M. A.; Singh, V. P.; Tripathi, D. K.; Alam, P.; Alyemeni, M. N., Eds. Academic Press: 2018; pp 407-414.
44. Martinez, K. B.; Mackert, J. D.; McIntosh, M. K., Chapter 18 - Polyphenols and Intestinal Health. In *Nutrition and Functional Foods for Healthy Aging*, Watson, R. R., Ed. Academic Press: 2017; pp 191-210.
45. Rashmi, H. B.; Negi, P. S., Phenolic acids from vegetables: A review on processing stability and health benefits. *Food Research International* **2020,** *136*, 109298.
46. Kumar, N.; Goel, N., Phenolic acids: Natural versatile molecules with promising therapeutic applications. *Biotechnology Reports* **2019,** *24*, e00370.
47. Heleno, S. A.; Martins, A.; Queiroz, M. J. R. P.; Ferreira, I. C. F. R., Bioactivity of phenolic acids: Metabolites versus parent compounds: A review. *Food Chemistry* **2015,** *173*, 501-513.
48. Mahajan, M.; Kuiry, R.; Pal, P. K., Understanding the consequence of environmental stress for accumulation of secondary metabolites in medicinal and aromatic plants. *Journal of Applied Research on Medicinal and Aromatic Plants* **2020,** *18*, 100255.
49. Kurhekar, J. V., Chapter 17 - Antimicrobial lead compounds from marine plants. In *Phytochemicals as Lead Compounds for New Drug Discovery*, Egbuna, C.; Kumar, S.; Ifemeje, J. C.; Ezzat, S. M.; Kaliyaperumal, S., Eds. Elsevier: 2020; pp 257-274.
50. Anantharaju, P. G.; Gowda, P. C.; Vimalambike, M. G.; Madhunapantula, S. V., An overview on the role of dietary phenolics for the treatment of cancers. *Nutrition Journal* **2016,** *15* (1), 99.
51. Shahidi, F.; Ambigaipalan, P., Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. *Journal of functional foods* **2015,** *18*, 820-897.
52. Francenia Santos-Sánchez, N.; Salas-Coronado, R.; Hernández-Carlos, B.; Villanueva-Cañongo, C., Shikimic Acid Pathway in Biosynthesis of Phenolic Compounds. In *Plant Physiological Aspects of Phenolic Compounds*, 2019.
53. Mustafa, N.; Verpoorte, R., Chorismate derived C6C1 compounds in plants. *Planta* **2005,** *222*, 1-5.
54. Widhalm, Joshua R.; Dudareva, N., A Familiar Ring to It: Biosynthesis of Plant Benzoic Acids. *Molecular Plant* **2015,** *8* (1), 83-97.
55. Wildermuth, M. C., Variations on a theme: synthesis and modification of plant benzoic acids. *Current Opinion in Plant Biology* **2006,** *9* (3), 288-296.
56. Pokotylo, I.; Kravets, V.; Ruelland, E., Salicylic Acid Binding Proteins (SABPs): The Hidden Forefront of Salicylic Acid Signalling. *International journal of molecular sciences* **2019,** *20* (18), 4377.
57. Hayat, Q.; Hayat, S.; Irfan, M.; Ahmad, A., Effect of exogenous salicylic acid under changing environment: A review. *Environmental and Experimental Botany* **2010,** *68* (1), 14-25.
58. Rivas-San Vicente, M.; Plasencia, J., Salicylic acid beyond defence: its role in plant growth and development. *Journal of Experimental Botany* **2011,** *62* (10), 3321-3338.
59. Liu, X.; Rockett, K. S.; Korner, C. J.; Pajerowska-Mukhtar, K. M., Salicylic acid signalling: new insights and prospects at a quarter-century milestone. *Essays in biochemistry* **2015,** *58*, 101-13.

60. Li, A.; Sun, X.; Liu, L., Action of Salicylic Acid on Plant Growth. *Frontiers in Plant Science* **2022,** *13*.
61. Khan, M. I. R.; Fatma, M.; Per, T. S.; Anjum, N. A.; Khan, N. A., Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. *Frontiers in plant science* **2015,** *6*.
62. Vasyukova, N. I.; Ozeretskovskaya, O. L., Induced plant resistance and salicylic acid: A review. *Applied Biochemistry and Microbiology* **2007,** *43* (4), 367-373.
63. Khan, M. S. S.; Islam, F.; Chen, H.; Chang, M.; Wang, D.; Liu, F.; Fu, Z. Q.; Chen, J., Transcriptional Coactivators: Driving Force of Plant Immunity. *Frontiers in Plant Science* **2022,** *13*.
64. Kumar, D., Salicylic acid signaling in disease resistance. *Plant Science* **2014,** *228*, 127-134.
65. Czerpak, R.; Bajguz, A.; Piotrowska, A.; Polecka, M., Effect of isomers of hydroxybenzoic acid on the growth and metabolism of Chlorella vulgaris beijerinck (chlorophyceae). *Acta Societatis Botanicorum Poloniae* **2001,** *70* (4), 253-259.
66. Juurlink, B. H. J.; Azouz, H. J.; Aldalati, A. M. Z.; AlTinawi, B. M. H.; Ganguly, P., Hydroxybenzoic acid isomers and the cardiovascular system. *Nutrition Journal* **2014,** *13*, 10.
67. Mallek-Ayadi, S.; Bahloul, N.; Kechaou, N., Characterization, phenolic compounds and functional properties of Cucumis melo L. peels. *Food Chemistry* **2017,** *221*, 1691-1697.
68. Khan, S. A.; Chatterjee, S. S.; Kumar, V., Low dose aspirin like analgesic and anti-inflammatory activities of mono-hydroxybenzoic acids in stressed rodents. *Life Sciences* **2016,** *148*, 53-62.
69. Haman, C.; Dauchy, X.; Rosin, C.; Munoz, J.-F., Occurrence, fate and behavior of parabens in aquatic environments: A review. *Water Research* **2015,** *68*, 1-11.
70. Huang, C.-z.; Xu, L.; Jin-jing, S.; Zhang, Z.-h.; Fu, M.-l.; Teng, H.-y.; Yi, K.-k., Allelochemical p-hydroxybenzoic acid inhibits root growth via regulating ROS accumulation in cucumber (Cucumis sativus L.). *Journal of Integrative Agriculture* **2020,** *19* (2), 518-527.
71. Uddin, M. N.; Robinson, R. W., Allelopathy and resource competition: the effects of Phragmites australis invasion in plant communities. *Botanical studies* **2017,** *58* (1), 29.
72. Kamaya, Y.; Tsuboi, S.; Takada, T.; Suzuki, K., Growth stimulation and inhibition effects of 4-hydroxybenzoic acid and some related compounds on the freshwater green alga Pseudokirchneriella subcapitata. *Archives of Environmental Contamination and Toxicology* **2006,** *51* (4), 537-541.
73. Tanna, B.; Mishra, A., Chapter 2 - Metabolomics of Seaweeds: Tools and Techniques. In *Plant Metabolites and Regulation Under Environmental Stress*, Ahmad, P.; Ahanger, M. A.; Singh, V. P.; Tripathi, D. K.; Alam, P.; Alyemeni, M. N., Eds. Academic Press: 2018; pp 37-52.
74. Richard-Forget, F.; Atanasova, V.; Chéreau, S., Using metabolomics to guide strategies to tackle the issue of the contamination of food and feed with mycotoxins: A review of the literature with specific focus on Fusarium mycotoxins. *Food Control* **2021,** *121*, 107610.
75. Aszyk, J.; Byliński, H.; Namieśnik, J.; Kot-Wasik, A., Main strategies, analytical trends and challenges in LC-MS and ambient mass spectrometry–based metabolomics. *TrAC Trends in Analytical Chemistry* **2018,** *108*, 278-295.
76. La Barbera, G.; Capriotti, A. L.; Cavaliere, C.; Montone, C. M.; Piovesana, S.; Samperi, R.; Zenezini Chiozzi, R.; Laganà, A., Liquid chromatography-high resolution mass spectrometry for the analysis of phytochemicals in vegetal-derived food and beverages. *Food Research International* **2017,** *100*, 28-52.
77. Wang, X. J.; Ren, J. L.; Zhang, A. H.; Sun, H.; Yan, G. L.; Han, Y.; Liu, L., Novel applications of mass spectrometry-based metabolomics in herbal medicines and its active ingredients: Current evidence. *Mass Spectrometry Reviews* **2019,** *38* (4-5), 380-402.
78. Reis, V. A. T.; Duarte, A. C., Analytical methodologies for arsenic speciation in macroalgae: A critical review. *TrAC Trends in Analytical Chemistry* **2018,** *102*, 170-184.
79. Martins, M.; Ventura, S. P. M., Chapter 12 - Emerging seaweed extraction techniques using ionic liquids. In *Sustainable Seaweed Technologies*, Torres, M. D.; Kraan, S.; Dominguez, H., Eds. Elsevier: 2020; pp 287-311.

80. Deng, Z.-H.; Li, N.; Jiang, H.-L.; Lin, J.-M.; Zhao, R.-S., Pretreatment techniques and analytical methods for phenolic endocrine disrupting chemicals in food and environmental samples. *TrAC Trends in Analytical Chemistry* **2019,** *119*, 115592.
81. Altemimi, A.; Lakhssassi, N.; Baharlouei, A.; Watson, D. G.; Lightfoot, D. A., Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. *Plants* **2017,** *6* (4), 42.
82. Segarra, G.; Jáuregui, O.; Casanova, E.; Trillas, I., Simultaneous quantitative LC–ESI-MS/MS analyses of salicylic acid and jasmonic acid in crude extracts of Cucumis sativus under biotic stress. *Phytochemistry* **2006,** *67* (4), 395-401.
83. Acosta-Estrada, B. A.; Gutiérrez-Uribe, J. A.; Serna-Saldívar, S. O., Bound phenolics in foods, a review. *Food Chemistry* **2014,** *152*, 46-55.
84. Gullón, B.; Gagaoua, M.; Barba, F. J.; Gullón, P.; Zhang, W.; Lorenzo, J. M., Seaweeds as promising resource of bioactive compounds: Overview of novel extraction strategies and design of tailored meat products. *Trends in Food Science & Technology* **2020,** *100*, 1-18.
85. Oniszczuk, A.; Olech, M., Optimization of ultrasound-assisted extraction and LC-ESI–MS/MS analysis of phenolic acids from Brassica oleracea L. var. sabellica. *Industrial Crops and Products* **2016,** *83*, 359-363.
86. Yuan, Y.; Zhang, J.; Fan, J.; Clark, J.; Shen, P.; Li, Y.; Zhang, C., Microwave assisted extraction of phenolic compounds from four economic brown macroalgae species and evaluation of their antioxidant activities and inhibitory effects on α-amylase, α-glucosidase, pancreatic lipase and tyrosinase. *Food Research International* **2018,** *113*, 288-297.
87. Gros, Q.; Duval, J.; West, C.; Lesellier, E., On-line supercritical fluid extraction-supercritical fluid chromatography (SFE-SFC) at a glance: A coupling story. *TrAC Trends in Analytical Chemistry* **2021,** *144*, 116433.
88. Ospina, M.; Castro-Vargas, H. I.; Parada-Alfonso, F., Antioxidant capacity of Colombian seaweeds: 1. Extracts obtained from Gracilaria mammillaris by means of supercritical fluid extraction. *The Journal of Supercritical Fluids* **2017,** *128*, 314-322.
89. Charoensiddhi, S.; Franco, C.; Su, P.; Zhang, W., Improved antioxidant activities of brown seaweed Ecklonia radiata extracts prepared by microwave-assisted enzymatic extraction. *Journal of Applied Phycology* **2015,** *27* (5), 2049-2058.
90. Andrade-Eiroa, A.; Canle, M.; Leroy-Cancellieri, V.; Cerdà, V., Solid-phase extraction of organic compounds: A critical review (Part I). *TrAC Trends in Analytical Chemistry* **2016**.
91. Rajauria, G., Optimization and validation of reverse phase HPLC method for qualitative and quantitative assessment of polyphenols in seaweed. *Journal of Pharmaceutical and Biomedical Analysis* **2018,** *148*, 230-237.
92. Klejdus, B.; Plaza, M.; Snoblova, M.; Lojkova, L., Development of new efficient method for isolation of phenolics from sea algae prior to their rapid resolution liquid chromatographic-tandem mass spectrometric determination. *Journal of Pharmaceutical and Biomedical Analysis* **2017,** *135*, 87-96.
93. Mikami, K.; Mori, I. C.; Matsuura, T.; Ikeda, Y.; Kojima, M.; Sakakibara, H.; Hirayama, T., Comprehensive quantification and genome survey reveal the presence of novel phytohormone action modes in red seaweeds. *Journal of Applied Phycology* **2016,** *28* (4), 2539-2548.
94. González-Curbelo, M. Á.; Socas-Rodríguez, B.; Herrera-Herrera, A. V.; González-Sálamo, J.; Hernández-Borges, J.; Rodríguez-Delgado, M. Á., Evolution and applications of the QuEChERS method. *TrAC Trends in Analytical Chemistry* **2015,** *71*, 169-185.
95. Anastassiades, M.; Lehotay, S. J.; Stajnbaher, D.; Schenck, F. J., Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. *Journal of AOAC International* **2003,** *86* (2), 412-31.
96. Cunha, S. C.; Oliveira, C.; Fernandes, J. O., Development of QuEChERS-based extraction and liquid chromatography-tandem mass spectrometry method for simultaneous quantification of bisphenol A and tetrabromobisphenol A in seafood: fish, bivalves, and seaweeds. *Analytical and Bioanalytical Chemistry* **2017,** *409* (1), 151-160.

97. Misra, N. N.; Rai, D. K.; Hossain, M., Chapter 10 - Analytical techniques for bioactives from seaweed. In *Seaweed Sustainability*, Tiwari, B. K.; Troy, D. J., Eds. Academic Press: San Diego, 2015; pp 271-287.
98. Skoog, D. A.; West, D. M.; Holler, F. J.; Crouch, S. R., *Fundamentals of analytical chemistry*. Cengage learning: 2013.
99. Batool, A.; Menaa, F., Chapter 13 - Concentration and purification of seaweed components by chromatography methods. In *Sustainable Seaweed Technologies*, Torres, M. D.; Kraan, S.; Dominguez, H., Eds. Elsevier: 2020; pp 315-370.
100. Kumar, B. R., Application of HPLC and ESI-MS techniques in the analysis of phenolic acids and flavonoids from green leafy vegetables (GLVs). *Journal of Pharmaceutical Analysis* **2017,** *7* (6), 349-364.
101. Moldovan, R.-C.; Bodoki, E.; Servais, A.-C.; Crommen, J.; Oprean, R.; Fillet, M., Selectivity evaluation of phenyl based stationary phases for the analysis of amino acid diastereomers by liquid chromatography coupled with mass spectrometry. *Journal of Chromatography A* **2019,** *1590*, 80-87.
102. Kruve, A.; Rebane, R.; Kipper, K.; Oldekop, M.-L.; Evard, H.; Herodes, K.; Ravio, P.; Leito, I., Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part I. *Analytica Chimica Acta* **2015,** *870*, 29-44.
103. Chong, Y.-K.; Ho, C.-C.; Leung, S.-Y.; Lau, S. K. P.; Woo, P. C. Y., Clinical Mass Spectrometry in the Bioinformatics Era: A Hitchhiker's Guide. *Computational and Structural Biotechnology Journal* **2018,** *16*, 316-334.
104. Smoluch, M.; Grasso, G.; Suder, P.; Silberring, J., *Mass spectrometry : an applied approach*. Second edition / edited by Marek Smoluch, Giuseppe Grasso, Piotr Suder, and Jerzy Silberring. ed.; Wiley: 2019.
105. Gross, J. H., *Mass spectrometry : a textbook*. Third edition. ed.; Springer: 2017.
106. Maher, S.; Jjunju, F.; Taylor, S., Colloquium : 100 years of mass spectrometry: Perspectives and future trends. *Reviews of Modern Physics* **2015,** *87*, 113-135.
107. NATA, Guidelines for the Validation and Verification of Quantitative and Qualitative Test Methods. *National Association Testing Authorities - Validation and Verification of Quantitative and Qualitative Test Methods* **2018**, 1-31.
108. Kruve, A.; Rebane, R.; Kipper, K.; Oldekop, M.-L.; Evard, H.; Herodes, K.; Ravio, P.; Leito, I., Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part II. *Analytica Chimica Acta* **2015,** *870*, 8-28.
109. Taylor, P. J., Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clinical Biochemistry* **2005,** *38* (4), 328-334.
110. Cortese, M.; Gigliobianco, M. R.; Magnoni, F.; Censi, R.; Di Martino, P. D., Compensate for or Minimize Matrix Effects? Strategies for Overcoming Matrix Effects in Liquid Chromatography-Mass Spectrometry Technique: A Tutorial Review. *Molecules* **2020,** *25* (13).
111. Cappiello, A.; Famiglini, G.; Palma, P.; Pierini, E.; Termopoli, V.; Trufelli, H., Overcoming Matrix Effects in Liquid Chromatography−Mass Spectrometry. *Analytical chemistry* **2008,** *80* (23), 9343-9348.
112. Cappiello, A.; Famiglini, G.; Palma, P.; Trufelli, H., MATRIX EFFECTS IN LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY. *Journal of Liquid Chromatography & Related Technologies* **2010,** *33* (9-12), 1067-1081.
113. Trufelli, H.; Palma, P.; Famiglini, G.; Cappiello, A., An overview of matrix effects in liquid chromatography–mass spectrometry. *Mass Spectrometry Reviews* **2011,** *30* (3), 491-509.
114. Raposo, F.; Barceló, D., Challenges and strategies of matrix effects using chromatography-mass spectrometry: An overview from research versus regulatory viewpoints. *TrAC Trends in Analytical Chemistry* **2021,** *134*, 116068.
115. Kruve, A.; Leito, I.; Herodes, K., Combating matrix effects in LC/ESI/MS: The extrapolative dilution approach. *Analytica Chimica Acta* **2009,** *651* (1), 75-80.
116. Jimenez-Lopez, C.; Pereira, A. G.; Lourenço-Lopes, C.; Garcia-Oliveira, P.; Cassani, L.; Fraga-Corral, M.; Prieto, M. A.; Simal-Gandara, J., Main bioactive phenolic compounds in marine

algae and their mechanisms of action supporting potential health benefits. *Food Chemistry* **2021,** *341*, 128262.
117. Górka, B.; Wieczorek, P. P., Simultaneous determination of nine phytohormones in seaweed and algae extracts by HPLC-PDA. *Journal of Chromatography B* **2017,** *1057*, 32-39.
118. Hakim, M. M.; Patel, I. C., A review on phytoconstituents of marine brown algae. *Future Journal of Pharmaceutical Sciences* **2020,** *6* (1), 129.
119. Generalić Mekinić, I.; Skroza, D.; Šimat, V.; Hamed, I.; Čagalj, M.; Popović Perković, Z., Phenolic Content of Brown Algae (Pheophyceae) Species: Extraction, Identification, and Quantification. *Biomolecules* **2019,** *9* (6), 244.
120. Torres, P.; Santos, J. P.; Chow, F.; dos Santos, D. Y. A. C., A comprehensive review of traditional uses, bioactivity potential, and chemical diversity of the genus Gracilaria (Gracilariales, Rhodophyta). *Algal Research* **2019,** *37*, 288-306.
121. Santos, S. A. O.; Félix, R.; Pais, A. C. S.; Rocha, S. M.; Silvestre, A. J. D., The Quest for Phenolic Compounds from Macroalgae: A Review of Extraction and Identification Methodologies. *Biomolecules* **2019,** *9* (12), 847.
122. Abirami, R. G.; Kowsalya, S., Quantification and Correlation Study on Derived Phenols and Antioxidant Activity of Seaweeds from Gulf of Mannar. *Journal of Herbs, Spices & Medicinal Plants* **2017,** *23* (1), 9-17.
123. Alkhalaf, M. I., Chemical composition, antioxidant, anti-inflammatory and cytotoxic effects of Chondrus crispus species of red algae collected from the Red Sea along the shores of Jeddah city. *Journal of King Saud University - Science* **2021,** *33* (1), 101210.
124. Chakraborty, K.; Joseph, D., Antioxidant Potential and Phenolic Compounds of Brown Seaweeds Turbinaria conoides and Turbinaria ornata (Class: Phaeophyceae). *Journal of Aquatic Food Product Technology* **2016,** *25* (8), 1249-1265.
125. Ertani, A.; Francioso, O.; Tinti, A.; Schiavon, M.; Pizzeghello, D.; Nardi, S., Evaluation of Seaweed Extracts From Laminaria and Ascophyllum nodosum spp. as Biostimulants in Zea mays L. Using a Combination of Chemical, Biochemical and Morphological Approaches. *Frontiers in Plant Science* **2018,** *9*.
126. Farvin, K. H. S.; Surendraraj, A.; Al-Ghunaim, A.; Al-Yamani, F., Chemical profile and antioxidant activities of 26 selected species of seaweeds from Kuwait coast. *Journal of Applied Phycology* **2019,** *31* (4), 2653-2668.
127. Fouda, W. A.; Ibrahim, W. M.; Ellamie, A. M.; Ramadan, G., Biochemical and mineral compositions of six brown seaweeds collected from red sea at hurghada coast. **2019**.
128. Generalić Mekinić, I.; Šimat, V.; Botić, V.; Crnjac, A.; Smoljo, M.; Soldo, B.; Ljubenkov, I.; Čagalj, M.; Skroza, D., Bioactive Phenolic Metabolites from Adriatic Brown Algae Dictyota dichotoma and Padina pavonica (Dictyotaceae). *Foods* **2021,** *10* (6), 1187.
129. HAMED, S. M.; MESSIHA, N. A., SUPPRESSION OF BACTERIAL WILT DISEASE BY SOME MARINE MACROALGAL EXTRACTS ISOLATED FROM SAFAGA COAST OF RED SEA, EGYPT. *Egyptian Journal of Agricultural Research* **2018,** *96* (4), 1275-1289.
130. Jerez-Martel, I.; García-Poza, S.; Rodríguez-Martel, G.; Rico, M.; Afonso-Olivares, C.; Gómez-Pinchetti, J. L., Phenolic Profile and Antioxidant Activity of Crude Extracts from Microalgae and Cyanobacteria Strains. *Journal of Food Quality* **2017,** *2017*, 2924508.
131. Korzeniowska, K.; Łęska, B.; Wieczorek, P. P., Isolation and determination of phenolic compounds from freshwater Cladophora glomerata. *Algal Research* **2020,** *48*, 101912.
132. López, A.; Rico, M.; Santana-Casiano, J. M.; González, A. G.; González-Dávila, M., Phenolic profile of Dunaliella tertiolecta growing under high levels of copper and iron. *Environmental Science and Pollution Research* **2015,** *22* (19), 14820-14828.
133. Rico, M.; López, A.; Santana-Casiano, J. M.; Gonzàlez, A. G.; Gonzàlez-Dàvila, M., Variability of the phenolic profile in the diatom Phaeodactylum tricornutum growing under copper and iron stress. *Limnology and Oceanography* **2013,** *58* (1), 144-152.

134. Machu, L.; Misurcova, L.; Vavra Ambrozova, J.; Orsavova, J.; Mlcek, J.; Sochor, J.; Jurikova, T., Phenolic Content and Antioxidant Capacity in Algal Food Products. *Molecules* **2015,** *20* (1), 1118-1133.
135. Nkurunziza, D.; Sivagnanam, S. P.; Park, J.-S.; Cho, Y.-J.; Chun, B. S., Effect of wall materials on the spray drying encapsulation of brown seaweed bioactive compounds obtained by subcritical water extraction. *Algal Research* **2021,** *58*, 102381.
136. Vo Dinh, T.; Saravana, P. S.; Woo, H. C.; Chun, B. S., Ionic liquid-assisted subcritical water enhances the extraction of phenolics from brown seaweed and its antioxidant activity. *Separation and Purification Technology* **2018,** *196*, 287-299.
137. Pangestuti, R.; Haq, M.; Rahmadi, P.; Chun, B.-S., Nutritional Value and Biofunctionalities of Two Edible Green Seaweeds (Ulva lactuca and Caulerpa racemosa) from Indonesia by Subcritical Water Hydrolysis. *Marine Drugs* **2021,** *19* (10), 578.
138. Sabeena Farvin, K. H.; Jacobsen, C., Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. *Food Chemistry* **2013,** *138* (2), 1670-1681.
139. Sabeena Farvin, K. H.; Jacobsen, C., Antioxidant Activity of Seaweed Extracts: In Vitro Assays, Evaluation in 5 % Fish Oil-in-Water Emulsions and Characterization. *Journal of the American Oil Chemists' Society* **2015,** *92* (4), 571-587.
140. Safafar, H.; Van Wagenen, J.; Møller, P.; Jacobsen, C., Carotenoids, Phenolic Compounds and Tocopherols Contribute to the Antioxidative Properties of Some Microalgae Species Grown on Industrial Wastewater. *Marine Drugs* **2015,** *13* (12), 7339-7356.
141. Semaida, A. I.; El-Khashab, M. A.; Saber, A. A.; Hassan, A. I.; Elfouly, S. A., Effects of Sargassum virgatum extracts on the testicular measurements, genomic DNA and antioxidant enzymes in irradiated rats. *International Journal of Radiation Biology* **2022,** *98* (2), 191-204.
142. Sirbu, R.; Stanciu, G.; Tomescu, A.; Ionescu, A. M.; Cadar, E., Evaluation of antioxidant and antimicrobial activity in relation to total phenolic content of green algae from Black Sea. *Rev. Chim* **2019,** *70*, 1197-1203.
143. Sumayya, S.; Lubaina, A.; Murugan, K., Phytochemical, HPLC and FTIR Analysis of Methanolic Extract from Gracilaria dura (C Agardh) J Agardh. *Journal of Drug Delivery and Therapeutics* **2020,** *10* (3), 114-118.
144. Waghmode, A.; Khilare, C., RP-HPLC profile of major phenolics from brown marine macro algae. *J Appl Pharm* **2018,** *10* (2), 1-5.
145. Zaharudin, N.; Salmeán, A. A.; Dragsted, L. O., Inhibitory effects of edible seaweeds, polyphenolics and alginates on the activities of porcine pancreatic α-amylase. *Food Chemistry* **2018,** *245*, 1196-1203.
146. Agregán, R.; Munekata, P. E. S.; Franco, D.; Dominguez, R.; Carballo, J.; Lorenzo, J. M., Phenolic compounds from three brown seaweed species using LC-DAD–ESI-MS/MS. *Food Research International* **2017,** *99*, 979-985.
147. Allwood, J. W.; Evans, H.; Austin, C.; McDougall, G. J., Extraction, Enrichment, and LC-MSn-Based Characterization of Phlorotannins and Related Phenolics from the Brown Seaweed, Ascophyllum nodosum. *Marine Drugs* **2020,** *18* (9), 448.
148. Goiris, K.; Muylaert, K.; Voorspoels, S.; Noten, B.; De Paepe, D.; E Baart, G. J.; De Cooman, L., Detection of flavonoids in microalgae from different evolutionary lineages. *Journal of Phycology* **2014,** *50* (3), 483-492.
149. Grina, F.; Ullah, Z.; Kaplaner, E.; Moujahid, A.; Eddoha, R.; Nasser, B.; Terzioğlu, P.; Yilmaz, M. A.; Ertaş, A.; Öztürk, M.; Essamadi, A., In vitro enzyme inhibitory properties, antioxidant activities, and phytochemical fingerprints of five Moroccan seaweeds. *South African Journal of Botany* **2020,** *128*, 152-160.
150. Gupta, V.; Kumar, M.; Brahmbhatt, H.; Reddy, C. R. K.; Seth, A.; Jha, B., Simultaneous determination of different endogenetic plant growth regulators in common green seaweeds using dispersive liquid–liquid microextraction method. *Plant Physiology and Biochemistry* **2011,** *49* (11), 1259-1263.

151. Klejdus, B.; Kopecký, J.; Benešová, L.; Vacek, J., Solid-phase/supercritical-fluid extraction for liquid chromatography of phenolic compounds in freshwater microalgae and selected cyanobacterial species. *Journal of Chromatography A* **2009,** *1216* (5), 763-771.
152. Maghraby, Y. R.; Farag, M. A.; G Kontominas, M.; Shakour, Z. T.; Ramadan, A. R., Nanoencapsulated Extract of a Red Seaweed (Rhodophyta) Species as a Promising Source of Natural Antioxidants. *ACS Omega* **2022**.
153. Mahomoodally, M. F.; Bibi Sadeer, N.; Zengin, G.; Cziáky, Z.; Jekő, J.; Diuzheva, A.; Sinan, K. I.; Palaniveloo, K.; Kim, D. H.; Rengasamy, K. R. R., In Vitro Enzyme Inhibitory Properties, Secondary Metabolite Profiles and Multivariate Analysis of Five Seaweeds. *Marine Drugs* **2020,** *18* (4), 198.
154. Nørskov, N. P.; Bruhn, A.; Cole, A.; Nielsen, M. O., Targeted and Untargeted Metabolic Profiling to Discover Bioactive Compounds in Seaweeds and Hemp Using Gas and Liquid Chromatography-Mass Spectrometry. *Metabolites* **2021,** *11* (5), 259.
155. Olasehinde, T. A.; Olaniran, A. O.; Okoh, A. I., Phenolic composition, antioxidant activity, anticholinesterase potential and modulatory effects of aqueous extracts of some seaweeds on β-amyloid aggregation and disaggregation. *Pharmaceutical Biology* **2019,** *57* (1), 460-469.
156. Onofrejová, L.; Vašíčková, J.; Klejdus, B.; Stratil, P.; Mišurcová, L.; Kráčmar, S.; Kopecký, J.; Vacek, J., Bioactive phenols in algae: The application of pressurized-liquid and solid-phase extraction techniques. *Journal of Pharmaceutical and Biomedical Analysis* **2010,** *51* (2), 464-470.
157. Rajauria, G.; Foley, B.; Abu-Ghannam, N., Identification and characterization of phenolic antioxidant compounds from Brown Irish Seaweed Himanthalia elongata using LC-DAD-ESI-MS/MS. *Innovative Food Science & Emerging Technologies* **2016**.
158. Rodríguez-Bernaldo de Quirós, A.; Lage-Yusty, M. A.; López-Hernández, J., Determination of phenolic compounds in macroalgae for human consumption. *Food Chemistry* **2010,** *121* (2), 634-638.
159. Shen, P.; Gu, Y.; Zhang, C.; Sun, C.; Qin, L.; Yu, C.; Qi, H., Metabolomic Approach for Characterization of Polyphenolic Compounds in Laminaria japonica, Undaria pinnatifida, Sargassum fusiforme and Ascophyllum nodosum. *Foods* **2021,** *10* (1), 192.
160. Souza, B. W. S.; Cerqueira, M. A.; Martins, J. T.; Quintas, M. A. C.; Ferreira, A. C. S.; Teixeira, J. A.; Vicente, A. A., Antioxidant Potential of Two Red Seaweeds from the Brazilian Coasts. *Journal of Agricultural and Food Chemistry* **2011,** *59* (10), 5589-5594.
161. Stirk, W. A.; Tarkowská, D.; Gruz, J.; Strnad, M.; Ördög, V.; van Staden, J., Effect of gibberellins on growth and biochemical constituents in Chlorella minutissima (Trebouxiophyceae). *South African Journal of Botany* **2019,** *126*, 92-98.
162. Tanna, B.; Brahmbhatt, H. R.; Mishra, A., Phenolic, flavonoid, and amino acid compositions reveal that selected tropical seaweeds have the potential to be functional food ingredients. *Journal of Food Processing and Preservation* **2019,** *43* (12), e14266.
163. Vlaisavljević, S.; Rašeta, M.; Berežni, S.; Passamonti, S.; Tramer, F., Four selected commercial seaweeds: biologically active compounds, antioxidant and cytotoxic properties. *International Journal of Food Sciences and Nutrition* **2021,** *72* (6), 757-766.
164. Wekre, M. E.; Kåsin, K.; Underhaug, J.; Holmelid, B.; Jordheim, M., Quantification of Polyphenols in Seaweeds: A Case Study of Ulva intestinalis. *Antioxidants* **2019,** *8* (12), 612.
165. Zhong, B.; Robinson, N. A.; Warner, R. D.; Barrow, C. J.; Dunshea, F. R.; Suleria, H. A. R., LC-ESI-QTOF-MS/MS Characterization of Seaweed Phenolics and Their Antioxidant Potential. *Marine Drugs* **2020,** *18* (6), 331.
166. Aremu, A. O.; Masondo, N. A.; Rengasamy, K. R. R.; Amoo, S. O.; Gruz, J.; Biba, O.; Subrtova, M.; Pencik, A.; Novak, O.; Dolezal, K.; Van Staden, J., Physiological role of phenolic biostimulants isolated from brown seaweed Ecklonia maxima on plant growth and development. *Planta* **2015,** *241* (6), 1313-1324.
167. Ghimire, B. K.; Hwang, M. H.; Sacks, E. J.; Yu, C. Y.; Kim, S. H.; Chung, I. M., Screening of Allelochemicals in Miscanthus sacchariflorus Extracts and Assessment of Their Effects on Germination and Seedling Growth of Common Weeds. *Plants* **2020,** *9* (10), 1313.

168. Klejdus, B.; Vacek, J.; Lojková, L.; Benešová, L.; Kubáň, V., Ultrahigh-pressure liquid chromatography of isoflavones and phenolic acids on different stationary phases. *Journal of Chromatography A* **2008,** *1195* (1), 52-59.
169. Naveen, P.; Lingaraju, H. B.; Chandrappa, S.; Anitha, S.; Prasad, K. S., Development and Validation of Veratric Acid in Tabebuia avellanedae using Liquid Chromatography-Electrospray Ionization-Mass Spectrometry/Mass Spectrometry-Multiple Reaction Monitoring-Based Assay Coupled with 1,1-Diphenyl-2-Picrylhydrazyl Method. *Pharmacognosy Research* **2020,** *12* (2), 107-111.
170. Olech, M.; Pietrzak, W.; Nowak, R., Characterization of Free and Bound Phenolic Acids and Flavonoid Aglycones in Rosa rugosa Thunb. Leaves and Achenes Using LC–ESI–MS/MS–MRM Methods. *Molecules* **2020,** *25* (8), 1804.
171. Olmo-García, L.; Kessler, N.; Neuweger, H.; Wendt, K.; Olmo-Peinado, J. M.; Fernández-Gutiérrez, A.; Baessmann, C.; Carrasco-Pancorbo, A., Unravelling the Distribution of Secondary Metabolites in Olea europaea L.: Exhaustive Characterization of Eight Olive-Tree Derived Matrices by Complementary Platforms (LC-ESI/APCI-MS and GC-APCI-MS). *Molecules* **2018,** *23* (10), 2419.
172. Sánchez-Patán, F.; Bartolomé, B.; Martín-Alvarez, P. J.; Anderson, M.; Howell, A.; Monagas, M., Comprehensive Assessment of the Quality of Commercial Cranberry Products. Phenolic Characterization and in Vitro Bioactivity. *Journal of Agricultural and Food Chemistry* **2012,** *60* (13), 3396-3408.
173. Koo, Y. M.; Heo, A. Y.; Choi, H. W., Salicylic Acid as a Safe Plant Protector and Growth Regulator. *Plant Pathol J* **2020,** *36* (1), 1-10.
174. Hasanuzzaman, M.; Nahar, K.; Bhuiyan, T. F.; Anee, T. I.; Inafuku, M.; Oku, H.; Fujita, M., Salicylic Acid: An All-Rounder in Regulating Abiotic Stress Responses in Plants. In *Phytohormones-Signaling Mechanisms and Crosstalk in Plant Development and Stress Responses*, InTech: 2017.
175. Horváth, E.; Szalai, G.; Janda, T., Induction of Abiotic Stress Tolerance by Salicylic Acid Signaling. *Journal of Plant Growth Regulation* **2007,** *26* (3), 290-300.
176. Arfan, M.; Athar, H. R.; Ashraf, M., Does exogenous application of salicylic acid through the rooting medium modulate growth and photosynthetic capacity in two differently adapted spring wheat cultivars under salt stress? *Journal of Plant Physiology* **2007,** *164* (6), 685-694.
177. El-Tayeb, M. A., Response of barley grains to the interactive e.ect of salinity and salicylic acid. *Plant Growth Regulation* **2005,** *45* (3), 215-224.
178. García-Pastor, M. E.; Zapata, P. J.; Castillo, S.; Martínez-Romero, D.; Guillén, F.; Valero, D.; Serrano, M., The Effects of Salicylic Acid and Its Derivatives on Increasing Pomegranate Fruit Quality and Bioactive Compounds at Harvest and During Storage. *Frontiers in Plant Science* **2020,** *11*.
179. Giménez, M. J.; Valverde, J. M.; Valero, D.; Guillén, F.; Martínez-Romero, D.; Serrano, M.; Castillo, S., Quality and antioxidant properties on sweet cherries as affected by preharvest salicylic and acetylsalicylic acids treatments. *Food Chemistry* **2014,** *160*, 226-232.
180. Hayat, S.; Fariduddin, Q.; Ali, B.; Ahmad, A., Effect of salicylic acid on growth and enzyme activities of wheat seedlings. *Acta Agronomica Hungarica* **2005,** *53* (4), 433-437.
181. Horváth, E.; Pál, M.; Szalai, G.; Páldi, E.; Janda, T., Exogenous 4-hydroxybenzoic acid and salicylic acid modulate the effect of short-term drought and freezing stress on wheat plants. *Biologia Plantarum* **2007,** *51* (3), 480-487.
182. Hussain, M. I.; Reigosa, M. J.; Al-Dakheel, A. J., Biochemical, physiological and isotopic responses to natural product p-hydroxybenzoic acid in Cocksfoot (Dactylis glomerata L.). *Plant Growth Regulation* **2015,** *75* (3), 783-792.
183. Lo'ay, A. A., Preharvest salicylic acid and delay ripening of 'superior seedless' grapes. *Egyptian Journal of Basic and Applied Sciences* **2017,** *4* (3), 227-230.
184. Makandar, R.; Nalam, V. J.; Lee, H.; Trick, H. N.; Dong, Y.; Shah, J., Salicylic acid regulates basal resistance to Fusarium head blight in wheat. *Molecular Plant-Microbe Interactions* **2012,** *25* (3), 431-439.
185. Mucciarelli, M.; Gallino, M.; Maffei, M.; Scannerini, S., Effects of 3,4-dihydroxybenzoic acid on tobacco (Nicotiana tabacum L.) cultured in vitro. Growth regulation in callus and organ cultures. *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology* **2000,** *134* (2), 185-192.

186. Quan, N. T.; Xuan, T. D., Foliar application of vanillic and p-hydroxybenzoic acids enhanced drought tolerance and formation of phytoalexin momilactones in rice. *Archives of Agronomy and Soil Science* **2018,** *64* (13), 1831-1846.
187. Shin, H.; Min, K.; Arora, R., Exogenous salicylic acid improves freezing tolerance of spinach (Spinacia oleracea L.) leaves. *Cryobiology* **2018,** *81*, 192-200.
188. Siboza, X. I.; Bertling, I.; Odindo, A. O., Salicylic acid and methyl jasmonate improve chilling tolerance in cold-stored lemon fruit (Citrus limon). *Journal of Plant Physiology* **2014,** *171* (18), 1722-1731.
189. Singh, B.; Usha, K., Salicylic acid induced physiological and biochemical changes in wheat seedlings under water stress. *Plant Growth Regulation* **2003,** *39* (2), 137-141.
190. Stevens, J.; Senaratna, T.; Sivasithamparam, K., Salicylic Acid Induces Salinity Tolerance in Tomato (Lycopersicon esculentum cv. Roma): Associated Changes in Gas Exchange, Water Relations and Membrane Stabilisation. *Plant Growth Regulation* **2006,** *49* (1), 77-83.
191. Tareen, M. J.; Abbasi, N. A.; Hafiz, I. A., Postharvest application of salicylic acid enhanced antioxidant enzyme activity and maintained quality of peach cv. 'Flordaking' fruit during storage. *Scientia Horticulturae* **2012,** *142*, 221-228.
192. Wang, Y.; Liu, J.-H., Exogenous treatment with salicylic acid attenuates occurrence of citrus canker in susceptible navel orange (Citrus sinensis Osbeck). *Journal of Plant Physiology* **2012,** *169* (12), 1143-1149.
193. Wang, Z.; Ma, L.; Zhang, X.; Xu, L.; Cao, J.; Jiang, W., The effect of exogenous salicylic acid on antioxidant activity, bioactive compounds and antioxidant system in apricot fruit. *Scientia Horticulturae* **2015,** *181*, 113-120.
194. Wang, Z.; Zhang, J.; Wu, F.; Zhou, X., Changes in rhizosphere microbial communities in potted cucumber seedlings treated with syringic acid. *PLOS ONE* **2018,** *13* (6), e0200007.
195. War, A. R.; Paulraj, M. G.; War, M. Y.; Ignacimuthu, S., Role of salicylic acid in induction of plant defense system in chickpea (Cicer arietinum L.). *Plant Signaling & Behavior* **2011,** *6* (11), 1787-1792.
196. Wu, H. C.; du Toit, E. S.; Reinhardt, C. F.; Rimando, A. M.; van der Kooy, F.; Meyer, J. J. M., The phenolic, 3,4-dihydroxybenzoic acid, is an endogenous regulator of rooting in Protea cynaroides. *Plant Growth Regulation* **2007,** *52* (3), 207-215.
197. Zhang, J.; Li, D.-M.; Sun, W.-J.; Wang, X.-J.; Bai, J.-G., Exogenous p-hydroxybenzoic acid regulates antioxidant enzyme activity and mitigates heat stress of cucumber leaves. *Scientia Horticulturae* **2012,** *148*, 235-245.
198. Zhou, X.; Yu, G.; Wu, F., Responses of Soil Microbial Communities in the Rhizosphere of Cucumber (Cucumis sativus L.) to Exogenously Applied p-Hydroxybenzoic Acid. *Journal of Chemical Ecology* **2012,** *38* (8), 975-983.
199. Cao, Z.-Y.; Sun, L.-H.; Mou, R.-X.; Zhang, L.-P.; Lin, X.-Y.; Zhu, Z.-W.; Chen, M.-X., Profiling of phytohormones and their major metabolites in rice using binary solid-phase extraction and liquid chromatography-triple quadrupole mass spectrometry. *Journal of Chromatography A* **2016**.
200. Shields, A.; Shivnauth, V.; Castroverde, C. D. M., Salicylic Acid and N-Hydroxypipecolic Acid at the Fulcrum of the Plant Immunity-Growth Equilibrium. *Frontiers in Plant Science* **2022,** *13*.
201. Llanes, A.; Andrade, A.; Alemano, S.; Luna, M., Alterations of Endogenous Hormonal Levels in Plants under Drought and Salinity. *American Journal of Plant Sciences* **2016,** *7*, 1357-1371.
202. Seasol Seasol Commercial Seaweed Concentrate https://seasolhortandag.com/products/seasol-commercial/.
203. Seasol Seasol Commercial Seaweed Concentrate Product Analysis. https://www.seasol.com.au/wp-content/uploads/2017/03/Product-Analysis-Seasol-Commercial-Seaweed-Concentrate-April-2017.pdf.
204. Seasol Seasol Seed Raising and Cutting Potting Mix. https://www.seasol.com.au/soils-potting-mixes/products/seasol-seed-raising-cutting-potting-mix/.
205. Doerner, P. J. C. S. H. P., Phenotypic analysis of Arabidopsis mutants: quantitative analysis of root growth. **2008,** *2008* (3), pdb. prot4960.

206. Huang, P.; de-Bashan, L.; Crocker, T.; Kloepper, J. W.; Bashan, Y. J. B.; Soils, F. o., Evidence that fresh weight measurement is imprecise for reporting the effect of plant growth-promoting (rhizo) bacteria on growth promotion of crop plants. **2017,** *53* (2), 199-208.
207. Collins, D. A Study of Salicylic Acid in Commercial Kelp Extracts. Thesis, Victoria University, Melbourne, Australia, 2016.
208. Wishart, D. S.; Feunang, Y. D.; Marcu, A.; Guo, A. C.; Liang, K.; Vázquez-Fresno, R.; Sajed, T.; Johnson, D.; Li, C.; Karu, N.; Sayeeda, Z.; Lo, E.; Assempour, N.; Berjanskii, M.; Singhal, S.; Arndt, D.; Liang, Y.; Badran, H.; Grant, J.; Serra-Cayuela, A.; Liu, Y.; Mandal, R.; Neveu, V.; Pon, A.; Knox, C.; Wilson, M.; Manach, C.; Scalbert, A., HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Res* **2018,** *46* (D1), D608-d617.
209. Ostrowski, W.; Wojakowska, A.; Grajzer, M.; Stobiecki, M., Mass spectrometric behavior of phenolic acids standards and their analysis in the plant samples with LC/ESI/MS system. *Journal of Chromatography B* **2014,** *967*, 21-27.
210. Liu, G. D.; Zhao, Y. W.; Li, Y. J.; Wang, X. J.; Si, H. H.; Huang, W. Z.; Wang, Z. Z.; Ma, S. P.; Xiao, W., Qualitative and quantitative analysis of major constituents from Dazhu Hongjingtian capsule by UPLC/Q-TOF-MS/MS combined with UPLC/QQQ-MS/MS. *Biomedical Chromatography* **2017,** *31* (6), 10.
211. Allen, F.; Greiner, R.; Wishart, D., Competitive fragmentation modeling of ESI-MS/MS spectra for putative metabolite identification. *Metabolomics* **2015,** *11* (1), 98-110.
212. Wang, F.; Liigand, J.; Tian, S.; Arndt, D.; Greiner, R.; Wishart, D. S., CFM-ID 4.0: More Accurate ESI-MS/MS Spectral Prediction and Compound Identification. *Analytical chemistry* **2021,** *93* (34), 11692-11700.
213. Tohma, H.; Gulcin, I.; Bursal, E.; Goren, A. C.; Alwasel, S. H.; Koksal, E., Antioxidant activity and phenolic compounds of ginger (Zingiber officinale Rosc.) determined by HPLC-MS/MS. *Journal of Food Measurement and Characterization* **2017,** *11* (2), 556-566.
214. Wang, H.; Zhou, H.; Horimoto, S.; Jiang, J.; Mayumi, T.; Hu, P., Quantitative determination of trimebutine maleate and its three metabolites in human plasma by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B* **2002,** *779* (2), 173-187.
215. Sánchez-Patán, F.; Monagas, M.; Moreno-Arribas, M. V.; Bartolomé, B., Determination of microbial phenolic acids in human faeces by UPLC-ESI-TQ MS. *J Agric Food Chem* **2011,** *59* (6), 2241-7.
216. Möller, M.; Du Preez, J. L.; Harvey, B. H., Development and validation of a single analytical method for the determination of tryptophan, and its kynurenine metabolites in rat plasma. *Journal of Chromatography B* **2012,** *898*, 121-129.
217. Forrest, C. M.; Kennedy, P. G. E.; Rodgers, J.; Dalton, R. N.; Turner, C.; Darlington, L. G.; Cobb, S. R.; Stone, T. W., Kynurenine pathway metabolism following prenatal KMO inhibition and in Mecp2+/− mice, using liquid chromatography-tandem mass spectrometry. *Neurochemistry International* **2016,** *100*, 110-119.
218. Blazics, B.; Papp, I.; Kéry, Á., LC–MS Qualitative Analysis and Simultaneous Determination of Six Filipendula Salicylates with Two Standards. *Chromatographia* **2010,** *71* (1), 61-67.
219. De Paola, E. L.; Montevecchi, G.; Masino, F.; Garbini, D.; Barbanera, M.; Antonelli, A., Determination of acrylamide in dried fruits and edible seeds using QuEChERS extraction and LC separation with MS detection. *Food Chemistry* **2017,** *217*, 191-195.
220. Agilent Polaris Reversed Phase HPLC Columns - Technical Information. https://www.agilent.com/en/product/small-molecule-columns/reversed-phase-hplc-columns/polaris#productdetails.
221. RESTEK Raptor C18 LC Columns (USP L1) - Technical Information. https://www.restek.com/row/products/columns/hplc--uhplc-columns/analytical-columns/43275/.
222. RESTEK Raptor Biphenyl LC Columns (USP L11) - Technical Information. https://www.restek.com/row/products/columns/hplc--uhplc-columns/analytical-columns/40873/.

223. Yang, M.; Fazio, S.; Munch, D.; Drumm, P., Impact of methanol and acetonitrile on separations based on π–π interactions with a reversed-phase phenyl column. *Journal of Chromatography A* **2005,** *1097* (1), 124-129.
224. Croes, K.; Steffens, A.; Marchand, D. H.; Snyder, L. R., Relevance of π–π and dipole–dipole interactions for retention on cyano and phenyl columns in reversed-phase liquid chromatography. *Journal of Chromatography A* **2005,** *1098* (1), 123-130.
225. Euerby, M. R.; Petersson, P.; Campbell, W.; Roe, W., Chromatographic classification and comparison of commercially available reversed-phase liquid chromatographic columns containing phenyl moieties using principal component analysis. *Journal of Chromatography A* **2007,** *1154* (1), 138-151.
226. Gobburi, A. L. P.; Kipruto, E. W.; Inman, D. M.; Anderson, D. J., A new LC-MS/MS technique for separation of gangliosides using a phenyl-hexyl column: Systematic separation according to sialic acid class and ceramide subclass. *Journal of Liquid Chromatography & Related Technologies* **2021,** *44* (1-2), 114-125.
227. Studzińska, S.; Bocian, S.; Siecińska, L.; Buszewski, B., Application of phenyl-based stationary phases for the study of retention and separation of oligonucleotides. *Journal of Chromatography B* **2017,** *1060*, 36-43.
228. Song, S.; Yin, W.; Sun, X.; Cui, B.; Huang, L.; Li, P.; Yang, L.; Zhou, J.; Deng, Y., Anthranilic acid from Ralstonia solanacearum plays dual roles in intraspecies signalling and inter-kingdom communication. *The ISME Journal* **2020,** *14* (9), 2248-2260.
229. Ma, Y.-J.; Guan, C.; Dong, Y.-J.; Yu, H., High-performance liquid chromatography utilization of ionic liquids as mobile phase additives for separation and determination of the isomers of amino benzoic acids. *Chinese Chemical Letters* **2016,** *27* (5), 749-752.
230. Zheng, J.; Polyakova, Y.; Row, K. H., Effects of Ionic Liquid as Additive and the pH of the Mobile Phase on the Retention Factors of Amino Benzoic Acids in RP-HPLC. *Journal of Chromatographic Science* **2007,** *45* (5), 256-262.
231. Deming, S. N.; Turoff, M. L., Optimization of reverse-phase liquid chromatographic separation of weak organic acids. *Analytical chemistry* **1978,** *50* (4), 546-548.
232. Cannazza, G.; Baraldi, M.; Braghiroli, D.; Tait, A.; Parenti, C., High-performance liquid chromatographic method for the quantification of anthranilic and 3-hydroxyanthranilic acid in rat brain dialysate. *Journal of Pharmaceutical and Biomedical Analysis* **2003,** *32* (2), 287-293.
233. Huh, H. W.; Song, H. Y.; Na, Y. G.; Kim, M.; Han, M.; Pham, T. M. A.; Lee, H.; Suh, J.; Lee, S. J.; Lee, H. K.; Cho, C. W., Bioanalytical Method Development and Validation of Veratraldehyde and Its Metabolite Veratric Acid in Rat Plasma: An Application for a Pharmacokinetic Study. *Molecules* **2020,** *25* (12).
234. Kato, Y.; Fujinaka, R.; Ishisaka, A.; Nitta, Y.; Kitamoto, N.; Takimoto, Y., Plausible Authentication of Manuka Honey and Related Products by Measuring Leptosperin with Methyl Syringate. *Journal of Agricultural and Food Chemistry* **2014,** *62* (27), 6400-6407.
235. Qin, Y.; Zhao, H.; Zhang, W.; Zhao, N.; Fan, P.; Zhang, L.; Zhang, H., A novel gradient LC-MS/MS method for simultaneous determination of trimebutine maleate and its two metabolites in human plasma. *Life Sci. J* **2013,** *10* (1), 2840-2849.
236. Zapała, L.; Kalembkiewicz, J.; Sitarz-Palczak, E., Studies on equilibrium of anthranilic acid in aqueous solutions and in two-phase systems: Aromatic solvent–water. *Biophysical Chemistry* **2009,** *140* (1), 91-98.
237. Fernando, I. S.; Kim, M.; Son, K.-T.; Jeong, Y.; Jeon, Y.-J. J. J. o. m. f., Antioxidant activity of marine algal polyphenolic compounds: a mechanistic approach. **2016,** *19* (7), 615-628.
238. Sudhakar, M. P.; Kumar, B. R.; Mathimani, T.; Arunkumar, K., A review on bioenergy and bioactive compounds from microalgae and macroalgae-sustainable energy perspective. *Journal of Cleaner Production* **2019,** *228*, 1320-1333.
239. Sabeena Farvin, K. H.; Jacobsen, C., Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. *Food Chemistry* **2013,** *138* (2–3), 1670-1681.

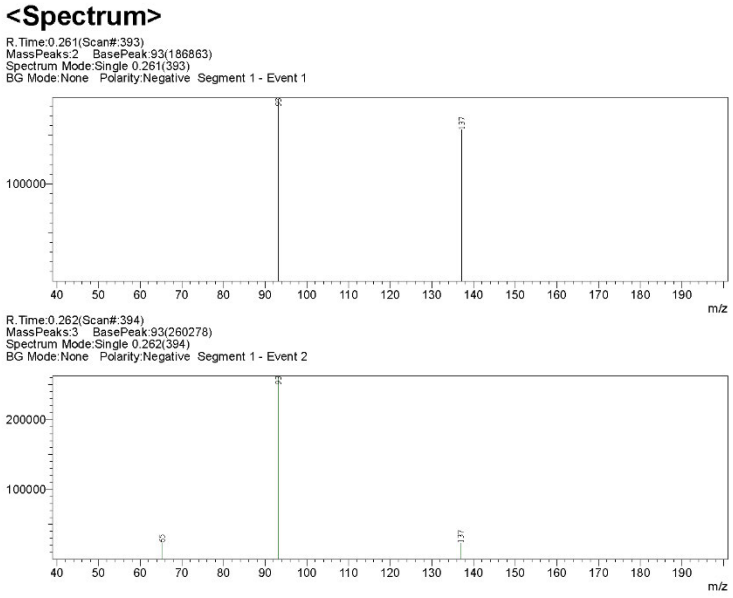
240. Kushveer, J. S.; Rashmi, M.; Sarma, V. V., Chapter 3 - Bioactive compounds from marine-derived fungi and their potential applications. In *Fungi Bio-Prospects in Sustainable Agriculture, Environment and Nano-technology*, Sharma, V. K.; Shah, M. P.; Parmar, S.; Kumar, A., Eds. Academic Press: 2021; pp 91-173.
241. Tay, S. A. B.; Macleod, J. K.; Palni, L. M. S.; Letham, D. S., Detection of cytokinins in a seaweed extract. *Phytochemistry* **1985,** *24* (11), 2611-2614.
242. Khadem, S.; Marles, R. J., Monocyclic Phenolic Acids; Hydroxy- and Polyhydroxybenzoic Acids: Occurrence and Recent Bioactivity Studies. *Molecules* **2010,** *15* (11), 7985-8005.
243. Zhang, W.; Wang, S.; Yang, J.; Kang, C.; Huang, L.; Guo, L., Glycosylation of plant secondary metabolites: Regulating from chaos to harmony. *Environmental and Experimental Botany* **2022,** *194*, 104703.
244. Kakkar, S.; Bais, S., A Review on Protocatechuic Acid and Its Pharmacological Potential. *ISRN Pharmacology* **2014,** *2014*, 952943.
245. Chen, Q.; Zhu, B.; Sun, D.; Liu, W.; Sun, X.; Duan, S., The effect of protocatechuic acid on the phycosphere in harmful algal bloom species Scrippsiella trochoidea. *Aquatic Toxicology* **2020,** *227*, 105591.
246. Li, Z.-H.; Wang, Q.; Ruan, X.; Pan, C.-D.; Jiang, D.-A., Phenolics and plant allelopathy. *Molecules* **2010,** *15* (12), 8933-8952.
247. Srinivasulu, C.; Ramgopal, M.; Ramanjaneyulu, G.; Anuradha, C. M.; Suresh Kumar, C., Syringic acid (SA) – A Review of Its Occurrence, Biosynthesis, Pharmacological and Industrial Importance. *Biomedicine & Pharmacotherapy* **2018,** *108*, 547-557.
248. Chong, K. P.; Rossall, S.; Atong, M., In vitro antimicrobial activity and fungitoxicity of syringic acid, caffeic acid and 4-hydroxybenzoic acid against Ganoderma boninense. *Journal of Agricultural Science* **2009,** *1* (2), 15.
249. Chen, P.-N.; Hao, M.-J.; Li, H.-J.; Xu, J.; Mahmud, T.; Lan, W.-J., Biotransformations of anthranilic acid and phthalimide to potent antihyperlipidemic alkaloids by the marine-derived fungus Scedosporium apiospermum F41-1. *Bioorganic Chemistry* **2021,** *116*, 105375.
250. Aniszewski, T., Chapter 1 - Definition, typology, and occurrence of alkaloids. In *Alkaloids (Second Edition)*, Aniszewski, T., Ed. Elsevier: Boston, 2015; pp 1-97.
251. Doyle, S. M.; Rigal, A.; Grones, P.; Karady, M.; Barange, D. K.; Majda, M.; Pařízková, B.; Karampelias, M.; Zwiewka, M.; Pěnčík, A.; Almqvist, F.; Ljung, K.; Novák, O.; Robert, S., A role for the auxin precursor anthranilic acid in root gravitropism via regulation of PIN-FORMED protein polarity and relocalisation in Arabidopsis. *New Phytologist* **2019,** *223* (3), 1420-1432.
252. Czerpak, R.; Bajguz, A.; Białecka, B.; Wierzcholowska, L. E.; Wolańska, M. M., Effect of auxin precursors and chemical analogues on the growth and chemical composition in Chlorella pyrenoidosa Chick. *Acta Societatis Botanicorum Poloniae* **1994,** *63* (3-4), 279-286.
253. Yang, S. Y.; Park, M. R.; Kim, I. S.; Kim, Y. C.; Yang, J. W.; Ryu, C.-M., 2-Aminobenzoic acid of Bacillus sp. BS107 as an ISR determinant against Pectobacterium carotovorum subsp. carotovotrum SCC1 in tobacco. *European Journal of Plant Pathology* **2011,** *129* (3), 371-378.
254. Biotage EVOLUTE® EXPRESS AX - Technical Information. https://www.biotage.com/polymeric-spe-ax?p=literature.
255. Jurado, J. M.; Alcázar, A.; Muñiz-Valencia, R.; Ceballos-Magaña, S. G.; Raposo, F., Some practical considerations for linearity assessment of calibration curves as function of concentration levels according to the fitness-for-purpose approach. *Talanta* **2017,** *172*, 221-229.
256. Hewavitharana, A. K.; Tan, S. K.; Shaw, P. N., Strategies for the detection and elimination of matrix effects in quantitative LC-MS analysis. *LCGC North America* **2014,** *32* (1), 54-64.
257. Desai, N. C.; Senta, R. D., Simultaneous Rp-HPLC determination of salicylamide, salicylic acid and deferasirox in the bulk API dosages forms. *Journal of Taibah University for Science* **2015,** *9* (2), 245-251.
258. Rengasamy, K. R. R.; Amoo, S. O.; Aremu, A. O.; Stirk, W. A.; Gruz, J.; Šubrtová, M.; Doležal, K.; Van Staden, J., Phenolic profiles, antioxidant capacity, and acetylcholinesterase inhibitory activity of eight South African seaweeds. *Journal of Applied Phycology* **2015,** *27* (4), 1599-1605.

259. Lefevere, H.; Bauters, L.; Gheysen, G., Salicylic Acid Biosynthesis in Plants. *Frontiers in Plant Science* **2020,** *11*.
260. Naz, S.; Fatima, Z.; Iqbal, P.; Khan, A.; Zakir, I.; Noreen, S.; Younis, H.; Abbas, G.; Ahmad, S., Agronomic Crops: Types and Uses. In *Agronomic Crops: Volume 1: Production Technologies*, Hasanuzzaman, M., Ed. Springer Singapore: Singapore, 2019; pp 1-18.
261. Stella de Freitas, T. F.; Stout, M. J.; Sant'Ana, J., Effects of exogenous methyl jasmonate and salicylic acid on rice resistance to Oebalus pugnax. *Pest Management Science* **2019,** *75* (3), 744-752.
262. Abreu, M. E.; Munné-Bosch, S., Salicylic acid may be involved in the regulation of drought-induced leaf senescence in perennials: A case study in field-grown Salvia officinalis L. plants. *Environmental and Experimental Botany* **2008,** *64* (2), 105-112.
263. Kerchev, P.; van der Meer, T.; Sujeeth, N.; Verlee, A.; Stevens, C. V.; Van Breusegem, F.; Gechev, T., Molecular priming as an approach to induce tolerance against abiotic and oxidative stresses in crop plants. *Biotechnology Advances* **2020,** *40*, 107503.
264. Beigzadeh, S.; Maleki, A.; Heydari, M.; Khourgami, A.; Rangin, A., Ecological and physiological performance of white bean (Phaseolus vulgaris L.) affected by algae extract and salicylic acid spraying under water deficit stress. *Applied Ecology and Environmental Research* **2019,** *17* (1), 343-355.
265. El-Kareem, G.; El-Rahman, A., Response of Ruby Seedless grapevines to foliar application of seaweed extract, salicylic acid and roselle extract. *Hortsci J Suez Canal Univ* **2013,** *1*, 294-303.
266. Hassoon, A. S.; Ramadan, E. L.; Hussain, M. H., Effect Of Salicylic Acid & Seaweed Extract In The Content Of Sepals Of Some Active Medical Compounds For Several Varieties Of Roselle Hibiscus Sabdariffa L. *Journal of Multidisciplinary Engineering Science and Technology* **2017,** *4* (4), 7068-7073.
267. Misra, N.; Saxena, P., Effect of salicylic acid on proline metabolism in lentil grown under salinity stress. *Plant Science* **2009,** *177* (3), 181-189.
268. Idrees, M.; Naeem, M.; Aftab, T.; Khan, M. M. A.; Moinuddin, Salicylic acid restrains nickel toxicity, improves antioxidant defence system and enhances the production of anticancer alkaloids in Catharanthus roseus (L.). *Journal of Hazardous Materials* **2013,** *252-253*, 367-374.
269. Hayat, S.; Masood, A.; Yusuf, M.; Fariduddin, Q.; Ahmad, A., Growth of Indian mustard (Brassica juncea L.) in response to salicylic acid under high-temperature stress. *Brazilian Journal of Plant Physiology* **2009,** *21* (3), 187-195.
270. Pasternak, T.; Groot, E. P.; Kazantsev, F. V.; Teale, W.; Omelyanchuk, N.; Kovrizhnykh, V.; Palme, K.; Mironova, V. V., Salicylic Acid Affects Root Meristem Patterning via Auxin Distribution in a Concentration-Dependent Manner. *Plant Physiology* **2019,** *180* (3), 1725-1739.
271. Asao, T.; Hasegawa, K.; Sueda, Y.; Tomita, K.; Taniguchi, K.; Hosoki, T.; Pramanik, M. H. R.; Matsui, Y., Autotoxicity of root exudates from taro. *Scientia Horticulturae* **2003,** *97* (3), 389-396.
272. Asao, T.; Kitazawa, H.; Ban, T.; Pramanik, M. H. R.; Matsui, Y.; Hosoki, T., Search of Autotoxic Substances in Some Leaf Vegetables. *Journal of the Japanese Society for Horticultural Science* **2004,** *73* (3), 247-249.
273. Yang, W.; Zhu, C.; Ma, X.; Li, G.; Gan, L.; Ng, D.; Xia, K., Hydrogen Peroxide Is a Second Messenger in the Salicylic Acid-Triggered Adventitious Rooting Process in Mung Bean Seedlings. *PLoS ONE* **2013,** *8* (12), e84580.
274. Ali, O.; Ramsubhag, A.; Jayaraman, J., Biostimulant Properties of Seaweed Extracts in Plants: Implications towards Sustainable Crop Production. *Plants* **2021,** *10* (3), 531.
275. Rouphael, Y.; Colla, G., Synergistic Biostimulatory Action: Designing the Next Generation of Plant Biostimulants for Sustainable Agriculture. *Frontiers in Plant Science* **2018,** *9*.

APPENDIX A – SUPPLEMENTAL DATA FOR CHAPTER 3

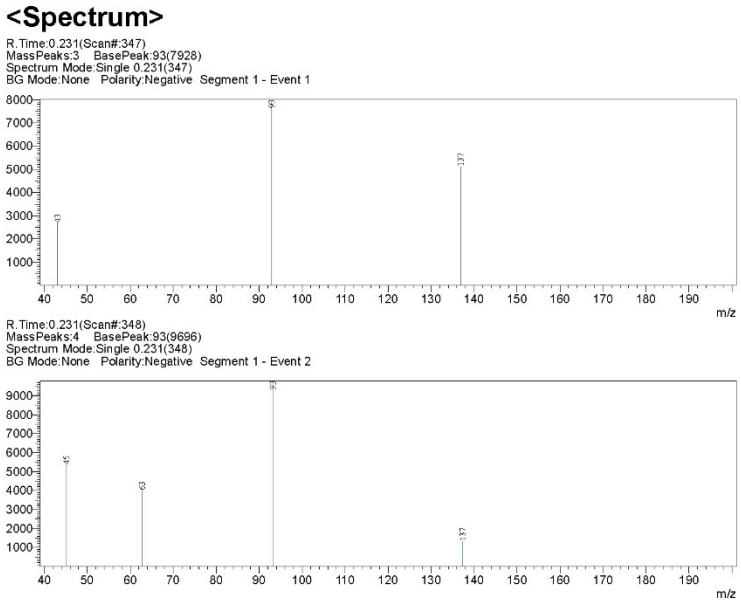
A.1 PRODUCT ION SCAN MS^2 MASS SPECTRA

2-hydroxybenzoic acid



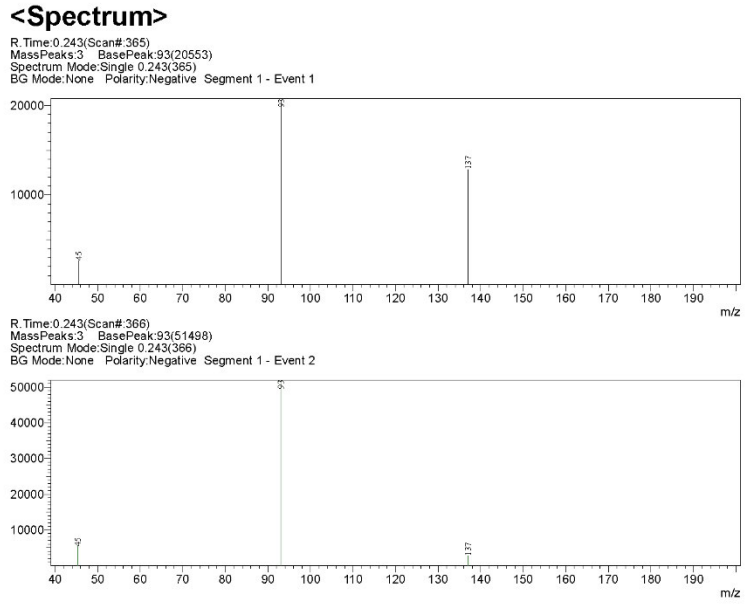
Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

3-hydroxybenzoic acid



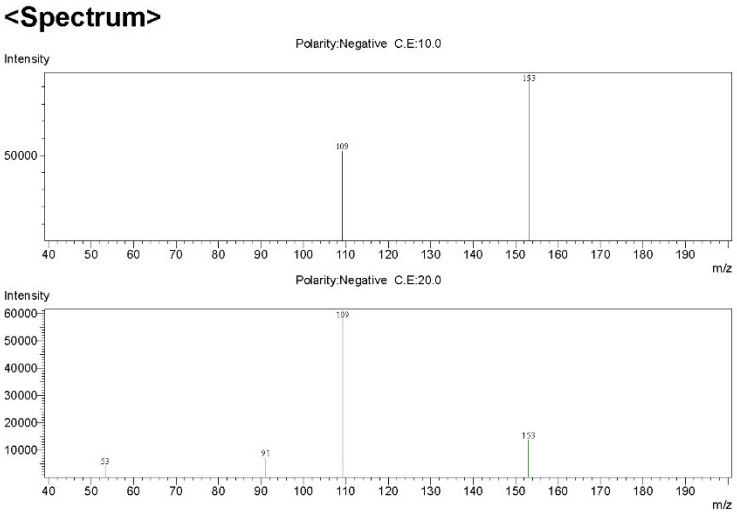
Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

4-hydroxybenzoic acid



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

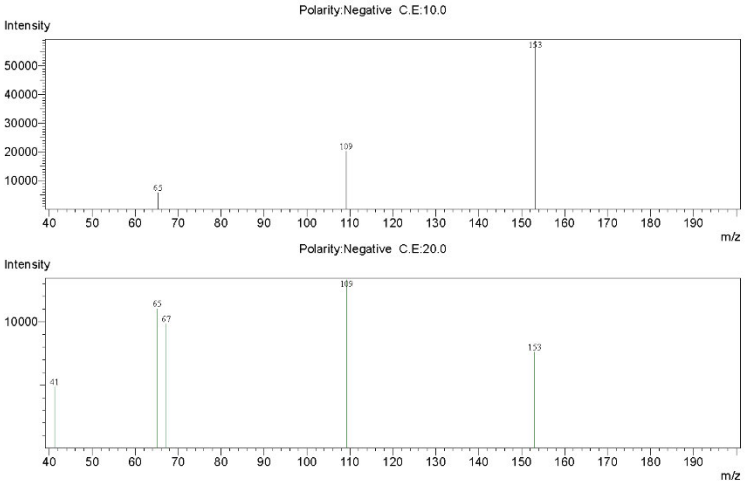
2,3-dihydroxybenzoic acid



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

2,4-dihydroxybenzoic acid

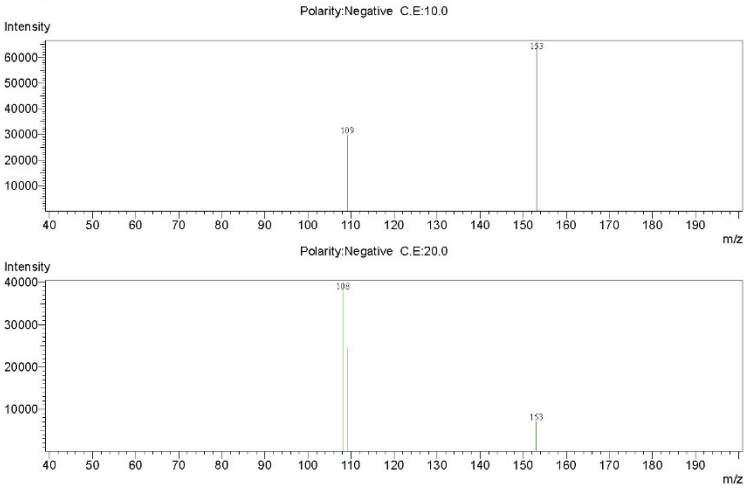
<Spectrum>



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

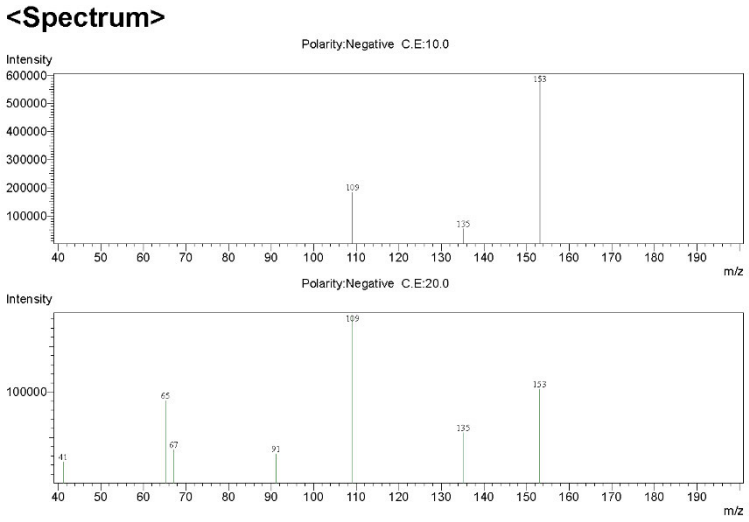
2,5-dihydroxybenzoic acid

<Spectrum>



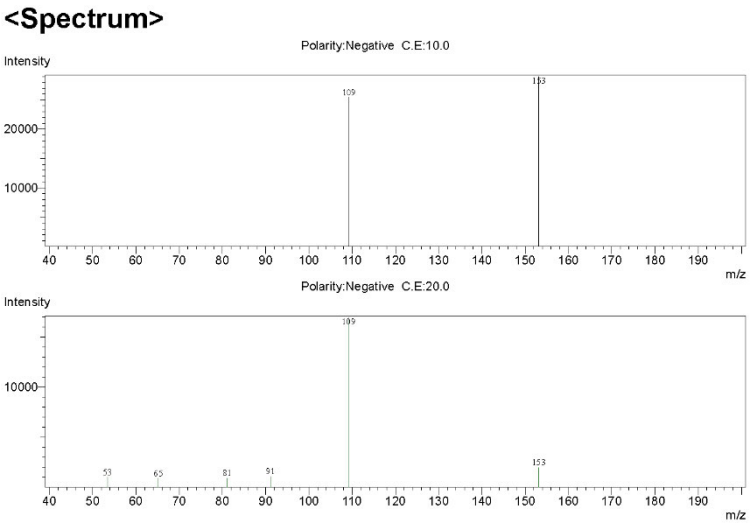
Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

2,6-dihydroxybenzoic acid



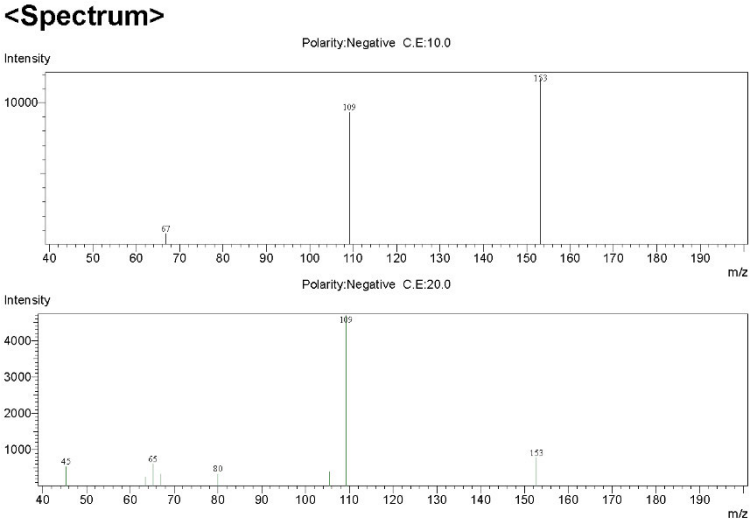
Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

3,4-dihydroxybenzoic acid



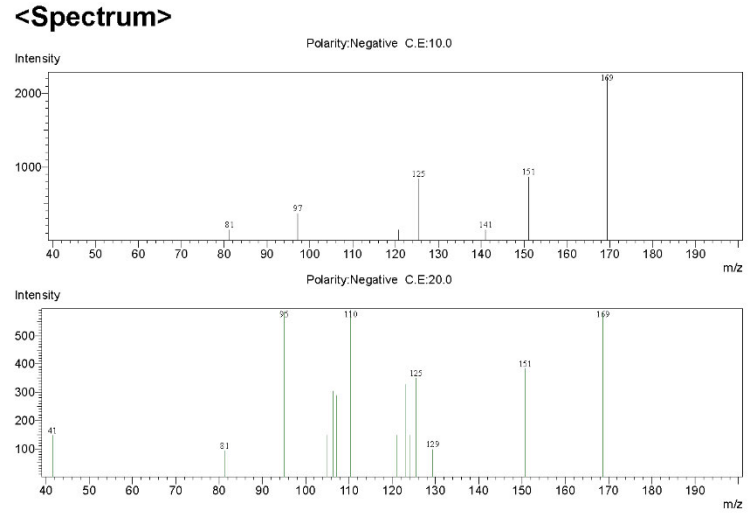
Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

3,5-dihydroxybenzoic acid



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

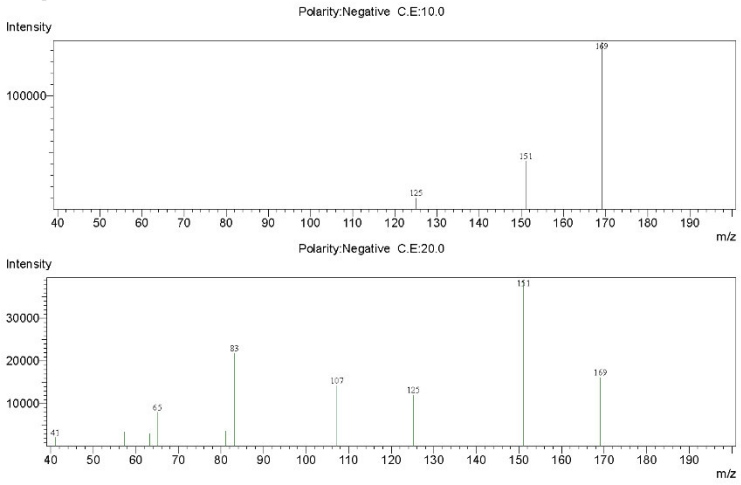
2,3,4-trihydroxybenzoic acid



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

2,4,6-trihydroxybenzoic acid

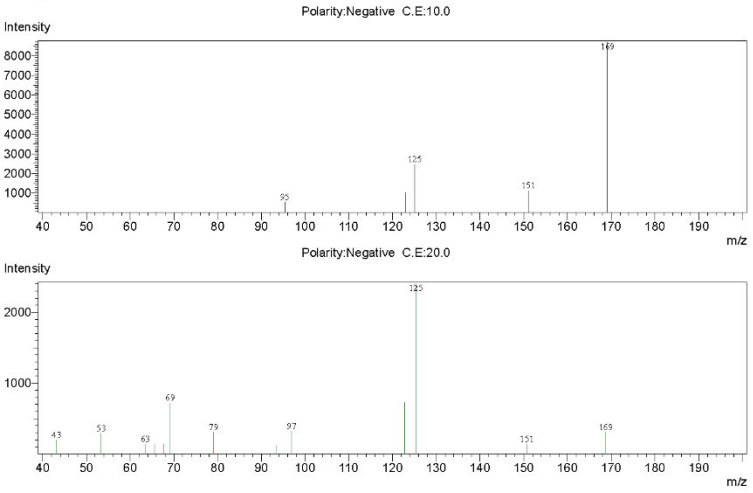
<Spectrum>



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

3,4,5-trihydroxybenzoic acid

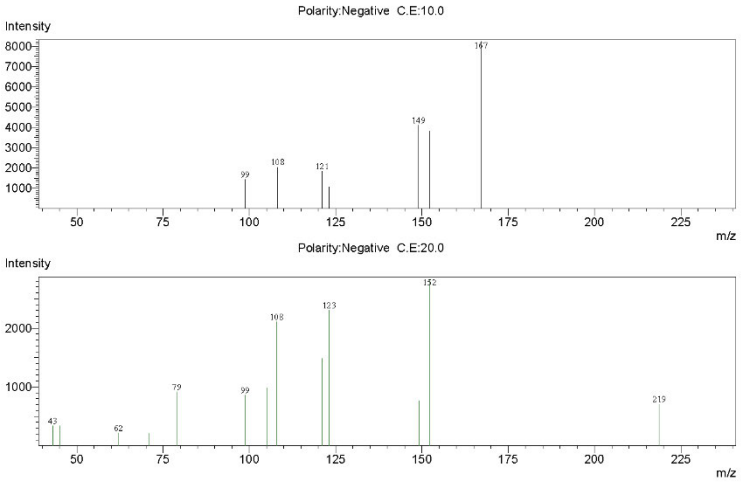
<Spectrum>



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

4-hydroxy-3-methoxybenzoic acid

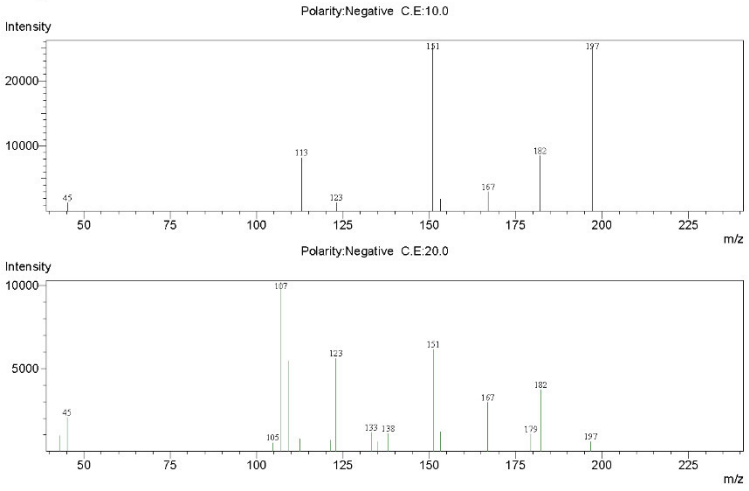
<Spectrum>



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

4-hydroxy-3,5-dimethoxybenzoic acid

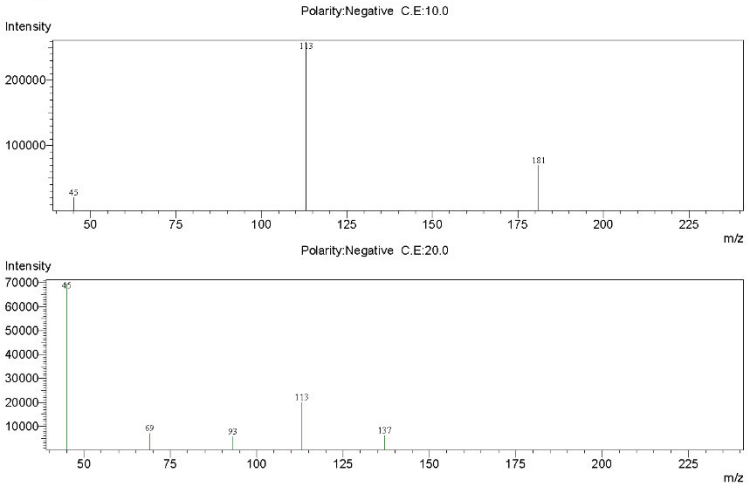
<Spectrum>



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

3,4-dimethoxybenzoic acid

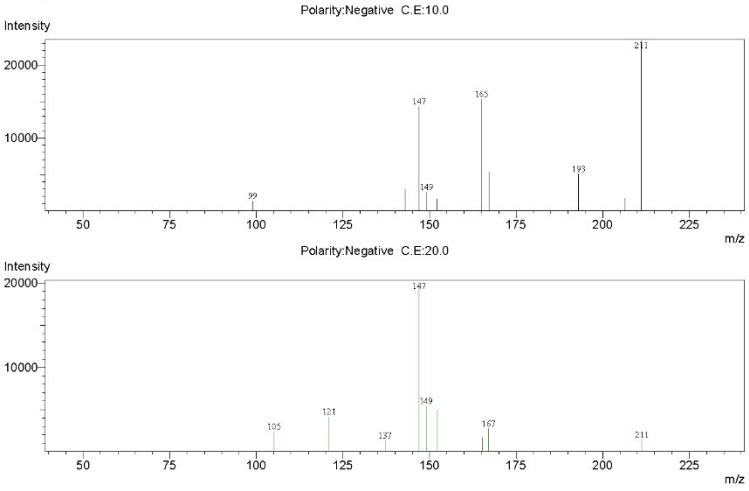
<Spectrum>



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

3,4,5-trimethoxybenzoic acid

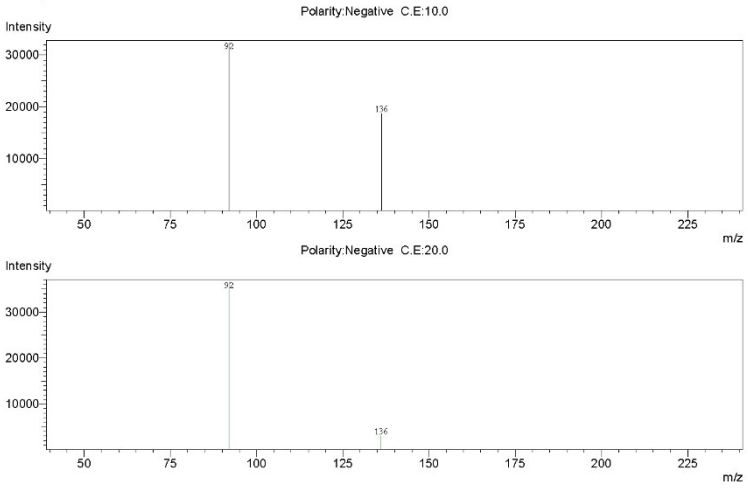
<Spectrum>



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

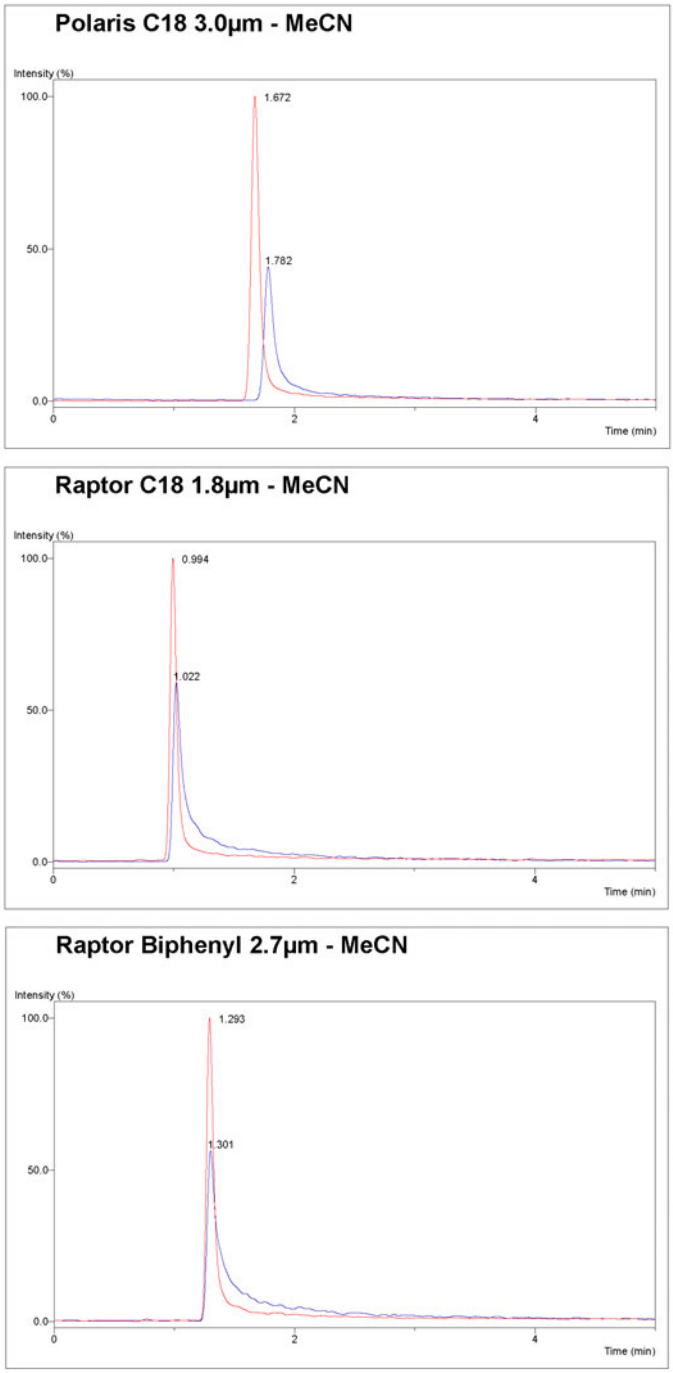
2-aminobenzoic acid

<Spectrum>

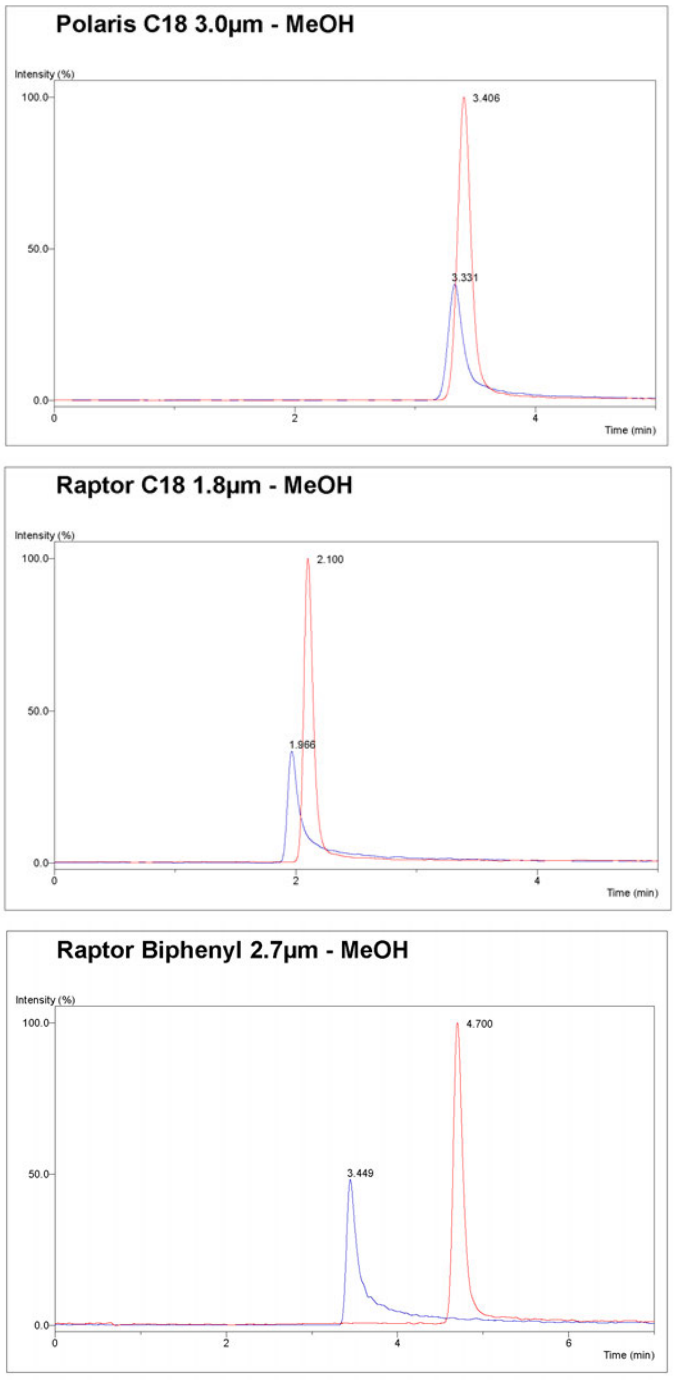


Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

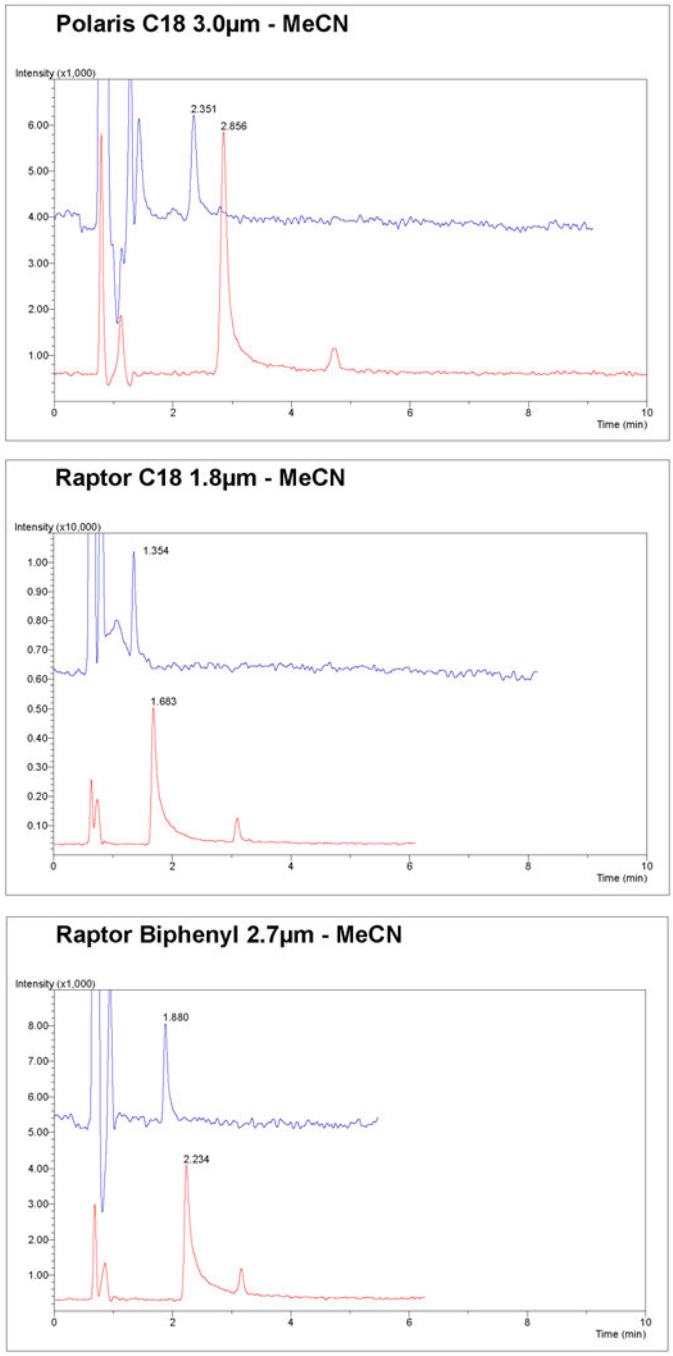
A.2 HYDROXYBENZOIC ACID RELATED DERIVATIVES LC INVESTIGATION CHROMATOGRAMS



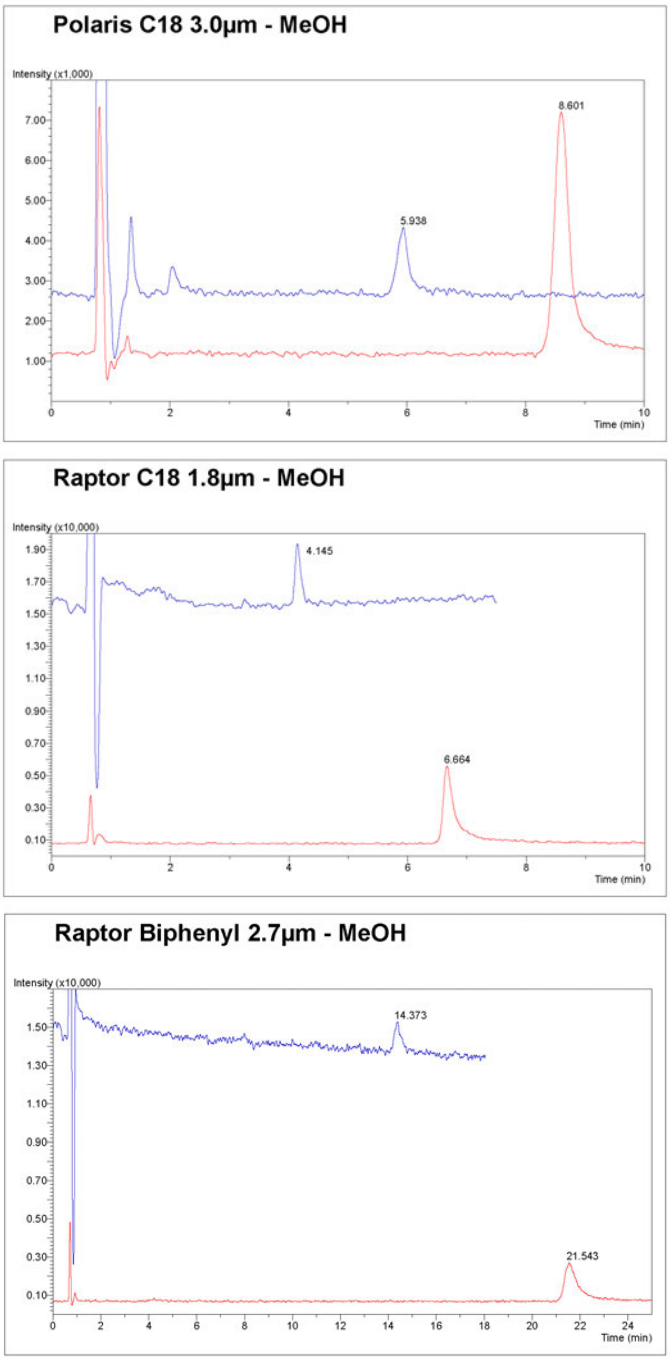
Chromatograms of vanillic (blue) and syringic (red) acids on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with an acetonitrile/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).



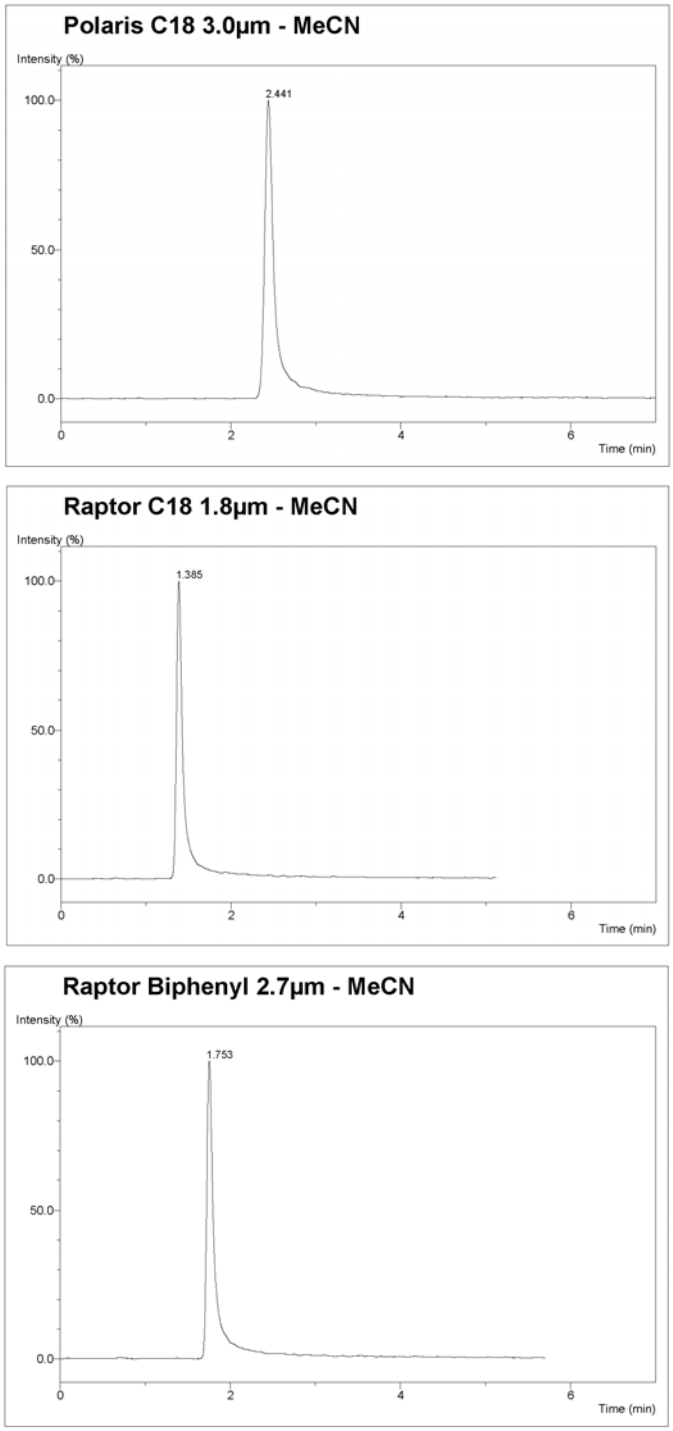
Chromatograms of vanillic (blue) and syringic (red) acids on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with a methanol/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).



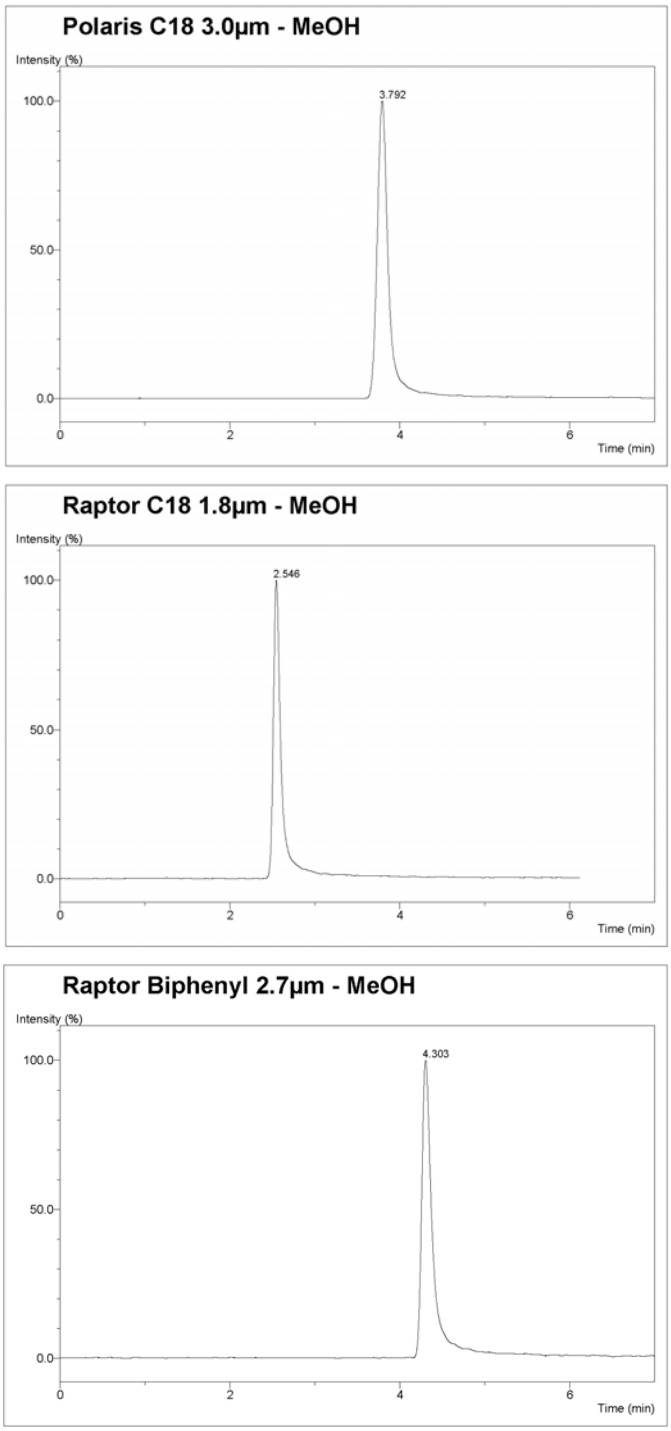
Chromatograms of veratric (blue) and eudesmic (red) acids on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with an acetonitrile/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).



Chromatograms of veratric (blue) and eudesmic (red) acids on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with a methanol/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).



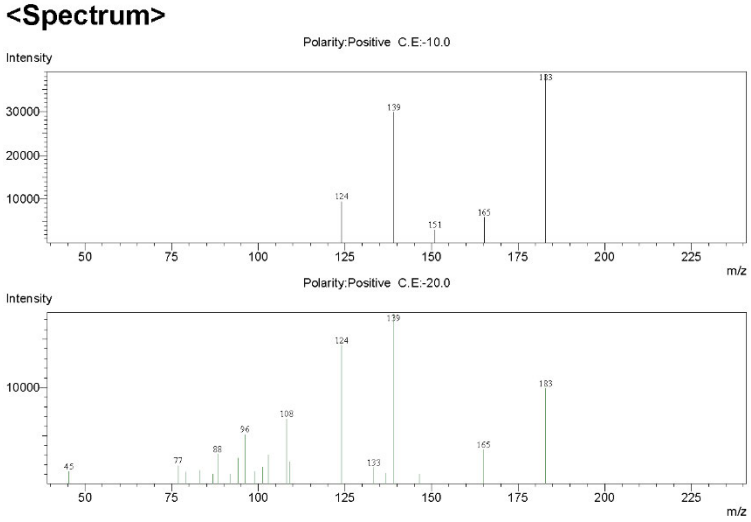
Chromatograms of anthranilic acid on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with an acetonitrile/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).



Chromatograms of anthranilic acid on the Polaris C18, Raptor C18, and Raptor Biphenyl columns respectively; all with a methanol/water mobile phase with formic acid modification run under the LC-MS/MS conditions outlined in (Chapter 2.6.2.3).

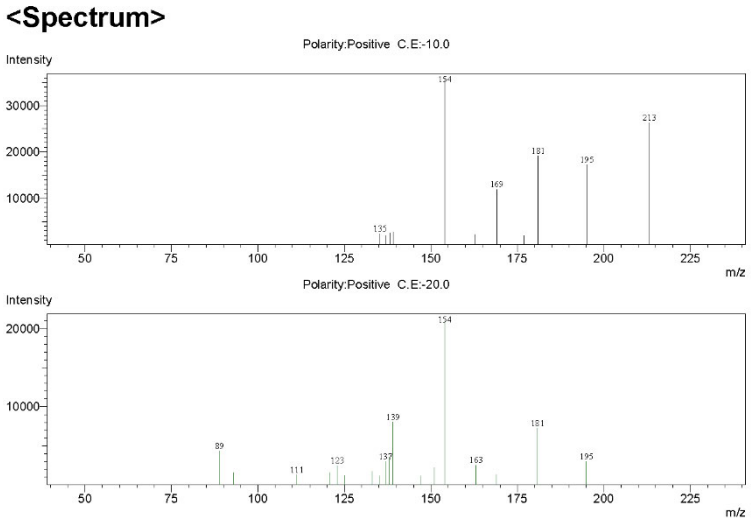
A.3 PRODUCT ION SCAN (POSITIVE MODE) MS^2 MASS SPECTRA

3,4-dimethoxybenzoic acid (Positive Ion Mode)



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

3,4,5-trimethoxybenzoic acid (Positive Ion Mode)



Mass spectra from product ion scans at collision energies of 10 V (top) and 20 V (below)

APPENDIX B – SUPPLEMENTAL DATA FOR CHAPTER 4

B.1 LINEARITY DATA

Linearity

2-hydroxybenzoic acid

Cal Level	Blank	1	2	3	4	5	6
	0	8980	18651	28076	39941	48813	58449
	0	8134	18088	28609	39889	48802	59945
	0	8092	18534	28901	39810	48736	60101
Average	0	8402	18424.33	28528.67	39880	48783.67	59498.33
%CV		5.96	1.61	1.47	0.17	0.09	1.53

3-hydroxybenzoic acid

Cal Level	Blank	1	2	3	4	5	6
	0	7692	16321	24491	33131	40926	51020
	0	7514	16716	25117	32612	41684	51012
	0	7588	16095	24892	32267	41773	50426
Average	0	7598	16377.33	24833.33	32670	41461	50819.33
%CV		1.18	1.92	1.28	1.33	1.12	0.67

4-hydroxybenzoic acid

Cal Level	Blank	1	2	3	4	5	6
	0	25092	49830	73795	96848	124432	153527
	0	24979	48799	73836	97795	126345	152921
	0	24946	47926	74774	97886	128288	152514
Average	0	25005.67	48851.67	74135	97509.67	126355	152987.3
%CV		0.31	1.95	0.75	0.59	1.53	0.33

B.2 RESPONSE FACTOR DATA

Response Factor Data

2-hydroxybenzoic acid

0	0		MEDIAN	L95%	U95%
10	8402	840.2	963.3144	915.1487	1011.48
20	18424.33	921.2167	963.3144	915.1487	1011.48
30	28528.67	950.9556	963.3144	915.1487	1011.48
40	39880	997	963.3144	915.1487	1011.48
50	48783.67	975.6733	963.3144	915.1487	1011.48
60	59498.33	991.6389	963.3144	915.1487	1011.48

3-hydroxybenzoic acid

0	0		MEDIAN	L95%	U95%
100	7598	75.98	82.33222	78.21561	86.44883
200	16377.33	81.88667	82.33222	78.21561	86.44883
300	24833.33	82.77778	82.33222	78.21561	86.44883
400	32670	81.675	82.33222	78.21561	86.44883
500	41461	82.922	82.33222	78.21561	86.44883
600	50819.33	84.69889	82.33222	78.21561	86.44883

4-hydroxybenzoic acid

0	0		MEDIAN	L95%	U95%
100	25005.67	250.0567	248.5867	236.1573	261.016
200	48851.67	244.2583	248.5867	236.1573	261.016
300	74135	247.1167	248.5867	236.1573	261.016
400	97509.67	243.7742	248.5867	236.1573	261.016
500	126355	252.71	248.5867	236.1573	261.016
600	152987.3	254.9789	248.5867	236.1573	261.016

B.3 RESIDUALS DATA

Residuals Data

2-hydroxybenzoic acid

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	-1002.642857	1002.643	1.436187697
2	9022.857143	-620.8571	-0.889317052
3	19048.35714	-624.0238	-0.893852992
4	29073.85714	-545.1905	-0.780931963
5	39099.35714	780.6429	1.118194439
6	49124.85714	-341.1905	-0.488721942
7	59150.35714	347.9762	0.498441813

3-hydroxybenzoic acid

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	-514.0714286	514.0714	1.134041407
2	7931.52381	-333.5238	-0.735753417
3	16377.11905	0.214286	0.000472714
4	24822.71429	10.61905	0.023425616
5	33268.30952	-598.3095	-1.319870618
6	41713.90476	-252.9048	-0.557907824
7	50159.5	659.8333	1.455592122

4-hydroxybenzoic acid

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	-1127.809524	1127.81	0.708051789
2	24240.71429	764.9524	0.480245902
3	49609.2381	-757.5714	-0.475612055
4	74977.7619	-842.7619	-0.529095616
5	100346.2857	-2836.619	-1.78086206
6	125714.8095	640.1905	0.401918943
7	151083.3333	1904	1.195353097

B.4 INSTRUMENT REPEATABILITY DATA

Repeatability

Retention Time

	2-hydroxybenzoic acid	**3-hydroxybenzoic acid**	**4 hydroxybenzoic acid**
	8.931	3.131	2.448
	8.937	3.126	2.446
	8.937	3.128	2.443
	8.921	3.125	2.445
	8.915	3.128	2.441
	8.940	3.124	2.448
	8.964	3.118	2.444
	8.913	3.122	2.445
	8.898	3.116	2.449
	8.917	3.119	2.443
Mean	8.927	3.124	2.445
S.D	0.018	0.005	0.003
CV	0.21	0.16	0.11

Peak Area

	2-hydroxybenzoic acid	**3-hydroxybenzoic acid**	**4 hydroxybenzoic acid**
	22178	14984	17234
	21608	15304	17281
	21750	15162	18705
	22080	15945	18121
	21939	15221	18065
	22220	16292	18516
	22748	15999	18724
	23044	16702	18296
	22510	15665	18244
	22834	16332	18642
Mean	22291.1	15760.6	18182.8
S.D	478.9	582.7	541.1
CV	2.1	3.7	3.0

B.5 INSTRUMENT DETECTION AND QUANTITATION LIMITS DATA

Mono LOD & LOQ

Signal/Noise

	2-hydroxybenzoic acid	3-hydroxybenzoic acid	4 hydroxybenzoic acid
	14.39	17.1	21.51
	12.53	17.38	20.28
	12.84	17.26	22.21
	14.56	21.15	24.94
	14.63	19.99	25.07
	14.36	20.5	23.89
	13.13	23.15	27.76
	16.2	22.59	26.44
	15.19	20.18	26.15
	16.32	22.42	26.63
Mean	14.42	20.17	24.49
S.D	1.30	2.27	2.46
CV	9.0	11.3	10.1
[x] ppb	25	250	100
LOD (3:1)	5.2	37.2	12.3
LOQ (9:1)	15.6	111.5	36.8

B.6 METHOD REPEATABILITY DATA

Method Repeatability

Peak Area

2-hydroxybenzoic acid	3-hydroxybenzoic acid	4-hydroxybenzoic acid
16675	14985	31645
16032	15796	31931
16012	14985	31940
15968	15214	32857
16102	14432	32506
16143	14327	32300
17033	15983	34696
16431	15431	34896
16718	15910	34124

Mean	16346	15229.22222	32988.33333
S.D	384.0	608.6	1254.5
CV	2.3	4.0	3.8

B.7 MATRIX EFFECTS AND RECOVERY DATA

MR 1

2-hydroxybenzoic acid

S.A Level	1	2	3	4
	9481	23662	38079	55802
	9339	24346	38738	54046
	9283	24166	38665	54320
Average	9410	24058	38494	54183
%CV	1.08	1.47	0.94	1.74

3-hydroxybenzoic acid

S.A Level	1	2	3	4
	9402	17744	24462	32975
	9641	17559	24785	31460
	9748	17401	24513	32089
Average	9597	17568	24624	32175
%CV	1.85	0.98	0.71	2.37

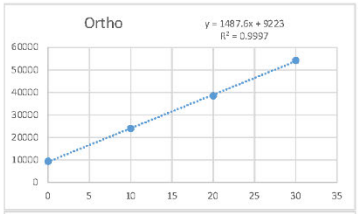
4-hydroxybenzoic acid

S.A Level	1	2	3	4
	15330	37223	57325	81913
	15464	37641	58336	81644
	15867	37828	58331	81135
Average	15554	37564	58334	81564
%CV	1.80	0.82	1.00	0.48

Dilution Factor	[x]	dil.f
Spiked Seasol Partition -	0.98	1
Load Solution Prep -	0.049	20.40816
S.P.E Load/Reconstitution -	0.065333	15
Standard Addition -	0.062067	16.11

Ortho

ppb	area
0	9410
10	24058
20	38494
30	54183

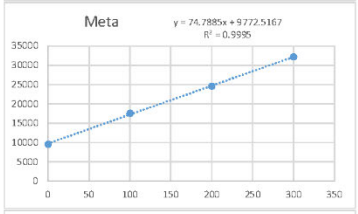


Slope	Y-Intercept
1488	9223

X-intercept	Concentration in Seasol	
-6.2	99.9	ppb

Meta

ppb	area
0	9597
100	17568
200	24624
300	32175

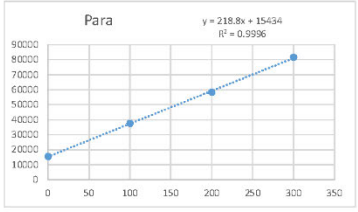


Slope	Y-Intercept
75	9773

X-intercept	Concentration in Seasol	
-130.7	2105.3	ppb

Para

ppb	area
0	15554
100	37564
200	58334
300	81564



Slope	Y-Intercept
219	15434

X-intercept	Concentration in Seasol	
-70.5	1136.5	ppb

MR 2

2-hydroxybenzoic acid

S.A Level	1	2	3	4
	16675	31174	45164	60357
	16032	31606	46475	61841
	16012	32017	45965	60747
Average	16354	31599	45868	61294
%CV	2.31	1.33	1.44	1.26

3-hydroxybenzoic acid

S.A Level	1	2	3	4
	14985	24091	31768	39791
	15796	23479	31565	41030
	14985	23382	31054	40218
Average	15255	23651	31667	40346
%CV	3.07	1.63	1.16	1.56

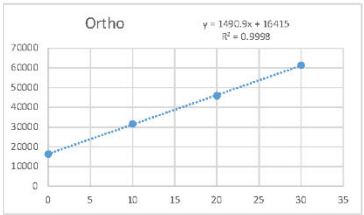
4-hydroxybenzoic acid

S.A Level	1	2	3	4
	31645	55487	79679	105820
	31931	55578	79354	104396
	31940	55895	79085	105765
Average	31839	55653	79220	105327
%CV	0.53	0.38	0.38	0.77

Dilution Factor	[x]	dil.f
Spiked Seasol Partition -	0.98	1
Load Solution Prep -	0.049	20.40816
S.P.E Load/Reconstitution -	0.065333	15
Standard Addition -	0.062067	16.11

Ortho

ppb	area
0	16354
10	31599
20	45868
30	61294

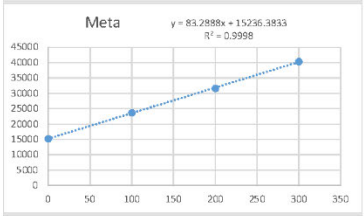


Slope	Y-Intercept
1491	16415

X-intercept	Concentration in Seasol	
-11.0	177.4	ppb

Meta

ppb	area
0	15255
100	23651
200	31667
300	40346

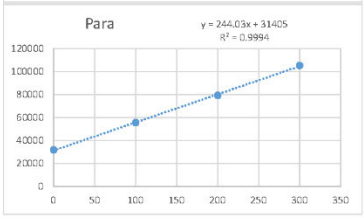


Slope	Y-Intercept
83	15236

X-intercept	Concentration in Seasol	
-182.9	2947.4	ppb

Para

ppb	area
0	31839
100	55653
200	79220
300	105327



Slope	Y-Intercept
244	31405

X-intercept	Concentration in Seasol	
-128.7	2073.5	ppb

MR 3

2-hydroxybenzoic acid

S.A Level	1	2	3	4
	15968	29728	45589	59019
	16102	30731	45167	60269
	16143	30678	45998	59594
Average	16035	30379	45585	59932
%CV	0.57	1.86	0.91	1.04

3-hydroxybenzoic acid

S.A Level	1	2	3	4
	15214	24056	30823	40296
	14432	23105	32115	39825
	14327	23208	31016	40095
Average	14658	23456	31469	40072
%CV	3.31	2.22	2.21	0.59

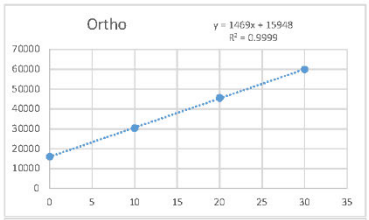
4-hydroxybenzoic acid

S.A Level	1	2	3	4
	32857	58332	83817	108968
	32506	57334	82724	109490
	32300	57083	83881	110063
Average	32554	57583	83303	109507
%CV	0.87	1.15	0.78	0.50

Dilution Factor	[x]	dil.f
Spiked Seasol Partition -	0.98	1
Load Solution Prep -	0.049	20.40816
S.P.E Load/Reconstitution -	0.065333	15
Standard Addition -	0.062067	16.11

Ortho

ppb	area
0	16035
10	30379
20	45585
30	59932

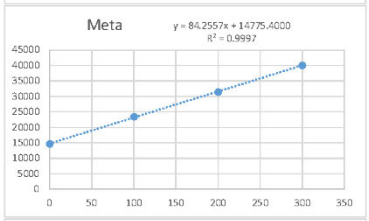


Slope	Y-Intercept
1469	15948

X-intercept	Concentration in Seasol	
-10.9	174.9	ppb

Meta

ppb	area
0	14658
100	23456
200	31469
300	40072

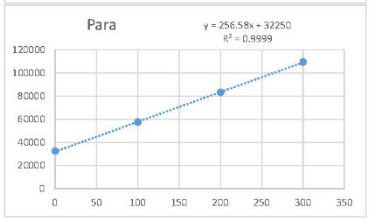


Slope	Y-Intercept
84	14775

X-intercept	Concentration in Seasol	
-175.4	2825.4	ppb

Para

ppb	area
0	32554
100	57583
200	83303
300	109507



Slope	Y-Intercept
257	32250

X-intercept	Concentration in Seasol	
-125.7	2025.1	ppb

MR 4

2-hydroxybenzoic acid

S.A Level	1	2	3	4
	17033	32255	47074	64075
	16431	31869	47397	64282
	16718	31437	47177	63709
Average	16732	31854	47287	63996
%CV	1.80	1.28	0.33	0.45

3-hydroxybenzoic acid

S.A Level	1	2	3	4
	15983	24861	33028	42676
	15431	24207	33027	43432
	15910	24003	33165	42128
Average	15775	24357	33096	42745
%CV	1.90	1.84	0.29	1.53

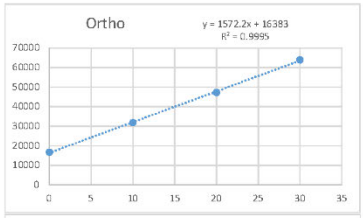
4-hydroxybenzoic acid

S.A Level	1	2	3	4
	34696	60841	88418	117296
	34896	61174	88324	121508
	34124	61292	89820	118393
Average	34572	61102	89820	119066
%CV	1.16	0.38	1.18	1.84

Dilution Factor	[x]	dil.f
Spiked Seasol Partition -	0.98	1
Load Solution Prep -	0.049	20.40816
S.P.E Load/Reconstitution -	0.065333	15
Standard Addition -	0.062067	16.11

Ortho

ppb	area
0	16732
10	31854
20	47287
30	63996

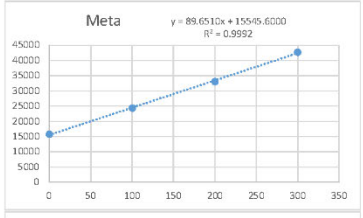


Slope	Y-Intercept
1572	16383

X-intercept	Concentration in Seasol	
-10.4	167.9	ppb

Meta

ppb	area
0	15775
100	24357
200	33096
300	42745

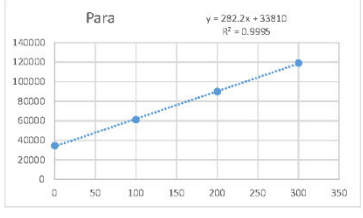


Slope	Y-Intercept
90	15546

X-intercept	Concentration in Seasol	
-173.4	2793.8	ppb

Para

ppb	area
0	34572
100	61102
200	89820
300	119066



Slope	Y-Intercept
282	33810

X-intercept	Concentration in Seasol	
-119.8	1930.3	ppb

B.8 RECOVERY CALCULATIONS TABLE

Recovery

	Blank **MR1**	*Spiked* **MR2**	*Spiked* **MR3**	*Spiked* **MR4**	Average Spiked	St Dev	CV	Spike	**% Recovery**
2-hydroxybenzoic acid	99.9	177.4	174.9	167.9	173.4	4.9	2.84	100.0	73.50
3-hydroxybenzoic acid	2105.3	2947.4	2825.4	2793.8	2855.5	81.1	2.84	1000.0	75.02
4-hydroxybenzoic acid	1136.5	2073.5	2025.1	1930.3	2009.6	72.8	3.62	1000.0	87.32

B.9 QUANTITATION DATA FOR MONOHYDROXYBENZOIC ACIDS

Quantitation

Ortho

S.A Level	1	2	3	4
	9189	25393	38365	54177
	9112	25393	38029	54453
	9295	25652	39192	54905
Average	9151	25479	38529	54679
%CV	1.00	0.59	1.55	0.67

Meta

S.A Level	1	2	3	4
	14168	24770	33988	43853
	15736	25244	33782	43315
	15571	24940	33430	43704
Average	15158	24985	33885	43624
%CV	5.68	0.96	0.83	0.64

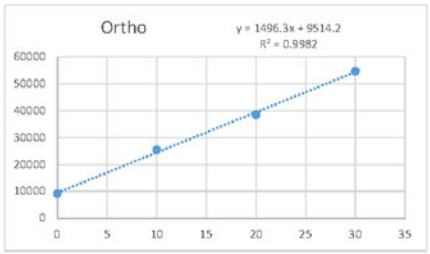
Para

S.A Level	1	2	3	4
	28066	57882	83835	115467
	26913	58420	84706	116825
	28087	58967	84369	114945
Average	27689	58423	84538	115746
%CV	2.43	0.93	0.52	0.84

Dilution Factor	[x]	dil.f
Seasol Partition -	1	1
Load Solution Prep -	0.05	20
S.P.E Load/Reconstitution -	0.066667	15
Standard Addition -	0.063333	15.79

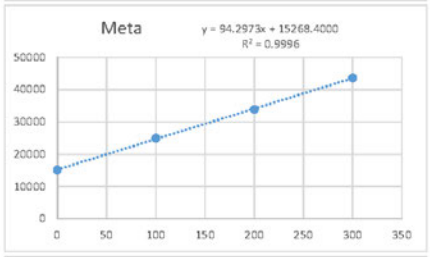
Ortho

ppb	area
0	9151
10	25479
20	38529
30	54679



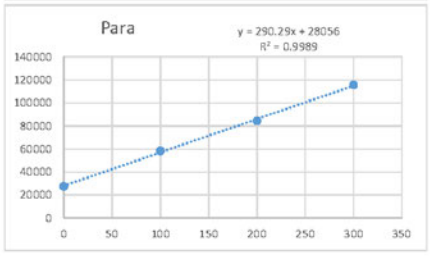
Meta

ppb	area
0	15158
100	24985
200	33885
300	43624



Para

ppb	area
0	27689
100	58423
200	84538
300	115746



2-hydroxybenzoic acid

0	9151	**y hat**	31959			
10	25479	**sum (xi - xbar)^2**	500			
20	38529	**|xE|**	6.358245462	-->	**100**	ppb
30	54679	**S xe**	0.728296854	-->	**11**	ppb
		dilution factor	0.063333333			

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999088585
R Square	0.998178001
Adjusted R Square	0.997267001
Standard Error	1010.817633
Observations	4

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1119529167	1119529167	1095.695288	0.000911415
Residual	2	2043504.575	1021752.288		
Total	3	1121572672			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*Upper 95.0%*
Intercept	9514.15	845.7107078	11.24988712	0.007808962	5875.350515	13152.95	5875.351	13152.95
X Variable 1	1496.348333	45.20513881	33.10128832	0.000911415	1301.846319	1690.85	1301.846	1690.85

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	9514.15	-363.65	-0.440612093
2	24477.63333	1001.7	1.213697602
3	39441.11667	-912.45	-1.105558927
4	54404.6	274.4	0.332473417

3-hydroxybenzoic acid

0	15158	**y hat**	29413			
100	24985	**sum (xi - xbar)^2**	50000			
200	33885	**|xE|**	161.9176223	-->	**2557**	ppb
300	43624	**S xe**	4.411414963	-->	**70**	ppb
		dilution factor	0.063333333			

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999822799
R Square	0.999645628
Adjusted R Square	0.999468443
Standard Error	280.7215702
Observations	4

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	444599353.7	444599353.7	5641.794434	0.000177201
Residual	2	157609.2	78804.6		
Total	3	444756962.9			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*Upper 95.0%*
Intercept	15268.4	234.8685164	65.00828733	0.000236542	14257.84234	16278.96	14257.84	16278.96
X Variable 1	94.29733333	1.255425028	75.11187945	0.000177201	88.89567541	99.69899	88.89568	99.69899

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	15268.4	-110.0666667	-0.480203874
2	24698.13333	286.5333333	1.250100696
3	34127.86667	-242.8666667	-1.059589771
4	43557.6	66.4	0.289692949

4-hydroxybenzoic acid					
0	27689	**y hat**	71599		
100	58423	**sum (xi - xbar)^2**	50000		
200	84538	**|xE|**	96.64927574	**1526**	ppb
300	115746	**S xe**	6.445579326	**102**	ppb
		dilution factor	0.063333333		

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999433971
R Square	0.998868263
Adjusted R Square	0.998302394
Standard Error	1544.948805
Observations	4

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	4213283576	4213283576	1765.194253	0.000566029
Residual	2	4773733.619	2386866.81		
Total	3	4218057309			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*Upper 95.0%*
Intercept	28055.88333	1292.596908	21.70505218	0.002115915	22494.28772	33617.48	22494.29	33617.48
X Variable 1	290.2855	6.909221099	42.01421489	0.000566029	260.557521	320.0135	260.5575	320.0135

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	28055.88333	-367.2166667	-0.291107853
2	57084.43333	1338.566667	1.061137207
3	86112.98333	-1575.483333	-1.248950856
4	115141.5333	604.1333333	0.478921502

APPENDIX C – SUPPLEMENTAL DATA FOR CHAPTER 5

C.1 EXPERIMENT 1 DATA

Root Length

Seasol control	Water control	2-hydroxy SA	3-hydroxy SA	4-hysroxy SA	2,3,4-hydroxy-SA	2,3,4-hydroxy-SA+Seasol
mm	mm	mm	mm	mm	mm	mm
32	9	3	23	7	6	57
39	2	6	18	3	2	73
59	11	5	17	24	2	119
49	15	4	25	23	1	97
50	29	8	1	6	4	84
66	6	11	19	30	7	78

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	295	49.16666667	155.7666667
Column 2	6	72	12	88.8
Column 3	6	37	6.166666667	8.566666667
Column 4	6	103	17.16666667	72.16666667
Column 5	6	93	15.5	131.5
Column 6	6	22	3.666666667	5.866666667
Column 7	6	508	84.66666667	455.4666667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	31494.95	6	5249.15873	40.02045213	2.98063E-14	2.371781196
Within Groups	4590.667	35	131.1619048			
Total	36085.62	41				

LSD Test

Significant Differences
b
cd
cd
c
cd
d
a

Root fresh weight

Seasol control	Water control	2-hydroxy SA	3-hydroxy SA	4-hysroxy SA	2,3,4-hydroxy-SA	2,3,4-hydroxy-SA+Seasol
g	g	g	g	g	g	g
0.42	0.08	0.02	0.14	0.08	0.07	0.34
0.39	0.04	0.07	0.12	0.01	0.04	0.39
0.33	0.10	0.08	0.15	0.16	0.08	0.40
0.38	0.09	0.05	0.17	0.15	0.02	0.36
0.33	0.12	0.04	0.08	0.02	0.07	0.45
0.44	0.09	0.11	0.17	0.13		0.50

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	2.29	0.381666667	0.002056667
Column 2	6	0.52	0.086666667	0.000706667
Column 3	6	0.37	0.061666667	0.001016667
Column 4	6	0.83	0.138333333	0.001176667
Column 5	6	0.55	0.091666667	0.004296667
Column 6	5	0.28	0.056	0.00063
Column 7	6	2.44	0.406666667	0.003506667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.822436098	6	0.137072683	70.27248522	1.01901E-17	2.380312704
Within Groups	0.06632	34	0.001950588			
Total	0.888756098	40				

LSD Test

Significant Differences
a
bc
c
b
bc
c
a

Root dryweight

Seasol control	Water control	2-hydroxy SA	3-hydroxy SA	4-hysroxy SA	2,3,4-hydroxy-SA	2,3,4-hydroxy-SA+Seasol
g	g	g	g	g	g	g
0.04	0.01	0.01	0.04	0.01	0.01	0.05
0.09	0.01	0.01	0.03	0.01	0.01	0.06
0.02	0.02	0.01	0.04	0.04	0.01	0.05
0.02	0.01	0.01	0.04	0.03	0.01	0.06
0.02	0.02	0.01	0.01	0.01	0.01	0.05
0.03	0.01	0.02	0.03	0.02	0.01	0.05

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	0.22	0.036666667	0.000746667
Column 2	6	0.08	0.013333333	2.66667E-05
Column 3	6	0.07	0.011666667	1.66667E-05
Column 4	6	0.19	0.031666667	0.000136667
Column 5	6	0.12	0.02	0.00016
Column 6	6	0.06	0.01	0
Column 7	6	0.32	0.053333333	2.66667E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.009281	6	0.001546825	9.725548902	2.63068E-06	2.371781196
Within Groups	0.005567	35	0.000159048			
Total	0.014848	41				

LSD Test

Significant Differences
b
d
d
bc
cd
d
a

Shoot Fresh Weight

Seasol control	Water control	2-hydroxy SA	3-hydroxy SA	4-hysroxy SA	2,3,4-hydroxy-SA	2,3,4-hydroxy-SA+Seasol
g	g	g	g	g	g	g
0.43	0.35	0.28	0.50	0.69	0.38	0.51
0.35	0.51	0.62	0.51	0.45	0.54	0.70
0.47	0.49	0.67	0.52	0.47	0.33	0.45
0.35	0.48	0.46	0.54	0.50	0.56	0.56
0.40	0.35	0.39	0.63	0.60	0.25	0.56
0.48	0.41	0.68	0.42	0.52	0.55	0.78

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	2.48	0.413333333	0.003226667
Column 2	6	2.59	0.431666667	0.005136667
Column 3	6	3.1	0.516666667	0.027226667
Column 4	6	3.12	0.52	0.0046
Column 5	6	3.23	0.538333333	0.008216667
Column 6	6	2.61	0.435	0.01763
Column 7	6	3.56	0.593333333	0.015186667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.161295	6	0.02688254	2.316794572	0.054724223	2.371781196
Within Groups	0.406117	35	0.011603333			
Total	0.567412	41				

LSD Test

Significant Differences
b
b
ab
ab
ab
b
a

Shoot Dry Weight

Seasol control	Water control	2-hydroxy SA	3-hydroxy SA	4-hysroxy SA	2,3,4-hydroxy-SA	2,3,4-hydroxy-SA+Seasol
g	g	g	g	g	g	g
0.09	0.07	0.04	0.09	0.13	0.05	0.10
0.05	0.10	0.10	0.11	0.08	0.09	0.13
0.07	0.09	0.10	0.12	0.07	0.08	0.09
0.05	0.09	0.08	0.11	0.10	0.10	0.10
0.07	0.07	0.05	0.13	0.11	0.04	0.12
0.08	0.07	0.10	0.08	0.10	0.10	0.13

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	0.41	0.068333333	0.000256667
Column 2	6	0.49	0.081666667	0.000176667
Column 3	6	0.47	0.078333333	0.000736667
Column 4	6	0.64	0.106666667	0.000346667
Column 5	6	0.59	0.098333333	0.000456667
Column 6	6	0.46	0.076666667	0.000666667
Column 7	6	0.67	0.111666667	0.000296667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.009957	6	0.001659524	3.955732123	0.004012861	2.371781196
Within Groups	0.014683	35	0.000419524			
Total	0.02464	41				

LSD Test

Significant Differences
c
bc
bc
a
ab
bc
a

C.2 EXPERIMENT 2 DATA

Root Length

Seasol control	Water control	2-hydroxy SA	3-hydroxy SA	4-hysroxy SA	2,3,4-hydroxy-SA	2,3,4-hydroxy-SA+Seasol
mm	mm	mm	mm	mm	mm	mm
90	2	5	10	9	2	85
75	5	7	11	5	2	78
65	2	8	11	3	4	72
65	7	8	8	6	3	70
80	6	3	8	8	2	62
65	7	8	10	10	4	68

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	440	73.33333333	106.6666667
Column 2	6	29	4.833333333	5.366666667
Column 3	6	39	6.5	4.3
Column 4	6	58	9.666666667	1.866666667
Column 5	6	41	6.833333333	6.966666667
Column 6	6	17	2.833333333	0.966666667
Column 7	6	435	72.5	64.7

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	38384.9	6	6397.484127	234.66754	9.33294E-27	2.371781196
Within Groups	954.1667	35	27.26190476			
Total	39339.07	41				

LSD Test

Significant Differences
a
bc
bc
b
bc
c
a

Root Fresh Weight

Seasol control	Water control	2-hydroxy SA	3-hydroxy SA	4-hysroxy SA	2,3,4-hydroxy-SA	2,3,4-hydroxy-SA+Seasol
g	g	g	g	g	g	g
0.336	0.118	0.140	0.195	0.123	0.081	0.614
0.451	0.103	0.080	0.145	0.148	0.114	0.487
0.620	0.060	0.202	0.124	0.090	0.090	0.534
0.403	0.100	0.170	0.149	0.114	0.091	0.521
0.473	0.086	0.190	0.140	0.099	0.088	0.698
0.483	0.095	0.118	0.119	0.141	0.094	0.667

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	2.766	0.461	0.0089996
Column 2	6	0.562	0.093666667	0.000382667
Column 3	6	0.9	0.15	0.0021456
Column 4	6	0.872	0.145333333	0.000731467
Column 5	6	0.715	0.119166667	0.000521367
Column 6	6	0.558	0.093	0.0001248
Column 7	6	3.521	0.586833333	0.007322967

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.462091952	6	0.243681992	84.32541984	2.46735E-19	2.371781196
Within Groups	0.101142333	35	0.002889781			
Total	1.563234286	41				

LSD Test

Significant Differences
b
c
c
c
c
c
a

Root Dry Weight

Seasol control	Water control	2-hydroxy SA	3-hydroxy SA	4-hysroxy SA	2,3,4-hydroxy-SA	2,3,4-hydroxy-SA+Seasol
g	g	g	g	g	g	g
0.044	0.020	0.030	0.059	0.047	0.044	0.162
0.046	0.020	0.010	0.049	0.054	0.045	0.105
0.149	0.010	0.093	0.042	0.040	0.032	0.130
0.066	0.020	0.088	0.052	0.039	0.037	0.139
0.087	0.020	0.050	0.041	0.032	0.029	0.175
0.104	0.020	0.020	0.034	0.055	0.035	0.192

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	0.496	0.082666667	0.001598267
Column 2	6	0.11	0.018333333	1.66667E-05
Column 3	6	0.291	0.0485	0.0012359
Column 4	6	0.277	0.046166667	7.97667E-05
Column 5	6	0.267	0.0445	8.27E-05
Column 6	6	0.222	0.037	4.12E-05
Column 7	6	0.903	0.1505	0.0010155

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.069148	6	0.011524603	19.82118482	6.15302E-10	2.371781196
Within Groups	0.02035	35	0.000581429			
Total	0.089498	41				

LSD Test

Significant Differences
b
d
c
cd
cd
cd
a

Shoot Fresh Weight

Seasol control	Water control	2-hydroxy SA	3-hydroxy SA	4-hysroxy SA	2,3,4-hydroxy-SA	2,3,4-hydroxy-SA+Seasol
g	g	g	g	g	g	g
1.042	0.555	0.593	0.609	0.408	0.305	1.076
1.002	0.637	0.452	0.360	0.640	0.635	0.877
0.985	0.460	0.690	0.394	0.684	0.414	0.959
0.823	0.512	0.540	0.584	0.335	0.564	1.024
0.793	0.282	0.567	0.371	0.368	0.489	1.072
0.952	0.476	0.449	0.322	0.423	0.501	1.165

Anova: Single Factor

LSD Test

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	5.597	0.932833333	0.010281367
Column 2	6	2.922	0.487	0.0141248
Column 3	6	3.291	0.5485	0.0083259
Column 4	6	2.64	0.44	0.0152996
Column 5	6	2.858	0.476333333	0.021827467
Column 6	6	2.908	0.484666667	0.013290667
Column 7	6	6.173	1.028833333	0.010116567

Significant Differences
a
b
b
b
b
b
a

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.152095	6	0.358682524	26.92050475	9.60374E-12	2.371781196
Within Groups	0.466332	35	0.013323767			
Total	2.618427	41				

Shoot Dry Weight

Seasol control	Water control	2-hydroxy SA	3-hydroxy SA	4-hysroxy SA	2,3,4-hydroxy-SA	2,3,4-hydroxy-SA+Seasol
g	g	g	g	g	g	g
0.122	0.082	0.082	0.089	0.083	0.084	0.123
0.124	0.085	0.082	0.089	0.087	0.085	0.109
0.112	0.081	0.087	0.086	0.081	0.083	0.115
0.110	0.084	0.083	0.086	0.084	0.082	0.121
0.112	0.082	0.082	0.083	0.084	0.082	0.121
0.121	0.081	0.086	0.083	0.087	0.083	0.131

Anova: Single Factor

LSD Test

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	0.701	0.116833333	3.77667E-05
Column 2	6	0.495	0.0825	2.7E-06
Column 3	6	0.502	0.083666667	5.06667E-06
Column 4	6	0.516	0.086	7.2E-06
Column 5	6	0.506	0.084333333	5.46667E-06
Column 6	6	0.499	0.083166667	1.36667E-06
Column 7	6	0.72	0.12	0.0000556

Significant Differences
a
b
b
b
b
b
a

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.010265	6	0.001710873	103.9893874	7.99528E-21	2.371781196
Within Groups	0.000576	35	1.64524E-05			
Total	0.010841	41				