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Ecometabolomic investigation of abiotically induced phytochemical change to invasive heather, *Calluna vulgaris* and its influence on the biocontrol agent *Lochmaea suturalis.*

A thesis presented in partial fulfilment of the requirement for

the degree of

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in

Ecology

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Summary

Invasive plants represent one of the most pressing environmental issues globally. They pose a serious threat to terrestrial ecosystem function, loss of biodiversity, economic wellbeing and food security. Biological control of invasive plants is a well-founded and in many cases successful management option against these threats but in some cases, the control agents fail and do not establish or are ineffective if they do. Relevant literature maintains that abiotic and biotic influences can be responsible for failed biocontrol programmes. However, a largely unexplored reason for these failures could involve abiotically induced changes to an invasive plants' biochemical phenotype, which may consequently enhance resistance against the biocontrol agent. Understanding invasive plant biochemical phenotypic change, and the environmental influences determining them, could provide valuable information to assist with control agent and site selection or to retrospectively elucidate failures or ineffectiveness.

Plant metabolomics studies plant biochemistry at the molecular level, providing a non-targeted characterisation and quantification of both known and unknown metabolites (the metabolome) in a particular tissue in response to its environment. This technique combines analytical chemistry, bioinformatics and multivariate statistics, allowing characterisation and identification of plant biochemical profiles and phenotypes. A literature review provides little evidence however that this technology is being applied to support weed biocontrol programmes. Heather beetle, *Lochmaea suturalis* (Thomson, 1866), introduced to control invasive heather *Calluna vulgaris* (L.) Hull in the Central Plateau (CP) region of New Zealand (NZ) was difficult to establish and displays variable effectiveness. This provides an ideal model system to apply metabolomic technology and to test hypotheses relating to potential changes in the heather metabolome in its invaded environment.

Using UHPLC-MS/MS based non-targeted metabolomics, I analysed primary and secondary metabolites of *C. vulgaris* from its native range in the United Kingdom (UK) and its introduced range (NZ), between which, differences in soil nutrients and ultraviolet light exist. I also explored secondary metabolite variation between sites that differ in soil nutrient profiles within each range. New Zealand samples had the greatest number of amplified metabolites, most notably defensive phenylpropanoids and evidence of upregulation of key biosynthetic pathways. Secondary metabolite variation within each range revealed differences between sites but found little correlation of phenylpropanoid levels being influenced by variable soil nutrients.

Ultra-violet (UV) radiation is a known driver of alterations to secondary metabolites in plants. On the CP, UV intensity is double that of the plants native range in the UK. Heather was experimentally exposed to 20% and 95% attenuation of UV and the secondary metabolite responses were recorded. This treatment demonstrated significant alterations to many compounds derived from the shikimate-phenylpropanoid pathway. Using plants from each treatment, bioassays were performed measuring prepupal weight and survival of *L. suturalis*. The results demonstrate significant UV-induced alterations to several compounds in heather but no significant difference in the beetle life history parameters.

Evidence exists that heather beetle performance and establishment improves with increased foliar nitrogen (N) in heather. A question remained whether this is due solely to increased foliar N or, additionally, to changes to the defensive metabolites of the plant. A field-based experiment using artificial fertilizer to increase soil nutrient availability was established. Foliar total carbon (C) and N, and primary and secondary metabolite responses in *C. vulgaris* foliage were quantified, revealing several amplified primary nitrogen containing metabolites and a decrease in ~ 68% of secondary phenylpropanoids. This result suggests

that nutrient stress increases the foliar C:N ratio resulting in increased carbon-rich defensive metabolites and decreased N-containing metabolites. This in turn reduces the efficiency of conversion of ingested food (ECI) index and thus is likely the main driver of poor performance of *L. suturalis* in the CP environment.

The results of these metabolomic investigations demonstrate the highly complex interactions that exist between abiotic parameters, plant metabolome and insect herbivore responses. Understanding these complexities could have an important role to play in future biological control of weeds programmes and perhaps be applied to improve success rates or retrospectively elucidate and/or resurrect problematic ones.

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Declaration

This thesis is based on publications, and the formatting style of each chapter follows the guidelines for the journal in which it has been published, or in which it has been submitted to or accepted for publication. Therefore, there are some inconsistencies in the layout and section headings between the chapters presented in this thesis.

Chapter 1

Introduction

Heather (*Calluna vulgaris*) at the invasion front on the Central Plateau sub-alpine region North Island NZ. Inset: An adult heather beetle (*Lochmaea suturalis*) the biocontrol agent, active on flowering heather during March. This biocontrol pairing is the model system studied throughout this thesis.

1.1 Invasive weeds and classical biocontrol

Globally, invasive alien plants are a serious threat to the terrestrial habitats they invade, potentially causing considerable economic loss in managed production systems (i.e., agriculture, horticulture, or forestry) and causing serious ecological perturbations to natural conservation reserves and traditional communal rangelands (Pimental et al. 2005). In many instances, all of these factors apply which may result in losses to biodiversity at the species, community or ecosystem level (Pyšek et al. 2012).

Classical biological control (the reuniting of specialist insects or pathogens with their co-evolved host plant) is often the only practical, economically viable and self-sustaining solution which, if implemented according to modern best practice, is low risk (Fowler et al. 2000, Hayes et al. 2013; Shepard et al. 2003). In conservation lands, biocontrol has often been the only viable solution to providing 1): effective long term weed control (Schwarzländer et al. 2018) and 2): preservation of the integrity of native plant communities. In contrast, chemical controls in these sensitive environments seldom achieves either of those goals (Peterson et al. 2020).

Biological control programmes usually take many years from initiation to release. For a single programme, hundreds of thousands of dollars are spent but cost-benefit analyses, particularly for production system weeds demonstrate considerable economic benefits with cost-benefit ratios often in excess of 100:1 (Fowler et al. 2024). Environmental weeds don't provide similar ratios, but advantages are usually clear when considering protection of biodiversity and ecosystem services (Fowler et al. 2024). Thus, any technological advances that can help ensure increased success rates in biocontrol will be of great benefit.

1.2 Poor establishment and performance of biocontrol agents

Many insect herbivores released as biocontrol agents, fail to establish or only achieve low population viability and therefore poor effectiveness, when reunited with their only possible co-evolved host plant in a new environment. This is one of the great conundrums of biological control of invasive weeds and as such is a key question motivating this thesis.

Some establishment failures are due to mismatches between the agent and its new environment (often termed environmental resilience) (Grevstad 1999; Lawton 1990; Peterson et al. 2007, 2011; Fowler et al. 2015). Environmental variability,

such as stochastic climatic events (Peterson et al. 2007, 2011; Fowler et al. 2015) the Allee effect and associated demographic stochasticity (Stephens 1999; Berec et al. 2007; Tobin et al. 2011) can all impose limitations or eventual extinctions on small populations. There is a paucity of literature however, considering the target plant itself and how changes in its physical or biochemical phenotype might act as a component Allee effect and impose extinctions on insect control agent populations (Tobin et al. 2011) or impair developmental or reproductive life stages thus limiting its population potential and effectiveness (Wittstock and Gershenzon 2002; Mazid et al. 2011; War et al. 2018; Yactayo-Chang et al. 2020).

1.3 Environment, plant biochemistry and the insect herbivore interface.

Plants produce an array of primary metabolites (essential for growth and reproduction) and secondary metabolites that provide multifunctional roles in primary metabolism and energy storage but are importantly, involved in plant defense and communication systems (Erb and Kliebenstein 2020). These metabolites often display a high degree of phenotypic plasticity in response to either abiotic (Cramer et al. 2011; Ramakrishna and Ravishankar 2011; Clavijo McCormick 2016; Yang et al. 2018; Shaar-Moshe et al. 2019) and/or biotic influences (Syrett 1983; Briese 1986; Schulz et al. 2019; Hartley et al. 2012; Schweiger et al. 2014; Stam et al. 2014; Maag et al. 2015). Thus, when a plant species invades a new environment, activation of highly complex interactive biochemical pathways can markedly alter its constitutive and/or induced phytochemical profile thus modifying its foliar nutritional value or its primary and secondary chemical defence systems (Wheeler and Schaffner 2013).

Several abiotic factors including drought, heat, cold, salinity and heavy metals are known to induce alterations to plant secondary metabolites. Heat and cold as well as soil moisture (including drought) induce responses to primarily maintain cellular osmolarity and homeostasis (Guy et al. 2008; Cramer et al. 2011;

Ramakrishna and Ravishankar 2011; Jorge et al. 2016; Yang et al. 2018). Such responses then, are variable both intra and inter-seasonally and therefore mostly transitory. Notwithstanding this, mean seasonal temperatures for the North Islands Central Plateau (CP) region closely match those of heathlands in the higher latitudes in the United Kingdom (UK) (Chapman and Bannister 1990). Soil moistures for the CP region and those selected for sampling in the UK are all considered free draining and neither range had experienced prolonged periods of dryness or higher rainfall than average prior to sampling for these studies. Temperature and soil moisture parameters then are considered less likely to be drivers of long-term phenotypic change to *C. vulgaris* in the CP region.

However, two parameters identified as possibly influencing the metabolome of heather in the CP region are ultra-violet (UV) light which differs significantly between the CP and all of the UK (Liley and McKenzie 2006) and soil nutrient availability (Chapman and Bannister 1990), also possibly differing between the two ranges. The literature revealed plants from many different families, respond to both these abiotic parameters and manifest as significant long-term changes to primary metabolites including nitrogen containing amino acids and derivatives of the TCA cycle. Significant changes also occur in secondary metabolites of the shikimate-phenylpropanoid pathway, the tricarboxylic acid cycle and mevalonic acid pathways.

Upregulation of these pathways result in amplification of phenylpropanoids, alkaloids and terpenes respectively (Bassman 2004; Ballaré et al. 2012) (Table 1.). All these secondary metabolite classes were of interest due to their known effects on insect herbivores (Rousseaux et al. 2001; Izaguirre et al. 2006; Kuhlmann and Müller 2010; Qi et al. 2018) thus providing a foundation for the hypotheses tested throughout this thesis.

	Primary Metabolites				Secondary metabolites		
	Amino acids	Carbohydrates	TCA cycle derivatives	Polyamines and Polyols	Phenyl-propanoids	Alkaloid N&S Comps	Terpenoids
Drought	✔	✔	✔	✓	✓	✓	
Heat	✔	✔		✓	✓	✓	✓
Cold	✓	✔	✔	✓	✓		
UV	✓				✔	✓	
Low N or P	✓		✓		✔	✔	✓

Table 1. General overview of metabolite classes that display amplified responses to five common abiotic stress parameters. Drought, heat and cold usually amplify primary metabolites associated with maintaining plant cellular and osmotic homeostasis. UV and soil nutrients responses generally amplify secondary metabolites which provide multifunctional roles in primary metabolism and energy storage but are also important in plant defense and communication systems. Large tick marks indicate the dominant responses while small ones, a lesser response.

Light quantity (intensity), light quality (the balance of photosynthetically active radiation wavelengths with those in the ultra-violet spectrum) and soil nutrients are parameters that upregulate the shikimic acid - phenylpropanoid pathway (Cramer et al. 2011; Bassman 2004; Singh et al. 2021, 2023), amplifying many phenylpropanoid derived secondary metabolites. Many of these, including phenolic acids, coumarins, chlorogenic acids, quinic acids, stilbenes and tannins can be highly effective defences against insect herbivores (Bassman 2004; Zhang and Bjorn 2009; Escobar-Bravo et al. 2017; Kumar et al. 2020).

Both primary and secondary compounds act as the key interface between plants and herbivorous insects, and alterations to their structure and/or concentration can directly influence herbivore survival and development (Bennett and Wallsgrove 1994; Wittstock and Gershenzon 2002; Howe and Jander 2008; War et

al. 2012, 2018). Altered phytochemical profiles of plants targeted for biological control, however, have seldom been considered as a cause of establishment failures, poor population growth rates or poor effectiveness.

1.4 Applying metabolomics to problematic biocontrol programmes

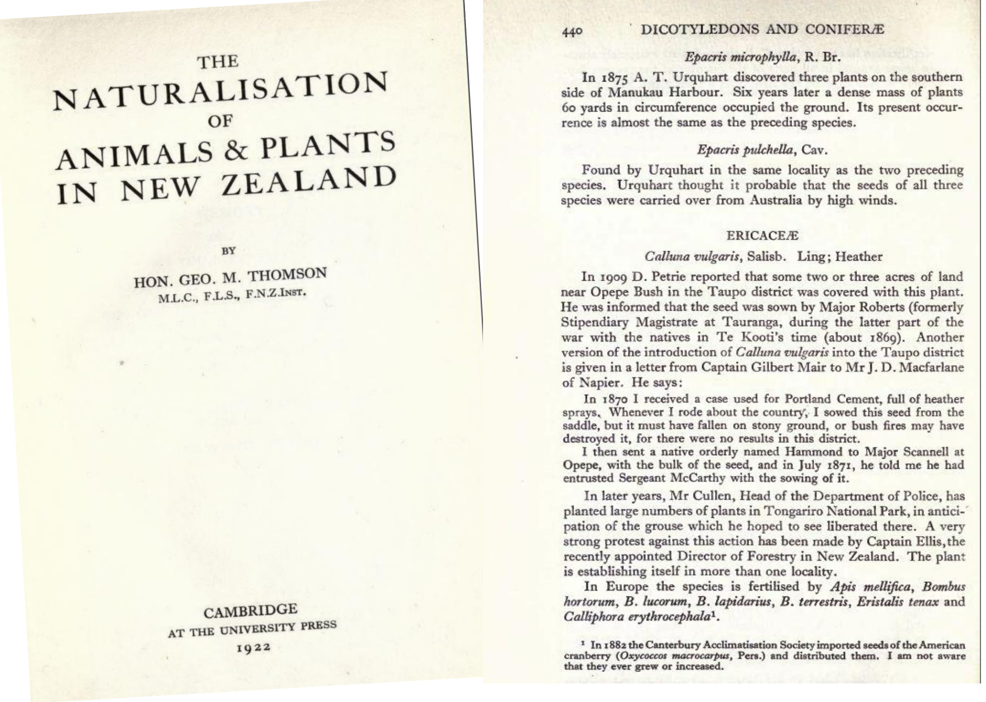
Recent advances in metabolomics makes it possible to study plant biochemistry at the molecular level, providing a non-targeted characterisation and quantification of all the metabolites (the metabolome) in a particular tissue in response to its environment (Hall 2006; Macel 2010; Arbona et al. 2013; Jorge et al. 2016a; Sampaio et al. 2016). These analyses generally use high throughput GC-MS or UHPLC-MS/MS chromatographic and mass spectral technology (Hall 2006; Bundy et al. 2009; Sardans et al. 2011; Obata and Fernie 2012; Arbona et al. 2013; Jorge et al. 2016; Sampaio et al. 2016; Subbaraj et al. 2019). In conjunction with computational packages which include algorithms to perform spectral processing functions such as, baseline correction, automatic peak detection, peak alignment of features in the *m/z* and chromatographic retention time and normalization, this technology is now available to a broad range of scientific researchers. Additionally, recent computer-driven user-friendly analytical and data mining packages are central to the integration, analysis and statistical interpretation of these usually vast data sets (Boudah et al. 2013; Alonso et al. 2015).

There is almost no published literature where metabolomics has been applied to biological control of invasive plant programmes either as a part of the pre assessment process or retrospectively for failed ones. This thesis focuses on applying metabolomics (more correctly ecometabolomics) to elucidate abiotically driven alterations to the invasive plant, heather, *Calluna vulgaris* (L.) Hull (Ericaceae), in New Zealand (NZ) and the establishment of a folivorous biocontrol agent. This programme was challenging, with very poor establishment rates and slow population growth rates and thus presents an opportunity to assess the

potential for this most recent of the “omics” technologies to assist with the science and success rates in classical weed biocontrol.

1.5 History and impact of heather in New Zealand

With support by the Prime Minister of the time, William Ferguson Massey, heather *C. vulgaris,* was deliberately introduced into Tongariro National Park in 1912 by the then Park Warden, John Cullen. The earliest records of this introduction are documented in the publication “The Naturalisation of Animals & Plants in New Zealand - 1922” (Fig. 1.). The aim was to replace 1000’s of acres of golden tussock with the heather of Celtic homelands to create a moorland for grouse (Bagnall 1982). Sixty-four sacks of seed were imported, most of which was broadcast sown, and 2000 heather seedlings were propagated then planted at Pukeonaki in the park.



THE
NATURALISATION
OF
ANIMALS & PLANTS
IN NEW ZEALAND

BY

HON. GEO. M. THOMSON
M.L.C., F.L.S., F.N.Z.INST.

CAMBRIDGE
AT THE UNIVERSITY PRESS
1922

440 DICOTYLEDONS AND CONIFERÆ

Epacris microphylla, R. Br.

In 1875 A. T. Urquhart discovered three plants on the southern side of Manukau Harbour. Six years later a dense mass of plants 60 yards in circumference occupied the ground. Its present occurrence is almost the same as the preceding species.

Epacris pulchella, Cav.

Found by Urquhart in the same locality as the two preceding species. Urquhart thought it probable that the seeds of all three species were carried over from Australia by high winds.

ERICACEÆ

Calluna vulgaris, Salisb. Ling; Heather

In 1909 D. Petrie reported that some two or three acres of land near Opepe Bush in the Taupo district was covered with this plant. He was informed that the seed was sown by Major Roberts (formerly Stipendiary Magistrate at Tauranga, during the latter part of the war with the natives in Te Kooti’s time (about 1869). Another version of the introduction of *Calluna vulgaris* into the Taupo district is given in a letter from Captain Gilbert Mair to Mr J. D. Macfarlane of Napier. He says:

In 1870 I received a case used for Portland Cement, full of heather sprays. Whenever I rode about the country, I sowed this seed from the saddle, but it must have fallen on stony ground, or bush fires may have destroyed it, for there were no results in this district.

I then sent a native orderly named Hammond to Major Scannell at Opepe, with the bulk of the seed, and in July 1871, he told me he had entrusted Sergeant McCarthy with the sowing of it.

In later years, Mr Cullen, Head of the Department of Police, has planted large numbers of plants in Tongariro National Park, in anticipation of the grouse which he hoped to see liberated there. A very strong protest against this action has been made by Captain Ellis, the recently appointed Director of Forestry in New Zealand. The plant is establishing itself in more than one locality.

In Europe the species is fertilised by *Apis mellifica*, *Bombus hortorum*, *B. lucorum*, *B. lapidarius*, *B. terrestris*, *Eristalis tenax* and *Calliphora erythrocephala*[1].

[1] In 1882 the Canterbury Acclimatisation Society imported seeds of the American cranberry (*Oxycoccos macrocarpus*, Pers.) and distributed them. I am not aware that they ever grew or increased.

Fig. 1. The earliest records of heather introductions in New Zealand are included in the above publication dated 1922.

Protests against this folly at the time came from the New Zealand Institute, the Minister of Forests and eminent botanists, but to no avail. Thus, Park Warden Cullen's efforts were very successful, and he certainly managed to change the landscape from golden tussock to a purple hue of heather, so successful, in fact, that within a hundred years the plant covered 50,000 hectares of the North Islands CP region (Chapman & Bannister 1990).

Heather is a highly invasive woody shrub, adapted to both wet and dry, acidic, low nutrient heathlands and is widely dispersed from the UK across Europe to the Ural Mountains and from Southern Spain to the high latitudes of Finland (Gil-Lopez et al. 2022). It now pervades the CP region from National Park in the West to the Three Kings range in the east and from Waiouru in the south to Turangi in the North at elevations from 380 to 1500 masl.

Fig. 2. A typical view of heather (foreground) dominating the sub-alpine flora of Tongariro National Park and the Central Plateau region. The rise below Mt Ngauruhoe (centre) is the small blown-out volcano Pukeonaki where heather was first planted in 1912.

In this region the native flora is a mosaic of sub-alpine tussock grasslands and shrubland dominated by *Chionochloa rubra* (Poaceae), *Dracophyllum subulatum* (Ericaceae), *Leptospermum scoparium* and *Kunzea ericoides* spp. (Myrtaceae). Heather, however, now dominates this community (Fig. 2.), negatively impacting *C. rubra* and displacing many small native and endemic understory shrubs and herbs in this fragile sub-alpine environment (Rogers and Leathwick 1994, 1996).

1.6 Biological control

In 1991 the Department of Conservation initiated a biocontrol programme to release heather beetle *Lochmaea suturalis* (Thomson, 1866) (Coleoptera: Chrysomelidae) into Tongariro National Park. Typical of Chrysomelidae, both larvae and adults are leaf chewers which cause severe defoliation of heather. In the native range, this regularly occurs in outbreak events resulting in large areas of severe damage to the host plant. This beetle is univoltine where summer emerged adults feed during autumn to accumulate abdominal lipid reserves before overwintering. They emerge in spring to feed on fresh heather growth then either fly to disperse or if environmental conditions are suitable, wing muscles undergo histolysis which induces reproductive organ development prior to mating. Eggs are laid in humid conditions at the soil leaf-litter interface beneath the heather canopy. On emergence, hatchlings climb to the canopy to feed and develop through three larval instars before dropping to the litter as pupae. There, they metamorphose to the adult stage and emerge in early summer (Rosenburgh and Marrs 2010).

Environmental impact assessment and host specificity testing were completed in NZ by Manaaki Whenua Landcare Research (MWLCR) prior to approval from the Environmental Risk Management Authority (ERMA) granting a permit to import into quarantine. Heather beetles were collected from sites at Oakworth Common

(Yorkshire) Rannoch Moor and Loch Linnhe in Scotland and sent to quarantine at MWLCR at Lincoln.

During the quarantine process an intracellular spore forming microsporidia was discovered, which resulted in severe line rearing of beetles and complete destruction of any infected lines. Eventually, 18 releases were made on the CP during summer 1996 - 1997. In the year 2000, the first and only established population was recorded which was derived solely from the Oakworth line. Fifty further releases on the CP were made from this one source before that population collapsed but only 5 of these releases established. These releases were also slow to establish, had slow population growth rates and the rate of spread at two of them took 6 yrs to radially expand 800m. Compare this rate with that which commonly occurs in the UK where heather beetle outbreaks can heavily damage hundreds of hectares or more in one season (Pakeman et al. 2002).

Several hypotheses have been tested, investigating reasons for this poor and unexpected *L. suturalis* establishment scenario. These include, “Is heather beetle establishment affected by the presence of predators, parasitoids or disease?” (Peterson et al. 2004). “Is there a climate mismatch between Oakworth and the CP which may impose overwintering mortality?” (Peterson et al. 2011). “Is poor performance the result of smaller body size and genetic bottlenecking from intensive line rearing in quarantine?” (Fowler et al. 2015). “Is foliar nitrogen of CP heather optimal for beetle growth and performance?” (Peterson et al. 2024).

These avenues of research have provided valuable insights into many of these questions, the most definitive being the impact on beetle life history performance and establishment rates due to low levels of foliar nitrogen. However, a question alluded to earlier and not yet addressed is: “Does heather on the CP have an altered defensive secondary metabolite profile which may impair heather beetle performance in this region”?

1.7 Aims and hypotheses

A lack of knowledge regarding the effects of abiotically induced alterations to defensive secondary metabolites in invasive plants and their effects on introduced biocontrol agents is a central question driving these investigations. Using the invasive weed heather *Calluna vulgaris* and its biocontrol agent *Lochmaea suturalis* as a model system, I explored if metabolomic technology is a technique that can determine differences in the secondary metabolite profile (the metabolome) between heather sourced from the invaded NZ CP range and that of Scotland in the UK native range.

This was designed to test two hypotheses, *H*1: *C. vulgaris* plants in the invaded NZ range are biochemically dissimilar to those of the native UK range and *H*2: The metabolite profile of *C. vulgaris* displays variation between sites within each range. This could verify whether heather beetles of the CP region may be exposed to different and variable levels of secondary metabolites between and within the two ranges of NZ and the UK.

A second aim, using ultra-violet (UV) attenuating screen technology with differing levels of attenuation and the application of metabolomics was to test the hypotheses, *H*3: that reduced intensity of ultra-violet induces an alteration of phenylpropanoid derived secondary metabolites in *C. vulgaris*? and *H*4: that altered levels of secondary metabolites in *C. vulgaris* will impact larval survival and prepupal liveweights of *L. suturalis*.

An earlier study (Peterson et al. 2024) demonstrated that life history performance parameters and establishment rates of heather beetle are impacted by low levels of foliar nitrogen in CP heather. There remained an unanswered question, however, which is whether increased performance is due solely to increases in foliar N or are defensive secondary metabolites simultaneously reduced as well?

Thus, we utilised metabolomics to test the hypothesis, $H5$: that application of fertilizer to soil will induce changes to the metabolite profile, foliar % N and the C:N ratio of *C. vulgaris*.

1.8 Thesis structure

This thesis consists of six Chapters. Chapter 1 is a general introduction. Chapter 2 is a published literature review and opinion piece. Chapters 3, 4 and 5 are data-based chapters focusing on the hypotheses discussed in the aims of the thesis. Chapter 6 is a general discussion and synthesis of the findings of the research embodied within. A summary for each chapter is presented below.

Chapter 1, provides an overview of the general concepts which are used to justify, guide and deliver the output required for this body of work. It provides a historical account and ecological implications of heather invasion, and the context of biological control programme since its initiation. The aims section includes the five hypotheses to be tested using metabolomic technology.

Chapter 2, published in the journal “Biological Control” (Barrett et al. 2021), brings to attention that alterations to a target plants defensive biochemical phenotype are rarely considered to be involved in biocontrol failures. It then posits that the application of metabolomics may be valuable to elucidate this possibility, thus adding another tool to the science supporting the biological control of weeds. Using a considerable body of literature it then sets out an explanation of the key concepts in metabolomic technologies and discusses a range of potential applications which may benefit weed biocontrol programmes.

Chapter 3, published in “Scientific Reports” (Barrett et al. 2024), is the work examining significant differences in the primary and defensive secondary metabolite profiles of heather between plants sourced from the CP range and

those of Scotland UK. The paper posits the idea that this result may be due to differing abiotic factors. It further examines inter-site variation of secondary metabolites within each range as a function of soil nutrients and discusses the potential for abiotically induced metabolite changes to impair herbivorous insect performance.

Chapter 4, in press, "Journal of Chemical Ecology", describes changes in secondary metabolites related to the shikimate–phenylpropanoid pathway after exposing heather to different intensities of UV light. It also describes results from a larval bioassay using plants from the same UV treatments to assess if such changes affect, in any way, growth and survival of heather beetle larvae.

Chapter 5, published in the journal "Biological Invasions". Poor performance in insect biocontrol programmes has been, attributed to low host plant foliar nitrogen. A common response of plants growing in nutrient poor soils is reduced primary metabolites and foliar nitrogen content and an elevation of defensive secondary metabolites which may affect the efficiency of conversion of ingested food (ECI). A field trial, using fertilizer enhanced *C. vulgaris* foliage resulted in increased N based amino acids and a concomitant reduction of defensive secondary metabolites. This study reports this attempt to better understand the interaction of secondary metabolites in relation to increased foliar N in the improved life history performance of *L. suturalis* recorded in an earlier study.

Chapter 6, is a general discussion and conclusions section, providing an overview of results and highlights several areas that indicate the complexity of plant primary and secondary metabolite responses to different abiotic factors and insect responses to plant metabolites. It also discusses potential future work on different *L. suturalis* life stages that could provide more information regarding beetle population density parameters and on metabolites where little

understanding of defensive function exists. It ends with a brief section of conclusions.

Author contribution statements: These comprise Appendix D at the end of this thesis.

1.9 Ethical issues

This study has no associated human or animal ethics issues.

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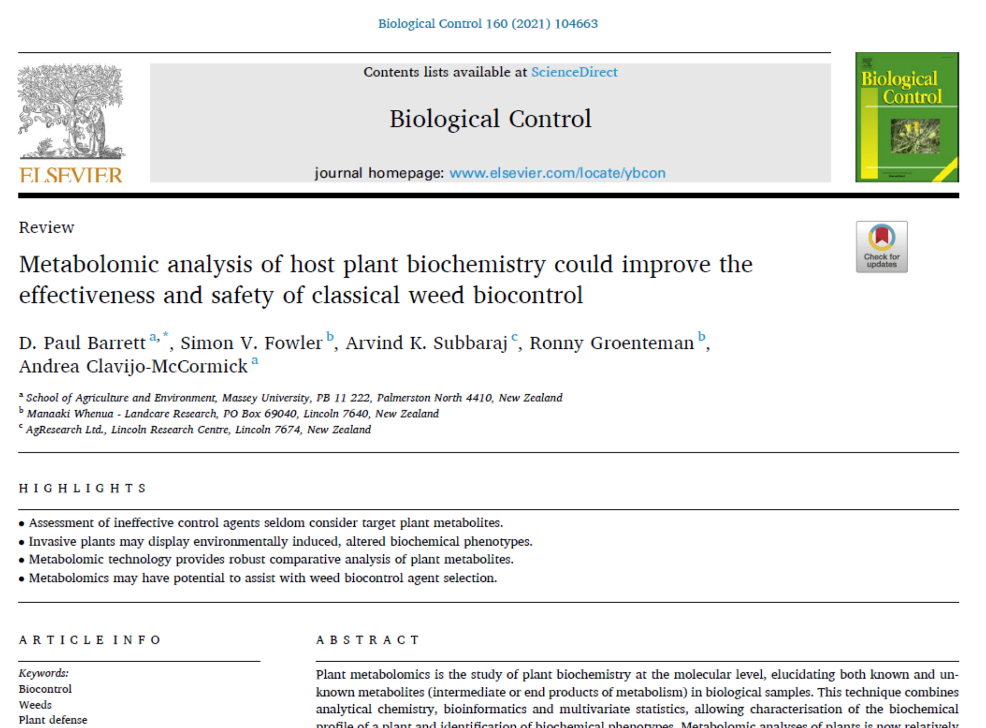
Chapter 2

Metabolomic analysis of host plant biochemistry could improve the effectiveness and safety of classical weed biocontrol.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Review

Metabolomic analysis of host plant biochemistry could improve the effectiveness and safety of classical weed biocontrol

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HIGHLIGHTS

- Assessment of ineffective control agents seldom consider target plant metabolites.
- Invasive plants may display environmentally induced, altered biochemical phenotypes.
- Metabolomic technology provides robust comparative analysis of plant metabolites.
- Metabolomics may have potential to assist with weed biocontrol agent selection.

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ABSTRACT

Plant metabolomics is the study of plant biochemistry at the molecular level, elucidating both known and unknown metabolites (intermediate or end products of metabolism) in biological samples. This technique combines analytical chemistry, bioinformatics and multivariate statistics, allowing characterisation of the biochemical profile of a plant and identification of biochemical phenotypes. Metabolomic analyses of plants is now relatively

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Abstract

Plant metabolomics is the study of plant biochemistry at the molecular level, elucidating both known and unknown metabolites (intermediate or end products of metabolism) in biological samples. This technique combines analytical chemistry, bioinformatics and multivariate statistics, allowing characterisation of the biochemical profile of a plant and identification of biochemical phenotypes. Metabolomic analyses of plants is now relatively commonplace, but a literature review showed little evidence that this technology is being applied to support weed biocontrol programmes. Understanding invasive plant biochemical phenotypes, and the biotic and abiotic influences determining that phenotype, could provide valuable information to assist with agent-selection decisions in weed biocontrol programmes, with potential to improve agent establishment rates and overall impacts on target weeds. Plant metabolomics could also further refine the determination of potential non-target taxa in host specificity testing, currently based on the centrifugal phylogenetic relatedness method. Metabolomics is a constantly evolving and powerful tool which can elucidate the complex biochemical interactions between plants and insects or pathogens. In this review, we explore how abiotic and biotic stressors may alter a plants biochemical phenotype, which may consequently affect insect herbivore performance. We provide a brief explanation of the key concepts in metabolomic technologies then examine a range of potential applications which may benefit weed biocontrol programmes.

Keywords: Biocontrol; Weeds; Plant defense; Secondary metabolites; Metabolomics

2.1 Introduction

Plant biochemical responses to abiotic stressors, herbivores and pathogens, collectively take the form of constitutive or inducible plant secondary compounds (PSC) which are now known to mediate interactions between competitors and mutualists, the effects of which can cascade through entire ecosystems (Iason et al. 2012). Metabolomics, the most recent of the "omics" technologies, can reveal the complexity of biochemical responses associated with phenotypic variation and/or the influence of environment or experimental treatments on biological systems (Macel et al. 2010; Iason et al. 2012; Arbona et al. 2013; Jorge et al. 2016a; Sampaio et al. 2016; Jorge and António 2018). An exciting potential application of this technology is its ability to reveal plant biochemical responses to abiotic (Obata and Fernie 2012; Arbona et al. 2013; Jorge and António 2018) as well as biotic stressors such as insect herbivores (Hartley et al. 2012; Schweiger et al. 2014; Maag et al. 2015) and plant pathogens (Hartley and Grange 2009; Lazebnik et al. 2014), which may be of interest to biocontrol practitioners.

Classical biological control of weeds is the reuniting of specialist co-adapted herbivores, usually insects or pathogens, with their original host plant, where that plant has established and become invasive in a new environment (Fowler et al. 2000). The ecological underpinning to this form of biological control is that the herbivore reduces plant vigour (Fowler et al. 2000) to a new equilibrium level, below that which causes either economic or environmental damage (Briese 2000). This management system is a viable and self-sustaining solution to controlling invasive plants in both managed and natural ecosystems (Pimental et al. 2005; Schwarzländer et al. 2018). If implemented according to best practice, it is low risk (Fowler et al. 2000; Fowler 2012; Hayes et al. 2013; Suckling and Sforza 2014; Hinz et al. 2019)), can preserve the integrity of plant communities (Peterson et al. 2020) and arrest the loss of biodiversity at the species, community, or

ecosystem level (Pyšek et al. 2012). Biocontrol programmes inevitably require many years to complete environmental risk assessment, agent selection, host range/specificity testing (McFadyen 1998; Briese 2004), and regulatory authorisation for importation and release of organisms (Sheppard et al. 2003a; Barratt 2010; Hayes et al. 2013; Ehlers et al. 2020).

Historically and currently, the main challenges to biocontrol agent selection is the ability to predict agent establishment, effectiveness i.e., impact on the target plant (McEvoy et al. 2012; Schwarzländer et al. 2018; Harms et al. 2020) and safety i.e., the probability of non-target impacts (Fowler et al. 2000; Paynter et al. 2018, 2020). There are well established protocols currently utilised to assist with these assessments, however, rarely is consideration given to host plant biochemistry. The biochemical phenotype determines the assimilable nutritional value and levels of defensive plant secondary compounds (PSC) of the plant, many of which can be altered by abiotic (Cramer et al. 2011; Khan et al. 2011; Ramakrishna and Ravishankar 2011; Clavijo McCormick 2016; Yang et al. 2018; Shaar-Moshe 2019) and biotic factors (Denno and Kaplan 2007; Pieterse and Dicke 2007; Schweiger et al. 2014). Such compounds can have a profound effect on insect performance and population dynamics (Bennett and Wallsgrove 1994; Wittstock and Gershenzon 2002; Howe and Jander 2008; War et al. 2012, 2018), both of which are key elements determining the establishment and effectiveness of a weed biocontrol agent (Zalucki and van Klinken 2006; McEvoy et al. 2012; Harms et al. 2020).

Altered host plant biochemical phenotype and how it impinges on the establishment and effectiveness of introduced biocontrol agents is poorly explored and we propose that metabolomics may be a valuable tool for investigating such changes and be of value when considering agent selection.

In this review, we explore how and why invasive plants are likely to display alterations to their biochemical phenotype in a new range, the complex effects of co-occurring biotic and abiotic factors on plant biochemistry, briefly describe what metabolomics is and consider how, with collaboration of plant biochemists, knowledge generated by this technology may apply to weed biocontrol in an effort to potentially improve agent establishment, effectiveness and safety.

2.2 Invading plants are likely to display altered biochemical phenotypes

Plants produce a wide array of primary metabolites (essential for growth and reproduction) and secondary metabolites (essential for ecological interactions and defence) which can display a high degree of phenotypic plasticity in many plant species. Induced by highly complex interactive biochemical pathways, metabolite profiles can be altered markedly by both abiotic (Cramer et al. 2011; Khan et al. 2011; Ramakrishna and Ravishankar 2011; Clavijo McCormick 2016; Yang et al. 2018; Shaar-Moshe et al. 2019) and biotic influences (Hartley and Grange 2009; Hartley et al. 2012; Schweiger et al. 2014; Stam et al. 2014; Maag et al. 2015). Thus, when a plant species invades a new environment, it is not unreasonable to expect that there may be alterations to its phytochemical profile, which may alter foliar nutritional value or constitutive and induced plant secondary chemical (PSC) defence systems (Wheeler and Schaffner 2013). Combined, these phytochemical compounds act as the key interface between plants and herbivorous insects, and changes in structure and/or concentration of these chemicals can directly influence herbivore survival and development (Bennett and Wallsgrove 1994; Wittstock and Gershenzon 2002; Howe and Jander 2008; War et al. 2012, 2018).

Literature focusing on the adaptation of invasive plants to new environments postulates two major hypotheses. One is that plant trait combinations pre-adapt species to become successful invaders (the pre-adaptation hypothesis),

manifested as phenotypic plasticity to abiotic and/or biotic stressors. The other postulates that successful plant invasion is the result of rapid evolutionary change (the post-invasion evolution hypothesis) due to similar stressors selecting for genetically based shifts (Müller-Schärer et al. 2004) ultimately leading to phenotypic change.

Abiotic factors which have been demonstrated to play a key role in driving biochemical phenotypic variation are amply documented. Osmotic stress (Tounekti et al. 2013; Rodziewicz et al. 2014; Yang et al. 2018), low temperatures, (Christie et al. 1994; Pedranzani et al. 2007; Moreira et al. 2014; Li et al. 2018; Rahman et al. 2020); high temperatures, (Rivero et al. 2001; Wahid et al. 2007; Yang et al. 2018) soil nutrients, (Gershenzon 1984; Tuomi et al. 1984; Waterman and Mole 1989; Wright et al. 2010; Ramakrishna and Ravishankar 2011; Sampedro et al. 2011; Kováčik and Klejdus 2014; Yang et al. 2018) quantitative light (Red: Far-red ratio) (McGuire and Agrawal 2005; Izaguirre 2006; Ballaré et al. 2012; Ballaré 2014; de Vries et al. 2017) and finally qualitative light (the balance of PAR, UV and UV-B) (Caldwell et al. 1983, 2007; Paul and Gwynn-Jones 2003; Roberts and Paul 2006; Demkura et al. 2010; Ballaré et al. 2011, 2012; Kuhlmann and Müller 2011; Dinh et al. 2013; Ballaré 2014; Escobar-Bravo et al. 2017) are all represented in the literature and have been demonstrated to alter constitutive and induced defensive plant secondary metabolites capable of influencing herbivore performance.

Biotic influences on phenotypic alteration in invading plants are thought to be driven following the release of the plant from its coevolved herbivores. Largely as a result of experimental observations from weed biological control programmes (Wheeler and Schaffner 2013), two hypotheses have emerged stating: i) phenotypic change is due to the "evolution of increased competitive ability" (EICA) hypothesis, as postulated by Blossey and Nötzold (1995); and ii)

the "shifting defence hypothesis" (SDH) postulated by Doorduin and Vrieling (2011).

The EICA proposes that evolutionary change in invasive plants is due to them being released from co-evolved specialist herbivores as the primary selective driver for shifts in growth and defence characteristics. This suggests, when plants are introduced into a new area, they reduce resource allocation into costly constitutive defences in favour of growth and reproduction (Blossey and Nötzold 1995; Wheeler and Schaffner 2013). Wheeler and Schaffner (2013) however conclude, evidence supporting the EICA is mixed, indicating a range of multidirectional plant responses were revealed when considering this mechanism and, work by Hornoy et al. (2011) as well as a recent meta-analysis found little evidence supporting this general hypothesis (Felker-Quinn et al. 2013).

The SDH is based on the premise that invasive plants once released from co-evolved specialist herbivores will be exposed to a new suite of generalists and will shift from producing expensive digestibility reducing compounds to cheaper toxic type compounds effective against the non-adapted generalists (Müller-Schärer et al. 2004). Using *Senecio jacobaea,* work by Joshi and Vrieling (2005) demonstrated increased pyrrolizidine alkaloids in plants from invaded regions which resulted in decreased protection against specialist herbivores adapted to the alkaloids and increased protection against generalists.

2.3 Multiple environmental influences on plant biochemistry will be complex

Regardless of the mechanism involved, a new environment is likely to cause plants to change their phenotype to adapt to novel conditions in a highly complex way involving morphological, physiological and biochemical changes (Shaar-

Moshe et al. 2019). At the biochemical level, numerous metabolites (Arbona et al. 2013; Krasensky and Jonak 2012) including volatile compounds (Effah et al. 2020) will be affected in both elastic (reversable) and plastic (irreversible) ways (Cramer et al. 2011; Shaar-Moshe et al. 2019). These responses involve stress sensing, signal transduction and the activation of stress-related genes and biosynthetic pathways (Jorge et al. 2016b). Where a region being invaded by an invasive plant differs either markedly or subtly in one or more biotic or abiotic parameters, we would predict that the species will undergo biochemical phenotypic change. However, the exact direction and magnitude of these changes and their impact on herbivore establishment are difficult to predict due to the interactive effects of such multiple co-occurring environmental factors.

The acclimation and response adaptations that induce responses to single or paired biotic and abiotic stresses have been well studied (Foyer et al 2016; Ashraf et al. 2018; Saddique et al. 2018). Most studies however are conducted using controlled conditions and limited to just two trophic levels. This does not account for the ecological complexity of plants growing (or invading) in naturally occurring ecosystems where they are simultaneously subjected to multiple stressors to which they must respond appropriately to establish and survive (Valladares et al. 2007a, 2007b; Clavijo McCormick 2016). Thus, there is a paucity of literature available on the dynamic aspects of secondary metabolite stress responses with multiple abiotic and either with or without simultaneous biotic stressors (Clavijo McCormick 2016; Foyer et al. 2016) under field conditions. However, plants have capacity to respond to a range of stresses via a plastic and delicately balanced response network that involves redox signalling pathways, stress hormones, growth regulators, plus calcium and protein kinase cascades (Cramer et al. 2011; Foyer et al. 2016). Therefore, the end metabolic product of an interaction is difficult to predict based on the knowledge of a plant's response to a single stressor.

For example, a field-based study by Virjamo et al. (2014) investigated the effects of elevated temperature, elevated UV-B, soil fertility and all combinations of these on growth and secondary metabolites of seedlings of Norway spruce *Picea abies.* Concentration of some compounds showed UV x temperature or UV x temperature x fertilization interactions and all treatments affected differently, the secondary metabolite profiles of the seedlings.

Feeding by herbivorous insects either of the same (Denno et al. 2000) or different feeding guilds (Mayer et al. 2002; Inbar et al. 1999), can induce changes to the biochemical defences of their host plant which in turn influences the plants interactions with its associated herbivore community (Denno and Kaplan 2007; Poelman and Dicke 2014; Stam 2014) further, these induced changes can be dependent on abiotic conditions such as nutrient availability (Han et al. 2019) or light (Ballaré 2012, 2014; de Vries et al. 2017; Escobar-Bravo et al. 2017).

Plants recognise and respond differently to every species of insect or pathogen attacker, producing a specific mix of metabolites directed at each and according to the type of tissue damage they inflict (Diezel et al. 2009; Bonaventure 2014). These defence responses are initiated via signal transduction pathways involving jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) phytohormones (Diezel et al. 2009; Erb et al. 2012; Pieterse et al. 2012; Thaler et al. 2012; Schweiger et al. 2014; Saddique et al. 2018). Leaf chewers and miners and necrotrophic pathogens generally activate the JA pathway, while sucking and piercing insects and biotrophic pathogens activate predominantly the SA pathway (Glazebrook 2005; Pieterse and Dicke 2007; Erb et al. 2012; Schweiger et al. 2014). Evidence also exists, showing plants infected with phytopathogens can systemically produce facilitative or negative responses towards feeding insects (Mayer et al. 2002; Rostás et al. 2003; Lazebnik et al. 2014). There is evidence of antagonism between these two biosynthetic pathways where SA suppresses the JA pathway (Pieterse and Dicke 2007; Diezel et al. 2009; Thaler et al. 2012; Schweiger et al.

2014), suggesting that response to one herbivore species or guild will render plants more vulnerable to the other, however, continuous crosstalk between these pathways appears to be most common in naturally occurring plant-insect/pathogen interactions (Pieterse and Dicke 2007; Schweiger et al. 2014).

There are many classes of metabolites which have a role in plant defence against insect herbivores and pathogens (Izaguirre et al. 2006; Ballaré 2014; Escobar-Bravo et al. 2017; Saddique et al. 2018). Among these are the phenylpropanoids, e.g., phenolic acids, coumarins, furano-coumarins, lignans, Isoflavonoids (Mazid et al. 2011), tannins (Barbehenn and Constabel 2011) and flavonoids, (e.g., Isoflavones, flavonols, flavones, and anthocyanins (Ashraf et al. 2018). Nitrogen-containing compounds include alkaloids, cyanogenic glycosides and nonprotein amino acids (Jamieson et al. 2017) and sulphur-containing compounds; glucosinolates, glutathione, phytoalexins, camalexin and defensins (Burow et al. 2008; Mazid et al 2011); and finally, terpenoids (monoterpenes, diterpenes, sesquiterpenes, triterpenes, polyterpenes (Mazid et al 2011) and iridoids and cardiac glycosides (Jamieson et al. 2017).

Individual metabolites may in isolation have no, or only a weak defensive effect against an herbivore or plant pathogen but can have a potent toxic or deterrent effect when part of a mixture (Gershenzon et al. 2012; Dyer et al. 2018). To add further complexity, additive and synergistic (Rasmann and Agrawal 2009) or even antagonistic effects (Liu et al. 2017) can occur between metabolites. Additive, being the sum of two (or more) combined effects thus providing greater toxicity or deterrence, and synergistic being the combined effects, which are greater than the additive sum of effects for each individual compound acting in isolation (Gershenzon et al. 2012; Richards et al. 2016; Dyer et al. 2018). Synergistic effects may be widespread across most plant families and can occur among both intraclass (Berenbaum et al. 1991; Segura et al. 1999; Dyer et al. 2003) and interclass compounds (Berenbaum and Neal 1985; Stermitz et al. 2000;

Hummelbrunner and Isman 2001; Akhtar and Isman 2003; Steppuhn and Baldwin 2007). The effects of these mixtures are thought to provide plants with the advantage of being able to target different types of herbivores and/or pathogens simultaneously (Gershenzon et al. 2012; Richards et al. 2016). Few studies exist reporting antagonistic effects of defensive secondary metabolites, i.e., where one metabolite reduces the effect of another, however Liu et al. (2017) demonstrated the antagonistic effects of several pyrrolizidine alkaloid (PA) free bases and chlorogenic acid (CGA) which resulted in reduced mortality of the western flower thrip *Frankliniella occidentalis* while in contrast, PA *N*-oxides and CGA produced synergistic affects which increased thrip mortality.

How abiotic or biotic stressors influence the balance of these secondary metabolite mixtures in invading plants is an area that remains largely unexplored. Small alterations to a mix of metabolites may be sufficient to affect host plant utilisation or selection by herbivores not only via direct effects involving deterrence and toxicity (Dickie 2000; Chapman 2003; Irmisch et al. 2014), but by mixes containing small alterations to ratios of volatile compounds necessary to trigger insect host finding or avoidance behaviours (Unsicker et al. 2009, Clavijo McCormick 2014; McCormick et al. 2019). It is reasonable to assume therefore, that subtle changes to the whole chemical composition, or perhaps a small number of compounds may play an important role in why a specialist insect or pathogen performs well or not, on a specific plant phenotype in its invaded range.

So, the complexity of the effect of multiple biotic and abiotic factors on plant metabolite responses and the possibility of synergistic or additive effects as a result, hints to the difficulty of translating results obtained under experimentally controlled conditions to field scenarios. It would be particularly challenging and costly to test individual factors of a new habitat and all the possible combinations to predict changes on the chemical behaviour of an invasive plant

and their effects on introduced biocontrol agents. Therefore, other approaches are needed to investigate the effects of combined biotic and abiotic factors on plant metabolism and metabolite diversity and metabolomics is a promising tool for this purpose.

2.4 What is Metabolomics?

Plant metabolomics in a non-targeted approach characterises and quantifies all identifiable plant metabolites (the metabolome) at the molecular level, from a particular tissue in response to its environment (Hall 2006; Macel 2010; Arbona et al. 2013; Jorge et al. 2016a; Sampaio et al. 2016). This non-targeted approach focuses on the entire metabolite profile, which having been subjected to multivariate analysis, has been discriminatively separated between for instance treatments, environments, phenotypes or genotypes. Alternatively, a targeted approach is more traditional, where the extraction, separation and detection methodologies are optimized (thus enhancing resolution), for a specifically chosen group of metabolites with similar properties (Hall 2018; Ribbenstedt et al. 2018).

In contrast to gene function, which is subject to epigenetic regulation and protein functions to translational modification, metabolites provide a direct "signature" of the results of all the biochemical process and their resultant compounds, thus carrying an imprint of all genetic, epigenetic and environmental factors, which provides a "link" or correlate between genotype and phenotype (Macel et al. 2010; Hartley et al. 2012; Jorge et al. 2016a; Krumsiek et al. 2016). Additionally, and in contrast to transcriptomics and proteomics, metabolomics has the advantage of not being dependent on the availability of published organism specific genome information for data analysis (Hall 2006; Jorge et al. 2016a).

Technology allowing high throughput DNA sequencing (genomics), mRNA expression profiling (transcriptomics), and protein profiling (proteomics) has provided tools that reveal highly complex molecular interactions involved in biological, evolutionary and ecological systems (Jorge et al. 2016a). More recently the ability to identify and quantify individual metabolites has developed through use of high throughput gas chromatography or high pressure liquid chromatography both coupled to high resolution mass spectrometry (GC-MS/MS and HPLC-MS/MS respectively), and nuclear magnetic resonance spectroscopy (NMR) technologies (Hall 2006; Bundy et al. 2009; Sardans et al. 2011; Obata and Fernie 2012; Arbona et al. 2013; Jorge et al. 2016a; Sampaio et al. 2016; Subbaraj et al. 2019). Similarly, the recent development of computer driven analytical and data mining packages is central to the integration, analysis, and interpretation of these usually, vast data sets. Computational packages either commercially provided or more recently, freely available on-line, include algorithms which perform an array of spectral processing functions including baseline correction, automatic peak detection, peak alignment of features in the *m/z* and chromatographic retention time and normalization (Boudah et al. 2013; Alonso et al 2015).

Metabolomics usually demands the skills and interpretive capability of plant biochemists familiar with metabolomic protocols and techniques who can provide valuable guidance with experimental design, understanding of technical limitations and appropriate data analysis. Sample collection, storage, extraction and processing prior to spectrometry, however, are within the skills of most researchers (Jorge et al. 2016a; Piasecka et al. 2019). To assist with analysis and interpretation of results, various online workflow tools are now evolving which cater for the novice user (Chong et al. 2018; Domingo-Almenara and Siuzdak 2020; Pang et al. 2020) which are generally well designed and intuitive. Online analytical packages such as XCMS, provide univariate e.g., *t*-test or ANOVA, or multivariate principal component analysis (PCA) or partial least squares (PLS) and

orthogonal PLS (O-PLS) statistical analyses from which can be derived data-driven hypotheses (Alonso et al. 2015). Then, using for example MetaboAnalyst 5.0, the investigator may obtain biomarker identification, metabolite quantification or pathway analysis, leading to biological or ecological interpretation (Alonso et al. 2015, Piasecka et al. 2019, Pang et al. 2020).

Converting mass spectrometry data into information relating to the biochemical composition of plants however is currently a significant bottleneck which demands considerable time and effort (Hall 2018). A major challenge is decoding the physico/chemical phenomena of ionized metabolites produced during mass spectrometry (Tsugawa 2018). Ongoing development of publicly accessible metabolite data repositories such as Metabolomics Workbench and MetaboLights (Spicer et al. 2017; Haug et al. 2020) however is continually improving the ability to interpret mass fragmentation patterns of ionized metabolites and identify these unknown structures or new compounds (Tsugawa 2018).

Associated with this identification bottleneck are a lack of authentic chemical standards required for unambiguous formula assignment and our ability to confirm actual metabolite identity (Hall 2018). This is an issue however for most biochemical analyses regardless of the platform or methodologies being applied but mass spectral databases such as METLIN (Guijas et al. 2018; Montenegro-Burke et al. 2020) and MassBank (Vinaixa et al. 2016) are continuously expanding, resulting in greatly improved metabolite conformation. Biological interpretation of metabolomic output can also be limited by the underrepresentation of some key groups of plant hormones such as auxins, cytokinins and gibberellins which restrict our ability to interpret observed phenotypic changes in the context of metabolite biosynthesis (Hall 2018). Notwithstanding this limitation, the measurement of phytohormones (e g., jasmonates, abscisic acid, salicylic acid and those mentioned above) allows identification of the key metabolites involved with signalling and communication pathways (during biosynthesis) between a

plant and its abiotic environment as well as biotic interspecific interactions including herbivory (Macel et al. 2010; Erb et al. 2012; Pieterse et al. 2012; Jorge et al. 2016b) and plant pathogens (Bari and Jones 2009).

So, metabolomic analysis allows us to investigate the central metabolism involved in regulation of the myriad of growth and developmental processes that allow plants to adapt to abiotic and biotic perturbations. Quantification and identification of known secondary and primary metabolites (e g. carbohydrates, amino acids, and organic acids) elucidates, how and to what extent plant metabolism readjusts to a changing environment (Krasensky and Jonak 2012; Obata and Fernie 2012). For further insight on plant metabolomics and its methods, we recommend reviews by Sumner et al. 2007; Hall and Hardy 2011; Sardans et al. 2011; Roessner and Dias 2013; Maag et al. 2015; Hall 2018.

As alluded to earlier, collaborative effort would generally be required to apply metabolomic technology to biocontrol programmes which inevitably incurs additional costs. Costs of metabolomic technologies over the past decade have been significant, but as with genomic DNA sequencing and proteomics, are now rapidly trending down according to "Moore's law" (Anderson 2014; Check Hayden 2014) and becoming progressively more affordable. Costs will also reduce as understanding of the metabolite profile being investigated advances. Initial non-targeted analysis will provide identification of metabolites at the broadest level and their associated metabolic pathways. Then, having reduced that complexity by identifying major pathways and compounds of interest, a targeted and therefore considerably cheaper approach might be applied.

2.5 Potential applications of metabolomics in weed biocontrol

2.5.1 Matching target plant metabolomes at collection and release sites

For many classical biological control of weeds programmes, there are releases that either fail to establish or are ineffective when they do (McFadyen 1998; Fowler 2000; Schwarzländer et al. 2018). Establishment failures can be due to several factors but mismatches between the biological control agent (agent) and its new habitat or environment (often termed environmental resilience) (Grevstad 1999; Myers 2000), variable climate (Peterson et al. 2007, 2011; 2015; Fowler et al. 2015), demographic and environmental stochasticity, elevated emigration and Allee effects are all capable of limiting early population establishment (Grevstad 1999; Jonsen et al. 2007). While the literature provides a diverse range of ecologically and genetically valid reasons for agent establishment failure, we suggest, some of these failures may be due to changes in the biochemical phenotype of the target plant in its introduced range, a consideration for which there is a paucity of information.

Plants subjected to warmer drier geographic regions are most likely to display increases in soluble carbohydrates and amino acids involved in maintaining cellular osmotic balance (Cramer et al. 2011; Sardans et al. 2011; Jorge et al. 2016a; Ashrafi et al. 2018) while some phenylpropanoids are reduced (Wahid et al. 2007). Likewise, plants subjected to colder conditions tend to adjust levels of similar classes of osmolytes (Janska et al. 2010; Li et al. 2018; Rahman et al. 2020). These abiotic influences however are variable and transitory in nature, following seasonal patterns which plants from all temperate regions are exposed and adapted to and these compounds may have either positive or negative effects on insect herbivore performance. Two abiotic influences however that may induce fixed changes to a plant's metabolome, are soil nutrients (Gershenzon, 1984; Tuomi et al. 1994; Mazid 2011) and light (Ballaré et al. 2012; Ballaré 2014; Yang et al. 2018). Soil nutrients, (in unmanaged conservation lands) are relatively fixed, so induced changes are likely to result in fixed changes to the metabolome. Both quantity and quality of light, while diurnally and seasonally variable, is fixed in relation to wherever the plant occurs on the globe, with major variation

particularly in UV, between the northern and southern hemispheres (Seckmeyer et al. 2008; Liley and McKenzie 2006). Carbohydrate and amino acids are likely to be altered by nutrients and light but more importantly derivatives of the shikimate, tricarboxylic acid cycle (TCA) and mevalonic acid pathways which produce phenylpropanoids, alkaloids and terpenoids respectively, can be substantially altered (Bassman 2004).

Provided that sufficient replication from a number of geographically independent sites in each range is available, metabolomic analysis can provide robust evidence which could be used to determine any variation between metabolomes. This information might assist with the selection of agent collection and release sites allowing practitioners to pair as close as possible, sites with the most similar metabolite profiles in an effort to reduce any host plant defensive or nutritional challenges to a newly released agent, particularly while they are at the vulnerable early stage of establishment.

Box 1.

In New Zealand, the heather beetle *(Lochmaea suturalis)* (Coleoptera – Chrysomelidae) was released against the highly invasive shrub, heather *(Calluna vulgaris)* in 1996. On the North Islands Central Plateau, the beetle was difficult to establish and does not reach the population density (Peterson et al. 2007, 2011; 2015; Fowler et al. 2015), that it does in its home range in the United Kingdom (UK) and Europe (Pakeman et al. 2002; Rosenburgh and Marrs 2010). While the beetle is now established and effectively controlling heather, considerable effort has been put in, investigating possible mechanisms for this outcome. Earlier work focussed on potential top-down effects of predation and parasitoids (Peterson et al. 2004), intrinsic effects i.e., genetic bottlenecking and small body size (Fowler et al. 2015) and possible extrinsic effects i.e., long winters and late spring sub-zero frosts (Peterson et al. 2011). Later, bottom-up effects investigating heather foliar nitrogen levels revealed low concentrations in plants from this region (Peterson et al. 2011, 2015) (Fig. 1) Using non-targeted HPLC-MS/MS and the online analytical platforms XCMS and MetaboAnalyst 5.0, we investigated plant secondary metabolites of heather, which shows significant regulation of several phenylpropanoid pathways and the isoquinoline alkaloid pathway of the metabolomes between heather in the UK and New Zealand, indicating differing levels of plant secondary defensive compounds between the home range and invaded range respectively (unpublished data). Metabolomic analysis therefore, is an informative way of identifying these multifaceted changes to plant biochemistry which could provide information to better predict, or retrospectively assess, both establishment and impact outcomes.

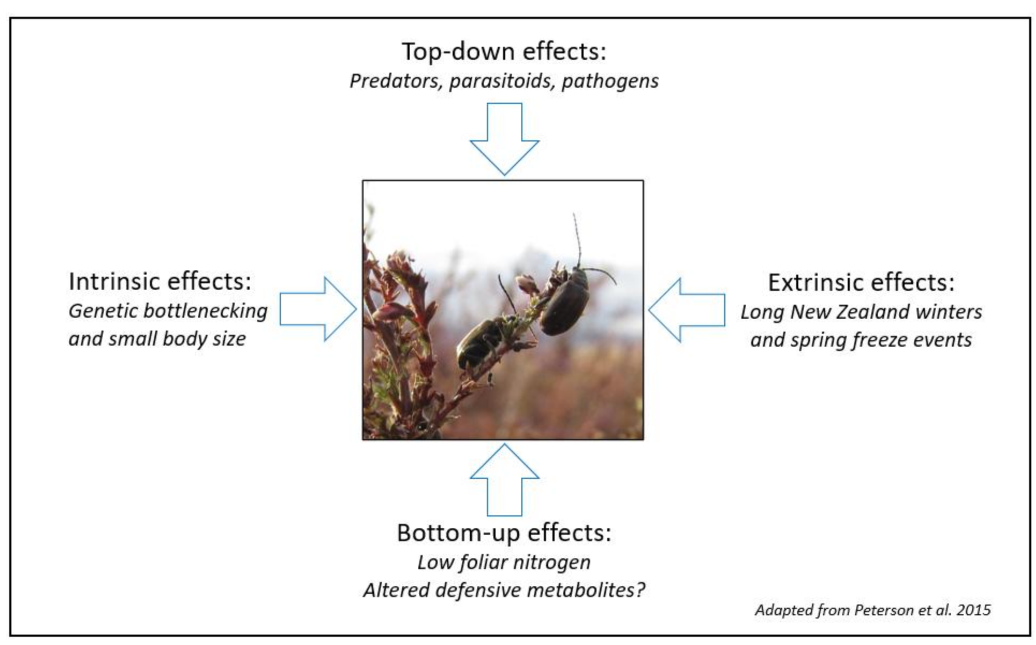

Fig. 1 Hypotheses for poor Heather Beetle performance in New Zealand.

2.5.2 To determine the greatest plant defensive response to a single agent

Selection of effective agents prior to release is key to successful biocontrol of invasive plants but predicting agent performance and impact in a new environment is difficult. Understanding population dynamics of the plant in its native range and the type and degree of damage required to reduce plant density is an approach used to predict agent efficacy (Sheppard 2003b). We suggest, understanding the plants biochemical response to an insect herbivore species or guild using metabolomic analysis may provide additional information for the agent selection process.

Chemical defences to protect plants against herbivores and pathogens, are induced "on demand" which can result in dramatically elevated levels of these secondary metabolites (Steppuhn and Baldwin 2008) and are thought to incur fitness costs to the plant (Karban and Baldwin 1997). The magnitude of this cost can be dependent on many interacting environmental variables including the

type of defensive compound being produced and nutrient availability (Koricheva 2002).

Plants elicit defences which can be species-specific to a particular herbivore or pathogen, or to a particular feeding guild, via the JA or SA induction pathways, thus producing an appropriate "cocktail" or mixture of compounds directed at the attacker or attackers (Kessler and Baldwin 2004; Howe and Jander 2008; Schweiger et al. 2014).

Traditionally a trade-off has been envisioned between resources allocated to primary metabolites required for growth and development and those to the biosynthesis and storage of such secondary metabolites. Recently this idea has been challenged, with evidence that secondary metabolites serve an auxiliary role which also includes functions associated with primary metabolism (Neilson et al. 2013). The cost of this induced defence may significantly reduce plant fitness and plant population dynamics and play a role in the success or failure of weed biocontrol (Runyon and Birdsall 2016). Under controlled conditions, we may expect a single attacking species to induce from the plant a particular profile of compounds and by using a metabolomic assessment, we can measure this response both qualitatively and quantitively. This should provide an opportunity to assess the magnitude of a potential agents' effect on the plant. Notwithstanding the resource allocation trade-off debate, a large defensive metabolite response may indicate a plant suffering greater stress. If the analysis did indeed show reduced allocations or biosynthetic impediment to primary metabolite production, that may indicate an even greater degree of stress on the plant. Such information of course won't definitively predict the actual impact of an agent, given our inability to predict the population dynamics of an agent in advance but it might at least indicate which agent may have potential for the greatest impact (Runyon and Birdsall 2016).

2.5.3 To identify supplementary or additional agents

In recent years, literature focusing on the idea that plants mediate the insect community or the presence or absence of species or feeding guilds on the plant via JA, ET and SA induced secondary defensive compounds has gained considerable traction (Ohgushi 2005, 2008; Denno and Kaplan 2007; Kaplan and Denno 2007; Utsumi 2010; Soler et al. 2012; Lazebnik et al. 2014; Poelman and Dicke 2014; Kinahan et al. 2020). Communities mediated via the plant can include herbivore – herbivore (Ohgushi 2005, 2008; Han et al. 2019), herbivore – pathogen/ microbe (Wittstock and Gershenzon 2002; Biere and Bennet 2013; Lazebnik et al. 2014) and herbivore – mycorrhizae/ endophyte (Hartley et al. 2012; Katayama et al. 2011; Gilbert and Johnson 2015) interactions. This plant mediated manipulation of herbivores, can occur during feeding occurrences that involve either simultaneously spatially separated, temporally separated or taxonomically distinct species of herbivores (Ohgushi 2005).

Plant defensive metabolite responses are initiated via signal transduction pathways as alluded to earlier, however SA is known to have an antagonistic effect on JA responsive gene expression thus suppressing the JA response (Pieterse and Dicke 2007; Caarls et al. 2015). In naturally occurring plant-herbivore systems however, continuous crosstalk (Pieterse and Dicke 2007; Schweiger et al. 2014) between the two pathways, maintains a delicately balanced response that keeps all attackers under control, thus maintaining a tolerable herbivore community. There is a small amount of evidence though that suggests in some systems the plant response induced by one herbivore may be positive for another herbivore i.e., facilitative where preference and/or performance is enhanced (Kessler and Halitschke 2007; Soler et al. 2012) perhaps as a result of synergistic effects as alluded to earlier. Alternatively, it may be negative, i.e., a vaccination effect (Kessler and Baldwin 2004; Schweiger et al. 2014) where the

plant responses induced by one herbivore reduce preference and/or performance of another.

Little knowledge exists however regarding how, once in a new range, host plant hormonal crosstalk responds to high densities of a single, specialist herbivore without the influence on the plant of the associated suite of herbivores and pathogens from the plants native range. Potentially, this “novel, artificial” plant insect pairing, could create a facilitative environment such that if an established agent “A”, perhaps either a piercer-sucker or pathogen, up-regulates high levels of SA which in turn suppresses JA, might this create a niche for species “B” e g. a chewer or miner (see Lazebnik 2014; Pineda et al. 2017) which could benefit from the reduced JA pathway defensive metabolites? The corollary of this could be however, that if facilitative associations between species either of the same or different guilds are more common than currently thought, selected agents could be being removed from a facilitative commensalist thus rendering them possibly more difficult to establish and/or ultimately be less effective in their new range.

Plant-insect ecologists using metabolomic techniques which focus on plant responses to a single specialist species, either for species being considered as potential agents or retrospectively where an agent is established but having little impact on the target plant could be beneficial to the agent selection process. Biological control of weeds programmes therefore, offer a great opportunity to study these associations and further elucidate the role of secondary metabolites and the role, if any, of facilitative or negative effects in mediating the composition and phenology of invertebrate herbivore communities.

2.5.4 Augmenting host range and specificity testing

Modern agent selection practice includes exhaustive host specificity and potential host range testing of phylogenetically related plant taxa from the invaded range, which provides a well-tested, reliable method of assessing the

safety of potential control agents for release (Sheppard et al. 2003b; Briese 2004) The premise being that the host range of specialist insects in the majority of cases are constrained by morphological and biochemical similarities of phylogenetically related plant taxa (Rasmann and Agrawal 2011; Rapo et al. 2019) Insight into plant-insect associations based on host plant chemical similarity rather than phylogenetic relatedness has recently been provided by Wheeler et al. (2021) where components of the volatile secondary compounds of six *Lygodium* fern sp. better predicted the oviposition choice of *Neomusotima conspurcatalis* caterpillars than phylogenetic relatedness. Similarly, Becerra (1997) and Becerra and Venable (1999), who used the Chrysomelid genus *Blepharida* to explain the beetles macroevolutionary host shifts on *Bursera sp. (*Burseraceae).

Recently, work by Rapo et al. (2019) demonstrated that for the specialist weevil *Ceutorhynchus cardariae,* being assessed as a potential biocontrol agent, a no choice feeding experiment, measuring feeding intensity, revealed plant biochemical phenotype (i.e., similarity of the glucosinolate profile) to be a more reliable indicator of feeding preference on the tested potential host plants than phylogenetic relatedness to the target host plant. This demonstrated that for species with phylogenetically-disjunct host ranges like *C. cardariae,* host range can be determined more reliably by phenotypic similarity among its potential hosts than by relatedness per se. (Rapo et al. 2019). The authors provided no indication of survival and performance of this weevil on the favoured plants however and measurements of feeding intensity alone, in a no choice experiment does not necessarily imply the insect will target those plants in natural field conditions. This work utilised targeted biochemical profiling of glucosinolates which served to elucidate the hypothesis being tested, however we now understand that herbivore defence systems predominantly consist of mixtures of PSC's many of which are synergistic (Wittstock and Gershenzon 2002; Dyer et al. 2003; Steppuhn and Baldwin 2007; Gershenzon et al. 2012). Non-targeted

metabolomic profiling offers the opportunity to test plants at the broadest possible spectrum of metabolites (Hartley et al. 2012; Jorge et al. 2016a; Sampaio et al. 2016) which may identify sufficient biochemical similarity, to augment phylogenetic relatedness protocols. This may improve host range safety testing by adding to the test list, taxa that might otherwise be overlooked because they are phylogenetically distant (Wheeler 2005; Wheeler and Schaffner 2013).

Application of metabolomics might be particularly valuable in host range testing scenarios where it is not always possible to test all phylogenetically related, or other unrelated taxa of interest, due to constraints on obtaining a particular plant species for testing. This may be due to importation or border restrictions, or the species being protected or rare, or indeed difficult to transplant or propagate into testing facilities. Thus, metabolomics could be used as a proxy which could rule a plant unavailable for testing, ”in”, as a potential host. We concur with Rapo et al. (2019) that biochemical profiling could augment the current centrifugal phylogenetic method used to select plants for specificity testing (Wapshere 1974; Briese 2003) and play an important role in identifying non-target plant species at risk of attack by introduced agents.

2.6 Discussion

Introduced biocontrol agents can fail to establish or, when they do, achieve little impact on the target species. We suggest some of these failures may be due to changes in the target plant's biochemical phenotype in their new invaded range. Metabolomic analysis offers a way of identifying any multifaceted changes and deciphering whether particular classes of compounds may be sufficiently altered, which may impair insect performance.

We present a suite of potential applications that might assist with new and/or problematic biocontrol programmes. Metabolomic analysis of target plants in

the native and invaded ranges may provide insight into, as far as practicable, matching host plant biochemical phenotypes for collection and release sites of biocontrol agents. Where an agent has failed to establish or is underperforming, retrospective metabolomic profiling of the target plant in both its native and invaded ranges may reveal valuable insight into the possible biochemical mechanisms involved. Measurement of a potential agent's impact on host plant defence responses may help predict potential impact. As a starting point, retrospective testing of existing effective agents compared with ineffective ones, may reveal that higher levels of plant response correlate with greater impact.

Plant biochemical responses to specialist feeders (particularly inter-guild specialists) and the interplay between JA and SA responses to these specialists could offer insight, into the possibility of novel pairings of specialist agents. Such knowledge may also provide a foundation for the release of augmentative secondary agents and possibly resurrect programmes that have had limited success. Finally, there appears to be scope for metabolomic analysis to augment host range testing procedures, alluded to in recent publications (Sheppard et al. 2003b; Wheeler and Schaffner 2013; Rapo et al. 2019). Any additional information which can be used to identify potential non-target plant attack by released agents will further reduce uncertainty for practitioners and help appease the concern of critics towards the safety of biological control of weeds programmes.

The potential for metabolomics to assist with biocontrol of weeds programmes is multifaceted and would most certainly add complexity and costs to the processes involved. This technology, however, could be the core platform on which collaborative research between plant biochemists, plant pathologists, plant-insect ecologists and biocontrol practitioners could all benefit tremendously. The ideas we present are intended to stimulate discussion around

potential applications, there will be many more, but they do, we hope, illustrate the potential of this exciting and hugely powerful technology.

Declarations of interest: None

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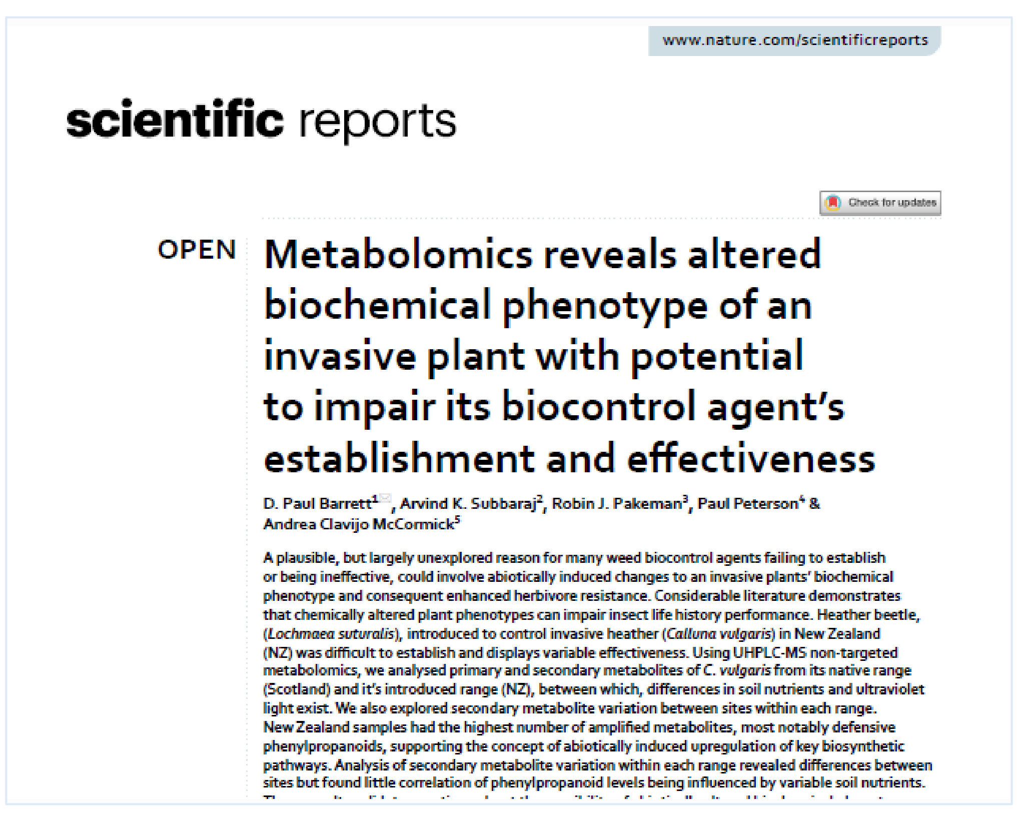
Chapter 3

Metabolomics reveals altered biochemical phenotype of an invasive plant with potential to impair its biocontrol agent's establishment and effectiveness.



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Metabolomics reveals altered biochemical phenotype of an invasive plant with potential to impair its biocontrol agent's establishment and effectiveness

D. Paul Barrett[1], Arvind K. Subbaraj[2], Robin J. Pakeman[3], Paul Peterson[4] & Andrea Clavijo McCormick[5]

A plausible, but largely unexplored reason for many weed biocontrol agents failing to establish or being ineffective, could involve abiotically induced changes to an invasive plants' biochemical phenotype and consequent enhanced herbivore resistance. Considerable literature demonstrates that chemically altered plant phenotypes can impair insect life history performance. Heather beetle, (*Lochmaea suturalis*), introduced to control invasive heather (*Calluna vulgaris*) in New Zealand (NZ) was difficult to establish and displays variable effectiveness. Using UHPLC-MS non-targeted metabolomics, we analysed primary and secondary metabolites of *C. vulgaris* from its native range (Scotland) and it's introduced range (NZ), between which, differences in soil nutrients and ultraviolet light exist. We also explored secondary metabolite variation between sites within each range. New Zealand samples had the highest number of amplified metabolites, most notably defensive phenylpropanoids, supporting the concept of abiotically induced upregulation of key biosynthetic pathways. Analysis of secondary metabolite variation within each range revealed differences between sites but found little correlation of phenylpropanoid levels being influenced by variable soil nutrients.

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Abstract

A plausible, but largely unexplored reason for many weed biocontrol agents failing to establish or being ineffective, could involve abiotically induced changes to an invasive plants' biochemical phenotype and consequent enhanced herbivore resistance. Considerable literature demonstrates that chemically altered plant phenotypes can impair insect life history performance. Heather beetle, *(Lochmaea suturalis),* introduced to control invasive heather *(Calluna vulgaris)* in New Zealand (NZ) was difficult to establish and displays variable effectiveness. Using UHPLC-MS non-targeted metabolomics, we analysed primary and secondary metabolites of *C. vulgaris* from its native range (Scotland) and it's introduced range (NZ), between which, differences in soil nutrients and ultraviolet light exist. We also explored secondary metabolite variation between sites within each range. New Zealand samples had the highest number of amplified metabolites, most notably defensive phenylpropanoids, supporting the concept of abiotically induced upregulation of key biosynthetic pathways. Analysis of secondary metabolite variation within each range revealed differences between sites but found little correlation of phenylpropanoid levels being influenced by variable soil nutrients. These results validate questions about the possibility of abiotically altered biochemical phenotypes in invasive plants, influencing weed biocontrol agent establishment and effectiveness, and show the potential for metabolomics in assisting future, or retrospectively analysing biological control programmes.

3.1 Introduction

Globally, invasive plants are a serious threat to the terrestrial habitats they invade, causing considerable economic loss in managed production systems such as agriculture, horticulture and forestry or ecological perturbations in environmentally sensitive natural systems including communal rangelands and conservation lands which provide essential ecosystem services (Pimentel et al. 2005). In most instances such intrusions cumulatively result in losses of production and biodiversity at the species, community, or ecosystem level (Pyšek et al. 2012).

For many of these ecosystems, the introduction of insect or pathogen biocontrol agents sourced from the plant's native range (classical biocontrol) offers a sustainable long-term management alternative for invasive plants (Hayes et al. 2013; Schwarzländer et al. 2018a). However, some biocontrol programmes do not achieve the desired goals due to agents either failing to establish (McFadyen 2000; Schwarzländer et al. 2018a), being ineffective once they are established (McClay and Balciunas 2005; Raghu and Dhileepan 2005) or display variable effectiveness between sites (Falla et al. 2023). While evidence suggests biocontrol agent population establishment is likely influenced by many environmentally driven factors (Grevstad 1999; Jonsen et al. 2007; Harms et al. 2020), there is a paucity of literature considering target plant biochemistry and how changes in its genotype or biochemical phenotype might act as a component allee effect and impose limitations or extinctions on control agent establishment, particularly while at vulnerable low population numbers (Tobin et al. 2011). Likewise, literature addressing target plant biochemistry on agent ineffectiveness or variable inter-site effectiveness is sparse (but see Falla et al. 2023).

In natural habitats, plants as sessile organisms are simultaneously subjected to multiple stressors to which appropriate responses are required for

establishment, growth, and survival (Valladares et al. 2007a; Valladares et al. 2007b; Clavijo McCormick 2016). Considerable evidence relating to abiotic and biotic stresses on plants exists (Obata and Fernie 2012; Foyer et al. 2016; Sharma et al. 2019), demonstrating that plants are capable of responding to such stresses via a plastic and finely balanced response network involving activation of stress responsive genes which regulate phytohormone production, redox signalling pathways, growth, and calcium and protein kinase cascades, resulting in changes to both primary and secondary metabolism (Foyer et al. 2016; Cramer et al. 2011; Verma et al. 2016), all of which are key endogenous components in mediating plant stress responses.

Plant responses resulting from adaptive changes driven by the genotype under changed environmental conditions can manifest either independently or as a combination of morphologically, physiologically, or biochemically altered phenotypes broadly termed phenotypic plasticity (Pigliucci 2005; Van Kleunen et al. 2010; Shaar-Moshe et al. 2019). Phenotypic plasticity is thought to be a common characteristic of invasive plants (Hauvermale and Sanad 2019), but experimental evidence investigating this is equivocal (Godoy et al. 2011), citing it is often limited to the early stages of establishment (Palacio-López and Gianoli 2011) and constrained within the ecological limits of the sites studied (Valladares et al. 2007b; Lázaro-Nogal et al. 2015; Bufford and Hulme 2021).

Nevertheless, some recent publications have demonstrated abiotically induced alterations to the metabolome of invasive plants when compared with their conspecifics in the native range. Such alterations generally result in increased levels of various defensive secondary metabolites (Wolf et al. 2011; Skubel et al. 2020; Medina van Berkum et al. 2023), suggesting that this may not be an uncommon phenomenon.

Heather, (*Calluna vulgaris*) is an invasive shrub introduced from Europe now widely established on the North Islands Central Plateau (CP) of New Zealand (NZ), where it impacts this fragile sub-alpine environment (Effah et al. 2020a, 2020b; 2022). The heather beetle (*Lochmaea suturalis*) (Coleoptera: Chrysomelidae) sourced and introduced from the United Kingdom (UK) in 1996 as a biocontrol agent, required multiple releases over several years before establishment, does not achieve the population densities of its home range in the UK and Europe and currently displays variable effectiveness within the CP region. A long-term research effort has investigated several factors to explain these scenarios with only low foliar nitrogen in CP heather providing a definitive explanation (Peterson et al. 2004, 2024; Fowler et al. 2015; Barrett et al. 2021), but potential changes in plant defensive biochemistry had not been explored. We hypothesize that the plastic nature of plant biochemical responses induced potentially by abiotic factors may cause introduced biocontrol agents, to encounter plants with altered defensive biochemistry compared to those plants from where the agent was sourced. We aim therefore to explore the biochemical phenotype of this plant in the invaded range (NZ) and its native range (UK), from where the biocontrol agent was sourced and we further aimed to explore such phenotypic variability between sites within each range.

In the CP, mean seasonal temperatures for this higher altitude region, while displaying slightly greater daily variation of maximums and minimums, are very closely matched to the higher latitudes of Scotland in the UK. The CP region however experiences a longer winter period (3 – 4 weeks) than that of northern UK latitudes (Peterson et al. 2007), but spring and early summer mean monthly temperatures, i.e., the growing period for *C. vulgaris*, are very closely matched. We assume therefore that *C. vulgaris* in this region experiences climatic conditions very similar to its northern hemisphere conspecifics. We hypothesize that two abiotic parameters of this region that may induce long term permanent changes to the biochemical profile of *C. vulgaris* are soil nutrients and light.

Soil nutrient availability (depending on the underlying geology) and long term increased chemical defences in plants (most notably phenylpropanoids such as polyphenolics, flavonoid glycosides, flavones and coumarins), are well documented (Yang et al. 2018; Gershenzon 1984; Mazid et al. 2011; Wright et al. 2010; Sampedro et al. Kováčik and Klejdus 2014). Soils of heather dominated heathlands in the UK and Europe range from free draining to wet, are acidic and low in nutrients, especially phosphate and nitrogen (De Graaf et al. 2009). Similarly, the young volcanic soils of the CP region are generally free draining but with levels even lower in phosphate and nitrogen (Peterson et al. 2024; Hewitt 2010).

Light quantity (intensity) and quality (the balance of PAR, UV-A and UV-B) which is dependent on latitude and altitude (Liley and McKenzie 2006; Seckmeyer et al. 2008), also induce long term changes to the plant biochemical phenotype by modulating the jasmonate (JA) and abscisic acid (ABA) dependant pathways thus inducing Shikimate – phenylpropanoid derived flavonoids and phenolic acids. These metabolites play an important role in plant photoprotection but have also been shown to enhance plant defences against insect herbivores (Ballaré et al. 2012; Rousseaux et al. 2001; Izaguirre et al. 2006; Qi et al. 2018) or provide systemically acquired resistance to biotrophic pathogens (Fu and Dong 2013; Ballaré 2014). Light intensity and ambient UV are significantly different between the two regions, with peak summertime UV index figures of 12 to 13 at the North Island CP being approximately double that of higher latitude regions of the UK at 6 to 7 (Liley and McKenzie 2006; Seckmeyer et al. 2008). Furthermore, previous work demonstrating plasticity of secondary metabolites in *C. vulgaris* at varying altitudes (Monschein et al. 2010) and seasonally (Jalal et al. 1982) in Europe and in response to multiple abiotic factors in New Zealand (Effah et al. 2020c, 2020d), indicates that this plant species readily responds to changing parameters in its environment, making it perhaps an ideal model species to explore.

In recent years, high throughput mass spectral chromatographic technology in conjunction with online cheminformatic platforms has markedly progressed metabolomic analytical capability. Metabolomics makes it feasible to now study plant biochemistry at the molecular level, providing a targeted or non-targeted characterisation and quantification of currently identifiable metabolites (the metabolome), in a particular plant tissue in response to its environment or treatment (Barrett et al. 2021; Hall 2006; Macel et al. 2010; Arbona et al. 2013; Jorge et al. 2016; Sampaio et al. 2016).

In this study, we applied non-targeted metabolomics to investigate possible changes to both primary and secondary metabolites of *C. vulgaris* between its native range in Scotland (UK) and plants from the invaded range of the CP in New Zealand. Using unsupervised principal components analysis (PCA) our findings are exploratory only but do demonstrate clear differences in the plant metabolomes between the native and invaded ranges potentially linked to UV and soil nutrients.

It is beyond the scope of this study to independently assess the effects of these abiotic parameters (UV and soil nutrients) on the plant metabolome, as this requires controlled, experimental conditions. Testing the direct effects of altered foliar biochemistry on heather beetle performance is also beyond the scope of this study. These questions are now being addressed and will be presented in subsequent publications. However, we posit that our results validate the question of biochemically altered defences potentially exposing beetles at the CP sites to encounter host plants that are less assimilable than those in its native range. Furthermore, we propose that metabolomics is a powerful analytical tool that could be useful to assess biochemical changes in invasive plants due to differing abiotic influences, and such information could assist decision making in future biocontrol programmes or retrospectively elucidate unsuccessful ones.

3.2 Materials and Methods

3.2.1 Sampling

Foliage:

Five samples of mature heather (*Calluna vulgaris*) foliage were collected from each of four sites in Scotland (see Supplemental, S Fig. 2a), during the Northern Hemisphere summer from 29th June to 3rd July 2018. The sites were, Glensaugh (GS) Lat. 56.910605°, Lon. -2.569144°, alt. 323m, soils - Strichen, peaty gleyed podzols; Ballogie Estate (BE) Lat. 56.998852° Lon. -2.743927°, alt. 316m, soils - Countesswells, peaty gleyed podzols; Glenturret (GT) Lat. 56.415890° Lon. -3.912876°, alt 351m, soils – Gourdie, noncalcareous gleys with peaty gleys; Creag Meagaidh (CM) Lat. 56.933362° Lon. -4.527912°, Alt 290m, soils – Arkaig, peaty gleys with dystrophic semi-confined peat. During this period, most plants at GS, GT, and CM, were not flowering but at BE a small number had reached very early budburst. Plants with flowers were, where possible, avoided for sampling. Each sample comprised of equal quantities of 10 – 15 mm long fresh sprigs combined from three individual intertwined or adjacent mature plants. Each such sample was taken ≥ than 10m apart and all were immediately cryo-frozen in nitrogen vapour then stored at -80 C° until freeze drying and grinding.

Mature *C. vulgaris* plants were sampled from four sites from the Central Plateau region of the North Island, New Zealand (see Supplemental, S. Fig. 2b), during January 17th - 18th 2019 (Southern Hemisphere summer) using the same sampling and storage protocol. The sites were Mangaturuturu (MU) Lat. -39.303293° Lon. 175.390239°, alt. 817m, soil - Orthic Podzol; Waiouru (WU) Lat. -39.456172° Lon. 175.677246°, alt. 814m, soil - Orthic Allophanic; Quarry (QU) Lat. -39.431120° Lon. 175.685689°, alt. 881m, soil - Orthic Allophanic; Waihohunu (WH) Lat. -39.227263° Lon. 175.732654°, alt. 975m, soil - Tephric Recent. At all

sites, plants were at very early to early budburst with only a few flowers present, and again plants with flowers were avoided for sampling.

Soils and UV:

Five samples for soil nutrient analysis were collected from random positions in all sites. Each sample consisted of 3 soil cores each taken to a depth of 15cm and air dried until no change in mass. Cores were sieved through a 1mm precision sieve, then combined and analysed for Olsen P, Total N and pH. NZ samples were analysed by Hill Labs, Hamilton NZ and SC samples at the James Hutton Institute Laboratories, Aberdeen, UK. Phosphorus for Olsen P for both NZ and SC samples were measured on air dried soil using $NaHCO_3$ (0.5M; pH8.5) extractant. For Total N both NZ and SC samples were subjected to the Dumas combustion method then measured using a VarioMAX CN Macro Elementar analyser on NZ soils and a Thermo Flash EA 1112 elemental analyser (Thermo Fisher) on SC soils. For pH, both soils were slurried (1:2 v/v) soil:H_2O and analysed using glass bulb pH probes. Parameters used for *C. vulgaris'* exposure to ultra-violet for each range/region, are based on reviews of global summer noontime maxima of a standardised measurement of erythemal UV intensity known as the ultra-violet index (UVI) and with an adjustment for altitude (1000m asl) at the North Island Central Plateau sites (Effah et al. 2020c, 2020d; Vanicek et al. 2000).

Invertebrates:

To assess potential herbivore induced plant metabolite responses, using a beating tray and standardised beating protocol, we collected all invertebrates from the foliage of all plants at all sites and immediately preserved them in 70% ethanol. These were later sorted into family and their associated feeding guild, enumerated, then interrogated for Pearson correlation with phenylpropanoid compound intensities associated with their own site.

3.2.2 Foliage sample preparation for UHPLC-MS analysis

Foliage samples were freeze dried then stored for 2 weeks at -20 C° prior to grinding to ≈ 150 – 50 μm particle size before extraction. 50 ± 2.0 mg of ground sample were weighed into 2 mL microcentrifuge tubes and extracted in 800 μL of pre-chilled $CHCl_3$: MeOH (1:1 v/v) with internal standards comprising 1.6 mg L-1 of d5-L-tryptophan, d4-citric acid, d10-leucine, d2-tyrosine, d35-stearic acid, d5-benzoic acid, 13C2-glucose, and d7-alanine. Samples were vortexed for 2 min and kept at -20 C° for 1 hour after which 400 μL of H_2O was added and again vortexed for 2 min. Samples were then centrifuged for 15 mins (11000 RPM @ 4 C°) creating a biphasic layer. Two aliquots of 200 μL each of the upper, aqueous layer were transferred to 2ml microcentrifuge tubes and evaporated to dryness, under a continuous stream of nitrogen (30°C for 50 min) and stored @ -80 C° until reconstitution.

For semi-polar compounds, reconstitution for C18-LC-MS analysis, was in 200 μL of C_2H_3N:H_2O (1:9 v/v), vortexed for 1 min then transferred to a glass insert in an auto-sampler vial. For polar compounds reconstitution for HILIC-LC-MS analysis, was in 200 μL of C_2H_3N:H_2O (1:1 v/v), vortexed for 1 min then similarly transferred to a glass insert in an auto-sampler vial. A pooled mix of all samples was similarly prepared (n=7) and used as quality controls (QC) for each of the C18 and HILIC streams. These were evenly distributed (every 8th sample) to monitor any systematic effects on the corresponding analysis. Five extraction blanks were included at the beginning of and an amino acid standard (A9906; Sigma-Aldrich, NZ) at the beginning and end of the sampling sequences.

3.2.3 Chromatography and mass-spectrometry

Chromatography and tandem mass-spectrometry analysis of polar and semi-polar compounds were achieved using a Thermo LC–MS system (Thermo Fisher Scientific, Waltham, MA, USA) which consisted of an Accela 1250 quaternary

UHPLC pump, a PAL auto-sampler fitted with a 15,000 psi injection valve (CTC Analytics AG., Zwingen, Switzerland) and 20 µl injection loop, and an Exactive Orbitrap mass spectrometer with electrospray ionisation run in both positive and negative modes.

For semi-polar compounds, samples were cooled in the auto-sampler at 4 °C and a 2 µL aliquot was injected into a 1.9 µm Thermo Hypersil Gold C18 column (UPLC, 100 mm × 2.1 mm, Thermo Fisher Scientific, USA) at 25 °C with a gradient elution programme and a flow rate of 400 µL/min. The mobile phase was water with 0.1% formic acid (solvent A), and acetonitrile with 0.1% formic acid (solvent B). Using the Xcalibur software package provided by the manufacturer the gradient elution programme was: held at 5% B (0–0.5 min), 5–99% B (0.5–13 min), held at 99% B (13–15 min), returned to 5% B (15–16 min) and allowed to equilibrate for a further 4 min prior to the next injection. The first 1.5 min and the last 6 min of the chromatogram were diverted to waste. Mass spectral data were collected in profile mode over a mass range of m/z 60–1200, at a mass resolution setting of 25,000 with a maximum trap fill time of 100 ms. Samples were run in both positive and negative ionisation modes separately. Positive ion mode parameters were: spray voltage, 3.5 kV; capillary temperature, 325 °C; capillary voltage, 50 V, tube lens 120 V. Negative ion mode parameters were: spray voltage, −3.5 kV; capillary temperature, 325 °C; capillary voltage, −90 V, tube lens −80 V. The nitrogen source gas desolvation settings were the same for both modes (arbitrary units): sheath gas, 40; auxiliary gas, 10; sweep gas, 5 (Fraser et al. 2013).

For polar compounds, samples were cooled in the auto-sampler at 4 °C and a 2 µL aliquot was injected into a 5 µm ZIC-pHILIC column (100 mm × 2.1 mm, Merck Darmstadt, Germany) at 25 °C with a gradient elution programme and a flow rate of 250 µL/min. The mobile phase was acetonitrile with 0.1% formic acid (solvent A) and 16 mM ammonium formate in water (solvent B). The gradient elution

programme was: held at 97% A (0–1 min), 97–70% A (1–12 min), 70–10% A (12–14.5 min), held at 10% A (14.5–17 min), returned to 97% A (17–18.5 min) and allowed to equilibrate for a further 5.5 min prior to the next injection. The first 1.5 min and the last 5 min of the chromatogram were diverted to waste. Mass spectral data were collected in profile mode over a mass range of m/z 55-1100 at a mass resolution setting of 25,000 with a maximum trap fill time of 100 ms. Positive ion mode parameters were: spray voltage, 3.5 kV; capillary temperature, 325 ˚C; capillary voltage, 90 V, tube lens 120 V. Negative ion mode parameters were: spray voltage, −3.0 kV; capillary temperature, 325 ˚C; capillary voltage, −90 V, tube lens −100 V. The nitrogen source gas desolvation settings were the same for both modes (arbitrary units): sheath gas, 40; auxiliary gas, 10; sweep gas, 5 (Fraser et al. 2012).

3.2.4 Data analysis

Thermo derived .raw files for each stream i.e., C18 and HILIC in both positive and negative modes, were converted to mzML format using MSConvertGUI (Adusumilli and Mallick 2017), uploaded into MZmine (Pluskal et al. 2020) to determine the appropriate baseline noise threshold and then into XCMS online "xcmsonline.scripps.edu", for feature detection, alignment and exploratory data analysis (Domingo-Almenara and Siuzdak 2020). Feature detection parameters for C18 data were, m/z deviation 10 ppm, min and max peak width 5 and 20 respectively, mzdiff 0.001, s/n threshold 20, Prefilter intensity 1e4 and noise filter 4e4. For HILIC data the same parameters were 10ppm, 10 and 60, 0.001, 20, 1e4 and 3e4. After downloading the output, a series of procedures followed, converting raw mass spectrometry data into data matrices comprising *m/z*, retention time, and the corresponding ion intensity measurements suitable for statistical analysis.

Reduction of background variability in the full data matrix of each stream was performed using a QC vs Blank t-test thus allowing subtraction of those features with $p > 0.05$ values or t.stat values corresponding to any features high in the blanks. These data matrices were each uploaded into MetaboAnalyst ver. 6.0 (MA 6.0) (Pang et al. 2021) and data integrity checked to confirm the number of samples, number of peaks, missing values, and the number of treatment groups. No missing values were detected in any of the data sets. For filtration of variables showing low repeatability, the threshold to remove those with high percent relative standard deviation (RSD) was set at 30% to that of the QCs and the data normalised by auto-scaling (mean-centred and divided by the standard deviation of each variable) and Gaussian distribution confirmed so that feature mass intensities are comparable.

For each stream, we explored the entire data matrix at the site level for both the NZ and SC ranges by subjecting them to multiple principal component analysis (PCA) in MA 6.0. This indicated a degree of site cluster overlap within each range but a clear and consistent separation of clusters between the NZ and SC ranges (see Supplemental, S. Fig. 1). We therefore considered it appropriate to apply paired PCA to each streams data matrix at the NZ and SC level for the purpose of demonstrating those differences between the metabolomes of each range.

Using the data matrices at the NZ and SC range level, final analyses were performed using the one factor statistical platform in MA 6.0. Unsupervised paired PCA's were conducted on those $p < 0.05$ features remaining in each stream, to visualise and confirm the degree of separation between clusters (metabolomes) for each range. Then from these data matrices, features for annotation were achieved by applying a paired t-test with all features below the threshold value of FDR < 0.05 (Benjamini and Hochberg 1995) being retained for that purpose.

Annotations were conducted and confidence levels confirmed (Sumner et al. 2007) for each metabolite by interrogating the original .raw files using Xcalibur Freestyle. Formula matches were confirmed, and mass accuracy parameters were set within +/– 10.0 ppm. For HILIC level 1 confidence, m/z and rt. results were matched against a Grasslands AgResearch in house spectral standards library (GL), based on authentic standards run under the same chromatographic conditions. For all C18 and the remaining HILIC features, level 2 and 3 (parent ion plus at least one fragment and parent ion only, respectively) confidence levels were confirmed using the MassBank.eu (https://massbank.eu/) spectral database. Where the same annotated confirmed compound (ion) appeared in both + and – modes the one with the highest intensity was included in the final data table. If a compound appeared in both C18 and HILIC streams the criteria for inclusion in the table was if they were a secondary or primary metabolite respectively.

Using the metabolites covering all three levels of confidence from tables 1 and 2 and using the pathways analysis platform in MA 5.0 we conducted analyses to elucidate which pathways are enriched the most and provide the greatest impact on the data sets between each range.

To observe inter-site differences for secondary metabolites potentially due to soil nutrient status within each range, we ran multiple PCA on the C18 pos and C18 neg data matrices independently. Each feature in these data matrices was then subjected to one-way ANOVA in MA 6.0 to provide significant features for annotation. MA 6.0 also provides PERMANOVA analysis results to assess the significance of difference between PCA derived clusters. Annotated compounds were subjected to Minitab v 21.1.0 for Tukey post hoc allocation of significant differences. Phenylpropanoid compounds resulting from these analyses were graphed then individually subjected to Pearson correlation analysis to explore potential relationships with site soil nutrient status. The soil nutrient samples

being randomly located within each site are therefore not paired with each foliage sample, so the mean site values for Olsen P and Total N were used.

Soil analysis results from the NZ range for Olsen P were converted from volumetric mg lt^{-1} to gravimetric mg kg^{-1} (Taylor et al. 2018) to match the SC result output and together with Total N and pH were for inter-site statistics subjected to one-way ANOVA with Tukey post hoc allocation analyses. Between NZ and SC range statistics for the same parameters were achieved using t-tests. Both analyses were achieved using Minitab v 21.1.

3.3 Results

3.3.1 Analysis, pathways and annotations

Paired PCA plots for both C18 and HILIC streams in both ionisation modes display clear unsupervised clustering of all samples from New Zealand's Nth Is. Central Plateau (CP) and those of Scotland (SC) in the United Kingdom (UK), see Fig. 1. Separation between the NZ and SC clusters for C18 pos is explained by principal components 1 and 2 combined being 34.9% of the observed variance. These values are for C18 neg, 36.3%, HILpos, 44.6% and HILneg, 41.5% respectively. The MA 6.0 platform provides statistical testing to verify robustness of the clustering using PERMANOVA with 999 permutations. With (PERMANOVA); C18 pos, $F = 27.17$, $p = 0.001$; C18 neg $F = 14.77$, $p = 0.001$; HILIC pos $F = 38.95$, $p = 0.001$ and HILIC neg $F = 38.73$, $p = 0.001$ respectively, these results confirm the validity of the PCA analyses.

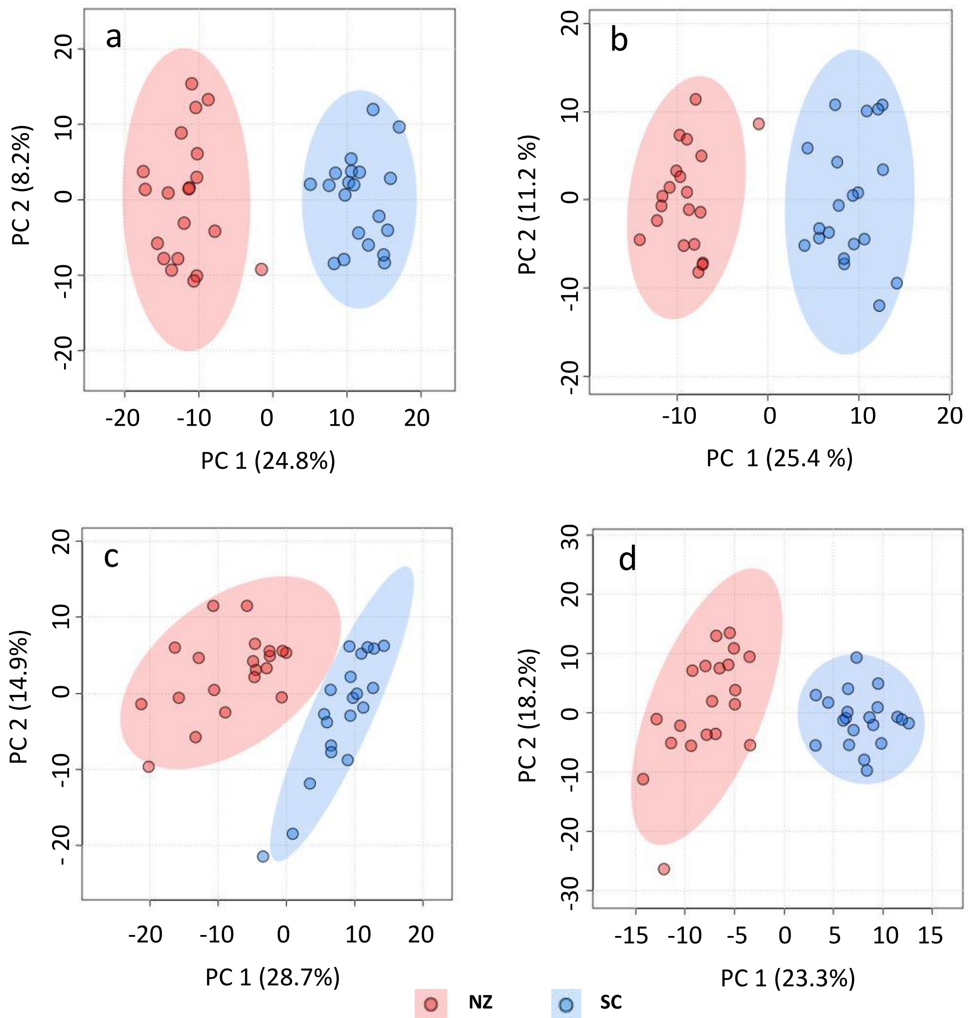

Fig. 1. Paired PCA plots including 95% confidence ellipses for each stream showing significant separation between New Zealand (NZ) and Scotland (SC) sites for each ionization mode. PC 1 and PC 2 scores for each, explain the observed variance between clusters. a = C18 pos, b = C18 neg, c = HILIC pos and d = HILIC neg. PERMANOVA (999 permutations) provides verification of the robustness of the cluster formations in all four analyses. C18 pos, $F = 27.17$, $p = 0.001$; C18 neg $F = 14.77$, $p = 0.001$; HILIC pos $F = 38.95$, $p = 0.001$ and HILIC neg $F = 38.73$, $p = 0.001$.

Interrogation of the .raw files resulted in 66 metabolites being annotated from the C18pos, C18neg, HILICpos and HILICneg streams combined. Twenty-two flavonoids, five coumarins, five hydroxycinnamic acids, two polyphenolics and one diarylheptanoid, all phenylpropanoids, were revealed from both C18 pos and neg streams combined. Fourteen of these were confirmed at level 2 with the remainder being level 3 (Table 1).

Of these 35 phenylpropanoids, 22 of them are amplified in the NZ samples compared to those of SC. Additionally, secondary metabolites including 4 benzoic acids; 2 phenols, 1 fatty acid plus 2 sesquiterpenoids, were identified from both streams (Table 2). For primary metabolites HILIC separation revealed 16 amino acids for which the Grasslands in house spectral standards library (GL) confirmed ten to be of confidence level 1, six to confidence level 2 and the remainder to level 3. Of these 16 amino acids, 14 are amplified in the NZ samples. Carboxylic acids, glycerolipids and organooxygen metabolites from both streams make up the remainder (Table 2). All compound annotation confirmation data for each table is available in Appendix A.

Table 1. Phenylpropanoid compounds annotated from all four streams. level 1 and 2 confidence compound names are in black type. Level 3 confidence names are in grey type and identified to sub class only. FC = Fold Change, U/D = up or down, CL = confidence level, Lib = GL library.

Class/Sub Class	Name	KEGG	Stream	FC	U/D	t.stat	CL	Lib	m/z	rt.	Fragments	
Flavonoid	Apigenin 7-O-glucoside	..	C18+	3.82	↑	4.56	2		433.11	4.06	433.073	
" " " "	Kaempferol 3-O-glucoside	C12249	C18+	1.64	↑	3.29	2		449.1052	5.10	287.0532	288.0579
" " " "	Kaempferol	C05903	C18+	1.37	↑	2.55	2		287.055	5.41	119.0337	
" " " "	Dihydrokeampherol	..	C18+	2.81	↑	13.29	2		289.0713	3.58	271.0592	272.0634
Flavone a.	Luteolin	C01514	C18+	2.07	↑	4.74	3		287.0549	5.10		
Flavone b.	Tricin	C10193	C18+	2.19	↑	4.50	3		331.0829	4.06		
Flavanonol	Dihydroquercetin	C01617	C18-	1.51	↑	4.41	3		303.0524	4.77		
Flavonoid-3-O-glucuronide	Quercetin 3-O-glucuronide	..	C18-	2.46	↑	4.13	3		477.069	4.89		
Flavonoid-3-O-glycoside	Syringetin 3-O-glucoside	..	C18-	96	↑	10.28	3		507.111	4.84		
Flavan a.	8-Prenylnaringenin	C18023	C18+	1.53	↑	6.61	3		341.1401	5.03		
Flavan b.	Eriodictyol	C05631	C18+	1.83	↑	8.05	3		289.0713	4.52		
Flavonol a.	Quercetin 7-methyl ether	C10176	C18+	2.53	↑	4.44	3		317.0647	5.03		
Flavonone	Eriodictyol 7-O-glucoside	..	C18+	5.06	↑	7.23	3		451.1225	4.06		
Isoflavonoid C-glycoside	Puerarin	C10524	C18+	3.76	↑	4.31	2		417.1185	4.71	399.1123	381.1027
											297.0768	
Flavonoid	Procyanidin B2	..	C18-	1.55	↓	-3.37	2		577.1309	4.29	407.0789	
" " " "	(+)-Epicatechin	C09728	C18-	1.33	↓	-5.23	2		289.0706	4.18	245.083	205.0519
											203.072	109.0299
" " " "	Isoquercitrin	C05623	C18+	1.15	↓	-2.97	2		465.1007	4.87	305.0553	
Flavonol b.	Kaempferide	C10098	C18+	4.51	↓	-7.71	3		301.0709	5.22		
Flavonol c.	Quercetin	C00389	C18-	1.15	↓	-2.83	3		301.0355	5.91		
Flavonol d.	Myricetin	C10107	C18-	1.82	↓	-2.79	3		317.0304	5.34		
Flavonoid glycoside	Kaempferol-3-glucoside-2''-p-coumaroyl	..	C18+	1.28	↓	-2.35	3		595.145	3.53		
Chalcone	Aspalathin	..	C18-	5.36	↓	-3.05	3		451.1257	3.94		
Coumarin	Scoparone	C09311	C18+	2.61	↑	9.83	2		207.0654	5.50	121.065	
Coumarin a.	Osthole	..	HIL+	2.93	↑	4.39	3		245.114	10.83		
7-Hydroxycoumarin	6-Methoxy-7-hydroxycoumarin	..	C18+	1.44	↑	3.21	3		193.0502	4.50		
Hydroxycoumarin a.	4-Hydroxycoumarin	C20414	C18+	1.2	↑	2.62	3		163.0384	3.82		
Hydroxycoumarin b.	6,7-Dihydroxycoumarin	..	C18+	4.76	↓	-7.80	3		179.0334	4.16		
Hydroxycinnamic acid	Caffeic acid	C01481	C18+	1.22	↑	2.65	2		181.0501	3.83	163.0386	164.024
" " " " " "	O-Coumaric acid	C01772	HIL+	1.29	↑	3.06	2		165.055	8.32	121.0633	
Hydroxycinnamic acid a.	2-O-sinapoylmalate	..	C18+	5.4	↓	-9.13	3		341.0853	4.17		
Hydroxycinnamic acid b.	5-Hydroxyferulic acid	C05619	HIL-	7.27	↓	-4.97	3		209.0457	8.63		
Cinnamyl alcohol	cis-o-Hydroxycinnamic acid	..	HIL-	8.02	↓	-7.22	1	GL	163.0394	7.01	119.0487	
Polyphenolic	Chlorogenic acid	C00852	C18-	1.13	↑	2.37	2		353.0871	3.82	191.0556	192.0589
" " " "	Pantothenic Acid	C00864	C18+	1.45	↑	5.38	2		220.119	3.52	202.1077	
Diarylheptanoid	Quercetin 3-Arabinoside	..	C18+	1.35	↓	-3.41	2		435.0933	5.08	303.0483	304.0539

Table 2. Primary and secondary compounds annotated from all four streams. level 1 and 2 confidence compound names are in black type Level 3 confidence names are in grey type and identified to sub class. FC = Fold Change, U/D = up or down, CL = confidence level, Lib = GL library.

Class/Sub Class	Name	KEGG	Stream	FC	U/D	t.stat	CL	Lib	m/z	rt.	Fragments	
Benzoic acid a.	Benzoic acid	C00180	C18+	1.46	↓	-4.96	3		123.0441	4.20		
Hydroxybenzoic acid	5-Hydroxysalicylic acid	..	C18+	1.77	↓	-4.23	3		155.0338	4.30		
Phenylacetic acid	Homogentisic acid	C00544	HIL+	2.44	↓	-4.19	3		169.0498	8.97		
Aminobenzoic acid	4-Aminobenzoic acid	C00568	HIL+	1.47	↑	3.56	3		138.0551	9.81		
Phenol	Phenol	C00146	HIL+	1.41	↑	2.36	3		95.0496	12.25		
Benzenediol	Pyrocatechol	C00090	HIL-	3.28	↓	-6.21	2		109.0285	8.88	108.0217	
Fatty acid	3-Hydroxy-3-methylglutaric acid	..	C18-	4.88	↓	-7.30	2		161.0443	1.54	101.0238	
Sesquiterpenoid	Isorhamnetin 3-O-glucoside	..	C18+	2.67	↑	5.04	2		479.1189	5.03	318.07	317.0655
Sesquiterpenoid a	Syringaldehyde	..	HIL-	1.78	↓	-3.86	3		181.0499	2.36		
Alpha-Amino Acid	L-Phenylalanine	C00079	HIL+	2.62	↑	9.64	1	GL	166.0867	9.79		
" " " " " "	L-Tyrosine	C00082	HIL+	1.71	↑	3.39	1	GL	182.0812	11.53	136.0762	
" " " " " "	Alanine	C01401	HIL+	1.32	↑	4.21	1	GL	90.0553	12.58		
" " " " " "	L-Threonine	C00188	HIL+	1.42	↑	3.87	1	GL	120.0659	12.77	102.0565	
" " " " " "	L-Serine	C00716	HIL+	1.56	↑	4.49	1	GL	106.0502	13.68		
" " " " " "	L-Glutamine	C00064	HIL-	2.38	↑	7.26	1	GL	145.061	13.21		
" " " " " "	Citrulline	C00327	HIL+	2.46	↑	7.48	2		176.1028	13.80	159.078	113.071
Alpha-Amino Acid a.	1-Aminocyclopropane-1-carboxylic acid	C01234	HIL+	1.57	↑	3.93	3		102.0555	12.77		
Amino Acid	L-Glutamate	C00025	HIL-	1.29	↑	3.18	1	GL	146.0451	13.12		
" " " "	L-Aspartic acid	C00049	HIL+	1.58	↑	4.39	1	GL	134.0449	14.07	116.0336	88.0387
Amino Acid a.	Betaine	C00183	HIL+	2.53	↑	6.76	3		118.0868	9.21		
Amino Acid b.	L-Pyroglutamic acid	..	HIL+	1.75	↑	6.35	3		130.0502	13.19		
Non-Protein Amino Acid	gamma-Aminobutyric acid (GABA)	C00334	HIL+	1.5	↑	3.76	1	GL	104.0708	12.01		
Amino acid conjugate	N-Fructosyl tyrosine	..	HIL+	1.38	↑	3.36	2		344.1349	8.33	187.1336	
Alpha-Amino Acid	L-Asparagine	C16438	HIL+	1.42	↓	-2.56	2		133.0614	13.58	87.0554	
" " " " " "	L-Tryptophan	C00078	HIL+	1.18	↓	-1.45	1	GL	205.0975	10.45	188.0725	
Tricarboxylic acid	cis-Aconitic acid	C00417	HIL-	1.84	↑	7.60	2		173.0086	9.46	129.0178	
Glycerolipid	Naringenin 7-O-glucoside	..	C18-	2.75	↑	4.02	2		433.1124	4.69	313.0657	
Alpha Hydroxy acid	2-Hydroxyisobutyric acid	..	HIL+	1.47	↓	-3.46	3		105.0552	9.16		
lactone	Gamma-Decalactone	..	C18+	1.54	↓	-3.00	2		171.1382	5.05	153.1275	135.1168
											154.1309	136.1202
Carbohydrate	Galactaric acid	C00879	HIL-	1.56	↑	4.14	3		209.0298	14.54		
Carbonyl compound	2-Hexenal	..	HIL+	1.38	↓	-2.33	3		99.0807	9.04		

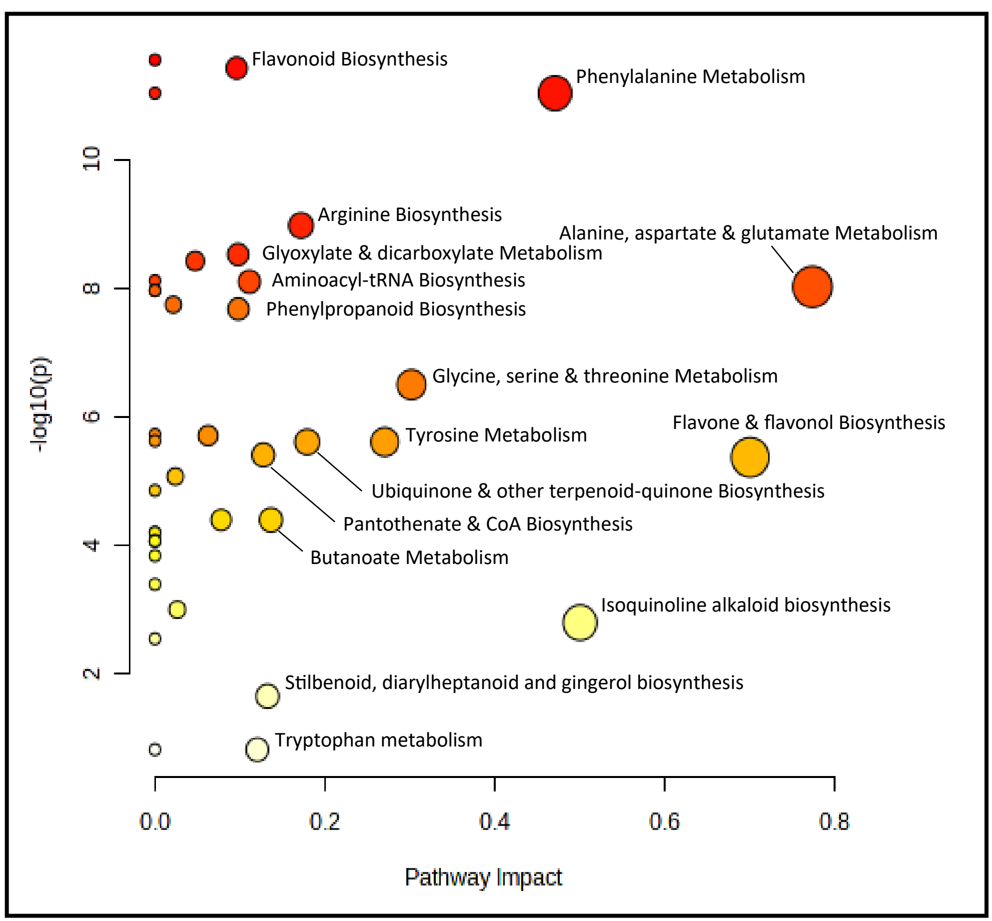

Fig. 2. Pathway enrichment analyses indicating the pathways having the greatest impact factor (x axis) and greatest p value of significance (y axis) between the two treatments (Ranges). Circle size indicates pathway impact value and colour the p value. All pathways with an impact factor ≥ 0.1 are identified. Those in white to pale yellow are represented by one compound only.

Pathway analysis identified those metabolic pathways to be enriched as indicated by the impact factor (x axis) and its p value of significance (y axis). Pathways with an impact factor ≥ 0.1 are labelled. The most significant of these were alanine, aspartate and glutamate metabolism which revealed the greatest enrichment value of 0.77 followed by flavone and flavonol biosynthesis 0.7 and Phenylalanine metabolism 0.47. Note: The Isoquinoline alkaloid biosynthesis pathway is dependent on one compound only i.e., L-Tyrosine (Fig. 2).

3.3.2 Inter-site metabolite variance

Multiple PCA using C18 pos and neg matrices revealed some overlap of clusters between sites in both ranges (Fig. 3). For the NZ sites, in C18 pos MU and WH are clearly separated but display a degree of overlap for QU and WU with a combined value for PC 1 and PC 2 of 29% explaining the observed variance between these groups. Separation is less well defined except for site WH in the C18 neg matrix however with a PC combined value of 30.3%. The SC sites generally display more overlap but BE displays the greatest separation in the C18 pos stream with a PC value of 28.7%. Separation of the SC C18 neg are the least well defined with a PC value of 30.9%. Statistical testing using PERMANOVA (999 permutations) however provides verification of the robustness of the cluster formations in all four of these PCA analyses. (PERMANOVA): NZ C18 pos, $F = 28.23$, $p = 0.001$; NZ C18 neg $F = 15.87$, $p = 0.001$; SC C18 pos $F = 17.86$, $p = 0.001$ and SC C18 neg $F = 9.48$, $p = 0.001$.

Of the one-way ANOVA identified features that differ significantly (all with $p < 0.01$) between the four sites in both NZ and SC, thirty-one compounds were annotated. Seventeen of these are additional to those listed in Tables 1 and 2. The previously annotated compounds are marked with and asterisk, see Table 3. Of these additional seventeen, eleven are phenylpropanoids with the remainder comprising five benzenoids and one organooxygen compound.

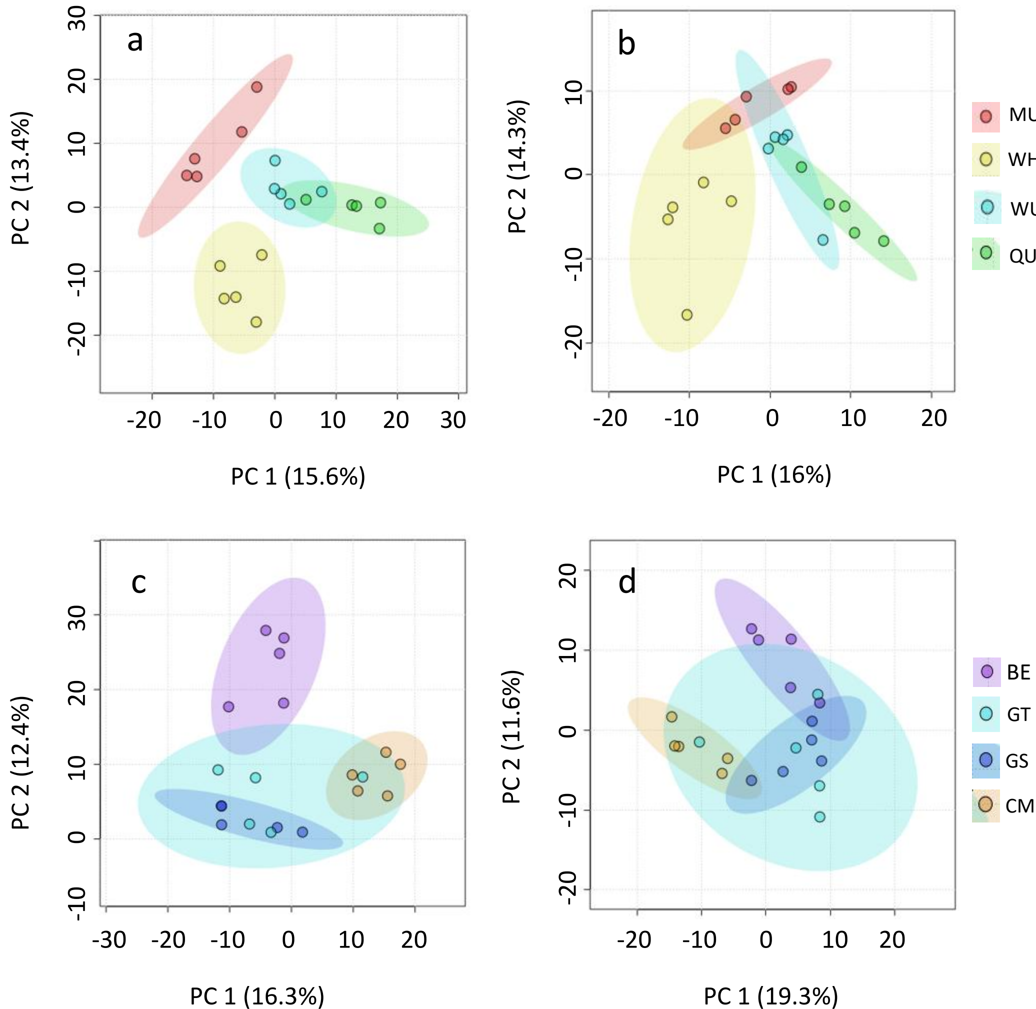

Fig. 3. Multiple PCA's of NZ and SC C18 pos and neg matrices showing clustering of sites in both ranges with 95% confidence ellipses. PC 1 and PC 2 scores for each, explain the observed variance between clusters. For NZ C18 pos (a) MU and WH clearly separate but with a degree of overlap for QU and WU. NZ C18 neg (b), separation is less well defined except for WH. SC C18 pos reveals BE, with the greatest separation but considerable overlap with the remaining three clusters. SC C18 neg separation is the least well defined. PERMANOVA (999 permutations) provides verification of the robustness of the cluster formations. For NZ C18 pos, $F = 28.23$, $p = 0.001$; NZ C18 neg $F = 15.87$, $p = 0.001$; SC C18 pos $F = 17.86$, $p = 0.001$ and SC C18 neg $F = 9.48$, $p = 0.001$. New Zealand CP sites are Mangaturuturu (MU), Waihohonu (WH), Waiouru (WU) and Quarry (QU). Scotland sites are Ballogie Estate (BE), Glenturret (GT), Glensaugh (GS) and Creag Meagaidh (CM).

Table 3. Compounds annotated from the C18 pos and C18 neg streams within the New Zealand (NZ) and Scotland (SC) ranges. Level 2 confidence compound names are in black type. Level 3 confidence names are in grey type and identified to sub class only. CL = confidence level, Compounds in common with table 1a and 1b denoted by * .

Class/Sub class	Name	Range	KEGG	Stream	CL	m/z	rt.	Fragments
Phenylpropanoids								
Flavonoid	Quercitrin	NZ	C01750	C18+	2	449.1084	4.09	287.057
" " "	Isoquercitrin *	NZ	C05623	C18+	2	465.1007	4.86	305.0553
" " "	Epigallocatechin	NZ	C12136	C18-	2	305.0651	4.00	221.0453 219.0654 261.0716 275.0559
Flavonol c.	Quercetin *	NZ	C00389	C18-	3	301.0355	5.91	
Cinnamic acid	trans-Cinnamic acid	NZ	C00423	C18+	2	149.0596	3.94	132.052 132.0593 131.416
Hydroxycinnamic acid	trans-Ferulic acid	NZ	C01494	C18+	2	195.0645	4.35	177.0529
Coumarin	7-methoxy-4-methylcoumarin	NZ	..	C18+	2	191.0705	3.94	119.0822 120.0617 120.0525
" " "	6-Methylcoumarin	NZ	..	C18+	2	161.0598	3.94	162.063 117.0698
Diarylheptanoid	Quercetin 3-Arabinoside *	NZ	..	C18+	2	435.0933	5.08	303.0483 304.0539
Chalcone	Phloretin	NZ	..	C18+	2	275.0906	5.40	107.0494
Benzenoids								
Methoxyphenol	4-methoxyphenol	NZ	..	C18+	2	125.06	3.94	110.063
" " " "	Eugenol	NZ	C10453	C18+	2	165.0908	4.99	135.0803
" " " "	Homovanillic acid	NZ	C05582	C18+	2	183.065	5.02	72.0807
" " " "	4-Hydroxybenzoic acid	NZ	C00156	C18-	2	137.0239	3.16	93.0341
Organooxygen Comp	Pantothenic Acid *	NZ	C00864	C18+	2	220.119	3.52	202.1077
Phenylpropanoids								
Flavonoid	(+)-Epicatechin *	SC	C09728	C18-	2	289.0706	4.18	245.083 205.0519 203.072 109.0299
" " "	Naringenin	SC	..	C18+	2	273.0761	4.20	123.0426
" " "	Kaempferol 3-O-glucoside *	SC	C12249	C18+	2	449.1052	5.10	287.0532 288.0579
" " "	Procyanidin B1	SC	..	C18-	2	577.131	4.57	425.0901 287.0585 125.025
Flavonol b.	Kaempferide *	SC	C10098	C18+	3	301.0709	5.22	
Flavonol c.	Quercetin *	SC	C00389	C18-	3	301.0355	5.91	
Hydroxycinnamic acid	p-Coumaric acid	SC	C00811	C18+	2	165.0546	3.94	127.9857 126.0209 119.0481
Hydroxycinnamic acid a.	2-O-sinapoylmalate *	SC	..	C18+	3	341.0853	4.17	
Cinnamic acid a.	4-Methoxycinnamic acid	SC	..	C18+	3	179.0702	4.18	
Hydroxycoumarin b.	6,7-Dihydroxycoumarin *	SC	..	C18+	3	179.0334	4.16	
Hydroxycoumarin c.	6-Methoxy-7-hydroxycoumarin *	SC	..	C18-	3	191.0349	4.63	
Benzenoids								
Benzenoid	Benzoic acid *	SC	C00180	C18+	3	123.0441	4.19	
" " " "	Gallic acid hexoside	SC	..	C18-	3	331.0671	3.30	
Benzoic acid	Homogentisic acid *	SC	C00544	C18-	3	167.0342	4.63	
Organooxygen Comp	Feruloyl quinic acid	SC	..	C18+	2	369.1191	4.60	177.0528 178.0604
Lactone	Gamma-Decalactone *	SC	..	C18+	2	171.1382	5.05	153.1275 135.1168 154.1309 136.1202

Ten phenylpropanoids for the NZ sites and eleven for the SC sites are graphically displayed in Fig. 4a and 4b respectively and were explored for Pearson correlation coefficient with site mean Olsen P and Total N values.

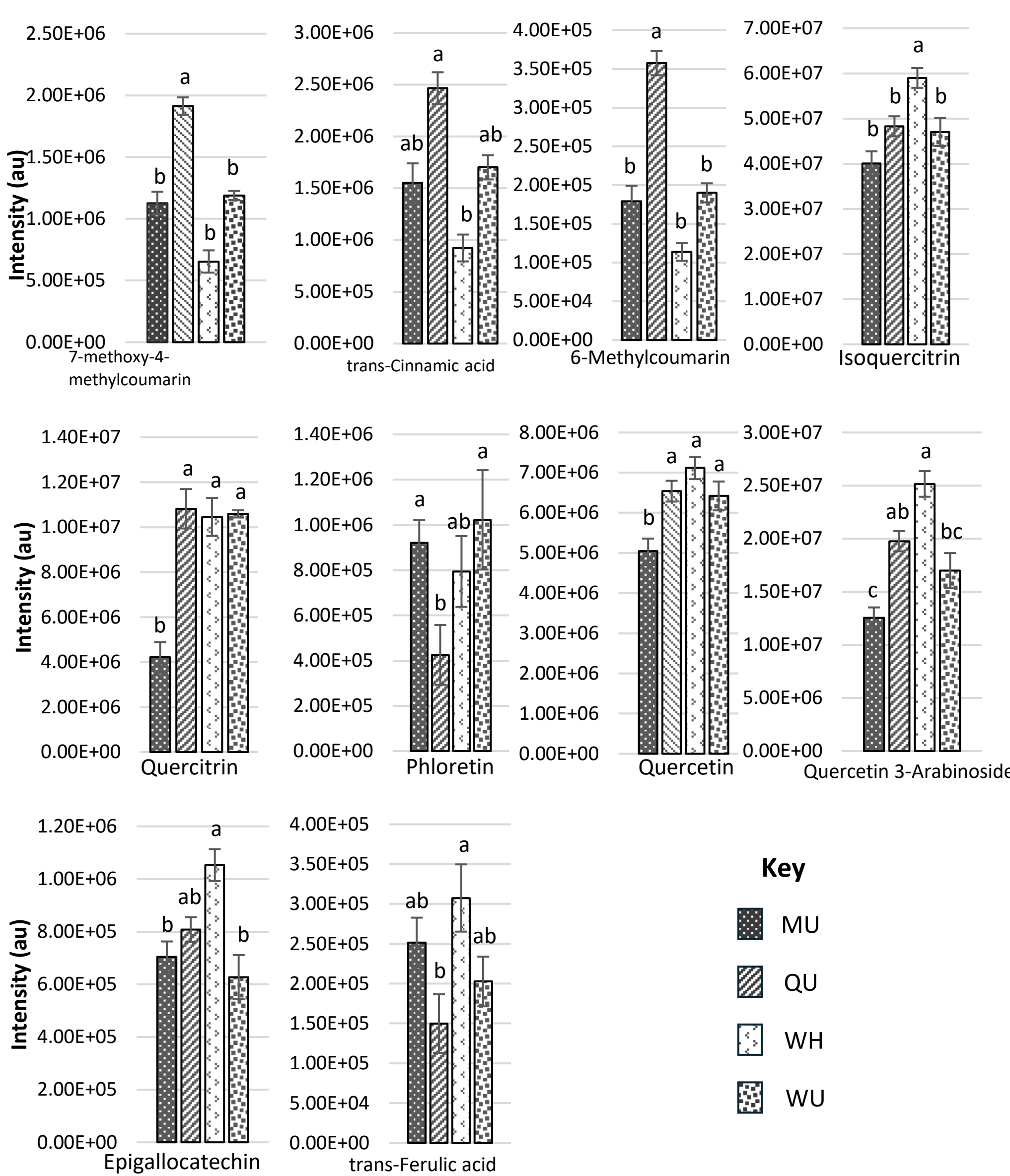

Fig. 4a. One-way ANOVA determined 10 phenylpropanoid compounds that differed significantly between the four New Zealand (CP) sites. Those sites sharing letters are not significant. All are $p < 0.01$. Sites are Mangaturuturu (MU), Quarry (QU), Waihohonu (WH) and Waiouru (WU).

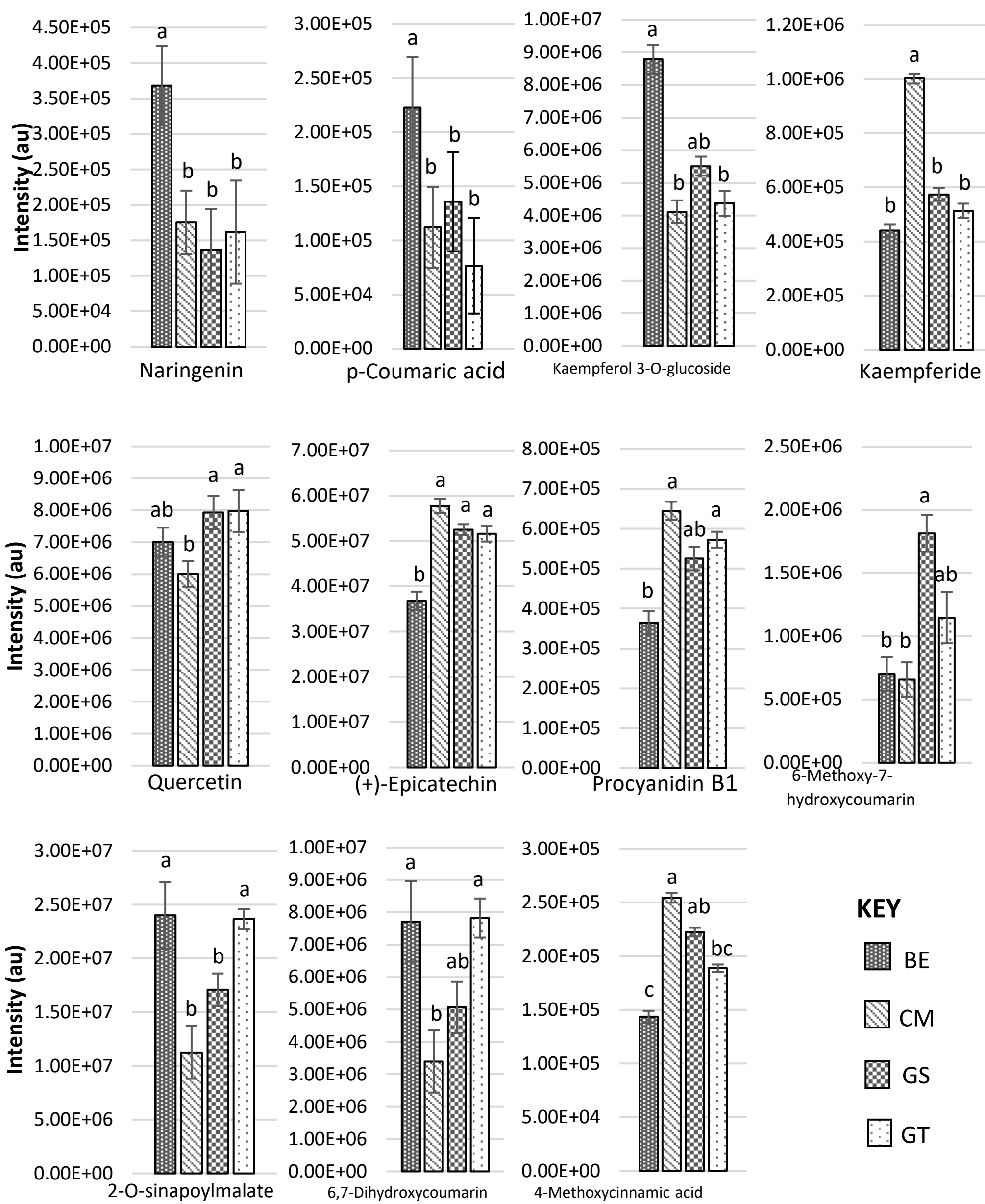

Fig. 4b. One-way ANOVA determined 11 phenylpropanoid compounds that differed significantly between the four United Kingdom (SC) sites. Those sites sharing letters are not significant, all are $p < 0.01$. Sites are Ballogie Estate (BE), Creag Meagaidh (CM), Glensaugh (GS) and Glenturret (GT).

There was little evidence either positive or negative for correlation between most of these phenylpropanoids. A moderate to weak negative but significant correlation does exist for the NZ compounds of trans-Ferulic acid (Olsen P, $r(18) = -0.43$, $p < 0.05$ and Total N, $r(18) = -.48$, $p < 0.05$) and Epigallocatechin (Olsen P, $r(18) = -0.43$, $p < 0.05$ and Total N, $r(18) = -0.58$, $p < 0.01$). In the SC compounds slightly stronger moderate negative correlation with Total N only, exist for 2-O-Sinapoylmalate (Total N, $r(18) = -0.58$, $p < 0.01$), 6,7-Dihydroxycoumarin (Total N, $r(18) = -0.61$, $p < 0.01$) and a positive correlation for 4-Methoxycinnamic acid (Total N, $r(18) = 0.59$, $p < 0.01$).

3.3.3 Invertebrate assessments

Phytophagous invertebrates were absent from the samples collected from the New Zealand CP sites. This was because the heather beetle biocontrol agent was not established at these sites and the only known New Zealand native spp. known to graze on *C. vulgaris* are the manuka beetle (*Pyronota festiva*) (Effah et al., 2022) and an unidentified lepidopteran leaf tying caterpillar. These two native spp. are generally only present in very low numbers earlier in the season. From the SC sites, foliage dwelling invertebrates sorted into taxonomic groups and feeding guild resulted in three major taxa being identified. These were, piercing/sucking Hemiptera, leaf chewing Coleoptera and leaf chewing Lepidoptera (see Supplemental, S. Table 1). Hemiptera were significantly different between sites with one-way ANOVA indicating GT significantly different from CM and BE but not GS, $F(3, 16) = 4.58$, $p = 0.017$. Coleoptera with numbers approximately an order of magnitude higher at site GT differed significantly from CM, BE and GS, $F(3, 16) = 10.72$, $p = 0.001$ and Lepidoptera showed no significant difference between any site $F(3, 16) = 3.04$, $p = 0.115$.

3.3.4 NZ and SC Soils

Soil nutrient analyses confirm soils of the 4 sites in SC to be relatively acidic and with very low levels of Olsen P and Total N. The volcanic soils of the CP in NZ however, while less acidic are even poorer in nutrient status with both Olsen P and Total N at extremely low levels, indeed Olsen P is at the limit of detection for this analytical method (Taylor et al. 2018). The paired t-test values between NZ and SC are highly significant for Olsen P $t(1, 19)$ 5.45, $p < 0.0001$; Total N $t(1, 19)$ 4.73, $p < 0.0001$, and pH $t(1, 19)$ -18.4, $p < 0.0001$ respectively. One way ANOVA for Olsen P levels at the NZ sites show no significant difference $F(3, 16) = 2.06$, $p = 0.1446$ while for Total N $F(3, 16) = 6.39$, $p = 0.0046$ and pH $F(3, 16) = 4.70$, $p = 0.015$, do show a significant difference between these means. For the SC sites Olsen P $F(3, 16) = 6.55$, $p = 0.0042$, Total N $F(3, 16) = 12.97$, $p < 0.0001$ and pH $F(3, 16) = 16.01$, $p < 0.0001$, all show a significant difference between the means, Table 4.

Table 4. Soil nutrient analysis values by site for Olsen P, Total N and pH with all Tukey post hoc levels of significance at $p < 0.05$. Those sites sharing letters are not significant. The New Zealand (NZ) and Scotland (SC) range values differ significantly by paired t-test and are indicated by * with $p < 0.0001$. Summer noontime average UV index for each country is also provided. The NZ Central Plateau UVI values are adjusted for 1000 masl). NZ sites are, Mangaturuturu (MU), Quarry (QU), Waihohonu (WH) and Waiouru (WU) and for SC are, Ballogie Estate (BE), Creag Meagaidh (CM), Glensaugh (GS) and Glenturret (GT).

Site	**Olsen P** (mg kg^{-1})	**Tot N** (%/w)	**pH**	**UVI**
MU	1.74	0.22 ^{b}	5.94 ^{ab}	
QU	2.17	0.27 ^{ab}	6.06 ^{a}	
WH	1.82	0.19 ^{b}	5.74 ^{b}	
WU	2.63	0.35 ^{a}	5.86 ^{ab}	
$\bar{x}$ NZ	**2.09**	**0.26**	**5.9**	**12-13**
BE	9.36 ^{b}	0.57 ^{b}	3.94 ^{b}	
CM	13.2 ^{ab}	1.31 ^{a}	4.31 ^{b}	
GS	25.82 ^{a}	1.64 ^{a}	4.03 ^{b}	
GT	7.41 ^{b}	0.3 ^{b}	4.82 ^{a}	
$\bar{x}$ SC	**13.95 ***	**0.96 ***	**4.28 ***	**6-7**

3.4 Discussion

Our results show significant differences in the biochemical profile of *Calluna vulgaris* plants growing in its native range in the UK compared to its conspecifics in NZ. A notable feature of this difference is the greater number of phenylpropanoids and amino acid metabolites that are amplified in the NZ range (Tables 1 and 2). According to the literature, both soil nutrients and ultraviolet light influence and upregulate the same major shikimate-phenylpropanoid pathway (Bassman 2004; Kumar et al. 2023) and the subsequently dependent flavonoid and flavone/flavonol biosynthesis pathways. We provide good evidence for upregulation of all these pathways, and the resultant amplification of many primary and secondary metabolites which suggest that *C. vulgaris* is responding to differing environmental factors in these two ranges.

New Zealand CP sites have poorer soil nutrient status compared to the SC sites. Literature demonstrating depleted or low soil nitrogen and phosphate availability, increasing the level of phenylpropanoid chemical defences in plants, is provided by several studies (Yang et al. 2018; Gershenzon 1984; Wright et al. 2010; Sampedro et al. 2011; Kováčik and Klejdus 2014). Nitrogen deficiency can induce the accumulation of foliar phenylpropanoid compounds by increasing the activity of phenylalanine ammonia-lyase (PAL), a key enzyme of the shikimic acid – phenylpropanoid pathway (Gershenzon 1984; Sampedro et al. 2011), resulting in increased levels of total phenolics (Kováčik and Klejdus 2014; Borges et al. 2017) as well as coumarins, anthocyanins, flavonoid glycosides, flavones, iso-flavones, and tannins (Gershenzon 1984; Kováčik and Klejdus 2014). Similarly, low levels of phosphate can increase foliar levels of caffeoylquinic acids, coumarins, anthocyanins and flavonoid glycosides (Gershenzon 1984; Sampedro et al. 2011). Many of these compounds appear to be amplified in our NZ samples, supporting the existing evidence of low soil nutrients enhancing chemical defences.

In contrast to soils, NZ has considerably higher ambient ultra-violet intensity levels than in the UK (Liley and McKenzie 2006; Seckmeyer et al. 2008). Changes in the light environment, particularly increased levels of UV-B, induce signal transduction pathways to regulate plant physiological activity (Yang et al. 2018; Roberts and Paul 2006) often resulting in soluble phenolic compounds, particularly phenolic acids, flavonoids, flavonol glycosides of kaempferol, quercetin and myricetin as well as hydroxy cinnamic esters to accumulate in plants, many of which play an important role in photoprotection and defence (Kotilainen et al. 2009; Kuhlmann and Müller 2010).

Considerable literature exists demonstrating that such UV-radiation induced phenylpropanoids can not only increase plant resistance to insects and impair insect herbivore performance (Mazid et al. 2011; Ballaré et al. 2012; Izaguirre et al. 2006) but also increase plant resistance to biotrophic pathogens (Fu and Dong 2013; Ballaré 2014). With reference to insect herbivores, several flavonoids (Singh et al. 2021) as well as chlorogenic acid (Leiss et al. 2009), several catechins (Li et al. 2022), caffeic and o-coumaric acid (Patton et al. 1997) have all been demonstrated to impair growth and survival rates, fertility, fecundity and population growth rates in a range of insect families and we found amplified levels of many of these compounds at our NZ sites.

Of the primary metabolites, the non-protein amino acid γ-Aminobutyric acid (GABA) is also upregulated in our samples. This compound accumulates under a range of abiotic stresses including soil nutrients and light and is known to function directly in plant immune responses to biotrophic pathogens and fungi as well as being a powerful neuromuscular and growth inhibitor against insect herbivores often causing ill thrift and death (Ramos-Ruiz et al. 2019; Tarkowski et al. 2020). It’s therefore conceivable , that elevated levels of some of the metabolites we have recorded in *C. vulgaris* in this study could render the assimilability of the host plant more challenging to *L. suturalis* in its new

environment. These amplified metabolites may contribute to a nutritional cascade, exacerbating the effects of low foliar N levels reported in another recent paper (Peterson et al. 2024) and may help explain the difficulties with the initial establishment of this control agent released to control *C. vulgaris*. in NZ.

When investigating differences between sites within the same range, we assume no variation in UV intensity exists and we know there is no influence of shade or aspect at either one, given all sites are in open shrubland communities. Therefore, we focused our analysis on response variables potentially linked to soil nutrients. While PCA revealed some overlap between sites (clusters) within each of the ranges, the one-way ANOVA revealed significant differences in phenylpropanoid compounds between some of those sites. For the CP sites we encountered significant differences in the intensities of ten phenylpropanoids with only trans-Ferulic acid and epigallocatechin however, having a negative correlation with both phosphate (Olsen P) and nitrogen (Total N) levels in those soils. Overall, there remains only weak evidence that soil nutrients in this region are influencing the intensities of the phenylpropanoids that we were able to positively annotate.

The SC sites revealed eleven phenylpropanoid compounds showing significant intensity differences between sites but similarly provide little evidence (notwithstanding the results for 2-O-Sinapoylmalate, 6,7-Dihydroxycoumarin and 4-Methoxycinnamic acid) from our Pearson correlation coefficients, that soil phosphate and nitrogen levels are greatly influencing phenylpropanoid intensities. We cannot, therefore, claim a causal effect as other factors and their interaction may also contribute to the observed metabolite changes. Regarding the variable effectiveness of *L. suturalis* at some of the New Zealand sites then, it remains uncertain if varying intensity of any phenylpropanoids could be involved. We suggest this question could be addressed with controlled dietary

experiments using some of the compounds that were amplified in the NZ range, that are known to impair insect performance.

While plant genetic variability is understood to influence the overall metabolite profile of plants, its effect on metabolite intensity appears equivocal. Literature indicates that for plants of the same species that vary genetically, metabolite intensity is influenced much more by abiotic parameters than genetic variability (Robinson et al. 2007; Frank et al. 2012; Neugart et al. 2018). We acknowledge that genetics may be a contributor to the observed metabolite differences and are currently addressing the degree of genetic variability of *C. vulgaris* between ranges and within sites but posit that differences in soil nutrients and UV-radiation are likely the strongest drivers for these results.

Support for both these abiotic influences altering the biochemical profile of *C. vulgaris* in NZ, is provided by recent field trials and a tunnel house experiment using UV attenuating screens (Effah et al. 2020a). From four sites on the CP region of the North Island, significant differences in the volatile organic compound (VOC) emissions of *C. vulgaris* were recorded between sites. Of the environmental variables collected i.e., soil nutrients, ambient daytime temperature, soil water content and soil temperature, the main contributing factor to these differences was soil nutrients (Effah et al. 2020b). Tunnel house manipulations of ultraviolet light using 20% and 95% attenuating screens and exposing mature and phenologically similar *C. vulgaris* plants for 75 days to this treatment, revealed significant differences between several VOC metabolites, demonstrating that this plant is also sensitive to differing levels of ultraviolet radiation. Which abiotic parameter may be driving the variances revealed in this study however requires controlled experiments manipulating nutrient availability and ultraviolet light both independently and combined, to provide quantitative data to confirm any direct effect of these parameters and further elucidate these results.

Average ambient temperatures vary little between our NZ and SC ranges and soil moisture contents (while we did not measure these) at the time of sampling were within normal summertime ranges, thus we expect little influence on metabolite profiles and intensities from those sources. Induced plant responses to differing insect herbivore communities, however, needed to be considered. The significantly high number of Hemiptera and Coleoptera at GT was the most likely to produce a positive correlation with high intensities of a given phenylpropanoid at that same site, but none were observed. Additionally, the PCA clusters for GS and CM show considerable overlap with GT, again suggesting that GT is not separating out as a result of any induced response to invertebrate herbivory. There was little evidence then of herbivory influencing the intensity measurements of secondary metabolites which may skew the NZ vs SC range or inter-site soil nutrient PCA results. Plant ontogeny can influence secondary metabolites which has been reported in a recent paper for VOC compounds in *C. vulgaris* (Effah et al. 2024), however all plants sampled at all sites in this study were mature and well developed, thus we expect any influence from that source to be negligible. For more in-depth understanding of constitutive metabolites of a successionally dynamic heather community displaying variable plant ontogeny, we also recommend further testing.

While we cannot ascribe direct causality from environmental factors, these results illuminate the potential use of metabolomic techniques for biological control of weeds. Once a potential agent has been selected, metabolomic assays may help determine sites with the closest matching target plant metabolomes, to potentially, both source and release the biocontrol agent. Such information may assist in reducing the chances of encountering difficult establishment or poor effectiveness scenarios. Additionally, it would be of interest to retrospectively compare plant metabolomes from both the source and release sites of already introduced control agents for both successful and unsuccessful programmes to explore to what extent the plant metabolome could be used as a predictor of

biocontrol agent success. Further ideas and potential applications have been extensively discussed in a recent review (Barrett et al. 2021).

3.5 Conclusions

Our results clearly demonstrate significant differences between the metabolomes of *C. vulgaris* plants occurring in its native range in Scotland (UK) and those in its invaded range of the Central Plateau of the North Island of New Zealand and suggest that UV-radiation and soil nutrients could be driving the observed differences. However, to assess direct causality further experiments under controlled conditions are necessary.

It is also evident for sites within each of the ranges we tested, significant variation occurs for many phenylpropanoids. Bioassays exploring biocontrol agent behaviour and preference and performance are required to understand if both inter and intra site variance in this and potentially other classes of compounds could be a driver of poor agent establishment rates or variable effectiveness.

Our non-targeted metabolomics approach to these investigations revealed not just secondary defensive metabolites, but the majority of the primary amino acid metabolites identified (in addition to those key precursors of the shikimate-phenylpropanoid pathway i.e., phenylalanine and tyrosine) are also amplified suggesting overall increased biosynthetic pathway upregulation of this plant in the New Zealand CP environment. Our results therefore add to the very limited literature documenting biochemical phenotypic change to invasive plants that have established in a new, abiotically different region.

We conclude, this study has provided a primer, that abiotically induced biochemical change may not be uncommon in invasive plants and validates our original question of the potential for biochemically modified phenotypes altering

plant defensive capacity and/or nutritional assimilability which may compromise specialist control agents that are reunited with their coevolutional host plant. The application of metabolomics therefore, may be a valuable tool to assist with determining such changes and help elucidate poor establishment and/or effectiveness scenarios in weed biocontrol.

Data Availability: All relevant .raw spectral data sets are available upon reasonable request.

Author contributions: DPB. Conceived the primary questions and objectives of the investigations. Carried out all sampling, processing of samples, annotation and statistical analysis. Secured funding. Primary author and writer of the manuscript. AKS. Advised on appropriate chromatographic analysis, supervised and ran all UHPLC-MS technical processes, advised regarding metabolomic analysis techniques and revised the manuscript. RJP. Advised and assisted with site selection and sample collection carried out in Scotland UK. Facilitated hosting and access to laboratory facilities at the James Hutton Institute, Aberdeen and revised the manuscript. PP. Has provided 2 decades worth of background research information on the Heather biocontrol program. Now recently has provided expertise on foliar N impacts on heather beetle control agent performance and other invertebrates associated with this program and revised the manuscript. ACM. Principal investigator and project supervisor. Advised on concept, design and interpretation of investigations, supported fieldwork sampling, secured funding and revised the manuscript.

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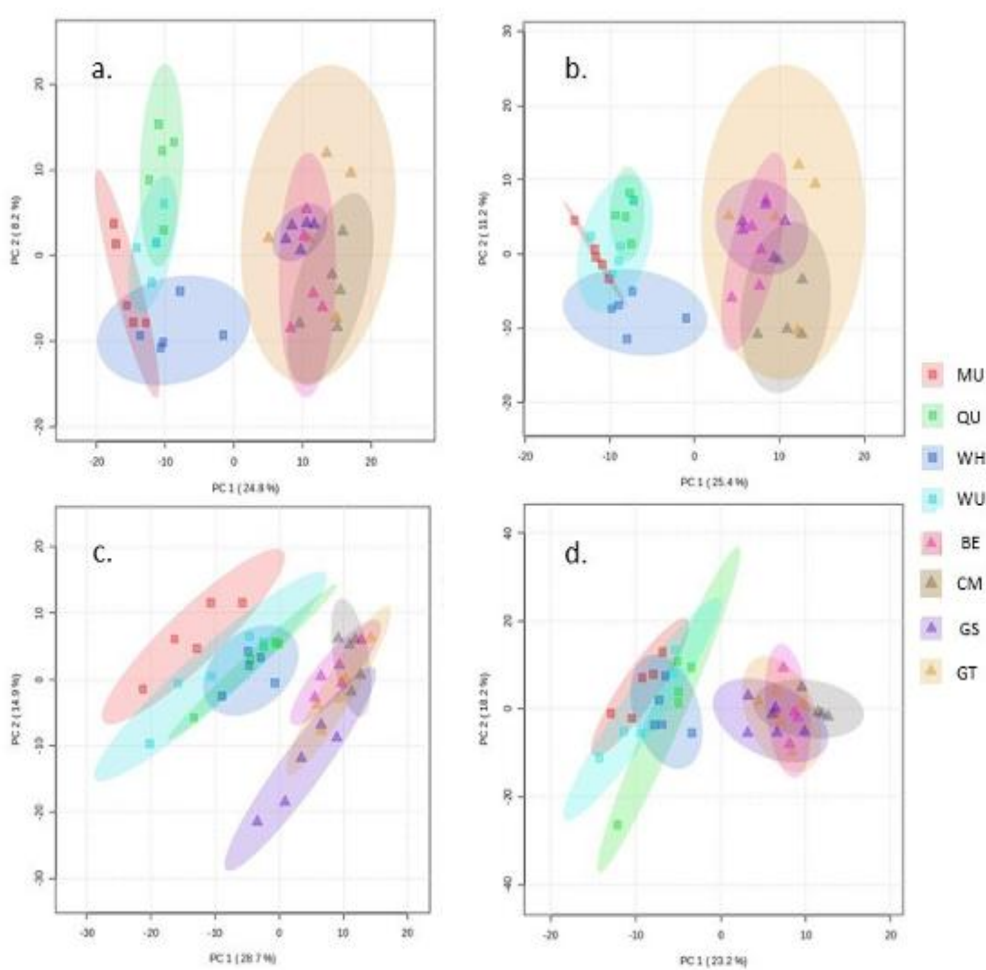
3.8 Supplemental Information

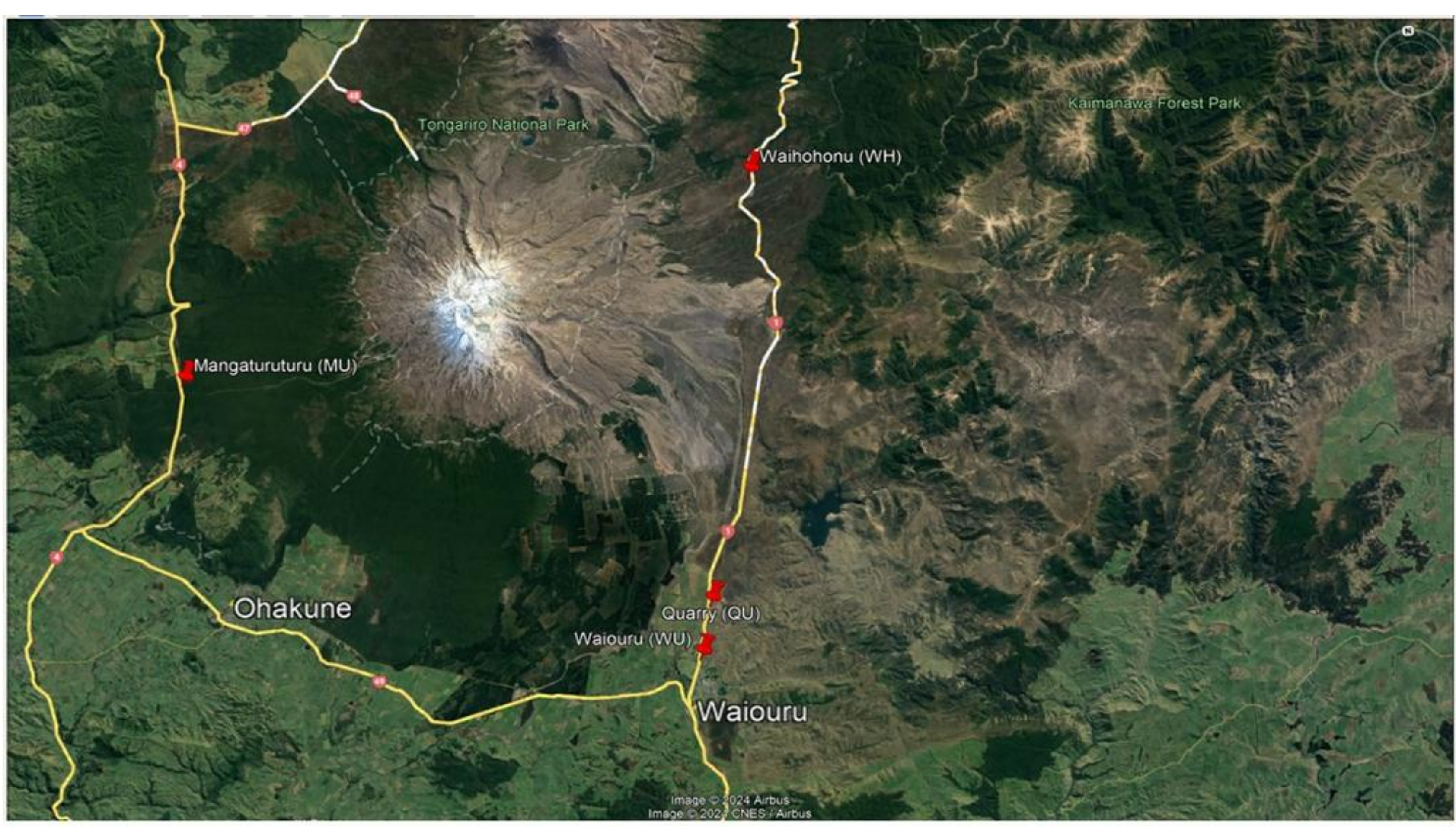


S. Fig. 1 Multiple PCA graphics for all NZ and SC sites in a. C18pos, b. C18neg, c. HILICpos and d. HILICneg. All demonstrate clearly separate clustering of the NZ and SC ranges. PC1 (x axis) and PC2 (y axis) demonstrate the observed variance between clusters. All analyses are validated using [PERMANOVA] p = 0.001. Square points represent New Zealand sites and triangles represent Scotland sites.

S. Table 1. Phytophagous invertebrate numbers recorded from the four SC sites. Hemiptera show significant differences between sites with GT except for GS and at GT, Coleoptera are significantly different from all other sites. Numbers sharing letters are not significantly different. Note: Phytophagous invertebrates were absent from the samples collected from the New Zealand CP sites.

Site	Hemiptera	Coleoptera	Lepidoptera
BE	10.0 ^{B}	2.2 ^{B}	1.3 ^{A}
CM	6.0 ^{B}	2.3 ^{B}	1.0 ^{A}
GS	16.2 ^{AB}	1.3 ^{B}	1.0 ^{A}
GT	47.8 ^{A}	23.2 ^{A}	4.5 ^{A}

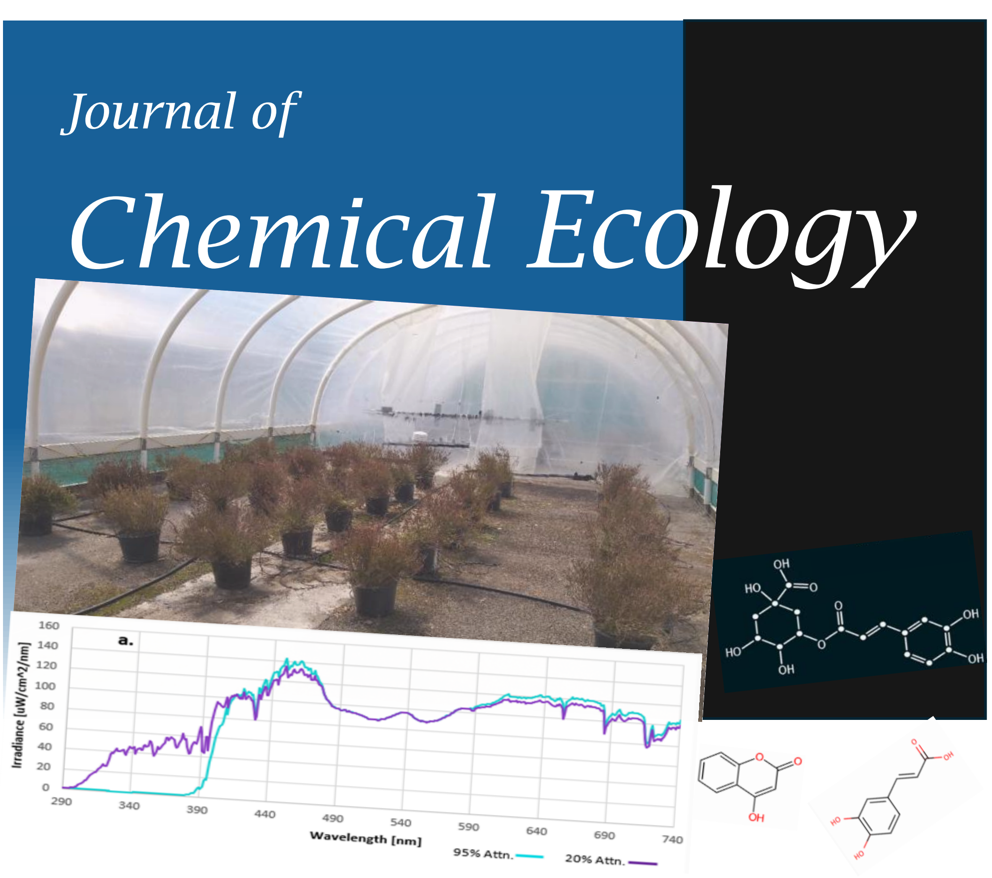
S. Fig. 2a Study sites located in Scotland UK. Altitudes, GS = 323, BE = 316, GT 351 and CM = 291 masl.



S. Fig. 2b Study sites located at New Zealand's Nth. Is. CP. Altitudes WU = 814, QU = 881, WH = 975, and MU = 817 masl.

Chapter 4

Ultra-violet induced biochemical changes in an invasive weed and their implications for plant-biocontrol agent interactions.



This chapter is written in the style suitable for and is in press in the *Journal of Chemical Ecology* but with minor structural and formatting modifications. It is *reproduced here with permission from Springer Nature* and is published as:

D. Paul Barrett, Arvind K. Subbaraj, Jason J. Wargent, Maria A. Minor, Paul Peterson, David J. Lun and Andrea Clavijo McCormick. Ultra-violet induced biochemical changes in an invasive weed and their implications for plant-biocontrol agent interactions.

Abstract—Introducing insect biocontrol agents sourced from a plant's native range is an effective, sustainable management strategy for invasive plants. However, not all biocontrol programmes achieve the desired outcome because control agents either fail to establish or are ineffective. Heather beetle *Lochmaea suturalis* (Coleoptera: Chrysomelidae), introduced from the United Kingdom (UK) to New Zealand (NZ) to control the invasive shrub *Calluna vulgaris* (heather), was difficult to establish and achieved poor population growth rates and expansion relative to its conspecifics in its native UK range. Poor performance in biocontrol is often attributed to various abiotic or biotic factors but seldom considers alterations to a target plants biochemical phenotype. A recent study revealed, heather has a significantly different biochemical profile in NZ compared with the UK, between which there is considerable difference in ultra-violet (UV) radiation. UV is known to drive plant biochemical change, including defensive secondary metabolites and we hypothesized that this factor could enhance heathers' defensive capability leading to poor biocontrol agent performance. Testing this hypothesis involved exposing heather plants to 20% and 95% UV attenuating screens and using metabolomics to measure plant secondary metabolite responses. Our results demonstrate significant alterations to many compounds derived from the shikimate-phenylpropanoid pathway. However, a bioassay revealed no impact on prepupal weight or larval survival of the biocontrol agent *L. suturalis*. We discuss and explore possible reasons for this outcome, the magnitude and impact of UV-induced biochemical changes on plant-insect interactions and the potential of metabolomics to support weed biocontrol.

Key Words—Biocontrol, metabolomics, ultra-violet, biochemical phenotype, invasive plants.

4.1 INTRODUCTION

Invasive plants can seriously threaten terrestrial habitats that they invade, with potential to cause considerable production and economic losses in managed ecosystems such as agriculture, horticulture or forestry. Similarly, ecological damage to environmentally sensitive ecosystems such as rangelands and conservation areas can impact essential ecosystem services (Rai et al. 2022) and cumulatively result in considerable losses to biodiversity at the species, community or ecosystem level (Roy et al. 2023).

For many of these ecosystems, the introduction of weed biocontrol agents sourced from the plant's native range (classical biocontrol) offers a sustainable long-term alternative for managing invasive plants (Hayes et al. 2013; Schwarzländer et al. 2018). However, not all biocontrol programmes achieve the desired outcome due to agents failing to establish (Hayes et al. 2013) or being ineffective if they do establish (McClay and Balciunas 2005; Raghu and Dhileepan 2005). Biocontrol agent population establishment is known to be influenced by abiotic (Grevstad 1999; Harms et al. 2020) and in some cases biotic factors (Syrett 1983; Briese 1986; Schulz et al. 2019). Literature exploring abiotically induced changes to target plant biochemical phenotypes which might impose limitations on biocontrol agent establishment or effectiveness is scarce (but see Falla et al. 2023; Barrett et al. 2024). Wheeler and Schaffner (2013) also review the importance of understanding the interplay between target plant phytochemistry and biocontrol agent responses, performance and safety. Despite this, only a small number of publications exist investigating target plant constitutive phytochemistry in host range testing and agent performance (Vrieling and de Boer 1999; Wheeler 2005; Wheeler et al. 2007, 2014; Rapo et al. 2019).

Plants perceive light and use it as essential environmental cues. Photosynthetically active radiation (PAR), defined as the 400 – 700 nm waveband

is essential for plant growth. They also detect a range of other quantitative (intensity per unit area) wavelengths which vary with time of day, season and/or latitude. These include the blue (450-495 nm), red (645-665 nm) and far-red (720-740 nm) wavebands. Qualitative wavelengths, that is, the balance of PAR, ultra-violet A (321-400nm) and ultra-violet B (291-320nm) similarly, act as important cues (Roberts and Paul 2006; Caldwell et al. 2007; Ballaré et al. 2012). Visible light, including red and blue wavelengths are sensed by plant photoreceptors e.g., phytochromes and cryptochromes (Wang et al. 2018), while ultra-violet B (UV-B) is sensed by the UV RESISTANCE LOCUS8 (UVR8) protein (Jenkins 2014). These elicit plant responses that modify growth, biochemical composition and morphology and regulate plant primary and secondary metabolism (Roberts and Paul 2006; Yang et al. 2018). Primary metabolites are linked to basic survival functions such as photosynthesis, growth or cell division, whereas secondary metabolites function in regulating primary metabolites and growth, but also importantly, they mediate ecological interactions with other organisms and the environment (Erb and Kliebenstein 2020).

Ultra-violet (UV) radiation influences the shikimic acid - phenylpropanoid metabolic pathway (Bassman 2004; Singh et al. 2021, 2023), resulting in changes to many phenylpropanoid derived metabolites such as flavonoids, flavonol glycosides and hydroxycinnamic acids (HCAs) (Kuhlmann and Müller 2010; Barnes et al. 2017). These compounds play an important role in photoprotection of plants, i.e. protecting against cellular DNA damage (Gill et al. 2015) while maintaining optimum photosynthetic capacity of the mesophyll (Wargent et al. 2015; Barnes et al. 2017). Additionally, UV-radiation can also affect other phenylpropanoid groups including phenolic acids, coumarins, chlorogenic acids (CGAs), quinic acids, stilbenes, terpenoids and tannins, many of which are involved in plant defence against insect herbivores (Bassman 2004; Zhang and Bjorn 2009; Escobar-Bravo et al. 2017; Kumar et al. 2020). This has been amply demonstrated in the literature where exposure of plants to high UV results in

impaired insect herbivore performance (Izaguirre et al. 2007; Kuhlmann and Müller 2010; Qi et al. 2018), or when exposure to reduced UV improves herbivore performance or increases feeding rates (Rousseaux et al. 2001; Ballaré et al. 2012; Dinh et al. 2013; Ballaré 2014).

Heather, *Calluna vulgaris* (L.) Hull, is a highly invasive shrub, well established on the North Islands Central Plateau (CP) of New Zealand (NZ). Previous work has demonstrated the plasticity of secondary metabolites in *C. vulgaris* at different altitudes with reference to varying light and soils (Monschein et al. 2010), as well as seasonality (Jalal et al. 1982) in Europe, and in response to multiple abiotic factors in the CP plants (Effah et al. 2020a, b). We also have good evidence that *C. vulgaris* foliage in NZ has significantly elevated levels of both primary and secondary metabolites when compared to levels found in *C. vulgaris* from Scotland in the United Kingdom (UK) (Barrett et al. 2024). In the sub-alpine CP environment, peak summertime UV index (UVI) measures as high as 14.0 whereas the same index throughout the UK is ~ 7.0 (Liley and McKenzie 2006). Thus, *C. vulgaris* is exposed to considerably higher levels of UV on the CP which may elevate the level of phenylpropanoid metabolites in these plants relative to those of the UK.

In 1996, heather beetle, *Lochmaea suturalis* (Thomson, 1866) (Coleoptera: Chrysomelidae), sourced from the UK was introduced into NZ as a biocontrol agent for *C. vulgaris* in the CP region. The beetle was difficult to establish and achieved poor population growth rates and expansion relative to those recorded in its UK range (Pakeman et al. 2002; Peterson et al. 2007, 2024; Rosenburgh and Marrs 2010; Fowler et al. 2015). Now however, two and a half decades after the first releases, beetle populations are well established and causing widespread damage to *C. vulgaris*. We posit that reduced assimilability due to altered phytochemistry in *C. vulgaris* in the CP region would conceivably impose strong selective pressure on *L. suturalis* and induce rapid eco-evolutionary adaptation

in this control agent (McEvoy et al. 2012; Szűcs et al. 2012, 2019) and result in greater population densities.

Thus, to help explain the poor early performance of this control agent, we sought to explore if elevated UV-radiation influences the secondary metabolome of *C. vulgaris.* Conversely, we might expect CP plants exposed to reduced levels of ultra-violet (with the application of UV attenuation screen technology) to display reduced levels of secondary phenylpropanoids and we hypothesize that artificially reducing exposure to UV-radiation will lead to a reduction in these metabolites in *C. vulgaris*. To test this hypothesis, we exposed potted *C. vulgaris* plants to 20% ['UV-transparent'] and 95% ['UV-opaque'] attenuating polyethylene screens over two growing seasons. Using ultra high performance liquid chromatography – mass spectrometry (UHPLC-MS) and non-targeted metabolomic techniques we measured the magnitude of change to the plant metabolome for each UV treatment. To determine any effect of altered metabolite levels on insect herbivore performance, foliage from plants in each treatment was used in a bioassay to test if larval survival and prepupal live weights of *L. suturalis* were affected.

4.2 MATERIALS AND METHODS

4.2.1 Plants and Experimental Conditions. In November 2020, heather plants, entirely free of *L. suturalis*, were collected from a field population at Waiouru on the North Islands Central Plateau (Lat. -39.455413, Lon. 175.677705) and planted into large (30 cm dia.) pots while keeping all roots and associated soil intact. On 7th Nov 2020, eighteen plants were placed into each of two tunnel house treatments at the plant growth unit of Massey University, Palmerston North, with sufficient spacing to avoid shading from neighbouring plants. The treatments exposed plants to two different intensities of UV using UV-modifying

polyethylene screens i.e. 95% attenuation (attn.) of UV ['UV-opaque' – Lumisol 018] and 20% attn. of UV ['UV-transparent' – Lumisol 019] (BPI Visqueen™, UK). On 15th Jan 2021, photosynthetically active radiation (PAR) and total UV irradiance for each screen were confirmed using a scanning UV-vis monochromatic spectroradiometer. During cloudless sunny conditions, 3 scans were recorded under each screen.

Average total UV irradiance (290-320 (UV-B) and 321-400 (UV-A) wavelengths combined) at 20% attn. and 95% attn. was 4178.8 and 336.7 $uW \cdot cm^{-2} \cdot nm^{-1}$, respectively. Therefore, total UV transmittance by the 95% attn. screen was considerably less (≤ 8.1% of total UV) than that of the 20% attn. screen. Average photosynthetically active radiation (PAR) (the 401-750 wavelengths), at 20% and 95% attn. was 783.6 and 802.7 $uW \cdot cm^{-2} \cdot nm^{-1}$ respectively, thus each were within 2.5% of PAR transmittance (see Supplemental Fig. 1a). During April (Southern hemisphere autumn) ambient UV significantly declines, thus on 1st April 2021, all plants were placed outside into ambient light, where UV levels continued to decrease during winter to ~ 1.0 - 2.0 $uW \cdot cm^{-2} \cdot nm^{-1}$ (McKenzie and Liley 2010) and then increase again in spring. All plants were placed back into their respective treatments on 1st Oct 2021. Thus, plants were exposed to two summer seasons of these UV attenuation treatments.

Temperature was logged continuously (Tinytag™ Plus 2, TGP-4020; Gemini Data Loggers (UK) Ltd.) for each of the attenuation and ambient outside treatments. Overall mean temperatures varied seasonally with the highest weekly mean of 25.45 °C which included the highest daily peak (42.2 °C) in the second week of January and the lowest mean of 6.6 °C during the last week of June 2021. The lowest daily peak of -1.8 °C occurred during the second week of July 2021 (see Supplemental Fig. 1b). All temperature data were subjected to a paired t-test (Minitab v 21.1.0.) to assess potential daytime temperature differences between

treatments. Temperature variance between the 20% and 95% attn. treatments was checked by assessing the recordings obtained between 8:00 am and 4:00 pm on the 10th, 20th (summer solstice) and 30th Dec 2020. A minor difference of ~ 1.17 °C was evident during the afternoon peak, but mean values throughout the three periods for 20% attn. (20.3 °C) and 95% attn. (20.5 °C) revealed no significant difference ($t(292) = -0.399$, $p = 0.69$) (see Supplemental Fig.2).

Humidity in each treatment was logged continuously using a Tinytag ™ Plus 2, TGP-4500 (Gemini Data Loggers (UK) Ltd.) logger. Data were analysed using Tinytag™ Explorer software and MS Excel. Humidity throughout the experimental period averaged 70.75%. Water was supplied individually to each plant every second day, using drip feed irrigation at the rate of ~ 1 L day^{-1} i.e., four times 250 ml $pot^{-1} day^{-1}$.

4.2.2 Metabolomic Analysis. Foliage samples for metabolomic analysis were collected on 14th Dec 2021, from the 18 plants in each treatment. Samples consisted of ~ 30 fresh current seasons sprigs approximately 2 cm in length, which were cut and immediately frozen on dry ice before being transferred to -80 °C for storage. All samples were freeze dried then stored for 2 weeks at -20 °C prior to grinding to 150 – 50 µm particle size before extraction. For extraction, 50 ± 0.5 mg of ground sample were weighed into 2 mL microcentrifuge tubes, each containing a 2.5mm glass bead. To each tube, 800 µL of pre-chilled chloroform:methanol ($CHCl_3$:CH_3OH; 1:1 v/v) was added and then homogenised for 5 mins at 25 Hz sec^{-1} using a Retsch MM400 Mill/TissueLyser and stored for 1 hr at -20 °C. Then, 400ml of HPLC grade H_2O was added to each tube, similarly homogenised, then centrifuged (Sigma 1-14K microcentrifuge) at 4 °C and 11000 RPM for 15 mins to create a biphasic layer. From each sample, 2 x 200 µl aliquots of the upper layer were pipetted into 2 mL microcentrifuge tubes for C18 and HILIC chromatography analysis. A final 200 µl aliquot was added to a 150 ml

tube, ultimately pooling every sample into a homogenous mix, then sub-aliquots (200 µl) of this mix were transferred into microcentrifuge tubes to use as quality control checks (QCs). All samples were then dried under a continuous flow of N_2 (3.5 L min^{-1}) at 38 °C for 60 min using a BT LabSystems sample concentrator, then immediately stored at -80 °C until reconstitution.

Reconstitution solvents which included an internal standard (cat no. MSK-QC-KIT) (Cambridge Isotope Laboratories Inc.) at the concentration of 10 µl ml^{-1} were prepared for C18 analysis in acetonitrile: water (CH_3CN:H_2O; 1:9, v/v) and for HILIC in acetonitrile:water (CH_3CN:H_2O; 1:1, v/v). Immediately prior to HPLC-MS analysis, all samples plus seven QCs were reconstituted by adding 200 µl of solvent, vortexed until dissolved and transferred by pipette to a 200 µl glass insert in an amber autosampler vial, capped and kept chilled until loading. The sequence was: five vials of reconstitution solvent only (blanks), one amino acid standard (A9906; Sigma-Aldrich, NZ), two QCs, 36 samples with a QC every 9th slot to finish with a final QC and an amino acid.

Chromatography and tandem mass-spectrometry analysis of polar and semi-polar compounds were achieved using a Thermo LC–MS system (Thermo Fisher Scientific, Waltham, MA, USA) which consisted of a Dionex UltiMate 3000 UHPLC system coupled with a high-resolution Q Exactive Focus Quadrupole-Orbitrap mass spectrometer utilising heated electrospray ionisation run in both positive and negative modes. For semi-polar compounds, samples were cooled in the auto-sampler at 4 ˚C and a 5 µL aliquot was injected into a 1.9 µm Thermo Hypersil GOLD™ C18 column (UHPLC, 100 mm × 2.1 mm, Thermo Fisher Scientific, USA) at 25 ˚C with a gradient elution programme and a flow rate of 350 µL min^{-1}. For polar compounds, samples were cooled in the auto-sampler at 4 ˚C and a 5 µL aliquot was injected into a 5 µm ZIC®-pHILIC column (100 mm × 2.1 mm, Merck Darmstadt, Germany) at 25 ˚C with a gradient elution programme and

a flow rate of 150 μL min^{-1}. All chromatography and mass spectral parameters are detailed in Supplemental Information (Doc. 1).

Thermo derived .raw files for each stream i.e., C18 pos and C18 neg and HILIC pos and HILIC neg modes, were converted to mzML format using MSConvertGUI (Adusumilli and Mallick 2017), uploaded into MZmine (Pluskal et al. 2020) to determine the appropriate baseline noise threshold and then into XCMS online https://xcmsonline.scripps.edu/ for feature detection, alignment and exploratory data analysis (Domingo-Almenara and Siuzdak 2020). Feature detection parameters for C18 data were: *m/z* deviation 10 ppm, min and max peak width 5 and 20 respectively, mzdiff 0.001, s/n threshold 20, prefilter intensity 1e4 and noise filter 2.5e4. For HILIC data the same parameters were 10 ppm, 10 and 60, 0.001, 20, 1e4 and 8e3. After downloading the output, raw mass spectrometry data were organised into data matrices comprising *m/z*, retention time and the corresponding ion intensity measurements for each feature making it suitable for statistical analysis.

Reduction of background variability in the data matrix for each stream was performed using a QC vs Blank t-test thus allowing subtraction of features with p values > 0.05, and secondly, t-values corresponding to any features high in the blanks. These data matrices were each uploaded into MetaboAnalyst 6.0 (MA ver 6.0) (Pang et al. 2021) and data integrity checked to confirm the number of samples, number of peaks, missing values, and the number of treatment groups. No missing values were detected in any of the data sets. For filtration of variables showing low repeatability, the threshold to remove those with high percent relative standard deviation (RSD) was set at 30% to that of the QCs and the data normalised by auto-scaling (mean-centred and divided by the standard deviation of each variable) and Gaussian distribution confirmed so that feature mass intensities are comparable.

For each stream, using MA ver 6.0, we explored these data matrices, and compared the 20% and 95% UV attenuated treatments by subjecting them to orthogonal partial least squares discriminant analysis (OPLS-DA); the separation between treatments was statistically confirmed using 20 permutations to provide acceptable R^2, Q^2, and p values. Features for annotation were identified by applying a paired t-test to these data matrices and retaining all the features below the false discovery threshold value of FDR < 0.05 (Benjamini and Hochberg 1995).

Annotations were conducted and confidence levels confirmed (Sumner et al. 2007) for each metabolite by interrogating the original .raw files using Xcalibur Freestyle. Formula matches were confirmed, and mass accuracy parameters were set within +/– 10.0 ppm. For all C18 features, confidence levels were set according to Sumner et al. (2007) with level 2 (parent ion plus at least one fragment) and level 3 (the parent ion only) being confirmed using the MassBank.eu (https://massbank.eu/) spectral database. Where the same annotated and confirmed compound (ion) appeared in both positive and negative modes, the one with the highest signal intensity (au) was included in the final data table. A literature search provided identification of those annotated compounds that were 1) considered as defensive metabolites with potential to influence the bioassay trial and 2) have demonstrated responses to experimentally manipulated UV exposures. For each annotated compound, fold change values were calculated and non-parametric Mann-Whitney *U*-tests used to investigate ranked distributional differences between treatments for each.

Using the intensity data of all the annotated compounds listed in Table 1 and the pathways enrichment analysis platform in MA ver 6.0, we identified the metabolic pathways that are significantly impacted (i.e. either upregulated or down regulated) by exposure to 95% UV. The upland cotton (*Gossypium hirsutum*) pathway library in MA ver.6 was used as a reference.

4.2.3 Larval Bioassay. Four days after the foliar samples were collected (18th Dec 2021) and using the same eighteen plants from each 20% and 95% UV attenuated treatments, a larval performance bioassay was conducted in the lab to assess if UV attenuation of *C. vulgaris* plant foliage impacted larval growth and mortality of the heather beetle *L. suturalis.* Here, our investigations are limited to the larval/pre-pupal life stages. Adults and larvae don't co-occur in natural field populations, so adults would require foliage of different phenological stages to measure parameters such as fecundity and overwintering lipid deposition. Thus, thirty-six (200mm L x 130mm W x 210mm H) mesh ventilated plastic cages were set up for 18 replicates each of 20% and 95% attenuated heather foliage. Foliage stems were mounted into Oasis ™ polystyrene plugs that protruded through the cage floor to contact water below and one individual *C. vulgaris* plant provided foliage for the entirety of each replicate. All 36 cages were held under constant 22 °C and illuminated with 5000 Lm LED grow lights at 16:8 hrs light:dark. Ten lab reared, unfed, hatchling larvae were placed onto the foliage in each cage and fresh *C. vulgaris* sprigs were added every three days while leaving the earlier foliage in place to avoid disturbing feeding larvae. Larvae were collected for weighing at the pre-pupal stage whereupon they drop to the cage floor and seek the humidity provided by the oasis plug. Pre-pupa were weighed (mg) using a Sartorius Quintix balance. The first collection occurred on the 3rd of Jan 2022 with the final one 10 days later. All larvae observed dead on the cage floor during the trial period were recorded and removed.

Mean prepupal weights were calculated for each replicate within each treatment. Survival for each replicate was calculated as the number of weighed prepupa subtracted from the original ten placed in each cage. Overall percent mortality for each treatment was also calculated. Both mean prepupal weight and survival data were subjected to non-parametric Mann-Whitney *U*-test to compare the

effects between treatments. We also tested if the bioassay prepupal weights differed from prepupa weights obtained from a natural field population. Using the non-parametric Kruskal-Wallis test we compared, prepupa weights from the two UV treatments and pre-pupa weight data collected from a field population on the CP close to where the experimental heather plants were originally sourced.

4.3 RESULTS

4.3.1 UV-radiation Induces Changes in the Secondary Metabolism of Heather. Our analyses show a clear separation between the metabolite profiles collected from *C. vulgaris* plants exposed for two summers to 20% (control) and the 95% UV-attenuation treatment. OPLS-DA (Fig. 1) revealed significant separation between the groups for the C18 positive stream, $R^2 = 0.99$, $Q^2 = 0.74$, $p < 0.05$ and for the C18 negative stream, $R^2 = 0.99$, $Q^2 = 0.82$, $p < 0.05$. No significant separation was observed for either of the HILIC positive or negative data sets (results not shown). A C18 column is considered the most suitable for analysing secondary metabolites due to its versatility in separating a wide range of semi-polar compounds. HILIC columns, however, are best suited to separate polar compounds and analyse primary metabolites (Xiao et al. 2012) which shows that under the tested conditions, secondary but not primary metabolism was influenced most by changes in UV-radiation.

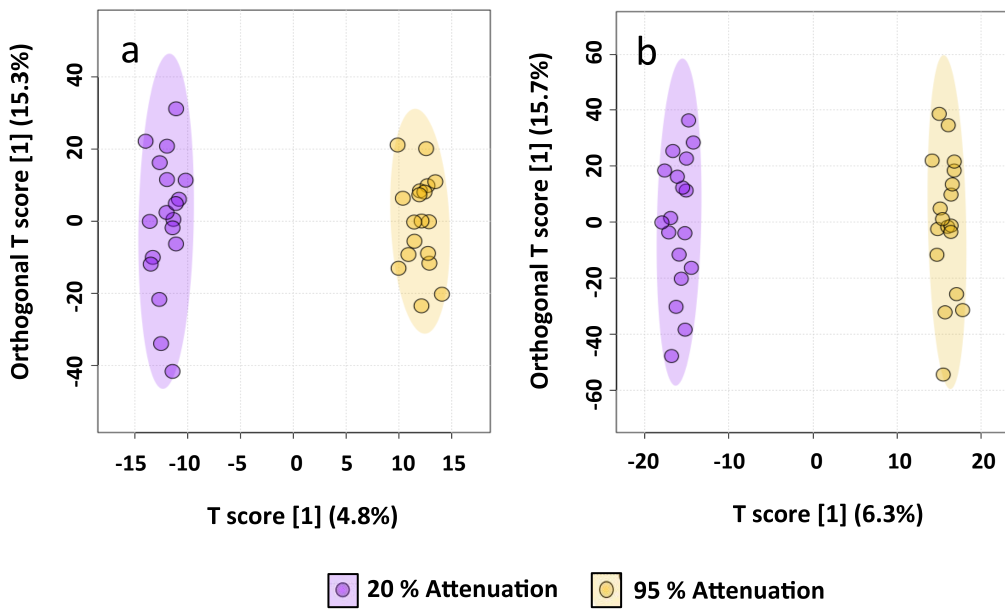


Fig. 1. Separation of *Calluna vulgaris* (heather) plant metabolite profiles after exposure over two summers of 20% (control) and the 95% UV attenuation. OPLS-DA results ($p < 0.05$) reveal complete separation for both (a) C18 positive and (b) C18 negative ionisation streams. Each cluster represents the eighteen plants (dots) in each attenuation treatment with 95% confidence ellipses.

4.3.2 UV-treatments influence the phenylpropanoid pathway and its associated defensive compounds. This analysis identified the phenylpropanoid and flavonoid biosynthesis pathways with impact factors of 0.078 and 0.015 and significance values of 10.94 and 10.17 -Log10(p), respectively, as being the two most impacted when exposed to 95% attenuation. Impact on the flavonoid biosynthesis pathway is upregulated, represented by the flavonoids and on the phenylpropanoid pathway downregulated, represented by hydroxycinnamic acids, coumarins and caffeoylquinic acids (see Table 1). Other pathways such as the ubiquinone/terpenoid and flavone/flavonol biosynthesis pathways from these data indicate minor yet significant impacts, both with values of < 0.001 (Fig. 2).

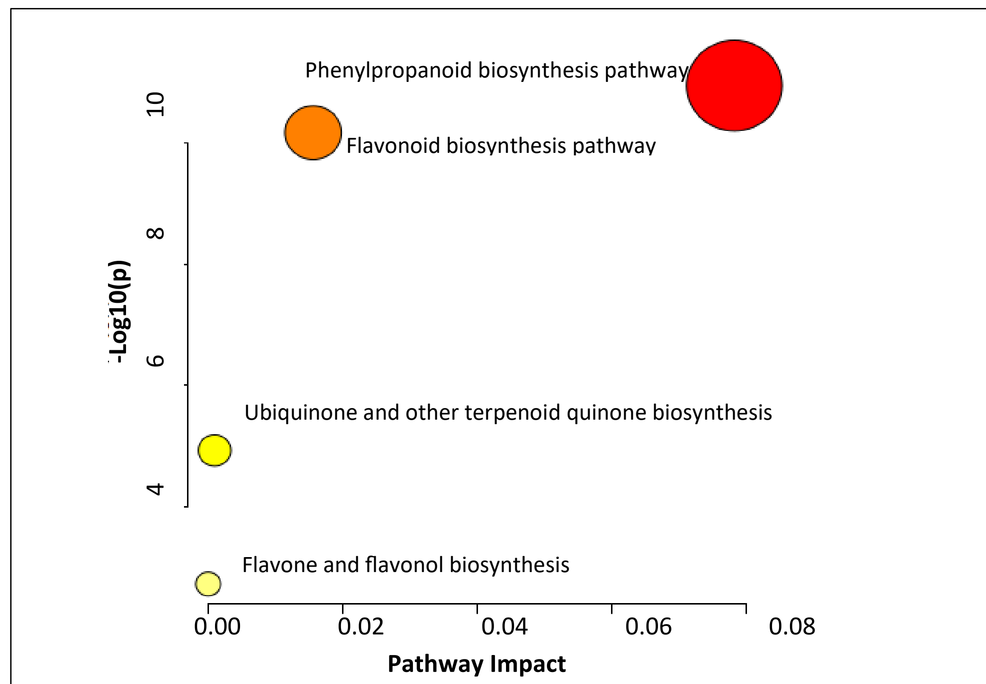


Fig. 2 Pathway enrichment analysis of *C. vulgaris* plants showing the pathways impacted when exposed to the 95% attenuation of UV treatment. Pathway impact value (x axis) and –log10(p) value of significance (y axis) indicate phenylpropanoid biosynthesis and the flavonoid biosynthesis pathways with impact factors of 0.078 and 0.015 and significance values of 10.94 and 10.17 -Log10(p), respectively. The size and colour of the dots represent the impact value, and the pathway *p* value respectively i.e. increasing *p* values change from yellow through to red.

From the C18 positive and negative streams combined, a total of eighteen compounds were annotated. Sixteen are derived from the Shikimate-phenylpropanoid pathway (see Table 1). Hydroxycinnamic acids, flavonoid glycosides, coumarins, caffeoylquinic acids and one each of a stilbene glycoside, cinnamyl alcohol and a flavonoid comprise this group. Two of the caffeoylquinic acids, 3-O and 5-O, each with the parent ion *m/z* 355.1021 were separated by the presence of the distinctive fragment *m/z* 135.043 belonging to 3-O-Caffeoylquinic acid (Yang et al. 2022). Two further compounds, a terpene lactone and an amino acid conjugate make up the remainder.

Table 1. Annotated metabolites from C18 pos and C18 neg streams. KEGG = that database's entry reference No.; Strm = column mode; R/A indicates if compound is reduced R,(⁻) or amplified A, (↑) in the attenuation treatments; CL = confidence level of annotation whereby level 2 is the parent ion and at least one co-eluted fragment and 3 is the parent ion only; ***m/z*** = mass to charge ratio; rt. = retention time. Antiherbivore defence (Herb.) References. a. Sing et al. 2021, b. Dixit et al. 2017, c. Hussain et al. 2018, d. Kundu and Vadassery 2019, e. Ramaroson et al. 2022. Ultra-violet (UV) References. f. Ruhland et al.2005, g. Chowdhray et al. 2021, h. Luis et al. 2007, i. Zhang and Bjorn 2009, j. Baker et al. 2020, k. Lavola et al. 1997, l. Holub et al. 2019.

Class/Sub Class	Name	KEGG	Strm	R/A	CL	*m/z*	rt.	Fragments	Herb. Ref.	UV. Ref.
Hydroxycinnamic acid	Caffeic acid	C01481	C18+	↓	2	181.0495	3.16	163.0381, 145.0278	a.	f.
Hydroxycinnamic acid	*p*-Coumaric acid	C00811	C18+	↓	2	165.0546	6.05	119.0509	b.	f.
Hydroxycinnamic acid	Rosmarinic acid	C01850	C18-	↓	3	359.0752	6.04			g. h. i.
Hydroxycinnamic acid	Sinapoyl malate		C18-	↓	3	339.0721	7.85			j.
Cinnamyl alcohol	4-Hydroxycoumarin	C20414	C18+	↓	3	163.0389	3.16			
Coumarin Glycoside	4-Methylumbelliferyl glucuronide	C11584	C18+	↓	3	353.0865	3.17			
Coumarin	Coumarin	C05851	C18+	↓	3	147.0440	7.60		c.	
Stilbene glycoside	3,4',5-Trihydroxystilbene-3-beta-D-glucoside		C18-	↓	3	389.1246	9.78			i.
Flavonoid glycoside	Kaempferol-3-Glucoside-2''-p-coumaroyl		C18+	↑	3	595.1444	1.87			
Flavonoid glycoside	Myricetin-3-Xyloside		C18-	↑	3	449.0733	9.04			
Flavonoid	Myricetin	C10107	C18+	↑	3	319.0446	9.03			
Quinic acid	Caffeoylquinic acid (3-O-caffeoyalquinic acid)	C00852	C18+	↓	2	355.1021	8.25	163.0370, 181.0470, 164.0410, 135.0430		
Quinic acid	1,5-Dicaffeoylquinic acid (Cynarine)		C18-	↓	2	515.1200	8.37	351.0718		
Quinic acid	Chlorogenic acid (5-O-caffeoylquinic acid)	C17147	C18+	↓	2	355.1021	3.16	163.0394, 181.0466, 164.0422, 165.0430	d. e.	k.
Quinic acid	D-(-)-Quinic acid (Quinic Acid)	C00296	C18-	↓	3	191.0556	8.24			
Quinic acid	3-Feruloylquinic acid		C18+	↑	2	369.1177	7.59	177.0528, 178.0604		l.
Terpene lactone	(-) - Bilobalidae	C07605	C18-	↓	3	325.0932	7.89			
Amino acid conjugate	N-Fructosyl Tyrosine		C18+	↓	3	344.1338	7.61			

Six of these compounds were confirmed at level 2 confidence based on parent and fragment ion matches with library spectra and the remainder at level 3 (i.e., parent ion only so identified to class/sub class with the named compound being the most probable) (Table 1). All annotated compound confirmation data is provided in Appendix B.

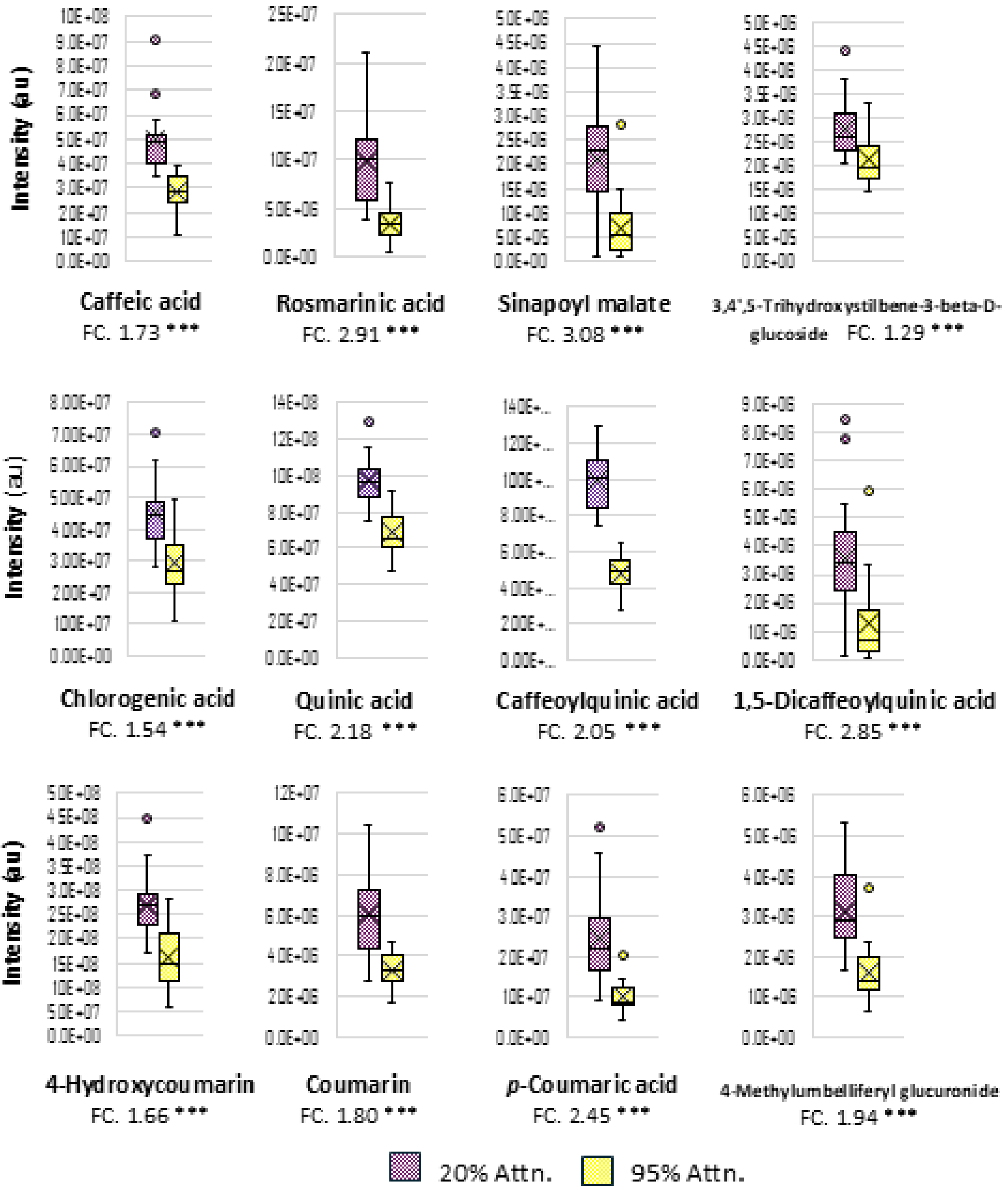


Fig. 3 Intensity (au) and fold change (FC) values for the 12 reduced phenylpropanoid and quinic acid derived compounds in *Calluna vulgaris* (heather) plants when exposed to 95% UV attenuation as compared to those exposed to 20% attenuation. ***$p < 0.001$, Mann-Whitney *U*-test. X = mean, — = median. A FC of 1 = 0 change, FC of 1.50 would = 50% reduction etc. All metabolites in the top row plus chlorogenic acid and quinic acid in the second row have been recorded in the literature, to respond to variation in ultra-violet light.

Mass spectral relative signal intensity of ions is expressed as arbitrary units (au). These intensities and the fold change (FC) ratio for each compound resulting from the 95% attenuation treatment as compared to the 20% control are provided in Fig.3 (for compounds reduced) and Fig. 4. (for compounds amplified). All relevant non-parametric statistics are presented in S. Table 1. Note: FC of 1 = 0 change, FC of 1.50 = 50% amplification or reduction, FC of 2.5 = 150% amplification/reduction etc.

The reviewed literature (annotated in Table 1) revealed that caffeic acid, rosmarinic acid, sina*p*oyl malate, 3,4',5-Trihydroxystilbene-3-beta-D-glucoside, chlorogenic acid and quinic acid have all been recorded as being influenced by UV in other plant species. Similarly, caffeic acid, *p*-coumaric acid, coumarin and chlorogenic acid, were identified in other studies as having anti-herbivore activity (Table 1).

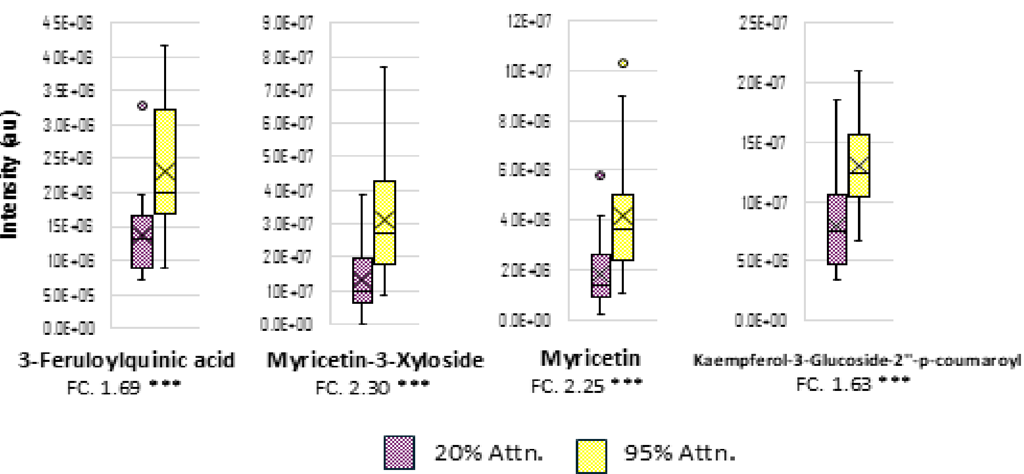


Fig. 4 Intensity (au) and fold change (FC) values for the 4 amplified phenylpropanoid and quinic acid derived compounds in *Calluna vulgaris* (heather) plants when exposed to 95% UV attenuation as compared to those exposed to 20% attenuation. ***$p < 0.001$, Mann-Whitney *U*-test. X = mean, — = median.

4.3.3 Larval Survival and Prepupal Weight are not Affected when Feeding on Foliage from Different Treatments. Mean prepupal weights and larval survival for *L. suturalis* feeding on foliage exposed to 20% or 95% attenuation of UV (n = 18 for both treatments) were very similar and revealed no significant difference between treatments (Fig. 5). Prepupal weights were 153.6 ± 5.75 mg for 20% attn. and 155.7 ± 11.1 mg for 95% attn., Mann-Whitney, U = 145.00, p = 0.580; larval survival (out of 10) was 8.8 ± 1.1 for 20% attn. and 8.5 ± 1.5 for 95% attn., U = 148.00, p = 0.642. Overall, the mortality rates were 12.2% and 15% for the 20% and 95% attenuation treatments respectively.

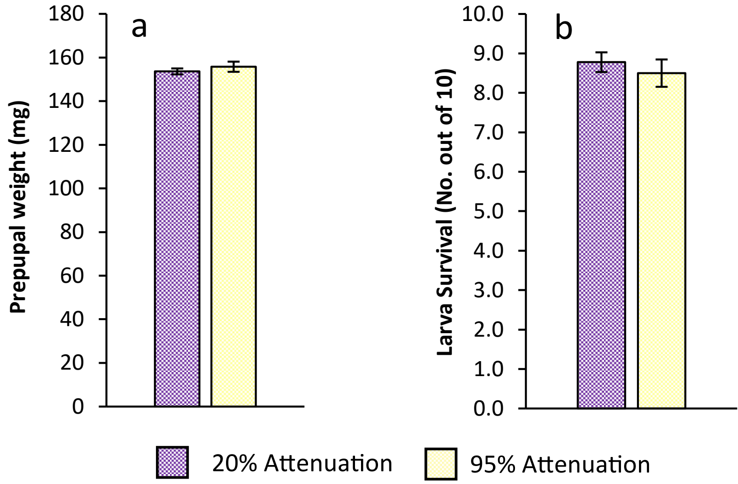


Fig. 5 Mean prepupal weight (mg) (a) and larval survival (b) of heather beetle (*Lochmaea suturalis*) feeding on 20% and 95% UV attenuated heather (*Calluna vulgaris*) foliage. No significant difference was observed between weights, $p > 0.05$ or survival, $p > 0.05$. Error bars = 1 SE.

4.4 DISCUSSION

Plant Metabolite Response to UV-radiation. Our results show that changes in UV-radiation influence the shikimate-phenylpropanoid secondary metabolism of *C. vulgaris*, primarily through the phenylpropanoid and flavonoid biosynthetic pathways both of which are dependent on *p*-coumaric acid as a key precursor but have displayed different responses. Under 95% attenuation of UV, hydroxycinnamic acids, coumarins, a stilbene glycoside and a number of caffeoylquinic acids, all catalysed by *p*-coumaroyl-CoA, shikimic acid and quinic acids via the phenylpropanoid biosynthesis pathway (Alcázar Magaña et al. 2021) are reduced. Conversely malonyl-CoA plus *p*-coumaroyl-CoA catalyse the biosynthesis of naringenin in the flavonoid pathway (Singh et al. 2021) leading to, under 95% attenuation, myricetin, myricetin-3-xyloside and kaempferol-3-glucoside-2''-p-coumaroyl remaining amplified (Table1). This suggests these particular flavonoids are possibly upregulated due to another stressor such as soil nutrient availability, thus maintaining their amplified state. The soils in which these trials were run are extremely low in both nitrogen (N) and phosphate (P) and there is evidence that many flavonoids are amplified and function as antioxidants involved in reducing reactive oxygen species induced by stress including low soil nutrients (Lillo et al. 2008; Patil et al 2024; Stewart et al. 2001). Support for this outcome is also provided from our own field trials at a CP site where the compound kaempferol-3-glucoside-2''-p-coumaroyl and other flavonoid glycosides were significantly reduced in *C. vulgaris* foliage after the application of N and P fertilizers (Barrett et al. In preparation).

The literature provides several reports (Table 1), showing responses of various plant species to UV (particularly the UV-B spectrum) which support our findings. For hydroxycinnamic acids, work with *Deschampsia antarctica* (É.Desv) using screen technology to manipulate UV-B showed decreased caffeic and *p*-coumaric acid concentrations when exposed to reduced levels of UV-B. This is

linked to the photoprotective function of these metabolites and their trade off with the growth parameters of total leaf area and leaf mass ratio in this plant (Ruhland et al. 2005). Rosmarinic acid, sinapoyl malate and the stilbene glycoside, resveratrol (3,4',5-Trihydroxystilbene-3-beta-D-glucoside), are all understood to be UV absorbing metabolites and respond to UV by accumulating in the upper epidermis of leaves which reduces the transmittance of damaging UV to the mesophyll (Baker et al. 2020; Luis et al. 2007; Zhang and Björn 2009). Quinic acid derived compounds, chlorogenic acid and 3-feruloylquinic acid are also documented as responding to and providing photo protection to *Betula pendula* (Roth), (birch) and *Hordeum vulgare* (L.) (barley) respectively (Lavola et al. 1997; Holub et al. 2019).

From our earlier studies (Barrett et al. 2024), chlorogenic acid, caffeic acid and 4-hydroxycoumarin are compounds that had significantly amplified in field-collected *C. vulgaris* samples from New Zealand CP sites where peak summertime UV index is ~ 14, compared to those samples from Scotland (SC) in the UK with an index of ~ 6-7. Fold change levels of these three compounds were higher in the CP samples at 1.13, 1.22 and 1.2-fold for chlorogenic acid, caffeic acid and 4-Hydroxycoumarin, respectively and we extrapolate that these compounds are elevated when exposed to high levels of UV in New Zealand's CP environment (Barrett et al. 2024). Additionally, earlier work by Effah et al. (2020b) also indicated the sensitivity of *C. vulgaris* to UV light, when exposure to different intensities of UV under the same attenuating screens induced changes in a range of sesquiterpene volatile metabolites. These results suggest that amplification of many phenylpropanoid compounds in *C. vulgaris* on the CP are likely being driven by elevated UV light.

Our findings align with and provide further evidence that secondary metabolites can be significantly altered in invasive plants that have established in a new environment (Wolf et al. 2011; Skubel et al. 2020; Medina van Berkum et

al. 2023). Changes in the light environment are known to alter plant physiological processes and the resulting biochemical phenotypes, which consequently can affect insect herbivore performance (Rousseaux et al. 2001; Kuhlmann and Müller 2010; Ballaré et al. 2012; Dinh et al. 2013; Ballaré 2014; Qi et al. 2018; Fallah et al. 2023). Where light parameters differ significantly between sites, regions or ranges, we suggest investigation of potential change to the biochemical phenotype of a host plant may deserve exploration.

Insect Responses to UV-related Metabolite Changes. We found no significant difference on the assessed life history parameters of the biocontrol agent *L. suturalis* after feeding on plants from each of the UV attenuation treatments. To validate this result, we also compared live weights from pre-pupae collected from a field population on the CP close to where the experimental heather plants were sourced originally. There was no significant difference (Kruskal-Wallis $H(2) = 0.77$, $p = 0.680$), in mean live weights of pre-pupae from that field population and the mean weights of pre-pupae obtained from either of the UV attenuated trials, confirming the lack of effect of the UV treated plants (see also S Fig. 3).

Three compounds recorded in this study that are known from the literature to be both altered by UV and involved in anti-herbivore defence are caffeic acid, chlorogenic acid and *p*-coumaric acid (Dixit et al. 2017; Singh et al. 2021). The literature provides little evidence that these three compounds impair performance in Coleopteran spp. but chlorogenic acid is a feeding deterrent for *Spodoptera exigua* (Hübner, 1808) feeding on honeysuckle *Lonicera maackii* (Rupr.) Maxim. Caffeic and chlorogenic acids are reported to inhibit larval growth and development of the caterpillar *Helicoverpa zea* (Boddie, 1850) when feeding on cotton *Gossypium hirsutum* (L.) and caterpillar growth of *Sesamia nanogriodes* (Lefebvre, 1827) is impaired by *p*-coumaric acid when feeding on corn *Zea mays*

(L.). However, unlike the specialist *L. suturalis,* these species are broad generalist (*S. exigua* and *H. zea*) or oligophagous (*S. nanogriodes*).

In a review of the specialist - generalist paradigm, Ali and Agrawal (2012) point out that while specialists are much better adapted to tolerate plant defences than generalists, at higher concentrations of many defensive compounds, few specialists are entirely immune to them. The specialist lepidopteran tobacco hornworm *Manduca sexta* (Linnaeus, 1763) showed no significant difference in pupal mass after feeding on a control diet containing no nicotine and one with 0.1 % wet weight nicotine, but a 5-fold increase (0.5%) in nicotine resulted in a significant reduction of pupal mass (Harvey et al. 2007). They also postulate that for FC increases at low to moderate levels, specialists can benefit from such compounds but at higher FC levels they will suffer deleterious effects. As an example, Richards et al. (2012) using the specialist buckeye caterpillar *Junonia coenia* (Hübner, 1822) demonstrated that diets with low concentrations of iridoid glycosides (ranging from 1 to 6-fold differences), caterpillar survival progressively increased but at concentrations equivalent to 8 to 10-fold, survival decreased significantly. It's therefore possible that the magnitude of change in our UV trials may have been insufficient to impact growth and survival in *L. suturalis* larvae.

Assuming any or all of these compounds i.e. caffeic acid, chlorogenic acid or *p*-coumaric acid may indeed impair *L. suturalis* larvae at higher concentrations, this raises the question of the magnitude of change induced by the 95% UV treatment. In our trials, the FC difference for the above three compounds were relatively low, i.e. 1.73, 1.54 and 2.45-fold, respectively. A FC reduction of these magnitudes would potentially provide an impetus to growth in a generalist insect herbivore but may be of little consequence to a specialist that has evolved the ability to tolerate variation in the concentration of defensive compounds and even use them as feeding stimulants, detoxify them pre- or post-ingestion, or

sequester them as defences against predators (Ali and Agrawal 2012; Jeckel et al. 2022; Kshatriya and Gershenzon 2024). Thus, the relatively small FC reduction in compounds of the 95% attn. treatment, appears to have little influence on the assimilability of *C. vulgaris* foliage for *L. suturalis*.

We acknowledge that the foliage presented to the larvae were cut stems. Severed stems can produce wound responses, including amplification of jasmonic and abscisic acids post severance (Da Costa et al. 2013), but may not be producing an appropriate herbivore damage response. Thus, the larvae may be exposed to constitutive compounds only, and not the full suite of induced defensive compounds and their potential synergistic effects (Barbehenn and Kochmanski 2013) as could be expected from a living plant. It's also possible that the mature heather plants collected from the CP field site, where they have had continuous exposure and are well acclimated to high natural levels of UV, had limited potential to respond to reduced UV during the attenuation period. Thus, exposing plants to controlled UV-intensities from seedling emergence and for longer periods may induce greater magnitudes of metabolite change and if used as live plants, provide more definitive results when testing herbivore responses.

Could Elevated Phenylpropanoids be Impairing L. suturalis on the CP? Using data from our earlier work, we established the fold change differences for thirty-five phenylpropanoid derived compounds in *C. vulgaris* between the CP (NZ) and Scotland (UK) (Barrett et al. 2024). Several flavonoid, coumarin and hydroxycinnamic acid compounds i.e. myricetin, kaempferol-3-glucoside-2''-p-coumaroyl, 4-Hydroxycoumarin, caffeic acid, o-coumaric acid (an isomer of *p*-coumaric acid) and chlorogenic acid, co-occur in both NZ vs UK data (Barrett et al. 2024) and in the attenuated UV trial results presented here. The fold changes for these compounds between NZ and UK ranged from 1.13 to 1.82 and for this UV trial were 1.54 to 2.45. These ranges are within those given above in Harvey et al. (2007) and Richards et al. (2012).

L. suturalis as a specialist herbivore is ubiquitous wherever *C. vulgaris* occurs in the UK and Europe and is a species with regular population outbreaks, causing complete destruction of all plants within the affected site over a wide range of habitats and degrees of latitude (Pakeman et al. 2002; Rosenburgh and Marrs 2010). Again, from our earlier work, we established for fifteen phenylpropanoid derived compounds, the fold change range of variation between four geographically and geologically different sites in a part of *C. vulgaris'* native range in Scotland, UK. These are all well-known flavonoids, coumarins and hydroxycinnamic acids with magnitude of inter site variation ranging from 1.57 to 7.29-fold. It seems that *L. suturalis* in its native range, is well adapted to a greater magnitude of phenylpropanoid variance, that it is not encountering on the CP or from our UV attenuation trial.

We postulate then, that at the magnitude of difference measured in our earlier work and this trial, there will be little effect of amplified phenylpropanoids on larval performance in the CP *L. suturalis* population. It may be, that the higher levels of phenylpropanoids recorded in CP *C. vulgaris* in Barrett et al. (2024), provide the plant with enhanced photoprotection, but don't necessarily enhance its anti-herbivore defensive capability against this specialist herbivore.

Nearly three decades of research have been accomplished since the release of *L. suturalis* against the invasive shrub *C. vulgaris* on the CP in New Zealand, which now provides a broad understanding of this biocontrol programme (Barrett et al. 2021). Publications include the effect of parasitoids and predators of *L. suturalis* eggs and larvae (Peterson et al. 2004), adult beetle body size and winter survival (Fowler et al. 2015) and comparing the efficacy of biocontrol vs. herbicides (Peterson et al. 2020).

The application of fertiliser to improve soil nutrient status and increase host plant foliar nitrogen with a consequent improvement in *L. suturalis* larval and adult performance parameters and establishment rates is the most recent (Peterson et al. 2024). This work links poor *L suturalis* performance and establishment with low foliar nitrogen of *C. vulgaris* foliage on the CP. We are now using metabolomics to provide insight into how increases in foliar nitrogen of *C. vulgaris* interacts with both primary nitrogen containing and secondary metabolites, to promote the demonstrated increase in *L. suturalis* performance and establishment.

The work covered in this report using metabolomic techniques, attempts to address an unanswered but fundamental question relating to plant biochemical defenses and their effects on this biocontrol agents' larval stages in the CP environment. We acknowledge there are other life stages that could potentially be affected by plant defences such as adult fecundity and overwintering fat body deposition. *L. suturalis* adults being univoltine however, generally don't co-occur with larvae on *C. vulgaris* during late spring and summer. Adults predominate during early spring leading up to oviposition and in autumn through flowering as next generation pre-overwintering adults, thus different phenological stages of *C. vulgaris* are required to test relevant adult life history parameters.

In conclusion, our trials using attenuating UV screen technology significantly altered the intensity of several phenylpropanoid metabolites in heather (*C. vulgaris*) with several metabolites being in common with similar trials reported for a range of plant species in the literature. Heather exposed to 95% attenuation of UV showed reduced intensities of metabolites. The corollary of this is that elevated UV (20% attenuation or ambient in natural field conditions) amplifies many phenylpropanoids which indicates an upregulation of the shikimate – phenylpropanoid pathway. This is what was expected and is further

supported by our results. Overall, we accept our hypothesis that exposure to artificially reduced ultra-violet light reduces phenylpropanoid metabolites of heather.

Results from the bioassays to determine if UV induced changes to the levels of defensive secondary metabolites result in differences in mean prepupal weight and larval survival of heather beetle *L. suturalis* larvae showed no significant effects. Given the adaptations of specialist insects to tolerate plant defences, and that there is considerable variability in the range of concentrations of metabolites both between plants and between sites, in both the native and invaded ranges, the magnitude of change to *C. vulgaris* plants exposed to the two UV attenuating treatments appears to be insufficient to have any effect on these life history parameters.

Notwithstanding that, we consider the use of metabolomics to have great potential to elucidate insect performance parameters as a function of plant metabolite variation and to provide valuable insights into the biochemistry of plant-biocontrol agent interactions. Where light or other abiotic parameters such as soil nutrients differ significantly between regions or ranges, we suggest investigation of potential change to the biochemical phenotype of a target host plant may deserve exploration. Such metabolomic assessments might be included early in a programme where biological control of a plant using insect herbivores is being considered or indeed retrospectively where a programme has proven to be ineffective. For investigation of UV induced phytochemical changes we encourage exposing plants to the experimental UV-intensities from seedling emergence and for extended periods of time to promote maximum potential for change and to use live plants when testing herbivore performance responses to those changes.

Finally, there are a multitude of questions which could be addressed using metabolomics (or more correctly, ecometabolomics) to assist with understanding fundamental ecological processes relating to biochemical phenotypic change in invasive plants. The application of metabolomics to address biochemical defensive effects on plant-insect interactions relating to biocontrol of weeds is in its infancy. We hope, however, the investigations reported here will prompt further use of this exciting technology in future weed biocontrol programmes and/or retrospectively to assess programmes that failed to meet expectations.

DECLARATIONS

Author Contributions—DPB, conceived all questions and objectives of the investigation. Conducted all experiments, sampling, processing, metabolomic analysis, interpretation and annotation of data. Secured funding. Primary author of the manuscript. AKS, advised on UHPLC-MS techniques, metabolomic data analysis and revised the manuscript. JJW advised on technical aspects of ultra-violet trials and measurements, data interpretation and revised the manuscript. PP provided advise on heather beetle rearing and bio-assay experimental setup, revised the manuscript. DJL ran all samples using UHPLC-MS and revised manuscript. MM, advised on aspects of experimental design, analysis and interpretation of data and revised the manuscript. ACM, principal investigator, secured funding. Advised on concept, design and interpretation of investigations, and revised the manuscript.

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Data availability—All relevant .raw spectral data sets, larval performance and annotated compound data are available upon reasonable request.

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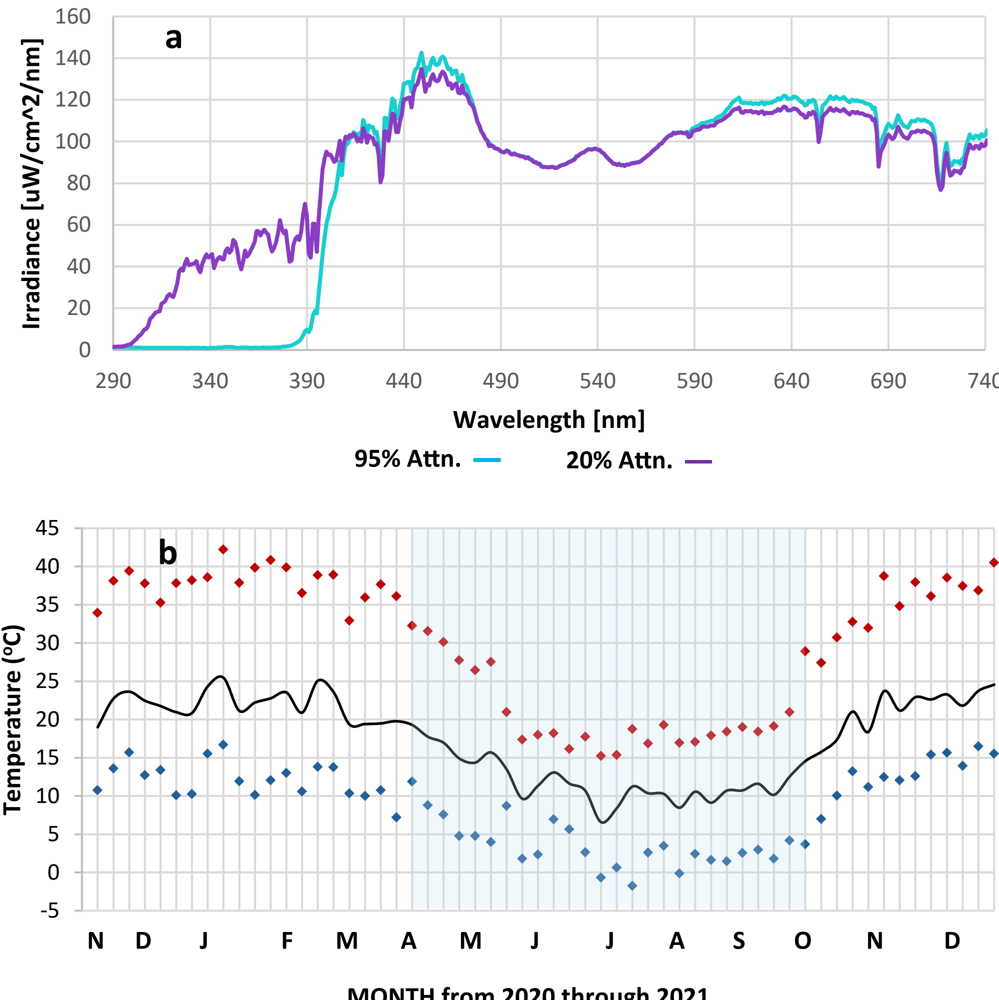
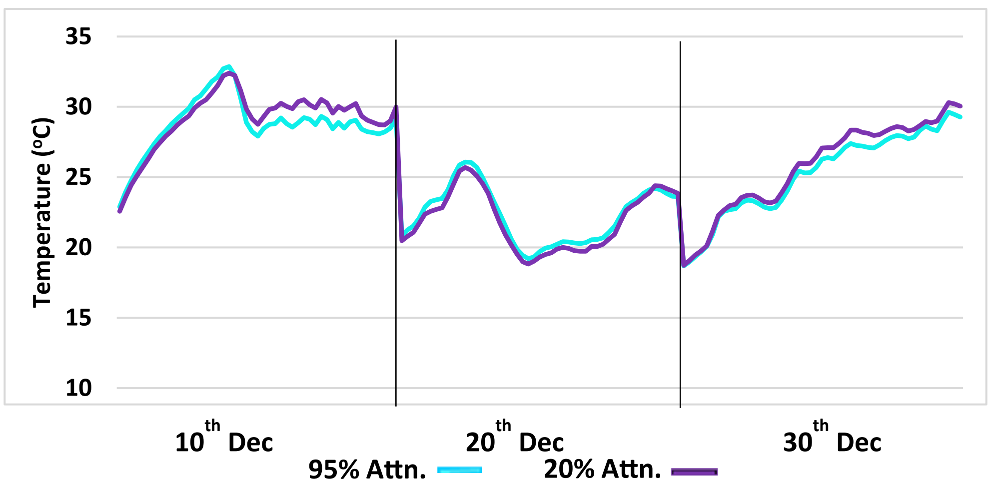
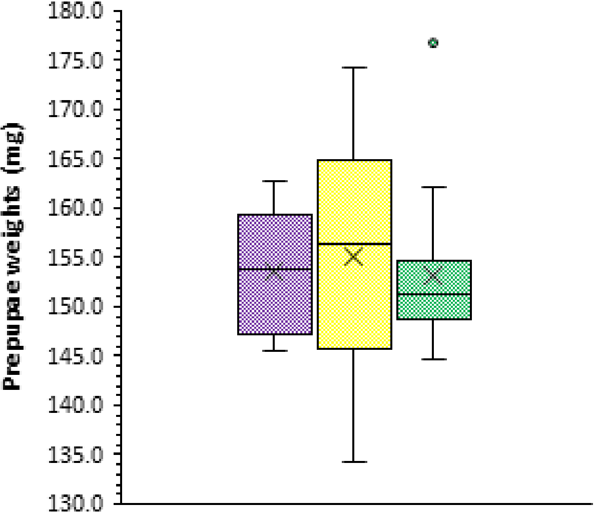
4.7 SUPPLEMENTAL FIGURES 1 a, b, 2 and 3



S. Fig. 1a, b. Scan results for the 20% and 95% attenuation treatments (a.) Average total UV irradiance i.e. 290-320 nm (UV-B) plus 321-400 nm (UV-A) wavelengths combined, show the 95% screen transmitting ≤ 8.1% of total UV transmitted by the 20% screen. Photosynthetically active radiation (PAR) i.e. 401-750 nm wavelength, shows each screen to be within 2.5% transmittance of the other demonstrating negligible difference in transmission of PAR. Temperature data logged continuously through the period of treatment (b.) shows the mean weekly temperature (black trace) and the single greatest maximum (red diamond) and minimum (blue diamond) temperature recorded during each week from November 2020 to end of December 2021. White background is the period under attenuation screens and blue background is the period when plants were outside exposed to ambient light and temperature.



S. Fig. 2 Temperature variation between 20% and 95% attenuation treatments recorded from three days in December 2020 (10th, 20th [summer solstice] and 30th). A minor difference of ≈ 1.17 °C exists during PM hours on the 10th and 20th but mean values throughout for 20% and 95% are 20.28 °C and 20.47 °C respectively with a t. test revealing no significant difference, $t.$ = -0.399, p = 0.69.



S. Fig. 3. Plotted data comparing mean *L. suturalis* prepupae weights from the 20% and 95% UV attenuated *C. vulgaris* (heather) foliage and those sampled off foliage from a CP field population (Fld Pop.). The Kruskal-Wallis test examining distributional differences between groups indicates no significant difference $H(2) = 0.77$, $p = 0.680$.

S. Table 1. Mann-Whittney distributional rank difference statistics for each compound annotated, displaying significant difference between the 20% and 95% UV attenuation treatments.

Compound	Mean Rank 20%	Mean Rank 95%	*U*	*z*	*p*
Caffeic acid	26.89	10.11	11.0	-4.7932	0.000002
p-Coumaric acid	26.06	10.09	26.0	-4.3187	0.000016
Rosmarinic acid	26.5	10.5	18.0	-4.5718	0.000005
Sinapoyl malate	24.61	12.39	52.0	-3.4961	0.00047
4-Hydroxycoumarin	25.8	11.2	30.0	-4.1921	0.000028
4-Methylumbelliferyl glucuronide	26.0	11.0	27.0	-4.287	0.000018
Coumarin	25.72	11.28	32.0	-4.1288	0.000036
3,4',5-Trihydroxystilbene-3-beta-D-glucoside	24.0	13.0	63.0	-3.148	0.00164
Kaempferol-3-Glucoside-2''-p-coumaroyl	12.17	24.83	48.0	-3.6226	0.000292
Myricetin-3-Xyloside	12.39	24.61	52.0	-3.4961	0.00047
Myricetin	12.67	24.33	57.0	-3.3379	0.00084
Caffeoylquinic acid	9.5	27.5	0.0	-5.1414	0.000001
1,5-Dicaffeoylquinic acid	24.39	12.61	56.0	-3.3695	0.00075
Chlorogenic acid	25.11	11.89	43.0	-3.7808	0.000156
D-(-)-Quinic acid	26.56	10.44	17.0	-4.6034	0.000004
3-Feruloylquinic acid	12.39	24.61	52.0	-3.4916	0.00047
(-) - Bilobalidae	25.17	11.83	42.0	-3.8125	0.00013
N-Fructosyl Tyrosine	27.44	9.56	1.0	-5.1096	0.000001

SUPPLEMENTAL INFORMATION DOC. 1.

Chromatography and Tandem Mass-spectrometry Conditions.

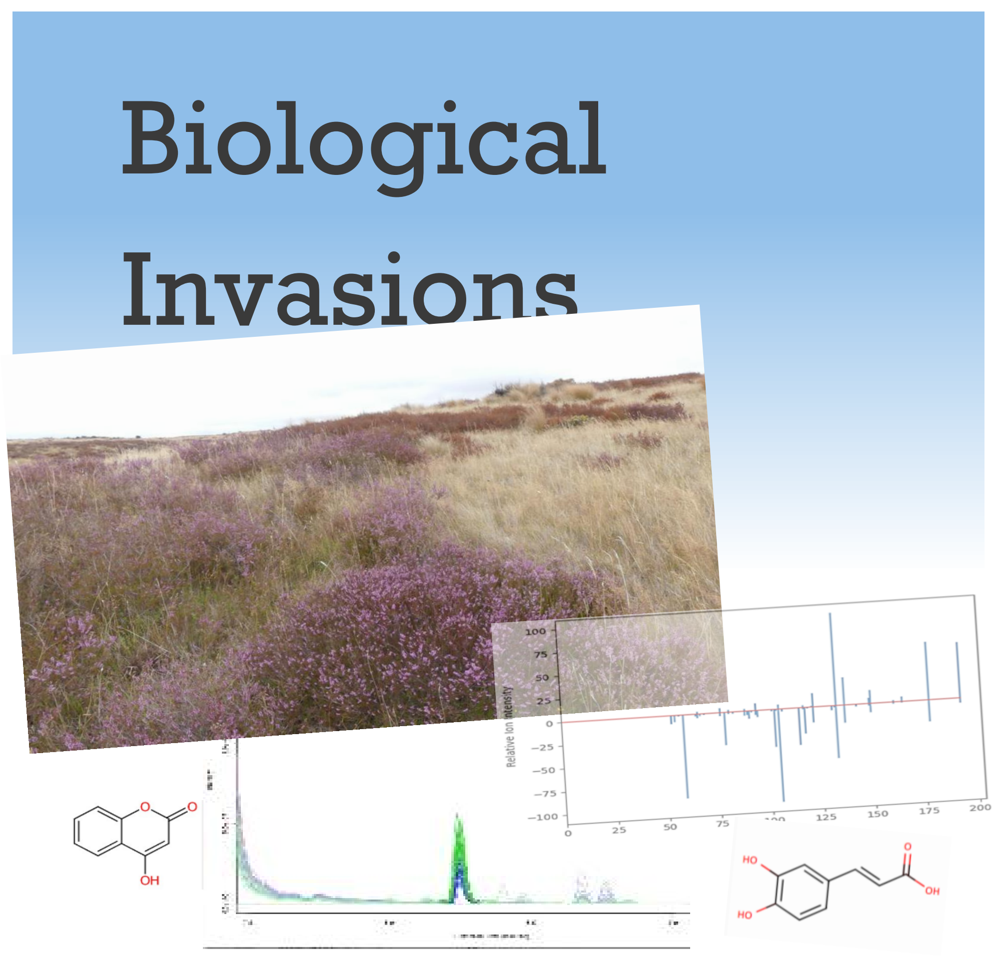
Chromatography and tandem mass-spectrometry analysis of polar and semi-polar compounds were achieved using a Thermo LC–MS system (Thermo Fisher Scientific, Waltham, MA, USA) which consisted of a Dionex UltiMate 3000 UHPLC system coupled with a high-resolution Q Exactive Focus Quadrupole-Orbitrap mass spectrometer utilising heated electrospray ionisation run in both positive and negative modes.

For semi-polar compounds, samples were cooled in the auto-sampler at 4 ˚C and a 5 μL aliquot was injected into a 1.9 µm Thermo Hypersil GOLD™ C18 column (UHPLC, 100 mm × 2.1 mm, Thermo Fisher Scientific, USA) at 25 ˚C with a gradient elution programme and a flow rate of 350 μL min^{-1}. The mobile phase was water with 0.1% formic acid (solvent A), and acetonitrile (C_2H_3N) with 0.1% formic acid (solvent B). Using the Xcalibur software package provided by the manufacturer the gradient elution programme was: held at 5% B (0–3 min), 5–80% B (3–13 min), held at 80% B (13–16 min), 80-100% B (16.0-16.5 min), held at 100 % B (16.5-18 min) returned to 5% B (18–19 min) and allowed to equilibrate for a further 4 min prior to the next injection. Full mass scan spectral data were collected in profile mode over a mass range of 60–900 *m/z*, at a mass resolution setting of 70,000 with a maximum trap fill time set to auto. ddMS^2 parameters were: resolution 17,500, isolation window 1.5 *m/z*, collision energy of 25 with a maximum trap fill time set to auto. Samples were run in both positive and negative ionisation modes separately. Positive ion mode parameters were: spray voltage 3.5 kV; capillary temperature 320 ˚C; auxiliary heater temperature 350 ˚C, sheath gas flow (N_2) 35, auxiliary gas 8, and sweep gas of 1 (arbitrary units). Negative ion mode parameters were the same except for spray voltage, −3.5 kV.

For polar compounds, samples were cooled in the auto-sampler at 4 ˚C and a 5 μL aliquot was injected into a 5 μm ZIC®-pHILIC column (100 mm × 2.1 mm, Merck Darmstadt, Germany) at 25 ˚C with a gradient elution programme and a flow rate of 150 μL min^{-1}. The mobile phase was 10mM NH_4COOH in water pH adjusted to ~ 4 with formic acid (solvent A) and 97% CH_3CN (solvent B). The gradient elution programme was: 95% B (0-2 min), 95–20% B (2–17 min), held at 20% B (17-19 min), returned to 95% B (19–19.5 min) and allowed to equilibrate for a further 9.5 min prior to the next injection. Full Mass scan spectral data were collected in profile mode over a mass range of *m/z* 60-900 at a mass resolution setting of 70,000 with a maximum trap fill time set to auto. $ddMS^2$ parameters were: resolution 17,500, isolation window 1.5 *m/z*, collision energy of 25 with a maximum trap fill time set to auto. Samples were run in both positive and negative ionisation modes separately. Positive ion mode parameters were: spray voltage 3.5 kV; capillary temperature 320 ˚C; auxiliary heater temperature 280 ˚C, sheath gas flow (N_2) 25, auxiliary gas 8, and sweep gas of 1 (arbitrary units). Negative ion mode parameters were the same except for spray voltage, −3.5 kV.

Chapter 5

Low foliar nitrogen and elevated defensive metabolites in the invasive weed* Calluna vulgaris *(heather) may impair biocontrol agent performance



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D. Paul Barrett, Maria A. Minor, Paul Peterson, Simon V. Fowler, Arvind K. Subbaraj, David J. Lun and Andrea Clavijo McCormick. Low foliar nitrogen and elevated defensive metabolites in the invasive weed *Calluna vulgaris* (heather) may impair biocontrol agent performance.

Abstract

Introducing insect biocontrol agents to manage invasive plants is an effective and sustainable management option. However, biocontrol agents sometimes fail to establish or are ineffective in the new range. Heather beetle *Lochmaea suturalis,* (Thomson, 1866) (Coleoptera: Chrysomelidae) sourced from the United Kingdom (UK), was introduced into New Zealand's North Island Central Plateau (CP) to control invasive *Calluna vulgaris* (L.) Hull (heather). The beetle was difficult to establish and the few populations that did, achieved poor population growth rates and expansion in contrast to the rapid rates associated with its conspecifics in the UK. We hypothesize that low soil nutrients such as nitrogen and phosphorous (N and P) on the CP may be linked to changes in *C. vulgaris'* primary nutritional and secondary defensive metabolites, leading to poor beetle performance. Testing this hypothesis involved application of N and P to soil in *C. vulgaris* field plots, measurement of foliar N and Carbon (C), plus using non-targeted metabolomics, exploring differences in the primary and secondary metabolites between treatments. Raising soil nutrients amplified several primary metabolites including N-based amino acids and concomitantly reduced the majority of phenylpropanoids, a secondary metabolite group containing many defensive compounds. This work seeks to better understand, how abiotic conditions in a new environment, influence invasive plant metabolism, potentially altering the efficiency of foliage assimilation which may impair biocontrol agent establishment and effectiveness. Such understanding may be applied to mitigate potential foliar deficiencies at release sites and contribute to improving success rates of biocontrol as a management strategy for invasive plants.

Key words: Invasive plants, *Calluna vulgaris*, foliar nitrogen, metabolomics, phenylpropanoids, biological control.

5.1 Introduction

Sustainable long-term management of invasive plants is often achieved using specialist phytophagous insects as classical biological control agents (Hayes et al. 2013; Schwarzländer et al. 2018). Not all biological control agents achieve the expected outcome due to either a failure to establish (McFadyen 2000; Hayes et al. 2013), by being ineffective or achieving only variable effectiveness when they do (McClay and Balciunas 2005; Raghu and Dhileepan 2005). Biocontrol agent establishment and/or effectiveness is thought to be influenced by abiotic (Grevstad 1999; Harms et al. 2020) and/or biotic factors (Syrett 1983; Briese 1986; Schulz et al. 2019). While target plant assimilability or nutritional value has been considered for some aquatic plants e.g. Room and Thomas (1986), the same parameters in terrestrial plants have, until recently, seldom been considered as a potential limitation on introduced insect control agents (but see Hinz and Müller-Schärer 2000; Van Hezewijk et al. 2008; Uyi et al. 2016).

Soil nutrient availability is known to influence the levels of both primary and secondary plant metabolites. For example, soil phosphate deficiency influences plants by reprogramming them from primary to secondary metabolism which can result in increased levels of a range of secondary metabolites such as phenylpropanoids (Gershenzon 1984; Malhotra et al. 2018; Wang et al. 2023).

Phenylpropanoids are the largest class of carbon (C) -rich plant secondary metabolites, which include phenolic acids (e.g. benzoic and cinnamic acids) and flavonoids (Kováčik et al. 2007; Ruan et al. 2010) plus flavones, coumarins (Kováčik and Klejdus 2014), quinic acids (Giorgi et al. 2009), anthocyanins, flavonoid glycosides, isoflavones (Gershenzon 1984), stilbenes, terpenoids and tannins, many of which are involved in plant defence against insect herbivores (Bassman 2004; Zhang and Bjorn 2009; Escobar-Bravo et al. 2017; Kumar et al. 2020). Thus, elevated levels of these compounds can result in impaired insect

herbivore performance (Izaguirre et al. 2007; Kuhlmann and Müller 2010; Qi et al. 2018), while reduced levels, can result in improved herbivore performance and/or increased feeding rates (Rousseaux et al. 2001; Ballaré et al. 2012; Dinh et al. 2013; Ballaré 2014).

Soil N deficiency, similar to P, imposes limits on plant metabolism which results in reduced growth and/or lower plant yields (Giorgi et al. 2009). This deficiency induces reprograming of primary and secondary metabolism with a reduction of N-rich amino acids, proteins and enzymes and an increase in C rich phenolic compounds and may also result in a shift of the plants carbon to nitrogen ratio (C:N) (Kováčik and Bačkor 2007; Rubio-Wilhelmi et al. 2012) . Nitrogen is an essential component of the insect diet but at low levels is a limiting factor for growth, survival and reproduction (Mattson 1980; Ren et al. 2022). The primary source of nitrogen for insects are plant proteins which comprise of amino acids — compounds with amino (-NH_2), carboxyl (-COOH) groups, and a specific chain — which, when assimilated are broken down into amino acid components and used as structural proteins, enzymes or receptor molecules (Kraus et al. 2022).

The C:N ratio in plant tissue is important for herbivores as reflected in the Efficiency of Conversion of Ingested food (ECI) model which implies that high ECI foods for herbivores have a higher concentration of N-containing amino acid and proteins coupled with adequate carbohydrates as a proportion of the concentration of C-rich defensive metabolites (Mattson 1980; Henn and Schopf 2001). Evidence also suggests that there are foliar N threshold values above which insect growth may increase with increasing concentration but below which they cease to develop because the N required to build structural proteins is insufficient (Mattson 1980). For example, the chrysomelid beetle *Paropsis atomaria* (Olivier, 1807) (Coleoptera: Chrysomelidae) feeding on *Eucalyptus blakelyi*, (Maiden) (Myrtales: Myrtaceae) larval weight gain decreased and development time increased when foliar N fell below 1.7% dry weight and at 0.8%

dry weight the larvae died before reaching 3rd instar (Ohmart et al. 1985; Ohmart 1991).

Heather beetle *Lochmaea suturalis* (Thomson, 1866) (Coleoptera: Chrysomelidae) was released in New Zealand in 1996 as a biocontrol agent for the highly invasive shrub, heather, *Calluna vulgaris* (L.) Hull. On the North Island Central Plateau most releases failed, and for those that did establish, population growth and expansion rates were poor relative to the rates recorded in its native UK range (Pakeman et al. 2002; Peterson et al. 2007, 2024; Rosenburgh and Marrs 2010; Fowler et al. 2015). This is in contrast to three releases in the Rotorua region where all three established on *C. vulgaris* with % foliar N approximately 50% higher than that of the CP region (Peterson et al. 2011). Now, some two and a half decades later, beetle populations in the CP region are well established and causing widespread damage to heather, and recent findings (Barrett unpublished data) suggests population densities are attaining levels closer to those recorded in the UK (Pakeman et al. 2002; Rosenburgh and Marrs 2010). Damage to *C. vulgaris* to most areas in this CP region is usually severe, but in some areas, beetle effectiveness is variable, with many plants remaining viable.

Soil types of the CP region are very low in N and extremely low in P which can impair the uptake of available soil N (Xia et al. 2023). Heather foliar N levels in the region recorded through the years 2008 to 2010 had a mean value of ~ 1.1 % N gm^{-1} DM (Peterson et al. 2015) whereas foliar N levels in the UK averaged ~ 1.48 % N gm^{-1} DM (Peterson et al. 2011). Previous trials with *L. suturalis* larvae and adults when fed foliage from N and P CP fertilised heather, resulted in improved larval survival and increased adult body size and female fecundity (Peterson et al. 2024). A series of field experiments using un-fertilised and fertilised heather plots at new release sites also demonstrated improved population growth rates and establishment success of *L. suturalis* in fertilized plots compared to unfertilized plots (Peterson et al. 2024). This indicates a direct link between soil

nutrients and biocontrol agent performance. However, how N and P fertilization changes the C:N ratio of CP heather foliage and the balance and interaction of N-containing primary and C-rich secondary metabolites on assimilation efficiency for *L. suturalis* remains unclear.

To explore these questions, we applied N and P fertilizer to naturally growing plants in a field-based experiment, and compared foliar N, C and primary and secondary metabolites, between fertilised and non-fertilised plants. For quantification of foliar N and C content we used a standard Dumas combustion method and for primary and secondary metabolites, UHPLC-MS/MS non-targeted metabolomics. The balance of primary N-containing and secondary metabolites was altered in line with the “growth-defence trade-off” principle for plants under nutrient stress and we discuss these results in the context of improved assimilability and improved control agent performance, and establishment rates. We posit that understanding abiotic influences on targeted invasive plants which result in altered assimilability may provide information with which to mitigate these changes before agent releases and improve biocontrol success rates and management outcomes.

5.2 Materials and Methods

5.2.1 Field experiment, sample collection and storage

On 22nd of Oct 2021 on the North Island CP, New Zealand, a site between Lat. -39.228816, Lon. 175.717081 and Lat -39.230145, Lon. 175.715263.with heather of similar age and architecture and no heather beetle present was established. Eight plots (four times two treatments), each 5x5 m^2 were set out, then three soil cores (30mm dia.) to the depth of 15cm were collected from random positions within each plot. These were analysed post setup, to assess any variation in soil nutrients between plots. After core collection, plots were randomly allocated to

fertiliser (treatment) or no fertilizer (controls) and seven *C. vulgaris* plants tagged within each. Fertiliser was applied as 325.5 g Urea and 1217.5 g Triple Superphosphate per 25 m^2 using the application protocol detailed in Peterson et al. (2024).

On 30th Dec 2021, sprigs of foliage ~ 20 mm long were collected from the tip of ~20-30 stems of each tagged plant in both treatments for foliar nitrogen (N), carbon (C) and metabolite analyses. These samples were immediately cryo-frozen in nitrogen vapour then stored at -80 °C. All plants in both treatments were sampled for herbivorous invertebrates to assess potential amplification of secondary metabolites induced due to insect herbivores. Further information and results are available in Supplemental Information Doc.1 (1.1).

5.2.2 Analytical protocols

Soil cores were air dried (18-25 °C) to zero mass change, passed through a 1mm Endicott precision sieve, homogenised and then analysed for pH, Olsen P (Ols P) and total N (tN) at Hills Laboratories Ltd., Hamilton, NZ. Phosphorus for Olsen P was obtained using $NaHCO_3$ (0.5M, pH8.5) extractant and total N obtained using the Dumas combustion method in a VarioMAX CN Macro Elementar analyser. For pH, soils were slurried (1:2 v/v) soil:H_2O and analysed using a glass bulb pH probe.

Before analysis, foliage samples were freeze dried, stored for 10 days at -20 °C, before grinding to ~150 – 50 µm particle size. For foliar total N and C analyses, 30 ± 0.5 mg of each ground sample was weighed, combined with an equal weight of tungsten (VI) oxide and pressed into foil envelopes for analysis. Using the Dumas combustion method protocol for foliage, samples were analysed at Massey University using a Vario Macro Cube Elementar analyser with furnace

temperature 1150 °C, reduction tube temperature 850 °C and helium flow 600 µl min^{-1}

Extraction of metabolites for UHPLC-MS/MS analysis are fully detailed in Supplemental Information Doc.1 (1.2). Briefly 50 ± 0.5 mg of ground sample was weighed into 2 mL microcentrifuge tubes, 800 µL of pre-chilled chloroform:methanol ($CHCl_3$:CH_3OH; 1:1 v/v) was added, then homogenised for 5 mins and stored for 1 hr at -20 °C. Then, 400ml of H_2O was added to each, homogenised again, and centrifuged to create a biphasic layer. 200 µl aliquots of the upper layer were pipetted into microcentrifuge tubes, one each for C18 and HILIC chromatography analysis and a final 200 µl aliquot added to larger tube to form a homogenous mix for quality controls (QCs). All samples were dried under a continuous flow of N_2 and immediately stored at -80 °C until reconstitution prior to analysis. Immediately prior to LC-MS analysis, all samples plus QCs were reconstituted by adding 200 µl of reconstitution solvent, vortexed until dissolved then transferred to an autosampler vial, capped and chilled until loading for analysis.

Chromatography and tandem mass-spectrometry analysis of polar and semi-polar compounds were achieved using a Thermo LC–MS system (Thermo Fisher Scientific, Waltham, MA, USA) consisting of a Dionex UltiMate 3000 UHPLC coupled with a high-resolution Q Exactive Focus Quadrupole-Orbitrap mass spectrometer utilising heated electrospray ionisation run in both positive and negative modes. For semi-polar compounds, samples were injected into a 1.9 µm Thermo Hypersil GOLD™ C18 column (UHPLC, 100 mm × 2.1 mm, Thermo Fisher Scientific, USA) at 25 °C and for polar compounds, injected into a 5 µm ZIC®-pHILIC column (100 mm × 2.1 mm, Merck Darmstadt, Germany) also run at 25 °C. Details of all chromatographic and mass spectral conditions are detailed in Supplemental Information Doc.1 (1.3)

Thermo derived .raw files for each stream i.e., C18 pos, C18 neg, HILIC pos and HILIC neg modes, were converted to mzML format using MSConvertGUI (Adusumilli and Mallick 2017), uploaded into MZmine (Pluskal et al. 2020) to determine the appropriate baseline noise threshold and then into XCMS online https://xcmsonline.scripps.edu/ for feature detection, alignment and exploratory data analysis (Domingo-Almenara and Siuzdak 2020). Feature detection parameters for C18 data were: *m/z* deviation 10 ppm, min and max peak width 5 and 20 respectively, mzdiff 0.001, s/n threshold 20, Prefilter intensity 1e4 and noise filter 2.5e4. For HILIC data the same parameters were 10ppm, 10 and 60, 0.001, 20, 1e4 and 8e3. After downloading the output, raw mass spectrometry data were organised into data matrices comprising the mean *m/z* and retention times and the corresponding ion intensity measurements for each feature making it suitable for statistical analysis.

5.2.3 Data analysis

We used non-parametric Kruskal-Wallis analysis to test for heterogeneity of soil nutrient parameters (pH, Ols P and tN) between the eight experimental plots prior to fertilization while foliar N levels were analysed using one-way ANOVA.

Analysis of primary and secondary metabolites involves a series of steps beginning with reduction of background variability in the data matrix for each stream using a QC vs Blank t-test thus allowing subtraction of features with p values > 0.05, and secondly, t- values corresponding to any features high in the blanks. These data matrices were each uploaded into MetaboAnalyst 6.0 (MA ver 6.0) (Pang et al. 2021) and data integrity checked to confirm the number of samples, number of peaks, missing values, and the number of treatment groups. No missing values were detected in any of the data sets. For filtration of variables showing low repeatability, the threshold to remove those with high percent relative standard deviation (RSD) was set at 30% to that of the QCs and the data

normalised by auto-scaling (mean-centred and divided by the standard deviation of each variable). Gaussian distribution was confirmed so that feature mass intensities are comparable.

For each stream, using MA ver 6.0, we explored these data matrices, and compared treatment (fertilizer) and control (no fertilizer) data by subjecting them to orthogonal partial least squares discriminant analysis (OPLS-DA); the separation between treatments was statistically confirmed using 20 permutations to provide acceptable R^2, Q^2 and p values. Based on a 95% confidence interval, one outlier (the same plant sample) was removed from all streams except for C18pos which had two removed, before final analysis. Features for annotation were identified by applying a paired t-test to these data matrices and retaining all the features less than the false discovery rate threshold value of $FDR < 0.05$ (Benjamini and Hochberg 1995).

Annotations were conducted and confidence levels confirmed (Sumner et al. 2007) for each metabolite by interrogating the original .raw files using Xcalibur Freestyle. Formula matches were confirmed, and mass accuracy parameters were set within 10 ppm (i.e. +/− 5.0). For all C18 features, confidence levels were set according to Sumner et al. (2007) with level 2 (parent ion plus at least one fragment) and level 3 (the parent ion only) being confirmed using the MassBank.eu (https://massbank.eu/) spectral database. Where the same annotated and confirmed compound (ion) appeared in both positive and negative modes, the one with the highest signal intensity (arbitrary units; au) was included in the final data table. For further verification of the annotated compounds and the potential to discover additional ones not available in the MassBank spectral library, a QC file was uploaded into XCMS-METLIN and spectral matches > 0.9 deemed level 2 confidence. Fold change values were calculated for all annotated compounds.

Using the intensity data of all the annotated metabolites listed in Tables 1 and 2 pathways enrichment analysis was performed using the platform in MA ver 6.0. Metabolic pathways that are significantly influenced by the fertilizer treatment were assessed using the upland cotton (*Gossypium hirsutum*) pathway library as a reference.

For all streams, a literature search provided information on the physiological or defensive functions of a range of both primary and secondary annotated compounds. We then used linear regression analysis to test for any predictive relationship between total foliar N and the intensity values for each compound identified as impacting the pathway analysis and also those identified from the literature as being involved in insect herbivore defence.

5.3 RESULTS

5.3.1 Soil nutrients and foliar N and C.

Analysis of the soil nutrient parameters sampled from all eight plots prior to establishment, showed very low total N levels in all plots (0.22 ± 0.04; min 15, max 32). Kruskal-Wallis analysis showed a significant difference, $H(7) = 15.38$, $p = 0.031$, between plots but post-hoc analysis (Dunns test with Bonferroni adjustment for multiple comparisons) could not reveal which plots differed at $\alpha = 0.05$. Olsen P revealed extremely low levels (2.04 ± 0.55; min 1, max 3) with no significant difference, $H(7) = 11.93$, $p = 0.103$, between plots. Similarly, pH levels (5.77 ± 0.12; min 5.60, max 6.00) between plots showed no significant difference, $H(7) = 9.96$, $p = 0.191$.

Mean foliar total N for fertilized plants (1.49% DW) was significantly higher than that for control plants (1.38% DW), $t(52) = -3.263$, $p = < 0.001$ (Fig. 1a). Despite the variability in soil N between plots, analysis of foliar total N as % dry weight (% DW) using one-way ANOVA demonstrated no significant difference between

the four plots within the treatment experiment (data not shown). The mean foliar carbon to nitrogen (C:N) ratio was 36.75 for control and 33.56 for fertilizer treatment, also significantly different, t(52) = 3.799, $p = < 0.001$, see Fig. 1b.

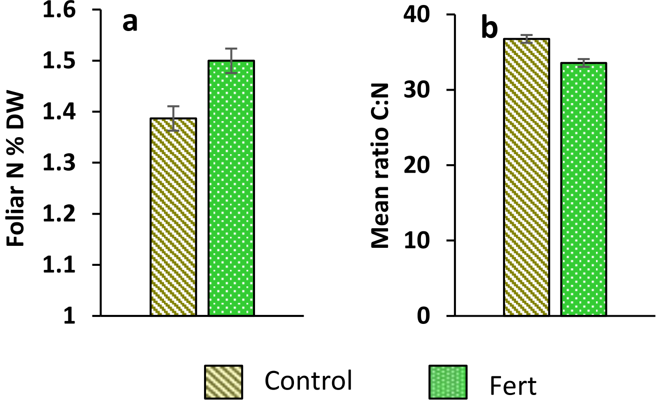

Fig. 1 Total N % dry weight level (a) and carbon to nitrogen (C:N) ratio (b) in *C. vulgaris* foliage from plants growing in control vs N and P fertilized soil (applied 10 weeks prior to sampling). Both foliar N and the mean C:N ratios differed significantly ($p < 0.001$) between treatments. Error bars = one SE.

5.3.2 Metabolite responses to treatments

OPLS-DA demonstrated significant separation between the metabolite profiles of *C. vulgaris* from the control (no fertilizer) and fertilizer groups. Confirmation statistics were for the C18 positive stream: $R^2 = 0.955$, $Q^2 = 0.715$, $p < 0.05$ and for C18 negative stream: $R^2 = 0.967$, $Q^2 = 0.735$, $p < 0.05$. For the HILIC positive stream, $R^2 = 0.969$, $Q^2 = 0.81$, $p < 0.05$ and the HILIC negative stream, $R^2 = 0.939$, $Q^2 = 0.769$, $p < 0.05$ (Fig. 2).

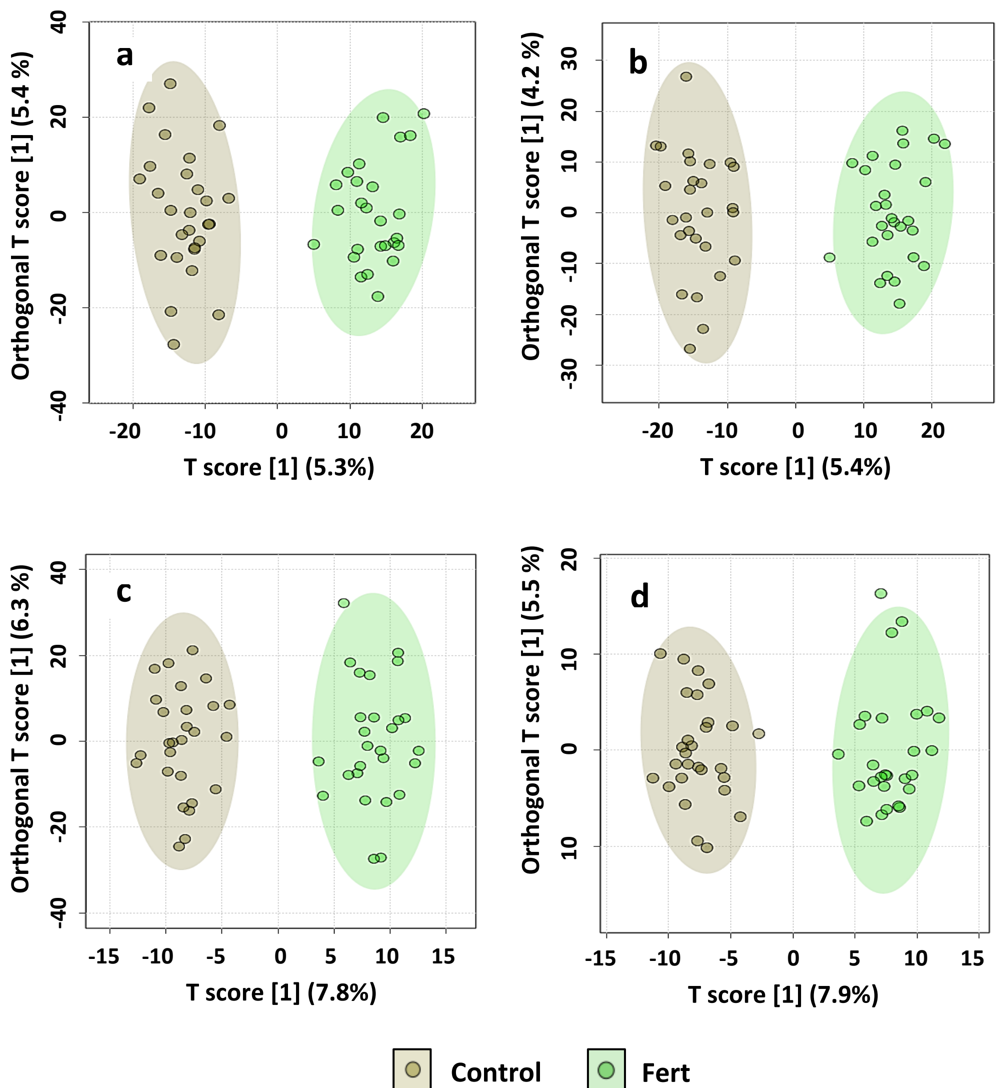

Fig. 2. Differences in metabolite profiles of heather (*Calluna vulgaris*) growing in control soil vs soil after N and P fertilizer (Fert) application 10 weeks prior to sampling. OPLS-DA for significant separation of clusters = $p < 0.05$. for all four HPLC-MS/MS streams. C18 pos (a), C18 neg (b), HILIC pos (c) and HILIC neg (d). Dots within each cluster represent the sampled plants in each treatment and ellipses represent 95% confidence. C18 pos control had two outliers removed and all others had one removed.

A total of thirty-five compounds were annotated from all four streams (Tables 1 and 2). Over both tables, thirteen compounds are allocated level two confidence and the remainder at level three. Of the level two compounds XCMS-METLIN provided three spectral matches not available via the MassBank library. All annotated compound confirmation data is provided in Appendix C.

Changes in metabolite intensity expressed as fold change (FC) implies that a FC of 1 = no change, FC of 1.50 = 50% amplification/reduction, or a FC of 2.5 = 150% amplification/reduction etc. Seven metabolites in the top section of Table 1 are N-containing compounds, five of which were amplified in the fertilizer treatment. Seven compounds in the top section of Table 1 are also primary metabolites identified from the HILIC streams, two of which are common carbohydrates i.e. galactaric acid and a,a-trehalose which are both similarly amplified. Four secondary compounds in the bottom section of Table 1 comprise two terpenes (including camphor), a chalcone and an aldehyde.

In Table 2, twenty-two phenylpropanoids show a range of phenolic glycosides, glucosides and various flavonoids, coumarins and cinnamic acids plus four benzenoids. The fertilizer treatment has resulted in fifteen (68%) phenylpropanoids having reduced intensities and seven being amplified with the four benzenoids all reduced.

Table 1. Primary and secondary metabolites including seven containing nitrogen ions, annotated from the HILIC and C18 streams. All t- test threshold values are FDR < 0.05. Superscript letters refer to plant physiological or effect on insect references, see footnote. P/S = Primary or secondary, KEGG = compound database, STR = stream, A/R = Amplified or reduced, t- stat. = t statistic, FC = fold change, CL = confidence level, m/z = Parent ion, rt. = retention time, Fragments with MS are annotated via XCMS-METLIN with a spectral match score > 0.9.

P/S	Class or sub class	Name	Formula	KEGG	STR	A/R	t- stat.	FC	CL	m/z	rt.	Fragments
1°	Pyrrolidine	2-Pyrrolidineacetic acid [a]	$C_6H_{11}NO_2$		HIL +	↓	-4.9223	2.17	2	130.0859	13.70	MS 1.0
1°	Pyridine	Pyridoxine	$C_8H_{11}NO_3$	C00314	HIL -	↓	-3.1595	1.35	3	168.0656	15.09	
1°	Alpha Amino Acid	L-Valine [b, e, f]	$C_5H_{11}NO_2$	C00183	HIL -	↑	3.0871	1.28	3	116.0706	14.72	
1°	Alcohol polyol	Pantothenic acid [c, d]	$C_9H_{17}NO_5$	C00864	HIL +	↑	8.4034	2.16	3	220.1172	13.70	
1°	Pyrrolidine	Pyrrolidine	C_4H_9N		HIL +	↑	2.9117	1.20	3	72.0809	14.75	
2°	Indole	1-methoxy-3-formylindole	$C_{10}H_9NO_2$		C18 +	↑	3.3148	1.90	2	176.0703	7.09	148.0778
2°	Phenylalanine deriv	N-Fructosyl phenylalanine	$C_{15}H_{21}NO_7$		C18 +	↑	3.1907	1.50	2	328.1383	9.50	311.1168, 311.1092
1°	Carbohydrate	Galactaric acid	$C_6H_{10}O_8$	C00879	HIL -	↑	6.547	1.60	3	209.0295	16.96	
1°	" " " "	a,a-Trehalose [g]	$C_{12}H_{22}O_{11}$	C01083	HIL +	↑	3.3114	1.15	3	343.1223	15.81	
2°	Monoterpenoid	Camphor [h, i]	$C_{10}H_{16}O$	C00808	C18 +	↓	-4.3251	2.18	3	153.1271	9.99	
2°	Chalcone	3,4,2',4',6'-Pentamethoxychalcone	$C_{20}H_{22}O_6$		C18 +	↓	-4.0992	1.58	3	359.1481	10.34	
2°	Terpene Lactone	Costunolide	$C_{15}H_{20}O_2$	C09382	C18 +	↑	4.5116	1.58	2	233.1531	10.14	137.0599 187.1482 214.1431
2°	Med-chain Aldehyde	trans-2-Hexenal	$C_6H_{10}O$	C08497	C18 +	↑	4.0411	1.72	3	99.0805	7.41	

Plant physiological references: Singh & Shaner, (1995) [b], Miret & Munné-Bosch, (2014) [c].
Effect on insect references: Huang et al., (2011) [a]; Levinson, et al., (1967) [d]; Kim & Mullin, (1998) [e]; Genc, (2006) [f]; Tellis et al.,(2023) [g]; Kleine & Müller, (2011) [h]; Lazarević et al., (2022) [i].

Table 2. Secondary phenylpropanoid and benzenoid compounds annotated from the HILIC and C18 streams. All t- test threshold values are FDR < 0.05. Superscript letters refer to references regarding defensive antiherbivore function, see footnote. Ph/Bz = Phenylpropanoid or Benzenoid, KEGG = compound database, STR = Stream, A/R = Amplified or reduced, t- stat. = t statistic, FC = fold change, CL = confidence level, m/z = Parent ion, rt. = retention time, Fragments with MS are annotated via XCMS-METLIN with a spectral match score > 0.9.

Ph/Bz	Class or sub class	Name		KEGG	STR	A/R	t- stat	FC	CL	m/z	rt.	Fragments
Ph	Flavonoid Glycoside	Naringin	$C_{27}H_{32}O_{14}$	C09789	C18 +	↑	3.3452	2.29	2	581.1849	9.11	563.1660 435.1210
Ph	" " " " "	Apigenin 7-O-glucoside	$C_{21}H_{20}O_{10}$	C04608	HIL -	↓	-2.9338	2.02	3	431.0978	2.51	
Ph	" " " " "	Quercetin 3-(6"-acetylglucoside)	$C_{23}H_{22}O_{13}$		C18-	↓	-3.0586	1.60	2	505.0989	10.01	301.0402 300.0304
Ph	" " " " "	Apigenin 7-glucuronide	$C_{21}H_{18}O_{11}$		C18-	↓	-3.0513	1.41	3	445.0776	10.10	
Ph	" " " " "	Kaempferol-3-O-Rhamnoside	$C_{21}H_{20}O_{10}$		C18 +	↓	-3.1122	1.80	2	433.1121	9.90	287.0511 288.0575 129.0539
Ph	Phenolic Glucoside	Dihydrobenzoic acid pentose	$C_{12}H_{13}O_8$		C18-	↓	-3.7425	1.96	3	285.0615	2.77	
Ph	Flavonol Glucoside	Kaempferol-3-Glucoside-2"-p-coumaroyl	$C_{30}H_{26}O_{13}$		C18-	↓	-3.9152	1.41	3	593.1303	6.84	
Ph	Flavonoid Glucoside	Myricetin-3-Xyloside	$C_{20}H_{18}O_{12}$		C18 +	↑	3.9611	1.57	2	451.0862	8.49	320.0488 319.0434
Ph	Flavone	Quercetin [j, k]	$C_{15}H_{10}O_7$	C00389	C18-	↓	-3.4841	1.51	3	301.0353	9.87	
Ph	" " "	Apigenin	$C_{15}H_{10}O_5$	C01477	C18 +	↓	-3.1233	1.70	3	271.0592	10.66	
Ph	Flavan	(+)-Gallocatechin	$C_{15}H_{14}O_7$	C12127	C18 +	↓	-3.3654	1.52	2	307.0807	6.30	MS 0.947
Ph	Isoflavonoid	Formononetin	$C_{16}H_{12}O_4$	C00858	C18-	↑	5.1222	1.81	3	267.0639	4.17	
Ph	Flavanonol	Dihydroquercetin	$C_{15}H_{12}O_7$	C01617	C18-	↑	3.2741	1.41	3	303.0509	7.00	
Ph	Flavone	Tricin	$C_{17}H_{14}O_7$	C10193	C18-	↑	3.265	2.96	3	329.0666	9.59	
Ph	Coumarin	3,4-Dihydrocoumarin	$C_9H_8O_2$	C02274	C18 +	↑	3.113	1.46	2	149.0594	9.50	103.0533
Ph	Hydroxycoumarin	Scopoletin [l]	$C_{10}H_8O_4$	C01752	C18 +	↑	3.6905	2.49	2	193.0492	7.49	MS 0.934
Ph	Cinnamic acid	4-Methoxycinnamic acid	$C_{10}H_{10}O_3$		HIL +	↓	-2.7308	1.43	3	179.0697	13.61	
Ph	Cinnamic acid	trans-Cinnamic acid [m]	$C_9H_8O_2$	C00423	C18-	↓	-3.4846	2.76	3	147.0443	8.46	
Bz	Benzenediol	3,4-Dihydroxymandelic acid	$C_8H_8O_5$	C05580	C18-	↓	-3.365	1.24	2	183.0292	8.91	139.0400 137.0242
Bz	Benzenoid	2,4,5-Trimethoxybenzaldehyde [n]	$C_{10}H_{12}O_4$		HIL +	↓	-2.7546	1.42	3	197.0802	13.63	
Bz	" " " "	2,5-dihydroxybenzoic acid	$C_7H_6O_4$	C00628	C18-	↓	-4.0177	1.49	2	153.0185	2.92	109.0294
Bz	" " " "	Diethylphthalic acid [o]	$C_{12}H_{14}O_4$	C14175	C18 +	↓	-7.6783	2.14	3	223.0961	6.00	

Effect on insect references: Wang et al., (2019) [j]; Gao et al., (2022) [k]; Westcott et al., (1992) [l]; Dixit et al., (2017) [m]; Gu et al., (2025) [n]; Huang et al., (2021) [o].

5.3.3 Pathway analyses show impact on flavone and flavonol biosynthesis

Pathway enrichment analysis identified five major pathways that were upregulated in the fertilized plants. The greatest impacts were on the flavone and flavonol biosynthesis pathway (with apigenin and quercetin as the main contributors) and on the biosynthesis of various plant secondary metabolite pathway (with scopoletin as the main contributor) each with an impact factor of 0.25. The pantothenate and CoA biosynthesis pathway (with pantothenic acid and L-valine as main contributors) was the next most impacted at 0.13, followed by the flavonoid biosynthesis pathway (with apigenin, taxifolin, quercetin and (+)-gallocatechin with a factor of 0.072. Vitamin B6 metabolism had the lowest impact factor of 0.024 due to the presence of pyridoxine. For further details on this analysis see Supplemental Information Doc. 1 (S. Fig. 1).

5.3.4 Relationships between foliar N and primary and secondary metabolites.

Simple linear regression analysis revealed significant positive correlations between total foliar N and two primary metabolites – pantothenic acid ($r^2 = 0.18$, $F(1,25) = 5.75$, $p = 0.02$ and $r^2 = 0.33$, $F(1,26) = 13.25$, $p = 0.001$) in fertilizer and control treatments, respectively) and L-valine ($r^2 = 0.32$, $F(1,25) = 11.78$, $p = 0.002$ and $r^2 = 0.38$, $F(1,26) = 16.31$, $p = 0.001$) in fertilizer and control treatments, respectively (Fig. 3a, b). For secondary metabolites, total foliar N was not significantly correlated with diethylphathalic acid in the fertilizer treatment but a significant negative correlation was evident in the control plants ($r^2 = 0.17$, $F(1,25) = 5.36$, $p = 0.02$ (Fig. 3d). A metabolite of real interest, trans-cinnamic acid, which is the key precursor of all phenylpropanoid derived metabolites, shows no correlation with % foliar N but displays considerably less variation with fertilizer treatment (Fig. 3c).

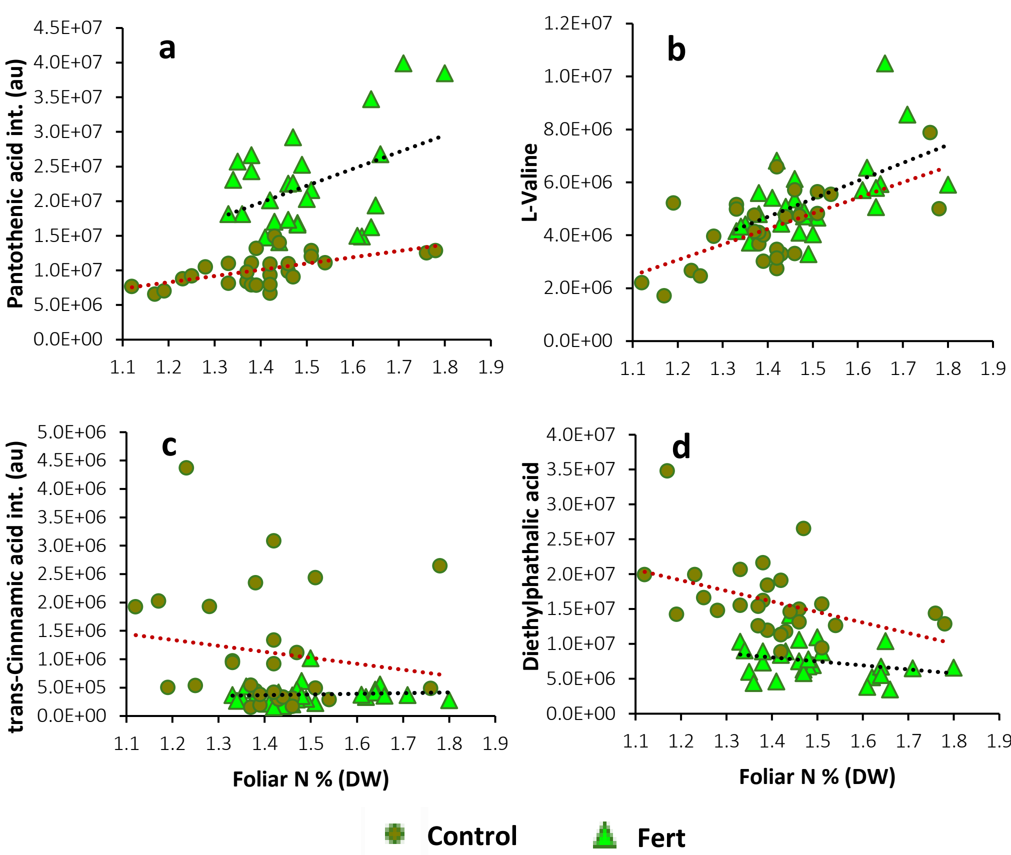

Fig. 3. Total foliar N (% dry weight) vs metabolite signal intensity, arbitrary units (au) in *Calluna vulgaris* growing in control soil vs soil after N and P fertilizer (Fert) application 10 weeks prior to sampling. Primary metabolites pantothenic acid (a) and L-valine (b) show a significantly positive correlation with foliar N in both treatments. The secondary metabolite trans-cinnamic acid (c) has no significant relationship with N but indicates a reduction in mean fold change of 2.76 and a marked reduction in variability in the fertilizer treatment. Diethylphthalic acid (d) is reduced in the fertilizer treatment by 2.14-fold change and shows a significant negative correlation with N in the control treatment. All listed R^2 values significant, $p < 0.05$.

5.4 Discussion

Soil fertilizer application affects the Calluna vulgaris *metabolome*

All four streams used in the HPLC-MS/MS analysis provided a comprehensive assessment of changes to both the primary and secondary metabolites associated with the two treatments. Our results show that application of fertilizer to the soil significantly influences the total foliar N content of *C. vulgaris*, with N % DW increases in the fertilizer treated plants (Fig. 1a). We also demonstrate a significant reduction in the C:N ratio (Fig. 1b) and changes to both of these parameters resulting from enhanced soil nutrients are in line with earlier reports from the literature (Carroll et al. 1999; Power and Collins 2010; Peterson et al. 2024). The mean foliage % N in the control treatments (1.38%) is in the higher range reported for this region (1.00 -1.42%) (Peterson et al. 2024) but was the only heather beetle-free site accessible for the experiment. Changes in foliar N and the C:N ratio with fertilizer application reported here do appear modest by comparison to earlier data (Peterson et al. 2024) but we contend this change sufficiently demonstrates the significant foliar metabolic effects in heather resulting from fertiliser application. The magnitude of foliar % N difference between fertilized and control treatments is ~ 7.4% which we consider to be biologically meaningful given the critical importance of foliar N for insect growth and development (Mattson 1980). Support for this can be extrapolated from the data of Fox and Macauley (1977). That study used various *Eucalyptus* spp. with foliar N differences ranging from 3-19%, all of which provided measurable differences in the efficiency of conversion of ingested food (ECI) for larvae of the chrysomelid leaf feeder *P. atomaria.*

A total of thirty-five compounds were annotated from these analyses which includes seven N containing metabolites, five of which display FC increases. Also, of the thirty-five, twenty-two (~ 68%) secondary metabolite phenylpropanoids

display a FC decrease in these C-rich compounds. Thus, an increase in N containing and a concomitant reduction in many of the C-rich compounds with fertilizer application reduced the C:N ratio in *C. vulgaris* foliage. This result is also consistent with the work reported by Power and Collins (2010) who demonstrated changes to the C:N ratio of *C. vulgaris* due to differential atmospheric N deposition in the UK.

Improved performance of the heather beetle *L. suturalis* has been linked to increased foliar N (due to atmospheric deposition) previously, from work done in the UK and Europe (Brunsting and Heil 1985; Bobbink and Heil 1993; Cuesta et al. 2008). Those reports, however, did not attempt to analyse, the metabolite profile of *C. vulgaris* (i.e., plant primary N-containing and secondary metabolites) to assess possible changes in foliage assimilability and gain deeper understanding of the mechanisms involved. It is well documented that plants are highly sensitive to nutrient supply and in either N, P or K nutrient stress, many, including woody shrubs, in line with the "growth-defense-trade-off" principle (Li et al. 2024), modulate phenylpropanoids and terpenoids. This response, results in elevated levels of secondary metabolites, thus increasing their resistance to herbivores (Gershenzon 1984; Zheng et al. 2021).

Variation of phenylpropanoids including quercetin and trans-Cinnamic acid (Barrett et al. 2024) and a range of volatile terpenoids (Effah et al. 2020) under varying soil nutrients, has previously been established for *C. vulgaris* in the NZ CP environment. When phenylpropanoids are elevated, trans-cinnamic acid, a key metabolite in the shikimate- phenylpropanoid pathway, can also be elevated (Kováčk and Bačkor 2007). Interestingly, this is seen in our results where trans-cinnamic acid in the control plants was elevated and highly variable, perhaps indicating the sensitivity to marginal levels of soil nutrients but markedly reduced and less variable with increased soil N and P (Fig. 3c). This trend was also apparent in camphor, a monoterpenoid synthesised via the entirely separate,

mevalonic acid pathway, again perhaps indicating high sensitivity to nutrient stress and suggesting that these metabolites in *C. vulgaris,* with further confirmation, might be useful bio-markers as indicators of nutrient stress.

Primary metabolite function and effects

Pathways analysis revealed pantothenic acid and L-valine as the two key metabolites that drive upregulation of the pantothenic CoA (Coenzyme A) biosynthetic pathway, but they are also important for insect nutrition. Pantothenic acid is a key precursor for CoA which has a central role in plant metabolism, especially for carbohydrate and fatty acid synthesis (Miret and Munné-Bosch 2014) but is also an important source of vitamin B5 which, if absent from an insect diet, severely impairs development and survival as demonstrated in the dermestid beetle *Dermestes maculatus* (De Geer, 1774) (Coleoptera: Dermestidae) (Levinson et al. 1967).

L-valine is an essential alpha amino acid which plays a central role in plant metabolism, growth and development and is a building block for proteins (Singh and Shaner 1995). It is also an essential amino acid for insect growth and development without which they fail to develop and, it has also been shown to be phagostimulatory to the western corn rootworm *Diabrotica virgifera* (LeConte, 1886) (Coleoptera: Chrysomelidae) (Kim and Mullin 1998; Genc 2006). Considerable FC reduction in the non-protein amino acid (NPAA), 2-pyrrolidineacetic acid was also evident for the fertilized plants (Table 1.) Many NPAAs are directly toxic to insect herbivores by obstructing primary metabolism and/or interfering with neurological processes. Additionally, NPAAs store nitrogen in a form that is metabolically unavailable to herbivores (Huang et al. 2011) and this FC decrease may potentially reduce the defensive capacity in fertilized *C. vulgaris.* The relatively small FC increase in a,a-trehalose may also be important as it is a carbohydrate which circulates in insect haemolymph

providing instant energy and is also associated with insect growth and development (Tellis et al. 2023).

Secondary metabolite effects and detoxification

Secondary metabolites in *C. vulgaris* were dominated by the twenty-two phenylpropanoid derived compounds which include four phenolic acid benzenoids. Of these phenylpropanoids, fifteen (68%) were reduced in the fertilizer treatment. The literature provides evidence that metabolites such as 2,4,5-trimethoxybenzaldehyde, camphor and the phthalic acid ester – diethylphthalic acid (Fig. 3d) – as well as trans-cinnamic acid, scopoletin and quercetin, all function as defensive metabolites against herbivorous insects.

The benzenoid 2,4,5-trimethoxybenzaldehyde has been shown to impact growth and development of *Chilo suppressalis* (Walker, 1863) (Lepidoptera: Crambidae), feeding on rice (*Oryza sativa*) (L) (Gu et al. 2025) and camphor is also considered a growth inhibitor and feeding deterrent (Kleine and Müller 2011). In a controlled trial, camphor significantly reduced the feeding rate of Colorado potato beetle (L*eptinotarsa decemlineata*) (Say, 1824) (Coleoptera: Chrysomelidae) at all concentrations when compared with controls (Lazarević et al. 2022). Less is known about diethylphthalic acid; however, phthalate esters are found in several plant families, have considerable antibiotic, insecticidal and allelopathic capabilities and therefore cannot be discounted from our assessment of *C. vulgaris'* defensive arsenal (Huang et al. 2021).

Trans-cinnamic acid is toxic to larvae of *Helicoverpa armigera* (Hübner, 1808) and *Spodoptera litura* (Fabricius, 1775) (lepidoptera: noctuidae) feeding on cotton (*Gossypium* sp.) (Malvales: Malvaceae) (Dixit et al. 2017). Scopoletin, a metabolite amplified in the fertilizer treatment is effective in reducing survival and mean weight of *Melanoplus sanguinipes* (Fabricius, 1798) nymphs (Orthoptera: Acrididae) (Westcott et al. 1992). Quercetin, a toxin, impairs growth, development

and survival in lepidopteran noctuid larvae, including larval development *of S. exigua*, pupation duration of *H. armigera* and *S. litura* and causes mortality in *S. frugiperda* (Gao et al. 2022). Larval mortality due to quercetin is also recorded in *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Lymantriidae) and *Oedaleus asiaticus* (Bei-Bienko, 1941) (Orthoptera: Acrididae) (Wang et al. 2019; Gao et al. 2022).

The toxicity of trans-cinnamic acid, scopoletin and quercetin to coleoptera species however is less certain. It is likely though, that enzymic proteins such as cytochrome P450, which catalyse reactions to detoxify terpinen-4-ol when ingested by *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Chrysomelidae), or glutathione-S-transferases (GST) that conjugate phenolic compounds in the emerald ash borer *Agrilus planipennis* (Fairmaire, 1888) (Coleoptera: Chrysomelidae) would be upregulated. These enzymes are released into the insect mid-gut in response to elevated phytotoxins and are likely a significant metabolic cost which can negatively impact insect performance (Rajarapu and Mittapalli 2013; Wang et al. 2019; Gao et al. 2022). A recent report by Kshatriya and Gershenzon (2024) suggests that cytochrome P450 and GSTs are a widespread strategy used by all major orders of insect herbivores (including specialists) for the detoxification of many plant metabolites including phenolic based compounds (Schuler 2011; Lu et al. 2021). Further, a report by Pokharel (2023), suggests that whether sequestration or detoxification is the resistance strategy used by insects to nullify plant defences there is likely a metabolic trade off and cost to growth and/or reproduction.

Effects on the ECI

The Efficiency of Conversion of Ingested food (ECI) model in relation to herbivorous insects, demonstrates that the highest ECI's for insect herbivores would be for those feeding on higher concentrations of N-containing amino acid

and proteins, coupled with adequate carbohydrates and low concentrations of defensive metabolites (Mattson 1980; Henn and Schopf 2001). For the primary metabolites, increased levels of pantothenic acid could be an important contributor to an improved ECI due not only to it providing N-containing amino acids but as a source of vitamin B5 critical to insect growth and development. Similarly, L-valine is likely to elevate the ECI by providing increased levels of amino acids. There is less certainty around the contribution of reduced levels of the NPAA 2-pyrrolidineacetic acid, however. Any N is likely to be inaccessible from this metabolite but with a FC reduction of 2.17 the potential for reduced toxicity may be relevant but requires controlled feeding trials focusing on the direct effects of this metabolite to gain a better understanding.

We are uncertain about the decrease in many other secondary metabolites and what degree of influence they may have on the ECI for *L. suturalis*. This is because the magnitude of fold reduction for most of these metabolites is relatively small. Fold difference changes of comparable magnitude for a similar range of secondary metabolites showed no effect in a bioassay measuring *L. suturalis* larval survival and pre-pupal weights (Barrett et al. unpublished data). It seems reasonable to assume that any reduction in the metabolic cost for detoxification for an insect herbivore should improve the conversion and assimilation efficiency of the food ingested. We posit therefore that amplification of N-containing primary metabolites in conjunction with ~ 68% decrease in carbon-rich phenylpropanoids in fertilized *C. vulgaris* should improve the ECI for *L. suturalis*. Such changes should increase the availability of N while at the same time reducing the metabolic costs of digestion for both larvae and adults and provide the impetus for increased larval growth and development and adult female fecundity as demonstrated in our earlier laboratory and field trials (Peterson et al. 2024).

5.5 Conclusions

There are previous reports demonstrating negative impacts of low foliar N in biocontrol agent performance in terrestrial weed biocontrol scenarios (Hinz and Müller-Schärer 2000; Wheeler 2001; Van Hezewijk et al. 2008; Uyi et al. 2016). The poor establishment and population growth of *L. suturalis* reported in Peterson et al. (2024) also confirmed a negative impact of low foliar N on this biocontrol agent in the CP environment. Changes to both primary N-containing and secondary metabolites identified with the application of metabolomics reported here, are the most likely drivers of this scenario. It is apparent that changes to target plant quality resulting in poor ECI's in the invaded environment could be an important factor to consider when predicting or assessing biocontrol agent success.

Early assessment and comparison of foliar total N and C between native and introduced ranges may provide important information to address any release site imbalances due to soil nutrient status and provide an enhanced ECI to improve introduced biocontrol agent establishment success. The population thereafter however has still to contend with poor quality foliage while dispersing. Our earlier metabolomics studies identified metabolites which may be involved in plant defence that are elevated in the CP range possibly due to the influence of high ultraviolet (UV) light compared to the native UK range (Barrett et al. 2024) and the results reported here revealed some of those are not reduced with fertilizer application. Thus, the initial poor performance of *L. suturalis* on the CP may well have been due to a combination of low foliar N and UV elevated defensive metabolites both contributing to the unexpected low population densities and variable effectiveness of this control agent. We contend therefore, the application of metabolomics may enhance our understanding of underlying failed or suboptimal biocontrol outcomes.

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Supplemental Information Doc. 1

1.1 Assessment of heather beetle impact on amplified metabolites

Heather beetle (HB) larvae were the only insect herbivores on the plants at time of sampling and were present in both treatments. These were sorted into 1st, 2nd or 3rd instar and enumerated. Eighteen plants covering both treatments had very low insect loading i.e. between two 1st instar and three 3rd instar larvae per plant. Costunolide, trans-2-Hexenal, naringin, myricetin-3-xyloside, formononetin, dihydroquercetin, tricin, 3,4-dihydrocoumarin and scopoletin are the seven secondary metabolites showing amplification (see Table 2). Paired t- tests using the mass spectral intensity data (au) for each of these secondary compounds, comparing the plants with insects and those with no insects showed no significant difference (all p = > 0.05) between those groups. Insect herbivory therefore was not associated with amplification of any of these metabolites.

1.2 Metabolite Extraction protocol for UHPLC-MS/MS analysis

For extraction of metabolites for UHPLC-MS/MS analysis, 50 ± 0.5 mg of ground sample was weighed into 2 mL microcentrifuge tubes, each containing a 2.5mm glass bead. To each tube, 800 µL of pre-chilled chloroform:methanol ($CHCl_3$:CH_3OH; 1:1 v/v) was added and then homogenised for 5 mins at 25 Hz sec^{-1} using a Retsch MM400 Mill/TissueLyser and stored for 1 hr at -20 °C. Then, 400ml of HPLC grade H_2O was added to each tube, similarly homogenised, then centrifuged (Sigma 1-14K microcentrifuge) at 4 °C and 11000 RPM for 15 mins to create a biphasic layer. From each sample, 2 x 200 µl aliquots of the upper layer were pipetted into 2 mL microcentrifuge tubes for C18 and HILIC chromatography analysis. A final 200 µl aliquot was added to a 150 ml tube, ultimately pooling every sample into a homogenous mix, then sub-aliquots (200 µl) of this mix were transferred into microcentrifuge tubes to use as quality

controls (QCs). All samples were then dried under a continuous flow of N_2 (3.5 L min^{-1}) at 38 °C for 60 min using a BT LabSystems sample concentrator and immediately stored at -80 °C until reconstitution.

Reconstitution solvents which included an internal standard (Cat. # MSK-QC-KIT) (Cambridge Isotope Laboratories Inc.) at the concentration of 10 µl ml^{-1} were prepared for C18 analysis in acetonitrile: water (CH_3CN:H_2O; 1:9, v/v) and for HILIC in acetonitrile:water (CH_3CN:H_2O; 1:1, v/v). Immediately prior to LC-MS analysis, all samples plus eight QCs were reconstituted by adding 200 µl of solvent, vortexed until dissolved and transferred by pipette to a 200 µl glass insert in an amber autosampler vial, capped and kept chilled until loading. The sequence was: five vials of reconstitution solvent only (blanks), one amino acid standard (A9906; Sigma-Aldrich, NZ), two QCs,56 samples with a QC every 8th slot to finish with a final amino acid.

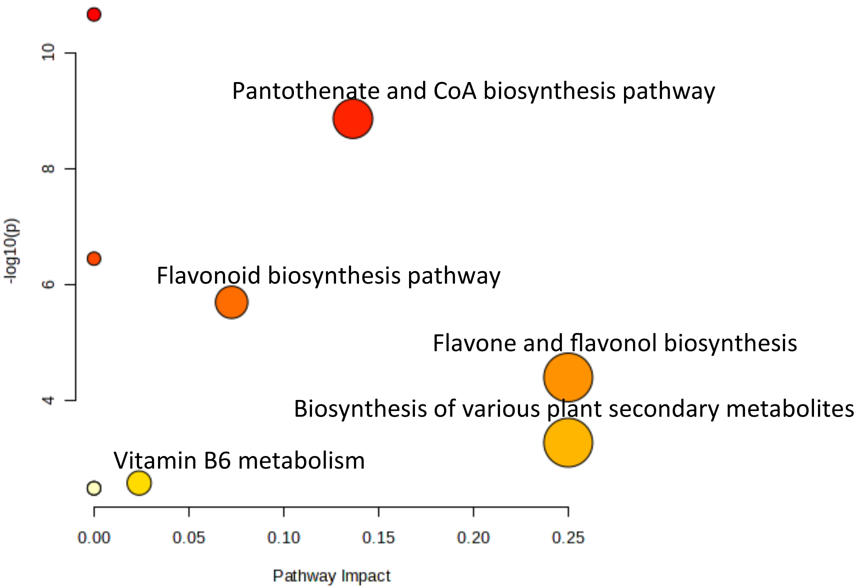
1.3 Chromatography and tandem mass-spectrometry conditions.

Chromatography and tandem mass-spectrometry analysis of polar and semi-polar compounds were achieved using a Thermo LC–MS system (Thermo Fisher Scientific, Waltham, MA, USA) consisting of a Dionex UltiMate 3000 UHPLC coupled with a high-resolution Q Exactive Focus Quadrupole-Orbitrap mass spectrometer utilising heated electrospray ionisation run in both positive and negative modes.

For semi-polar compounds, samples were cooled in the auto-sampler at 4 ˚C and a 5 μL aliquot was injected into a 1.9 µm Thermo Hypersil GOLD™ C18 column (UHPLC, 100 mm × 2.1 mm, Thermo Fisher Scientific, USA) at 25 ˚C with a gradient elution programme and a flow rate of 350 μL min^{-1}. The mobile phase was water with 0.1% formic acid (solvent A), and acetonitrile (C_2H_3N) with 0.1% formic acid (solvent B). Using the Xcalibur software package provided by the manufacturer the gradient elution programme was: held at 5% B (0–3 min), 5–80%

B (3–13 min), held at 80% B (13–16 min), 80-100% B (16.0-16.5 min), held at 100 % B (16.5-18 min) returned to 5% B (18–19 min) and allowed to equilibrate for a further 4 min prior to the next injection. Full mass scan spectral data were collected in profile mode over a mass range of 60–900 *m/z*, at a mass resolution setting of 70,000 with a maximum trap fill time set to auto. ddMS^2 parameters were: resolution 17,500, isolation window 1.5 *m/z*, collision energy of 25 with a maximum trap fill time set to auto. Samples were run in both positive and negative ionisation modes separately. Positive ion mode parameters were: spray voltage 3.5 kV; capillary temperature 320 °C; auxiliary heater temperature 350 °C, sheath gas flow (N_2) 35, auxiliary gas 8, and sweep gas of 1 (arbitrary units). Negative ion mode parameters were the same except for spray voltage, −3.5 kV.

For polar compounds, samples were cooled in the auto-sampler at 4 °C and a 5 µL aliquot was injected into a 5 µm ZIC®-pHILIC column (100 mm × 2.1 mm, Merck Darmstadt, Germany) at 25 °C with a gradient elution programme and a flow rate of 150 µL min^{-1}. The mobile phase was 10mM NH_4COOH in water pH adjusted to ~ 4 with formic acid (solvent A) and 97% CH_3CN (solvent B). The gradient elution programme was: 95% B (0-2 min), 95–20% B (2–17 min), held at 20% B (17-19 min), returned to 95% B (19–19.5 min) and allowed to equilibrate for a further 9.5 min prior to the next injection. Full Mass scan spectral data were collected in profile mode over a mass range of *m/z* 60-900 at a mass resolution setting of 70,000 with a maximum trap fill time set to auto. ddMS^2 parameters were: resolution 17,500, isolation window 1.5 *m/z*, collision energy of 25 with a maximum trap fill time set to auto. Samples were run in both positive and negative ionisation modes separately. Positive ion mode parameters were: spray voltage 3.5 kV; capillary temperature 320 °C; auxiliary heater temperature 280 °C, sheath gas flow (N_2) 25, auxiliary gas 8, and sweep gas of 1 (arbitrary units). Negative ion mode parameters were the same except for spray voltage, −3.5 kV.



S. Fig. 1. Pathway enrichment analysis for *Calluna vulgaris* metabolites resulting from the control and treatments plants. The x axis provides the pathway impact value, and the y axis indicates –log10(p) value of significance between the two treatments. The size and colour of the dot represent the impact value, and the pathway *p* value respectively i.e. increasing *p* values change from yellow through to red.

Chapter 6

General Discussion

An impressive result! Above: the view towards Mt. Ngauruhoe in Tongariro National Park showing the extent of heather invasion. Below, shows the grey-coloured twigs and litter (foreground and several hundred meters toward the mountain) are all that remain after heavy heather beetle damage. The lower image was taken September 2020 some 24 years after the first release of the biocontrol agent.

6.1 Rationale and main findings

6.1.1 Summary

This thesis explores how abiotic stressors may change the plant biochemical phenotype in an invasive weed, which in turn could potentially impact insect biocontrol agent establishment and effectiveness. The results suggest that understanding biochemical phenotypic changes in targeted invasive plants may benefit the planning, testing and implementation of weed biocontrol programmes or retrospectively elucidate establishment failures or poor effectiveness of introduced agents. This is now possible due to advances in metabolomics, which characterises the biochemical profile (the metabolome) of biological tissue in response to its natural or experimentally manipulated environment (Arbona et al. 2013; Jorge et al. 2016; Sampaio et al. 2016).

The biological control programme introducing heather beetle (*Lochmaea suturalis*) as a biocontrol agent against heather (*Calluna vulgaris*) in New Zealand (NZ) proved to be problematic in its early stages (Peterson et al. 2004, 2024; Fowler et al. 2015). This provided an ideal model system to apply metabolomic techniques to investigate biocontrol agent performance in relation to plant biochemical phenotypic change. This thesis (Chapter 3) compares the biochemical phenotype of the invasive plant (*Calluna vulgaris*) in its native United Kingdom (UK) range and in its invaded range on the Central Plateau (CP) of North Island, NZ. Chapter 4 compares, in a controlled experiment, changes to the metabolome of *C. vulgaris* after exposure to two intensities of ultra-violet (UV) radiation and an assessment of those changes on *L. suturalis* larval growth and survival. Chapter 5 assesses change in the primary and secondary metabolome of *C. vulgaris* after manipulating soil nutrient availability with the application of fertilizer in a field experiment and interprets how these changes may alter assimilability of the foliage for *L. suturalis*. In the next sections, I summarise the key findings of each chapter and discuss the overall results.

6.1.2 Chapter 2

Chapter 2 is a literature review focusing on the fundamental question of why some introduced biocontrol agents do not perform as expected given that they are highly specialised species with a close co-evolutionary history to their host or target plant. This scenario is not uncommon in classical biological control of weeds (McFadyen 2000; Hayes et al. 2013) yet target plant defensive biochemistry is rarely considered as a factor in these problematic programmes. The plant biochemical phenotype determines the level of primary nitrogen containing compounds, defensive plant secondary compounds and thus, the assimilable nutritional value of foliage. Such phenotypic change can be altered by several abiotic factors (Ramakrishna and Ravishankar 2011; Clavijo McCormick 2016; Yang et al. 2018; Shaar-Moshe 2019) which in turn, can profoundly affect insect life history parameters (Wittstock and Gershenzon 2002; Howe and Jander 2008; War et al. 2012, 2018). This is the foundation for the central question, "are abiotically induced alterations to the biochemical phenotype of *C. vulgaris* influencing heather beetle (*L. suturalis*) performance on the CP?" and consequently, generates all the hypotheses tested throughout this thesis. The published paper resulting from this chapter also provided several suggestions where metabolomics might contribute to the science of biological control to potentially improve successful outcomes.

6.1.3 Chapter 3

Based on the knowledge that UV light and soils differ between the NZ CP range and those of the UK, Chapter 3 investigated if *C. vulgaris* plants in NZ may be biochemically dissimilar to those in its native UK range, therefore exposing *L. suturalis* to different primary and secondary metabolites in the invaded CP range. The null hypothesis $H_0 1$: *C. vulgaris* plants in the invaded NZ range are not biochemically dissimilar to those of the native UK range was formulated to test

this, Thus, *C. vulgaris* plant samples sourced from Scotland (UK) and the CP (NZ) were analysed using UHPLC-MS non-targeted metabolomics but showed significant differences between samples from each range, hence we reject this null hypothesis. The CP samples had the higher number of amplified metabolites, both in the primary and secondary classes. Most primary metabolites were amplified in the CP samples compared with those from the UK, suggesting an increased level of metabolic activity for the plants in NZ range. Several of these amplified primary metabolites are known precursors of the shikimate–phenylpropanoid pathway and many of the downstream secondary metabolites annotated are known to have effective photoprotective (Gill et al. 2015; Wargent et al. 2015) and/or antiherbivore functions (Bassman 2004; Zhang and Bjorn 2009; Escobar-Bravo et al. 2017; Kumar et al. 2020).

These samples were also analysed for secondary metabolite variation within each range to explore if the metabolite profile of *C. vulgaris*, varies between sites within each range. Thus, the null hypothesis $H_0 2$, was the metabolite profile of *C. vulgaris* does not display variation between sites within each range.

The analyses revealed significant differences in individual metabolites between sites and thus we also reject that null hypothesis. This result provides evidence that metabolites do differ between sites, however overall, there was very little correlation with soil nutrient parameters between sites. Metabolite variability between sites could potentially be involved in inter-site variation of *L. suturalis* performance and therefore effectiveness between sites. Thus, the interaction of soil nutrient status and inter-site control agent effectiveness required additional exploration.

6.1.4. Chapter 4

Results discussed thus far, demonstrate the plasticity of secondary metabolites in *C. vulgaris* both between native and invaded ranges and within each range.

Similar plasticity in response has been demonstrated in this plant with reference to altitude, light variability, soils (Monschein et al. 2010) and seasonality (Jalal et al. 1982) in Europe, and in response to multiple abiotic factors in NZ (Effah et al. 2020a, 2020b). Evidence exists that UV radiation markedly induces alterations to the shikimic acid-phenylpropanoid metabolic pathway (Bassman 2004; Singh et al. 2021, 2023). This can result in changes to phenylpropanoid derived metabolites such as flavonoids, flavonol glycosides and hydroxycinnamic acids (Kuhlmann and Müller 2010; Barnes et al. 2017) which may be photoprotective (Gill et al. 2015) or in the form of phenolic acids, coumarins, chlorogenic acids, quinic acids, stilbenes or tannins, be involved in plant defence against insect herbivores (Bassman 2004; Zhang and Bjorn 2009; Escobar-Bravo et al. 2017; Kumar et al. 2020).

Our knowledge of the differences in UV intensity between the CP and most regions of the UK provided background information for the null hypothesis $H_0 3$, that reduced intensity of ultra-violet does not induce an alteration of phenylpropanoid derived secondary metabolites in *C. vulgaris.* This was addressed by exposing *C. vulgaris* plants to two levels of UV intensity and measuring the changes in secondary metabolites as well as larval survival and prepupal liveweights of *L. suturalis.*

Reducing ultra-violet radiation to less than 5% of ambient (>95% attenuation) demonstrated a significant response for a small number of secondary compounds and the consequent rejection of the null hypothesis. For seven of these compounds, confirmation that UV is potentially driving the changes is supported by the literature and a further four are also confirmed by the literature to have antiherbivore defensive capability. Interestingly no changes were recorded in the primary metabolite pathways. However, the bioassay conducted to test the null hypothesis, $H_0 4$, that altered levels of secondary metabolites in *C. vulgaris* do not affect larval survival and prepupal liveweights of *L. suturalis,*

showed no significant differences. Thus, we accept that changes in secondary defensive metabolites did not affect *L. suturalis* prepupal weights or larval survival. However, the magnitude of change to these metabolites was relatively small and therefore may be insufficient to affect insect life history parameters thus, the effect of UV-induced alterations to secondary metabolites of *C. vulgaris* foliage on *L. suturalis* performance in the CP environment remains uncertain.

6.1.5. Chapter 5

The young volcanic soils of the CP are very low in nitrogen (N) and extremely low in available phosphate (P), which for plants can result in nutrient stress and low total foliar N content (Xia et al. 2023). According to the "growth-defence trade-off" hypothesis, low foliar N levels result in higher levels of stored carbon (C)-rich secondary metabolites (Li et al. 2024). This is reflected in an increased C:N ratio, which in turn, can be reflected in a poorer efficiency of conversion of ingested food (ECI) index, a parameter often used to assess food assimilability in herbivores (Mattson 1980; Henn and Schopf 2001).

Nitrogen is an essential component of insect diets but is also a limiting factor for growth, survival and reproduction (Mattson 1980; Ren et al. 2022). Earlier work in Europe (Bobbink and Heil 1993; Cuesta et al. 2008) and on the CP (Peterson et al. 2024) provided confirmation that elevating foliar nitrogen in *C. vulgaris* results in increased performance for both larval and adult life stages of *L. suturalis,* as well as positively influencing *L. suturalis* establishment and population densities.

Questions surrounding how the profile and ratio of primary N containing and C rich secondary metabolites of *C. vulgaris* respond to soil nutrient status were addressed in a controlled field experiment. The null hypothesis being, H_05, that application of fertilizer to soil does not induce change to the metabolite profile, foliar % N and the C:N ratio of *C. vulgaris.* An assessment of this may help clarify

whether altered primary and secondary metabolite levels in CP heather and the possible impacts on *L. suturalis* performance is due to poor soil nutrient status or elevated intensity of ambient UV. The significant increase in foliar % N, and changed C:N ratio, plus amplifications of some primary N containing and reduction of many secondary metabolites, of *C. vulgaris* after fertilization implies we reject this null hypothesis. This result is in line with earlier reports from the literature (Carroll et al. 1999; Power and Collins 2010; Peterson et al. 2024) and is consistent with the concept that an increase in foliar N suggests a shift from secondary to primary metabolism resulting in a reduced C:N ratio (Wang et al. 2023).

Phenylpropanoids were the major group of secondary metabolites annotated and the literature confirms that many of those annotated function as defensive metabolites against herbivorous insects (Wang et al. 2019; Dixit et al. 2017; Gu et al. 2025). Reduction in intensity of ~ 68% of this group then suggests a reduced metabolic cost to detoxify or sequester these metabolites. Thus, amplification of N-containing primary metabolites in conjunction with the considerable reduction of carbon-rich phenylpropanoids in fertilized *C. vulgaris* should improve the ECI and assimilability of that foliage for *L. suturalis* and help explain the improved performance of the life stages reported in Peterson et al. (2024).

6.2 Integration of results and synthesis

This section has been structured around five main areas: 1) primary metabolite and 2) secondary metabolite responses; 3) soil nutrients vs UV as a driver of *L. suturalis* performance; 4) the complexity of metabolite responses and 5) limitations.

6.2.1 Primary metabolism responses

There were fundamental differences in the metabolic pathways detected in plants in the NZ vs UK data, the fertilizer trial and the UV trial.

The NZ vs UK results indicated differences in the alanine aspartate and glutamate metabolism pathways. These primary metabolites comprise a mix of sixteen amino acids with fourteen of them being amplified in the NZ plants. Two of the amino acids amplified in these data (e.g. L-phenylalanine and L-tyrosine) are known as key precursors of the shikimate-phenylpropanoid pathway which, when upregulated, suggests the amplification of secondary metabolism in response to abiotic stressors, potentially UV, soil nutrients or a combination of these.

In the fertilizer trial, the pantothenate acid and CoA biosynthesis pathways were the most impacted. Plants produced seven entirely different primary metabolites with one amino acid (L-valine) being identified from five that are N-containing compounds. This response of *C. vulgaris* in the fertilizer trial indicates that fertilization, as expected, promoted increased growth, higher total foliar N content (thus reduced C:N ratio) and amplification of a range of N-containing metabolites. This fundamental shift in pathway activity is seemingly the response to releasing *C. vulgaris* from nutrient stress.

Conversely, no response in the primary metabolites or pathway activity was recorded in the UV attenuation trial. This could be due to a reduction in the environmental variability of potential stressors in a controlled environment. These might include warmer and relatively stable temperatures, no wind, constant soil moisture or reduced competition and/or allelopathy. While primary metabolites would certainly be involved as precursors and catalysts of secondary metabolism, it seems that the only measurable response was in those secondary metabolites being directly influenced by the intensity of UV.

6.2.2 Secondary metabolism responses

In all trials, *C. vulgaris* plants shared three common secondary metabolism pathways. The flavone and flavonol biosynthesis and the flavonoid biosynthesis pathways were common to plants in all three studies, while the phenylpropanoid biosynthesis pathway was common to plants in the NZ vs UK and in the UV-attenuation studies. However, very few individual metabolites were shared across studies. The only secondary metabolite to occur in the plants of all three studies was kaemferol-3-glucoside-2"*p*-coumaroyl which was reduced in the CP sites and the fertilizer trial but amplified in the UV trial, suggesting this metabolite may be very sensitive to abiotic environmental perturbation.

From the list of annotated secondary metabolites, those in common to *C. vulgaris* in the NZ vs UK study and the UV attenuation trial were N-fructosyl tyrosine, myricetin, kaemferol-3-glucoside-2"*p*-coumaroyl, sinapoyl malate 4-hydroxycoumarin, caffeic acid and chlorogenic acid. All but sinapoyl malate have reversed their response when exposed to 95% attenuation of UV. Caffeic and chlorogenic acids, plus *p*-coumaric acid (the isomer of o-coumaric acid found in the CP plants) all amplified in the CP environment, have reduced intensities when exposed to the 95% UV attenuation treatment. Responses to UV by these three metabolites are reported in the literature (Lavola et al. 1997; Ruhland et al. 2005) which supports the notion that some secondary metabolites are responding to and being amplified due to elevated levels of UV in the CP environment.

Only four metabolites were in common between *C. vulgaris* in the NZ vs UK and in the fertilizer trial: apigenin 7-O-glucoside, dihydroquercetin, quercetin and kaemferol-3-glucoside-2"*p*-coumaroyl. Apigenin 7-O-glucoside was the only one to display reversed intensity in the fertilizer treatment, perhaps indicating little influence of the improved soil nutrient status on these four metabolites.

6.2.3 UV vs soil nutrients as drivers of L. suturalis performance

Of the eighteen secondary metabolites annotated from the UV attenuation trial, fourteen (88%) were reduced in intensity after exposure to the 95% attenuated UV treatment. While the literature indicates that many phenylpropanoid derived metabolites are involved in plant defence, only four of these (caffeic acid, *p*-coumaric acid, coumarin and chlorogenic acid) have actually been shown to have defensive capability against herbivores (Dixit et al. 2017; Hussain et al. 2018; Kundu and Vadassery 2019; Sing et al. 2021). A relatively small reduction in magnitude of those four metabolites may explain why no effect (improvement) was evident in either prepupal live weights or larval survival of the herbivore – *L. suturalis* – in this study.

It seems that the relatively small fold changes recorded in the UV attenuation trial are well within the metabolite variability that the beetle encounters in its original native environment. Thus, while there may be a reduction in the metabolic cost of detoxification (or sequestration), the small magnitude of change may not make any appreciable difference to the ECI and manifest as an improvement in growth and development for *L. suturalis*. Conversely, the magnitude of change in secondary metabolites recorded for CP *C. vulgaris* in the NZ vs UK study was also within the variability found in the native range and smaller than the 5 – 10 fold change required for impairment of growth and/or survival shown for other specialist insects (Harvey et al. 2007; Ali and Agrawal 2012; Richards et al. 2012). Thus, for secondary metabolites, heather plants in the CP do not appear to have enhanced defensive capability against *L. suturalis* sufficient to impair the beetle's performance. Further work is required to identify the metabolites that are actively involved in plant defence against *L. suturalis* and manipulate these using UV attenuation, before repeating similar bioassays.

In the fertilizer trial, heather plants produced a very different set of secondary metabolites as compared to the plants under UV-attenuation, demonstrating the complex nature of the same pathways being upregulated by different abiotic factors. This made a direct comparison for any effects imposed by secondary metabolites on *L. suturalis* larvae difficult. As with UV-treated foliage, decreased secondary metabolites should improve the ECI for *L. suturalis* but now, due to increased foliar N, a measurable response in insect performance becomes evident. The amplified levels of N-containing primary metabolites in the fertilized plants seem almost certainly to be promoting growth, survival and adult fecundity in *L. suturalis* as demonstrated in the Peterson et al. (2024) trials.

6.2.4 Complexity and unknowns of responses

Non-targeted metabolomics focuses only on the difference in signal intensity for a given metabolite between the environments or treatments that the plant has been subjected to. It is also limited to the metabolites deposited in online spectral libraries for annotation and therefore doesn't necessarily identify the entire metabolome associated with that plant. Some of these unknowns may be influential in herbivore or photoprotective defense but are not altered by changes to environmental or treatment conditions.

Unknown influences may also apply to some primary metabolites such as non-protein amino acids (NPAAs), which are involved in multiple functions including stress signaling, nutrient acquisition and allelopathy but also can be highly toxic to insect herbivores (Vranova et al. 2011; Rodrigues-Corrêa and Fett-Netto 2022). While little is known of its function as a defensive metabolite, the NPAA 2-pyrrolidineacetic acid was reduced with fertiliser application and may potentially be improving the ECI for *L. suturalis.* Conversely, the NPAA γ-aminobutyric acid (GABA) was amplified in the CP plants as compared to those of the UK but has not been reduced with fertilizer application. Thus, in the naturally occurring

unfertilized heather environment, it is possible that metabolites such as GABA, independent of secondary metabolites, may be active in the *C. vulgaris* defence system and continue to impact the performance of *L. suturalis* in the CP population.

Some secondary metabolites which may also play a role in herbivore defence, including flavonoids, coumarins and hydroxycinnamic acids, were amplified in the CP plants but not reduced with fertilization. There is also a possibility that the ECI following fertilization of CP plants may not be improved as much as it might be under atmospherically enhanced nitrogen conditions in the UK (Power and Collins 2010) where, according to our earlier study (Barrett et al. 2024) many primary and secondary metabolites seem to occur at lower concentrations.

The lack of metabolites in common between the heather plants across studies from this work is an indication of the highly complex nature of metabolic processes and their responses to a range of abiotic conditions. Added to this is the possibility of combined effects in the natural environment, where several abiotic factors may additively or synergistically affect the other. For example, both UV and soil nutrients upregulating primary metabolic pathways while at same time upregulating (or down regulating) the shikimate-phenylpropanoid pathway and related downstream metabolites. The recent reassessment of secondary metabolites functioning in multiple roles associated with primary metabolism (Erb and Kliebenstein 2020) supports the idea of considerable pathway interaction and crosstalk being induced by different abiotic stresses. Temperature, drought, UV, shade, salinity and heavy metals are examples of factors that amplify reactive oxygen species (ROS) causing oxidative damage in plants (Foyer et al. 2016; Escobar-Bravo et al. 2017; Kumar et al. 2023). ROS are highly reactive metabolites that interact with a vast array of biomolecules to induce interactive crosstalk and alteration of biochemical pathways to ultimately

reduce ROS and maintain homeostasis, defensive capability and overall plant integrity (Foyer et al. 2016).

Responses for *C. vulgaris* identified from these studies also do not include the potential influence of genetic variation between plants within each population or between populations of each range. Literature suggests, however, that in any given habitat abiotic factors can induce greater changes to metabolite variation than do genetic differences (Robinson et al. 2007; Frank et al. 2012). Since the studies detailed here, population genetic analyses of *C. vulgaris* in the CP and SC ranges have been completed. These indicate that for SC, genetic differentiation between the populations sampled is not detectable. That is, the populations comprise of random continuously outcrossing plants which is maintaining the same genotypic and allelic frequencies (V. Symonds pers. comm. 2026). Thus, in chapter 3 the significant differences in metabolite intensities between sites within the SC population are almost certainly driven by abiotic conditions. Further, while there is more genetic variation of *C. vulgaris* within the CP population there is no detectible pattern that would suggest the between site metabolite differences in this region are being influenced by genetic variables. Thus, abiotic influences are again, the most likely driver. Notwithstanding this however, genetic variability is a factor that perhaps should be considered when assessing metabolite change to multiple abiotic parameters, and if combined with transcriptomics, might provide additional information with which to isolate cause and effect for each abiotic parameter.

6.2.5 Limitations and future studies

The UV attenuation trial and the larval bioassay may not accurately represent plant and larval responses to the UV manipulated conditions. The field-collected plants used in the UV attenuation trials were mature, having spent all their life under ambient light conditions and would have been acclimated to those UV

intensities. It is uncertain if the relatively short exposure to substantially attenuated (>95%) UV intensity would impose the same changes in the metabolite profile as might occur in a plant grown from seedlings in the same experimental environment. Neither is there certainty that the 95% attenuation treated plants would have metabolite profiles and levels representative of those from the UK. Unfortunately, direct comparisons of these data are not possible, because samples for the UK analyses were run in a different HPLC-MS using slightly different conditions than the UV (and fertilizer) NZ samples.

The larval bioassay trial using *C. vulgaris* cuttings also may not be entirely representative. Plant stems, once severed, can initiate a cascade of stress responses in living plants and produce wound responses including amplification of jasmonic and abscisic acid post severance (Da Costa et al. 2013). How *C. vulgaris* cut stems respond after UV alteration of secondary metabolites, if at all, is not known, thus these trials may not have exposed larvae to the actual suite of defensive compounds, as well as their potential for synergistic affects, as could be expected from an intact living plant (Barbehenn and Kochmanski 2013). For this reason, bioassay trials should, wherever possible, be conducted using living plants. The compromise under such conditions, however, is the potential for loss of insects to the substrate, which for a multi-stemmed shrub like *C. vulgaris* would be challenging to prevent but not unachievable.

The insect life history parameters measured in this thesis focused just on larval performance, which provides only part of the equation when assessing this beetle's population dynamics in relation to its establishment and effectiveness. In addition to the adult parameters assessed in the field trial of Peterson et al. (2024) (i.e. fecundity), pre overwinter abdominal lipid accumulation in adults needs to be assessed. Evidence suggests that stochastic and highly variable temperatures prevalent in the CP environment challenge the overwintering capacity and survival of *L. suturalis* adults (Peterson et al. 2011). Overwintering

adults, as with many Northern Hemisphere insects utilize a freeze avoidance strategy reliant on cryoprotective compounds. Low lipid reserves induce early emergence and loss of cryoprotection and when coupled with spring freeze events makes this species vulnerable in the sub-alpine CP region. To assess the effect of foliar N, metabolites and assimilability on this overwintering adult stage, however, requires an entirely different set of phenologically more advanced plants (i.e., those at budburst to post flowering with a concomitantly altered biochemical phenotype) that prevail through the summer and autumn seasons leading into winter.

Finaly, recently gathered field survey data indicates that there may be another factor at play regarding *L. suturalis* performance and concomitant population growth. Evidence points toward a density-dependent positive feedback loop, whereby *L. suturalis* larvae growth and body weights are significantly improved when feeding on *C. vulgaris* plants that are suffering severe damage due to high larval densities. A hypothesis may be that the plant biochemical defence systems are being overwhelmed by high grazing pressure, leading to a substantial improvement in the ECI. Foliar samples taken for metabolomic and C:N ratio analysis from lightly damaged and heavily damaged plants have been collected and will be processed forthwith to verify if this is the case. If such a shift in the biochemical defense system of the plant is revealed, it will be interesting to observe the magnitude of those changes. That information may help to confirm the conclusion reached on the lack of effect in the bioassay trial conducted in Chapter 4. If this hypothesis stands, such information could have important and practical applications for future biocontrol release strategies and planning.

6.3 Conclusions

This work is among the first to apply non-targeted metabolomics to elucidate the role of changes in the plant biochemical phenotype, i.e. primary and secondary

metabolites, in classical biological control of an invasive plant. It demonstrates that such changes in the shrub *Calluna vulgaris* in the invaded NZ range are indeed exposing the insect control agent, *Lochmaea suturalis* to host plants that are biochemically dissimilar to those in its native UK range.

These analyses have revealed metabolites never before recorded in *C. vulgaris.* Little is known of the role of some of these, particularly the non-protein amino acids such as GABA which displays higher intensity in the NZ CP plants compared with those of the UK but was not reduced with elevated soil nutrients from fertilization. There are also several secondary metabolites including flavonoids, coumarins and hydroxycinnamic acids elevated in the CP plants which likewise were not reduced with improved soil nutrients. If in *C. vulgaris,* GABA and these secondary metabolites are active as defensive metabolites against *L. suturalis,* they could still be adding an additional impediment to this beetle's overall performance in the NZ environment.

Fold change in intensity values for almost all *C. vulgaris* secondary metabolites annotated in all three trials were generally low, and at this magnitude of change appear not to have any measurable effect on this specialist herbivore. However, elevation of foliar nitrogen and the concomitant reduction of some secondary metabolites following soil fertilizer application should improve assimilability and therefore, boost several life history parameters of this insect folivore. Thus, based on the data, low foliar nitrogen in *C. vulgaris* on the CP region appears to be a likely factor affecting this control agents' establishment, population density and effectiveness.

Metabolomics is key to understanding invasive plant metabolic responses associated with abiotic parameters, and this technology could have an important role to play in the practice of biological control of weeds. The results presented here demonstrate the highly complex interactions that exist between abiotic

parameters, plant metabolome and insect herbivore responses that would otherwise remain unknown. These are all areas of understanding which, in the future, may be used to better predict success or retrospectively elucidate challenges in biocontrol programmes.

6.4 References

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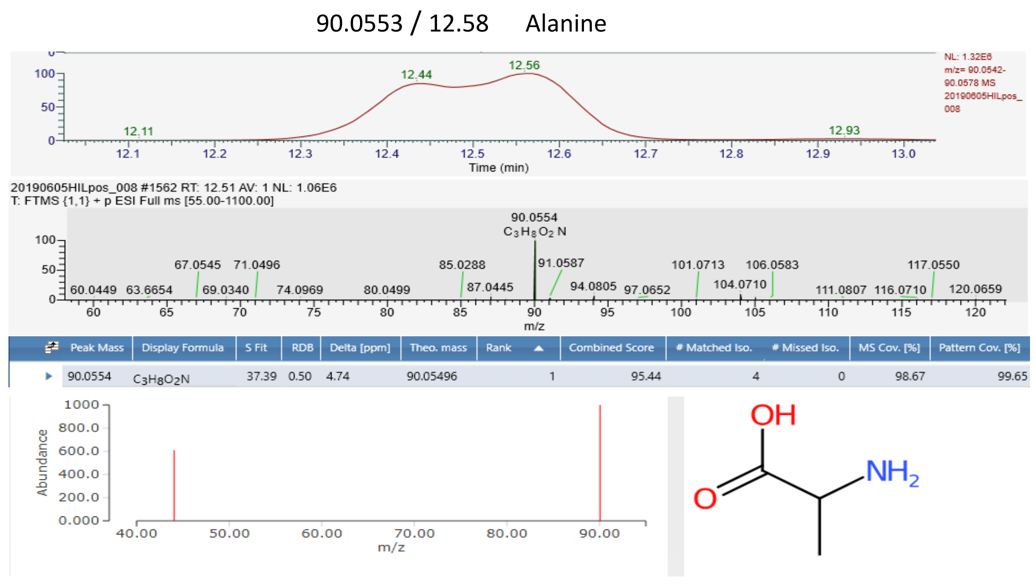
Appendix A:

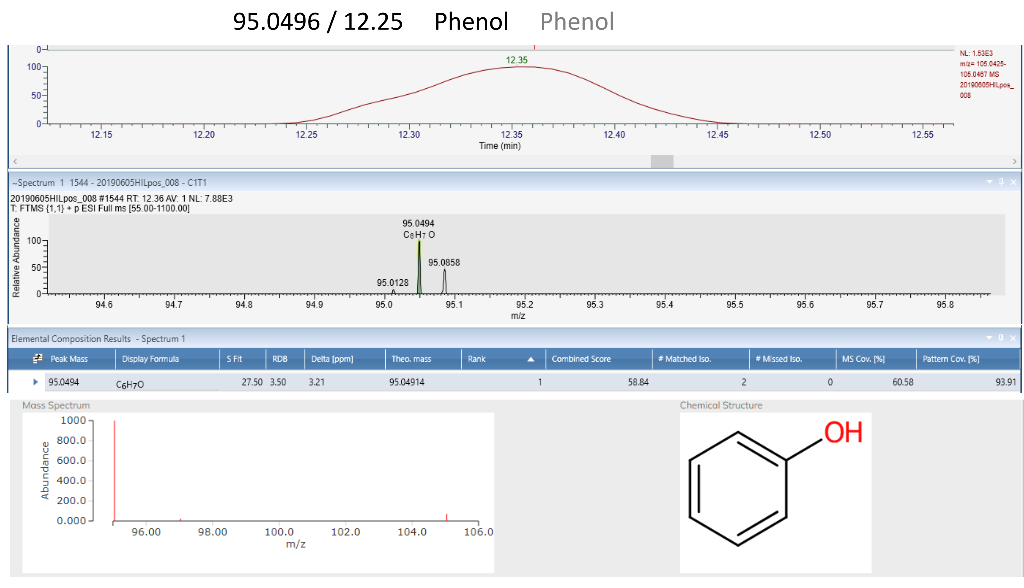
Chapter 3, Annotated compound confirmations.

All annotation confirmation data composes of screen images from 1: ThermoFisher — Excalibur “Freestyle”. In the top chromatogram is retention time (rt.). The spectrum beneath provides the m/z value and below that the chemical formula and mass error (Delta [ppm]). 2: The bottom field shows the mass spectral graphic in the online “MassBank” mass spectral library from which parent and fragment ion *m/z* abundance values have been derived and the chemical structure is also displayed. Compounds are ordered by ascending *m/z* value. Zooming in on each will reveal finer detail.

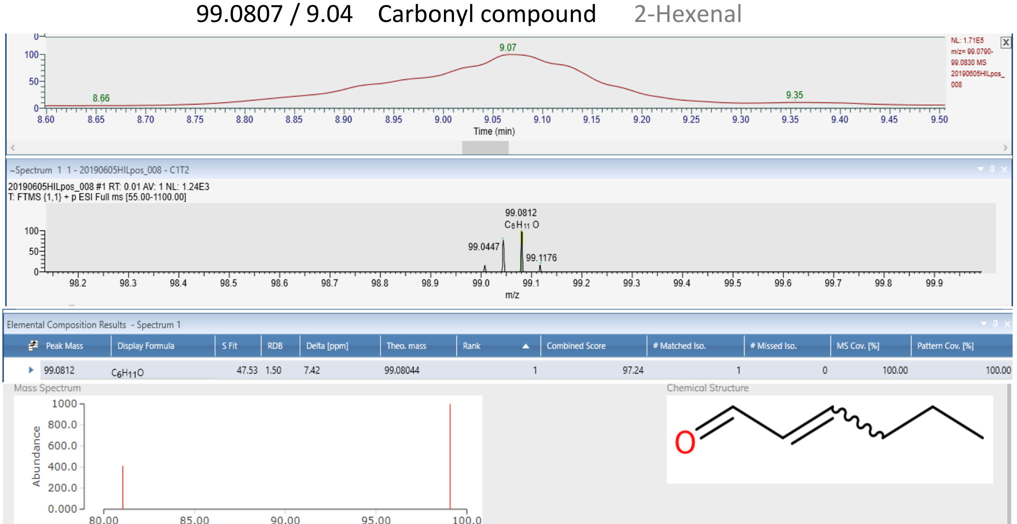
90.0553 / 12.58 Alanine



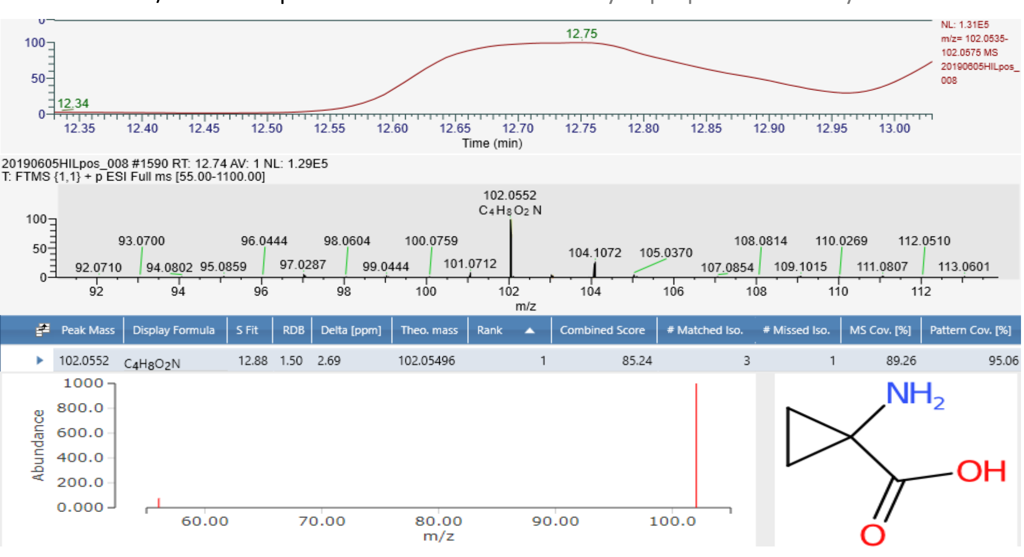
95.0496 / 12.25 Phenol Phenol



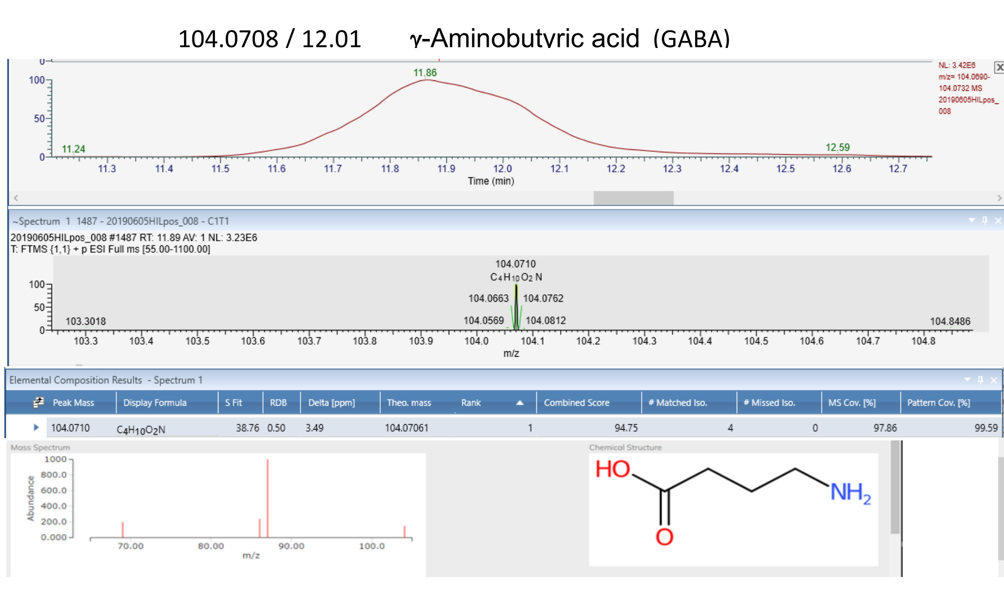
99.0807 / 9.04 Carbonyl compound 2-Hexenal



102.0555 / 12.77 Alpha-Amino Acid a. 1-Aminocyclopropane-1-carboxylic acid



104.0708 / 12.01 γ-Aminobutyric acid (GABA)



105.0552 / 9.16 Alpha Hydroxy acid 2-Hydroxyisobutyric acid

Peak Mass	Display Formula	S Fit	RDB	Delta [ppm]	Theo. mass	Rank	Combined Score	# Matched Iso.	# Missed Iso.	MS Cov. [%]	Pattern Cov. [%]
105.0550	$C_4H_9O_3$	17.23	0.50	3.32	105.05462	1	91.95	2	1	96.10	95.18

106.0502 / 13.68 L-Serine

20190605HILpos_008 #1702 RT: 13.60 AV: 1 NL: 3.05E5
T: FTMS {1,1} + p ESI Full ms [55.00-1100.00]

Peak Mass	Display Formula	S Fit	RDB	Delta [ppm]	Theo. mass	Rank	Combined Score	# Matched Iso.	# Missed Iso.	MS Cov. [%]	Pattern Cov. [%]
106.0503	$C_3H_8O_3N$	21.09	0.50	4.28	106.04987	1	93.99	4	0	98.04	99.05

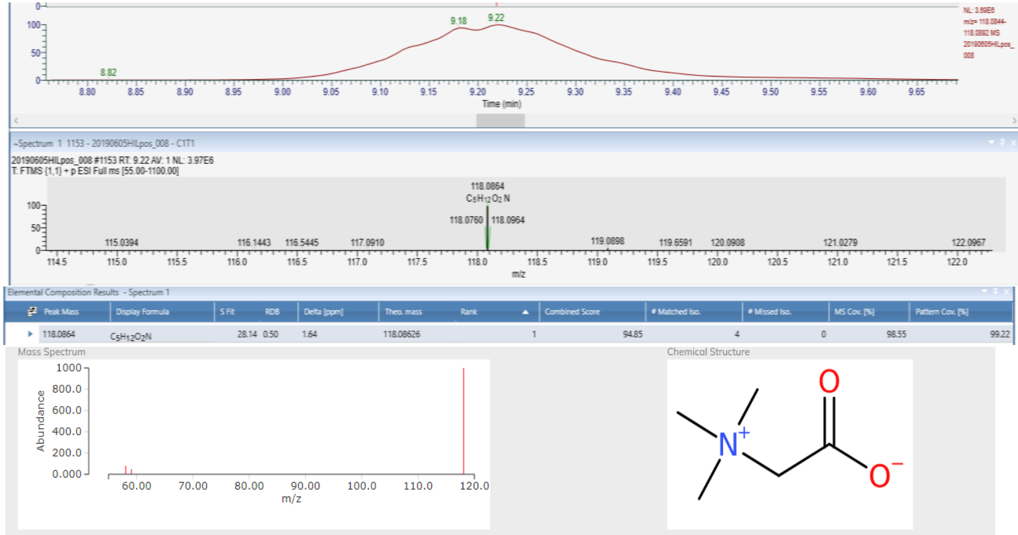
109.0285 / 8.88 Pyrocatechol

~Spectrum 1 1094 - 20190605 HILneg_008 - C1T1

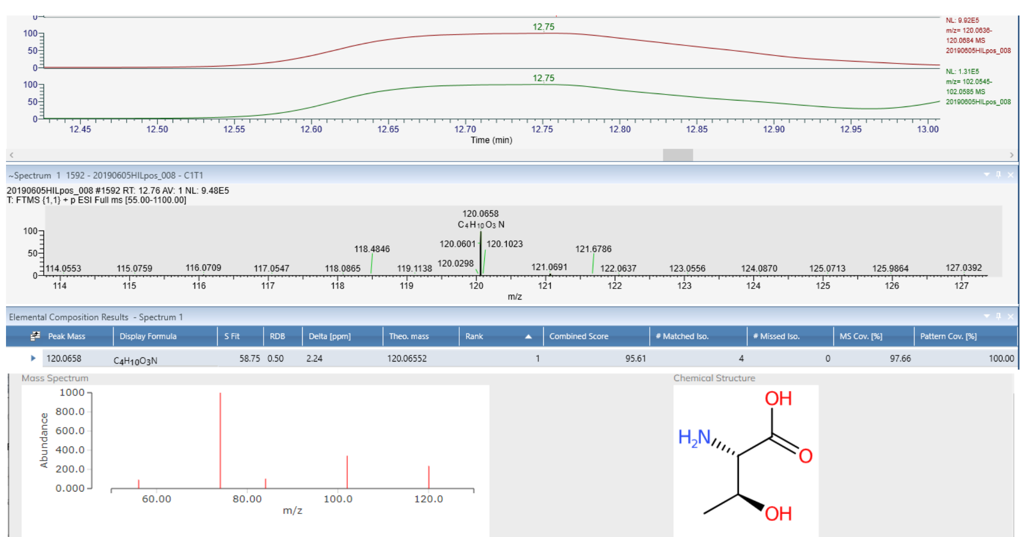
Elemental Composition Results - Spectrum 1

Peak Mass	Display Formula	S Fit	RDB	Delta [ppm]	Theo. mass	Rank	Combined Score	# Matched Iso.	# Missed Iso.	MS Cov. [%]	Pattern Cov. [%]
109.0285	$C_6H_5O_2$	20.88	4.50	-9.59	109.02950	1	63.76	2	1	66.14	99.56

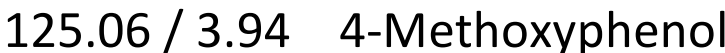
118.0868 / 9.21 Amino Acid a. Betaine



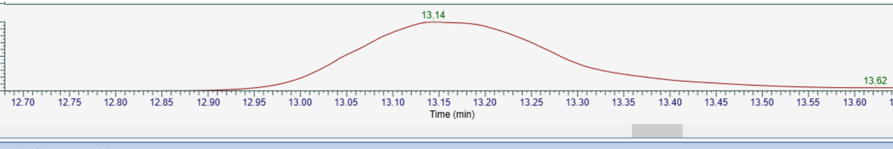
120.0659 / 12.77 L-Threonine

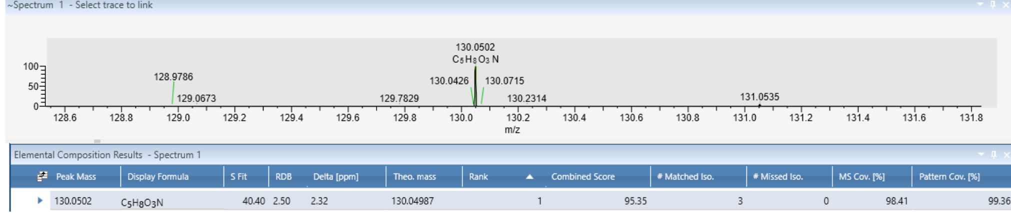


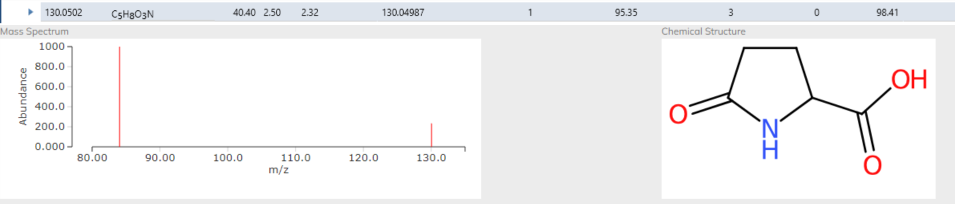
123.0441 / 4.20 Benzoic acid a. Benzoic acid

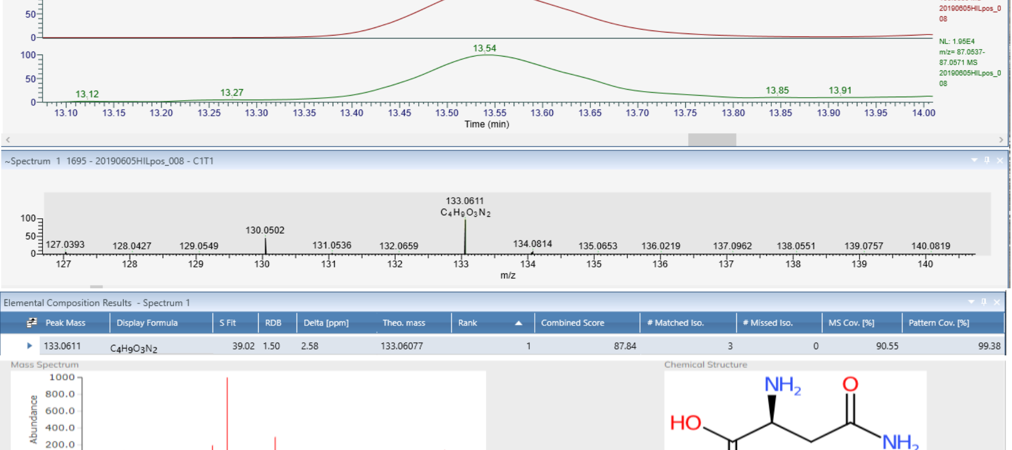


130.0502 / 13.19 Amino Acid b. L-Pyroglutamic acid

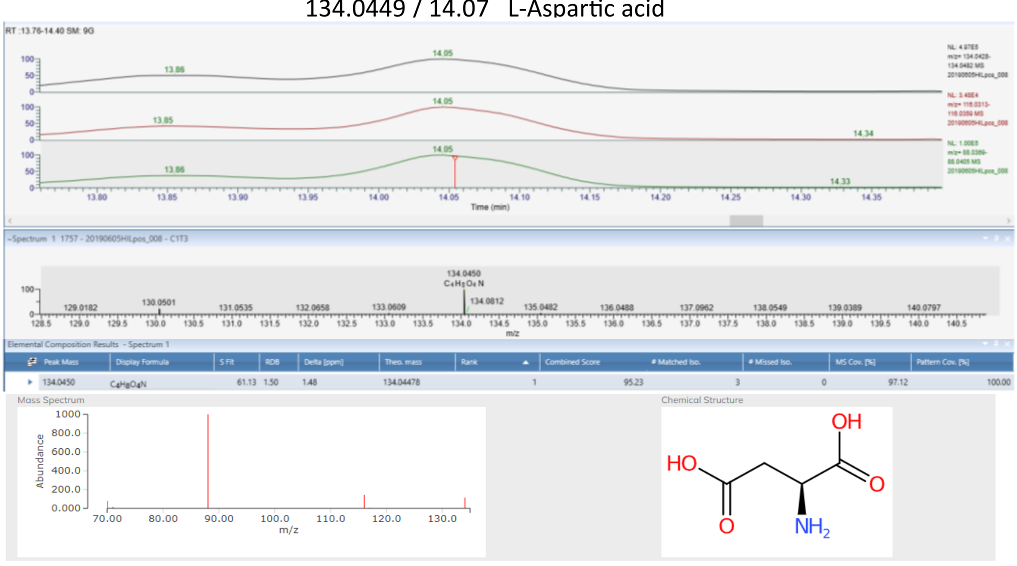




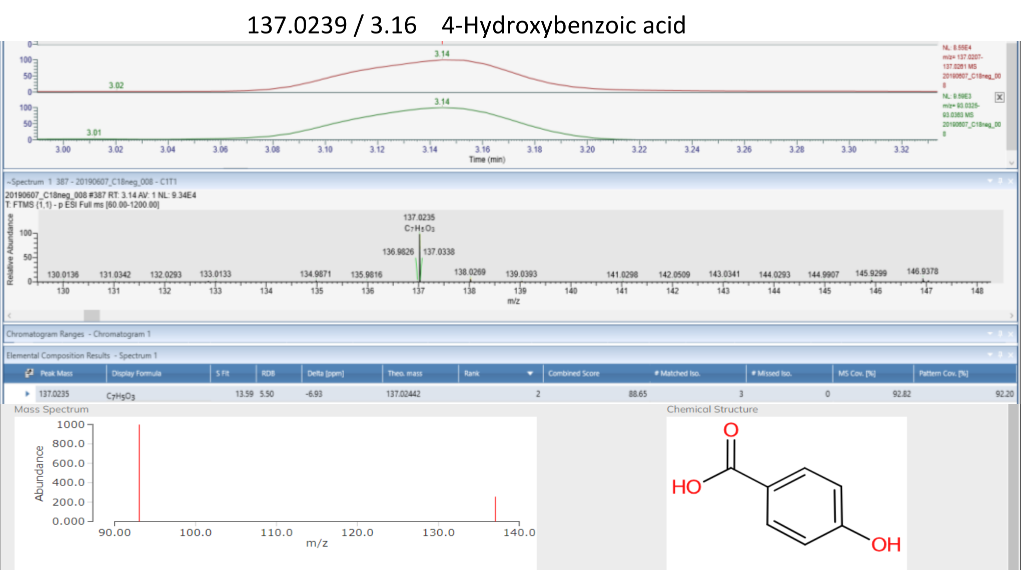


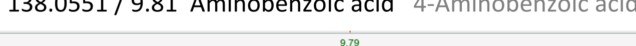


134.0449 / 14.07 L-Aspartic acid

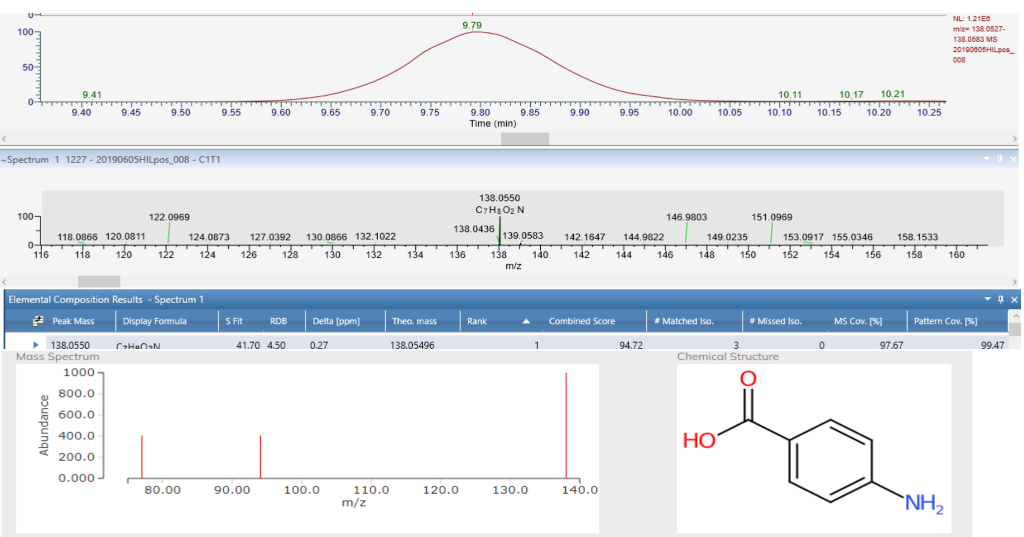


137.0239 / 3.16 4-Hydroxybenzoic acid

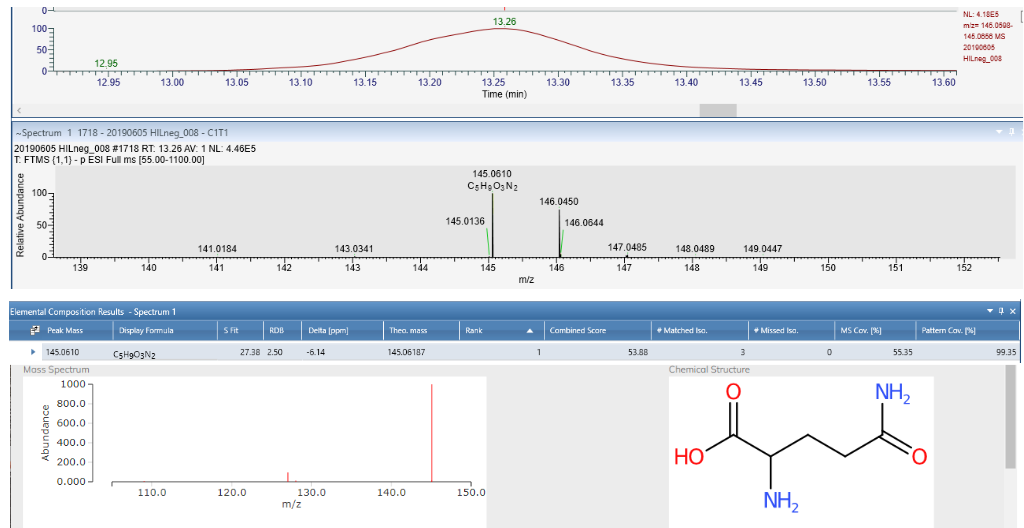




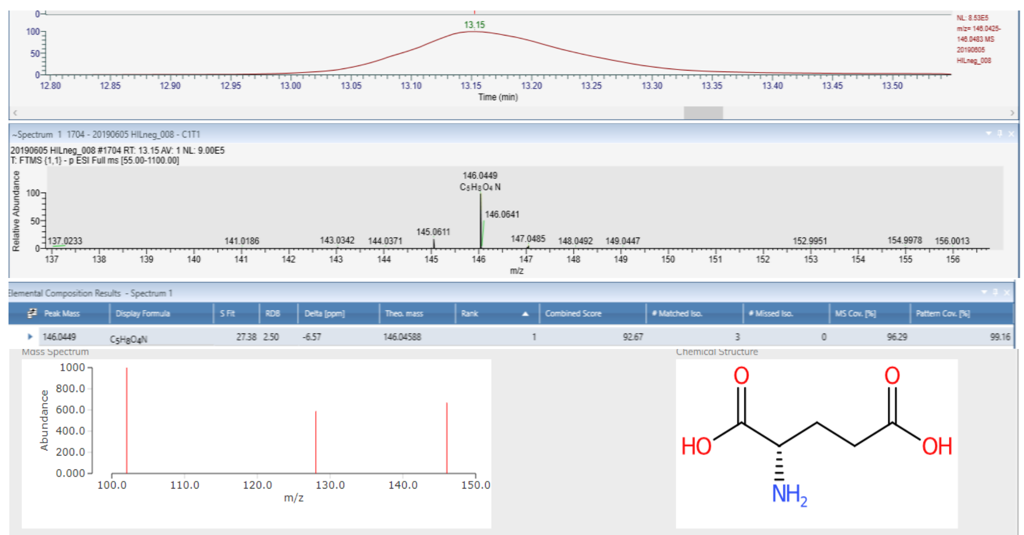
138.0551 / 9.81 Aminobenzoic acid 4-Aminobenzoic acid



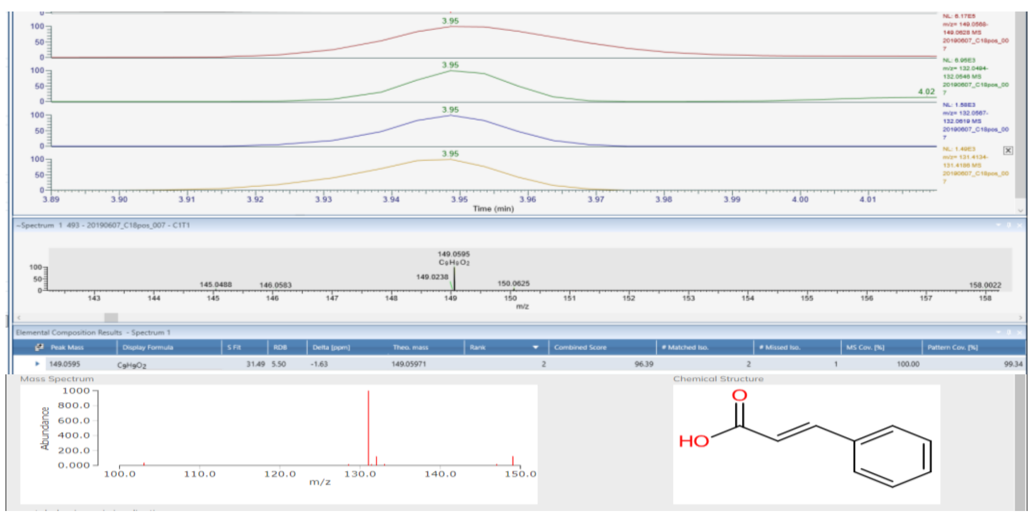
145.061 / 13.21 L-Glutamine



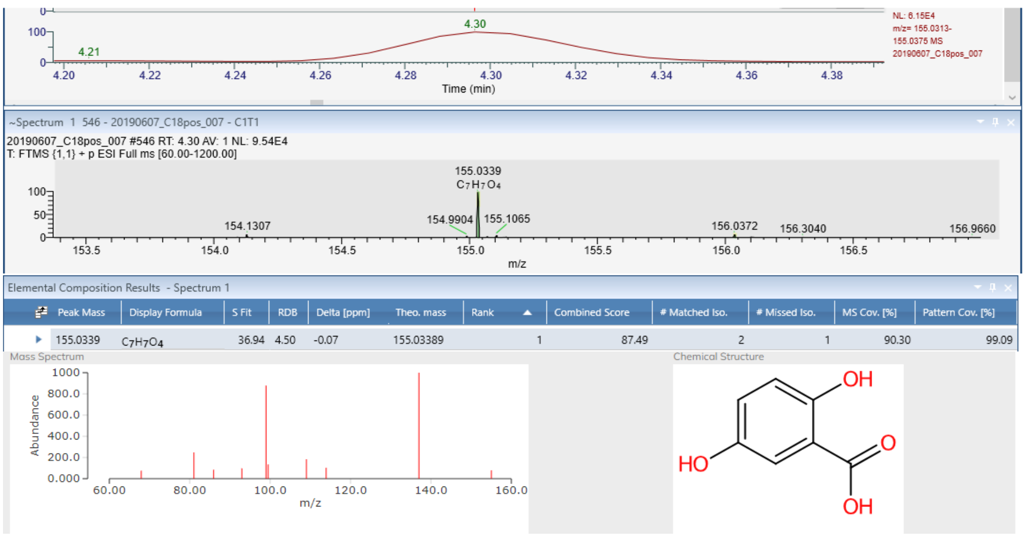
146.0451 / 13.12 L-Glutamate



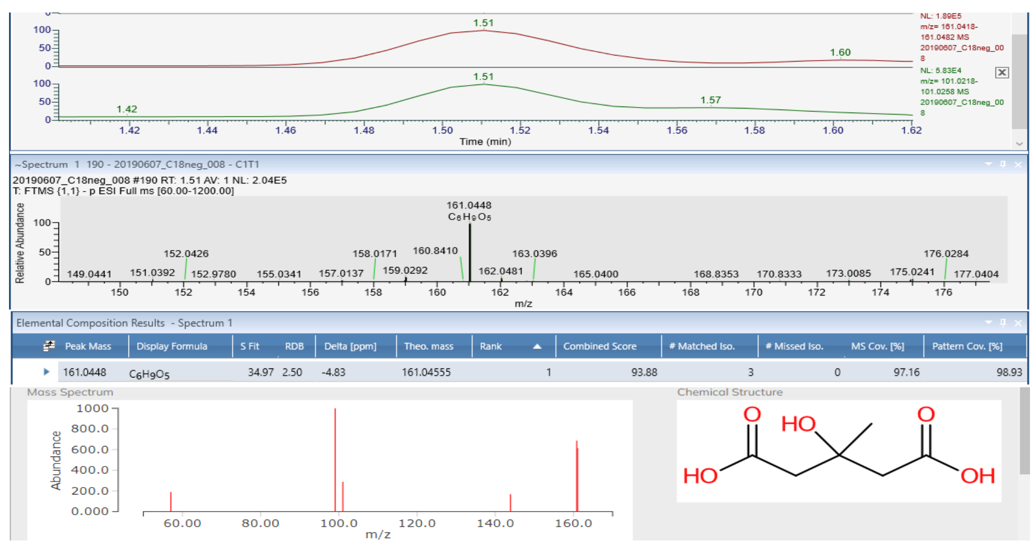
149.0596 / 3.94 trans-Cinnamic acid



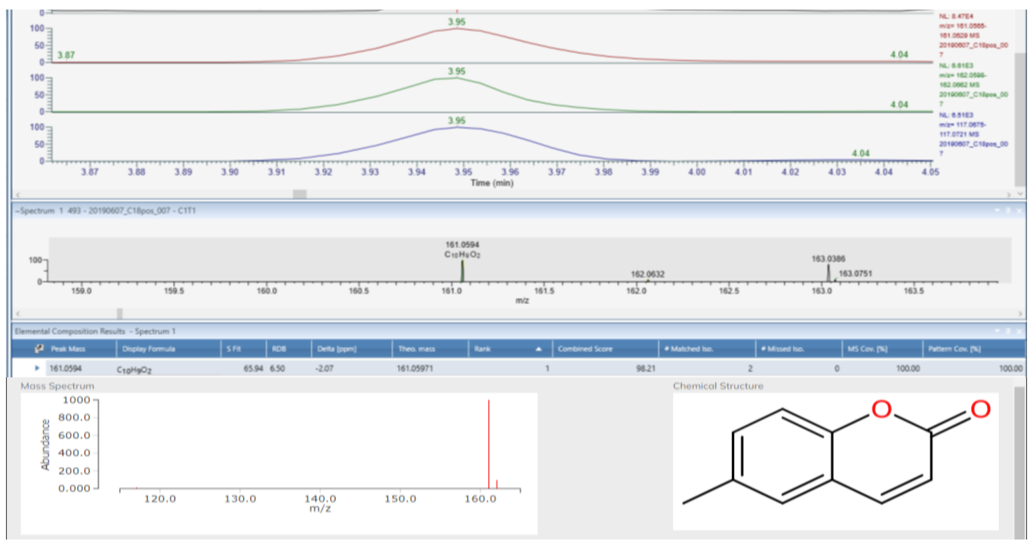
155.0338 / 4.30 Hydroxybenzoic acid 5-Hydroxysalicylic acid



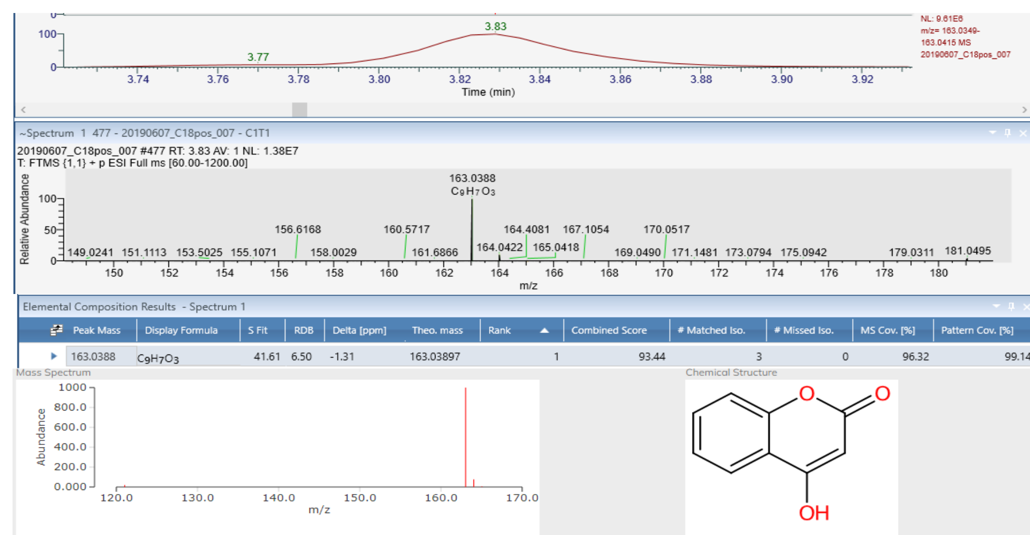
161.0443 / 1.54 3-Hydroxy-3-methylglutaric acid



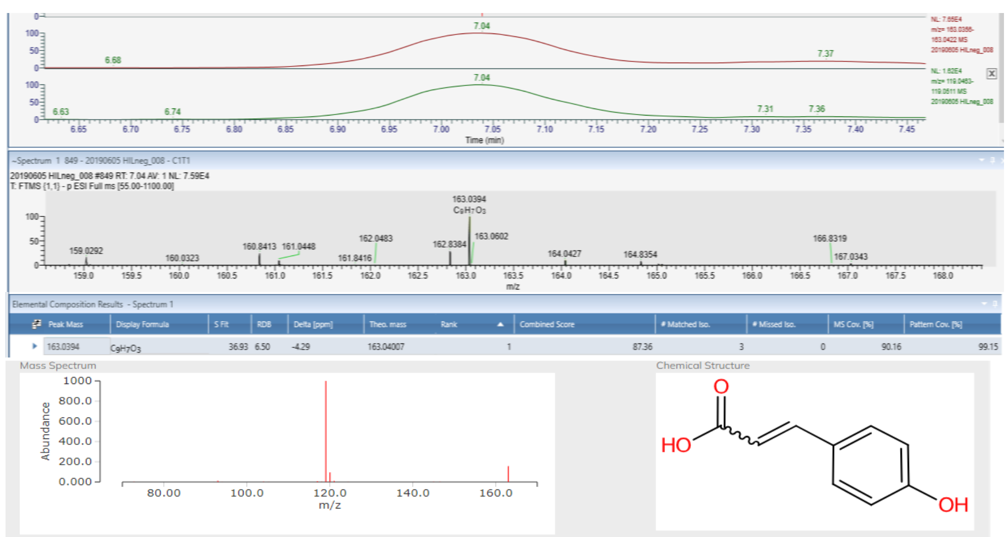
161.0598 / 3.94 6-Methylcoumarin



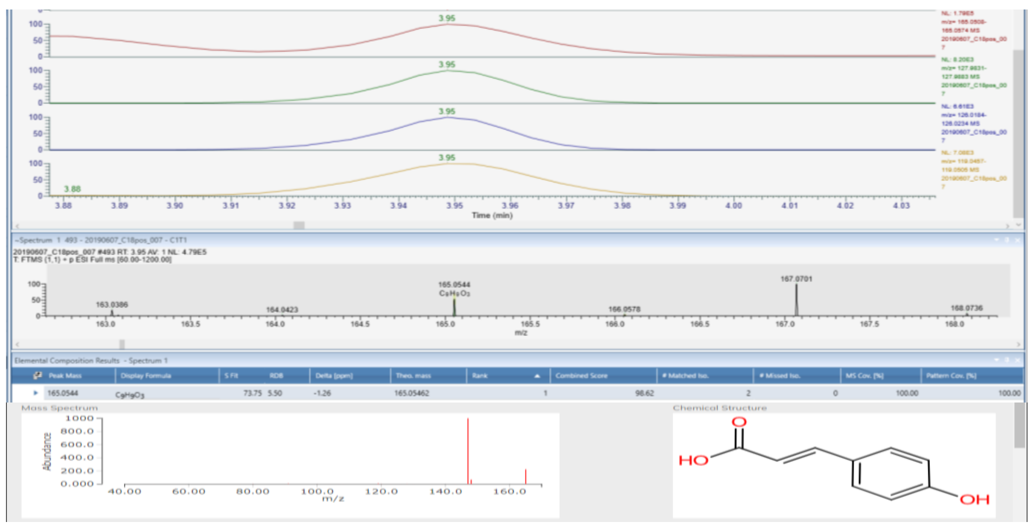
163.0384 / 3.82 Hydroxycoumarin a. 4-Hydroxycoumarin



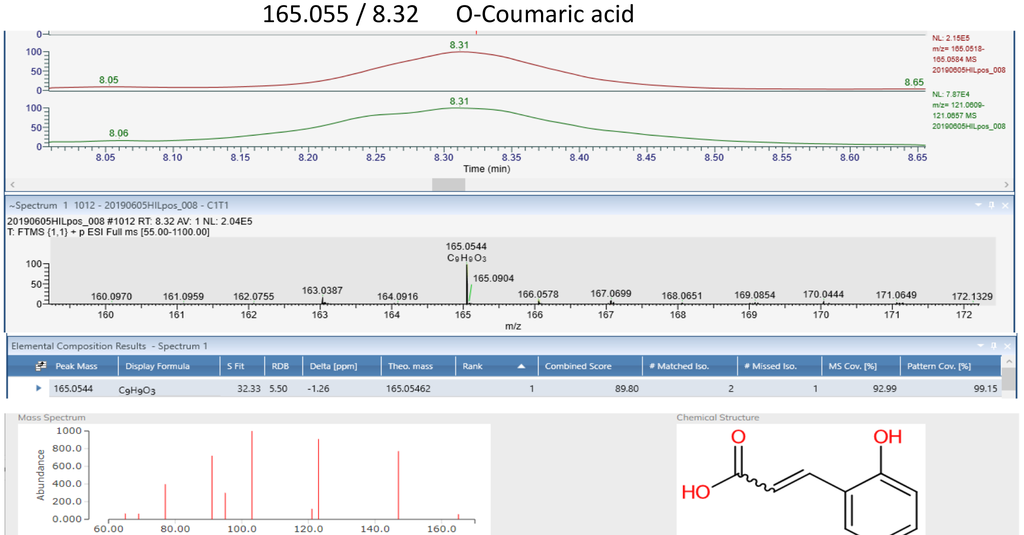
163.0394 7.01 cis-o-Hydroxycinnamic acid



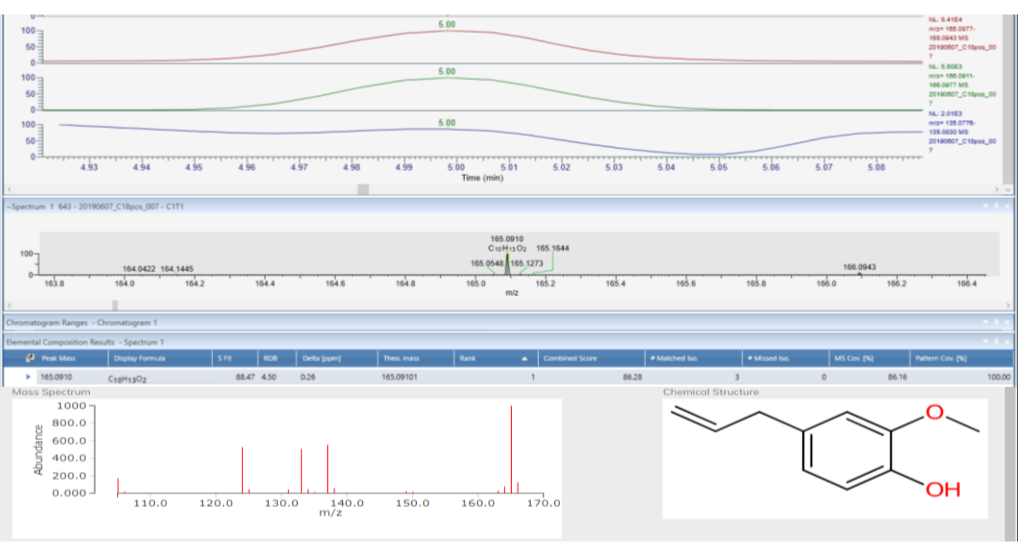
165.0546 / 3.94 p-Coumaric acid



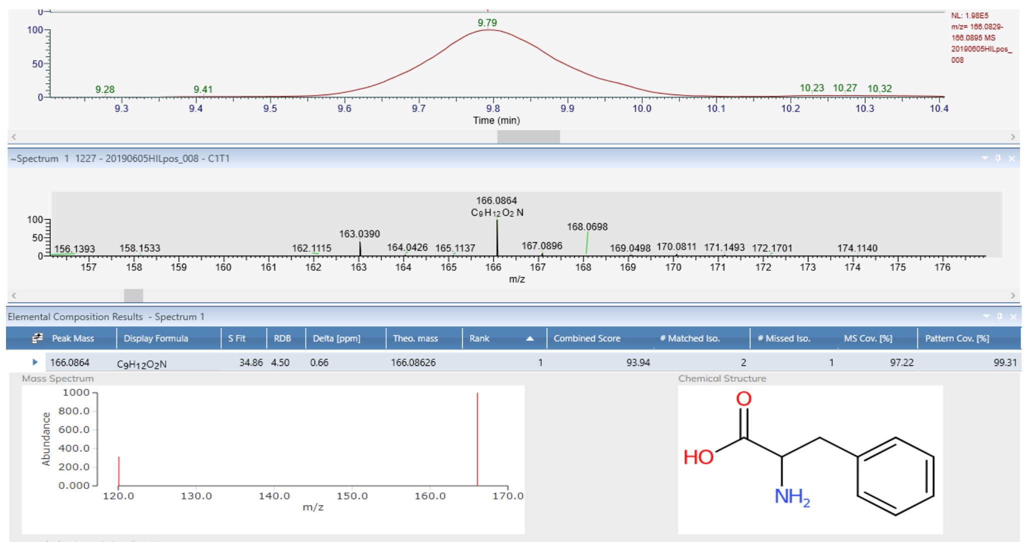
165.055 / 8.32 O-Coumaric acid



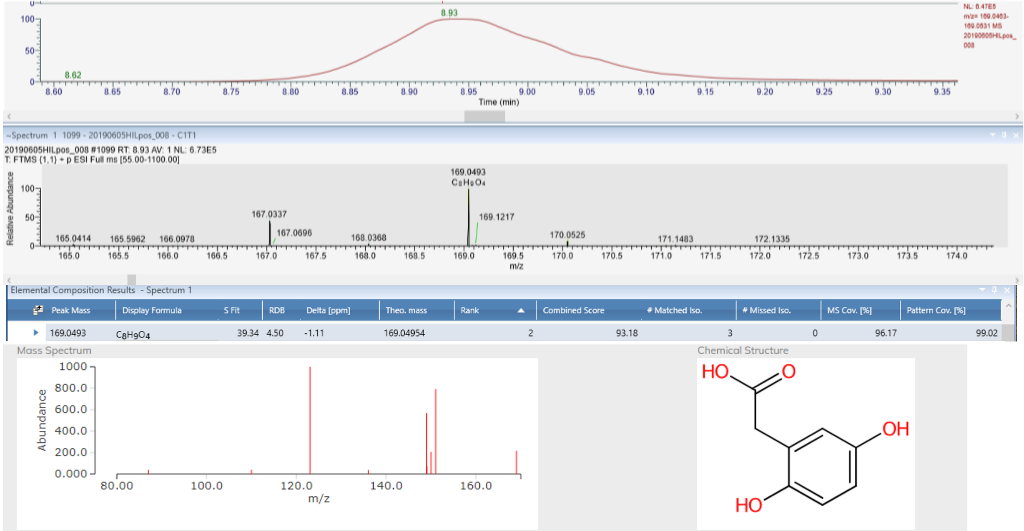
165.0908 / 4.99 Eugenol



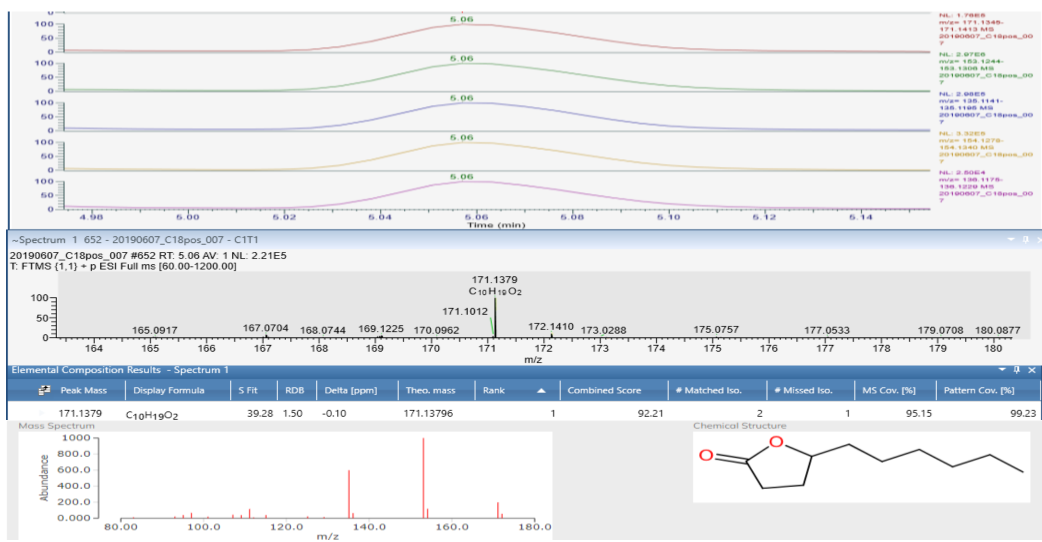
166.0867 / 9.79 L-Phenylalanine



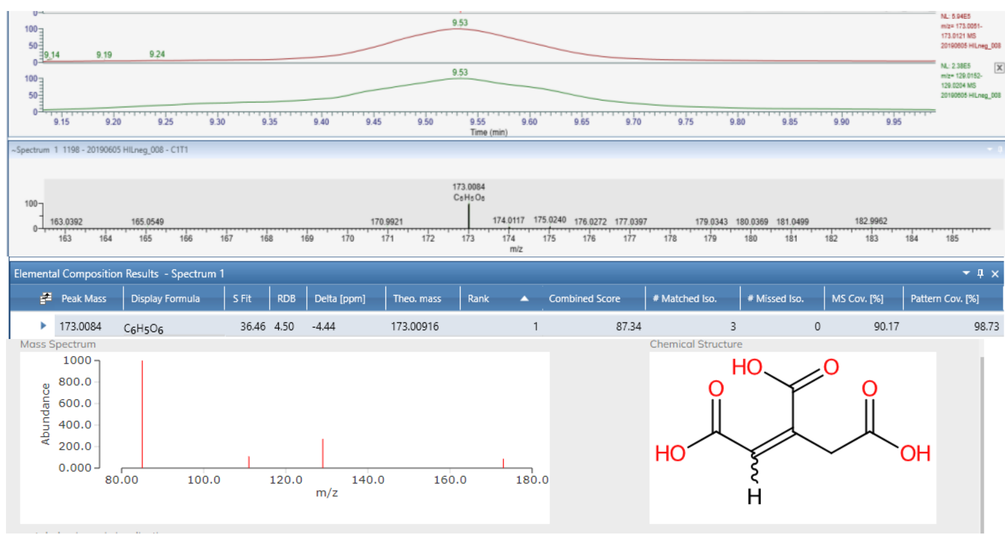
169.0498 / 8.97 Phenylacetic acid Homogentisic acid



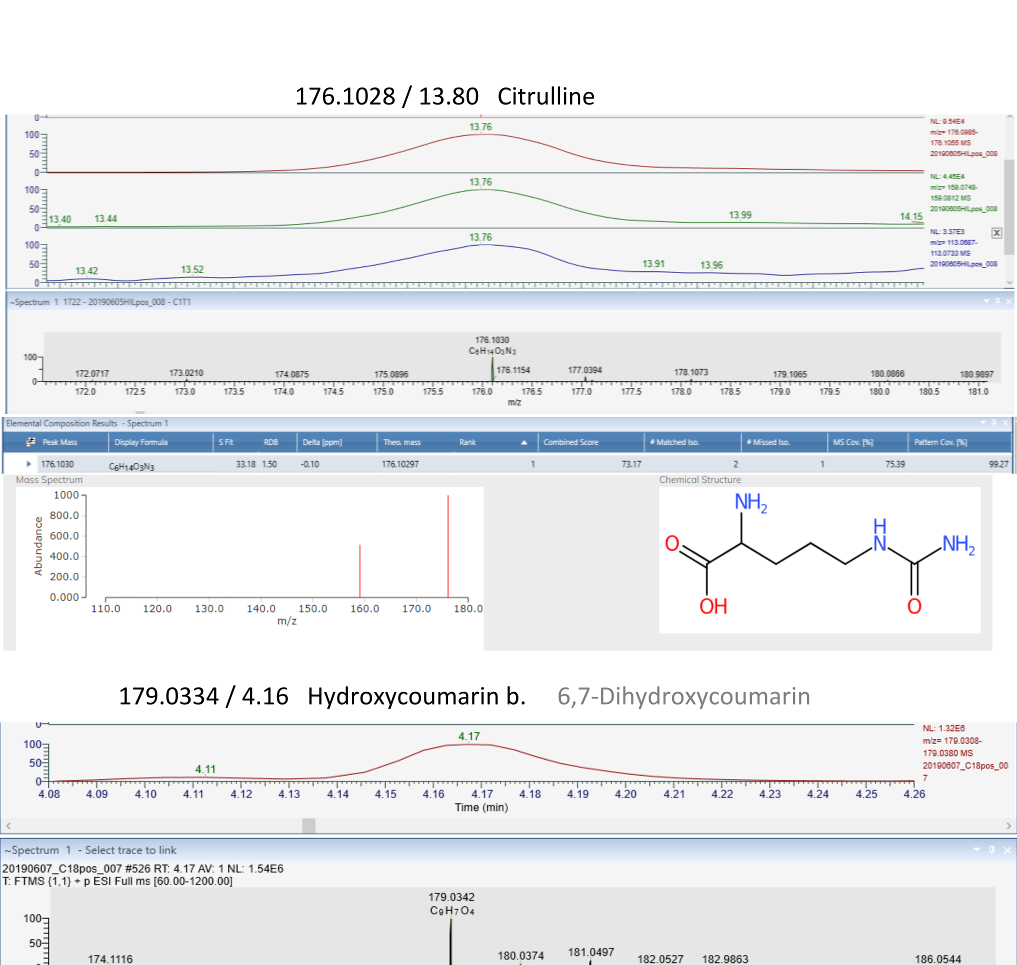
171.1382 / 5.05 Gamma-Decalactone



173.0086 / 9.46 cis-Aconitic acid

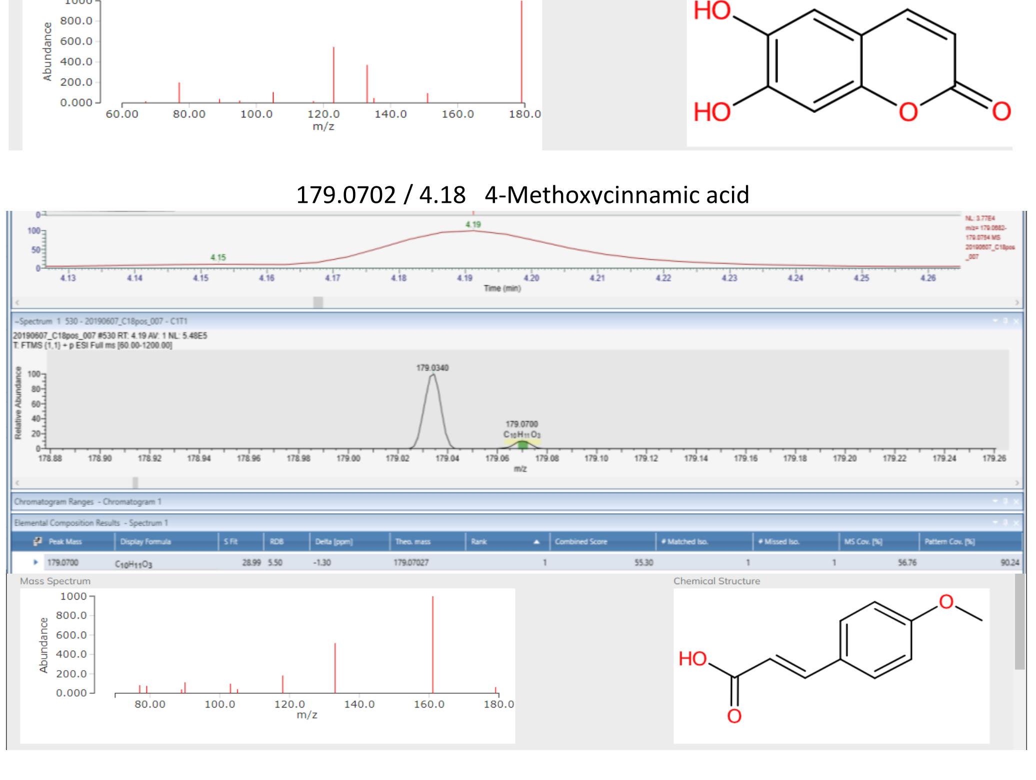


176.1028 / 13.80 Citrulline

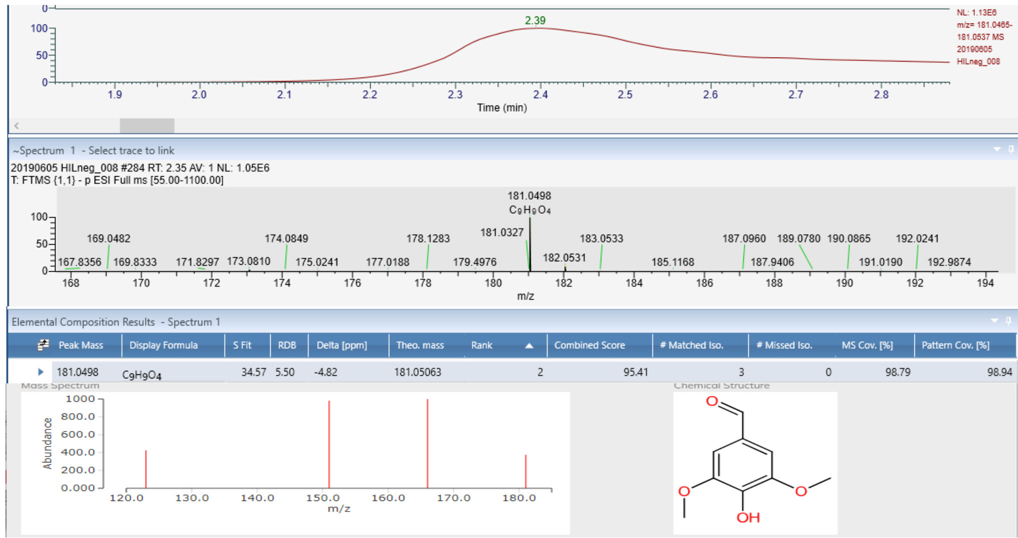


179.0334 / 4.16 Hydroxycoumarin b. 6,7-Dihydroxycoumarin

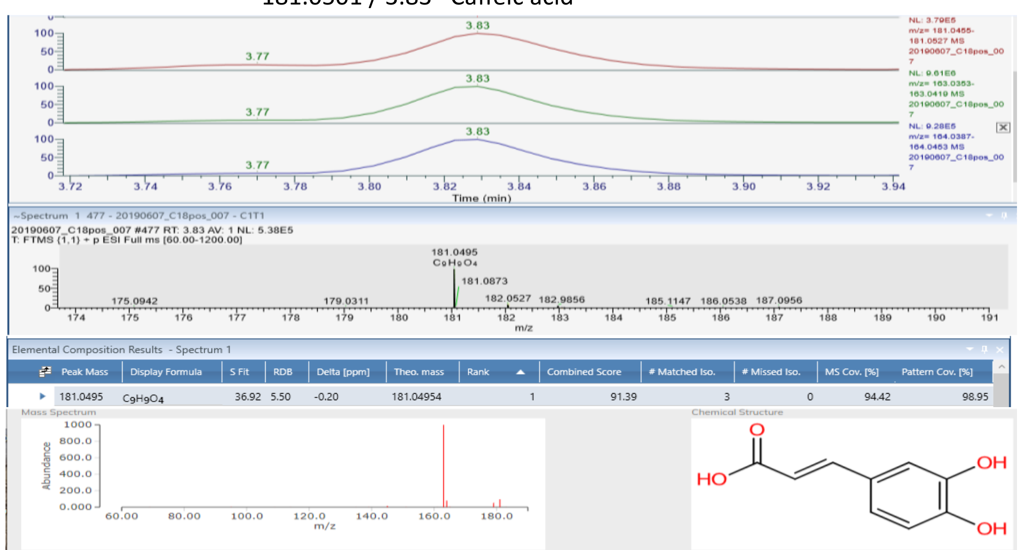
179.0702 / 4.18 4-Methoxycinnamic acid



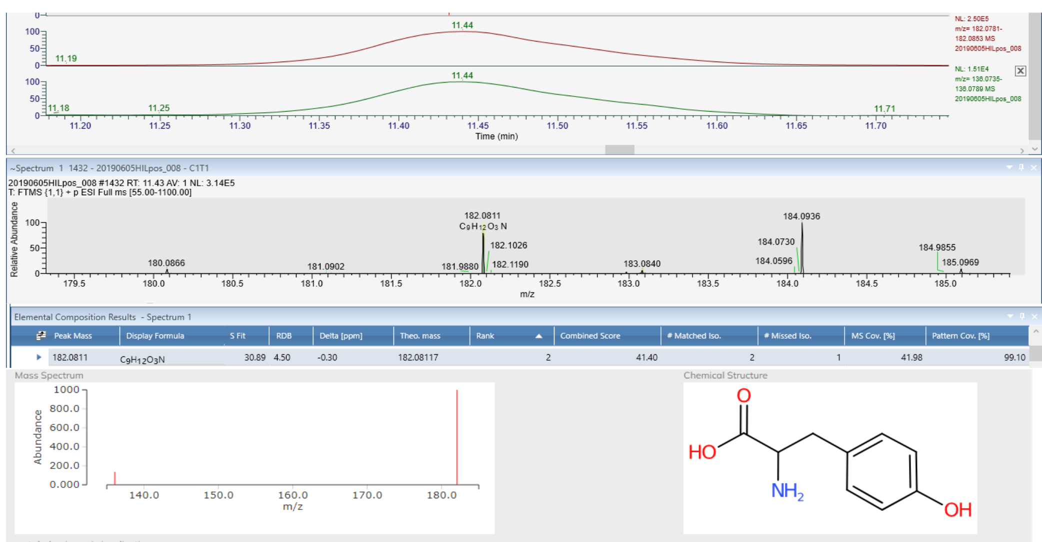
181.0499 / 2.36 Sesquiterpenoid a. Syringaldehyde



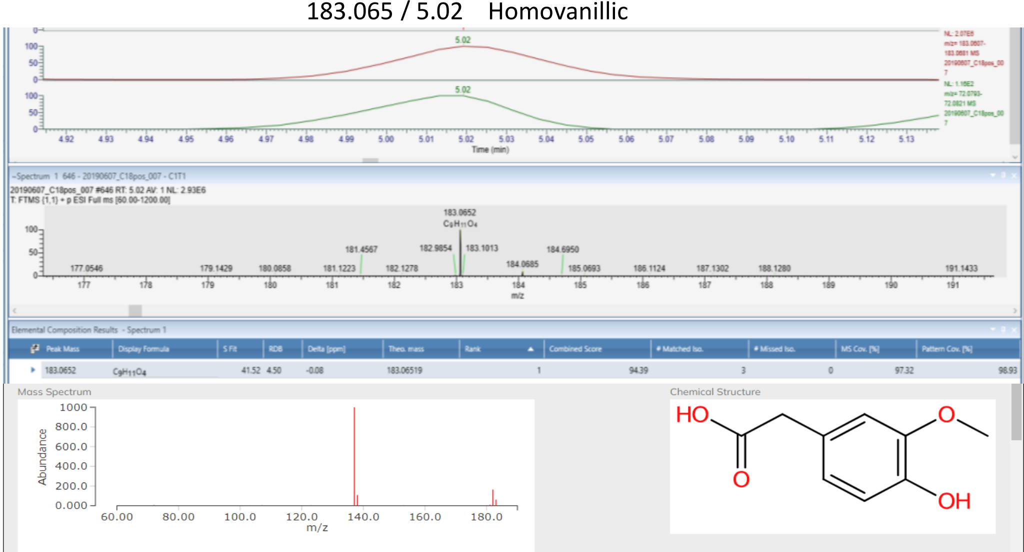
181.0501 / 3.83 Caffeic acid



182.0812 / 11.53 L-Tyrosine

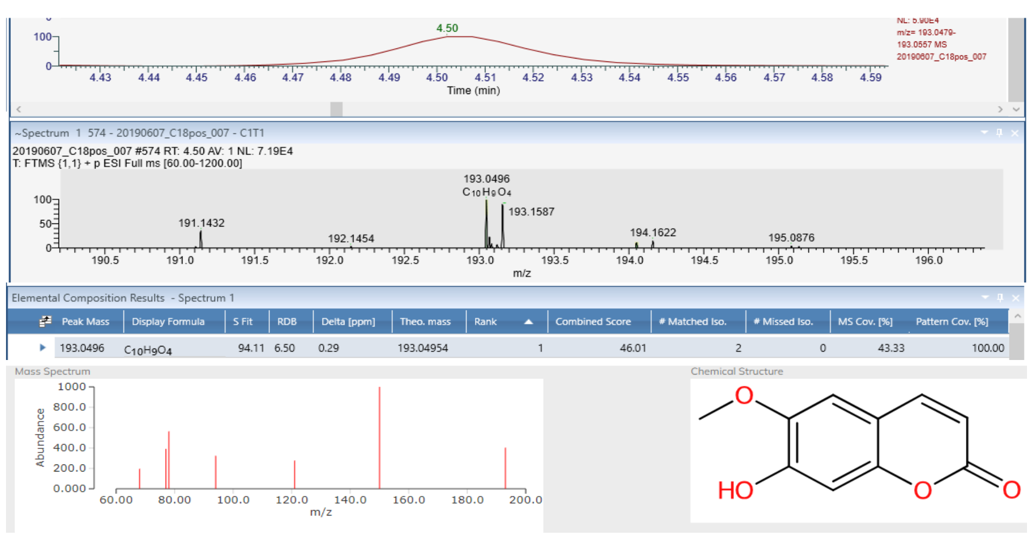


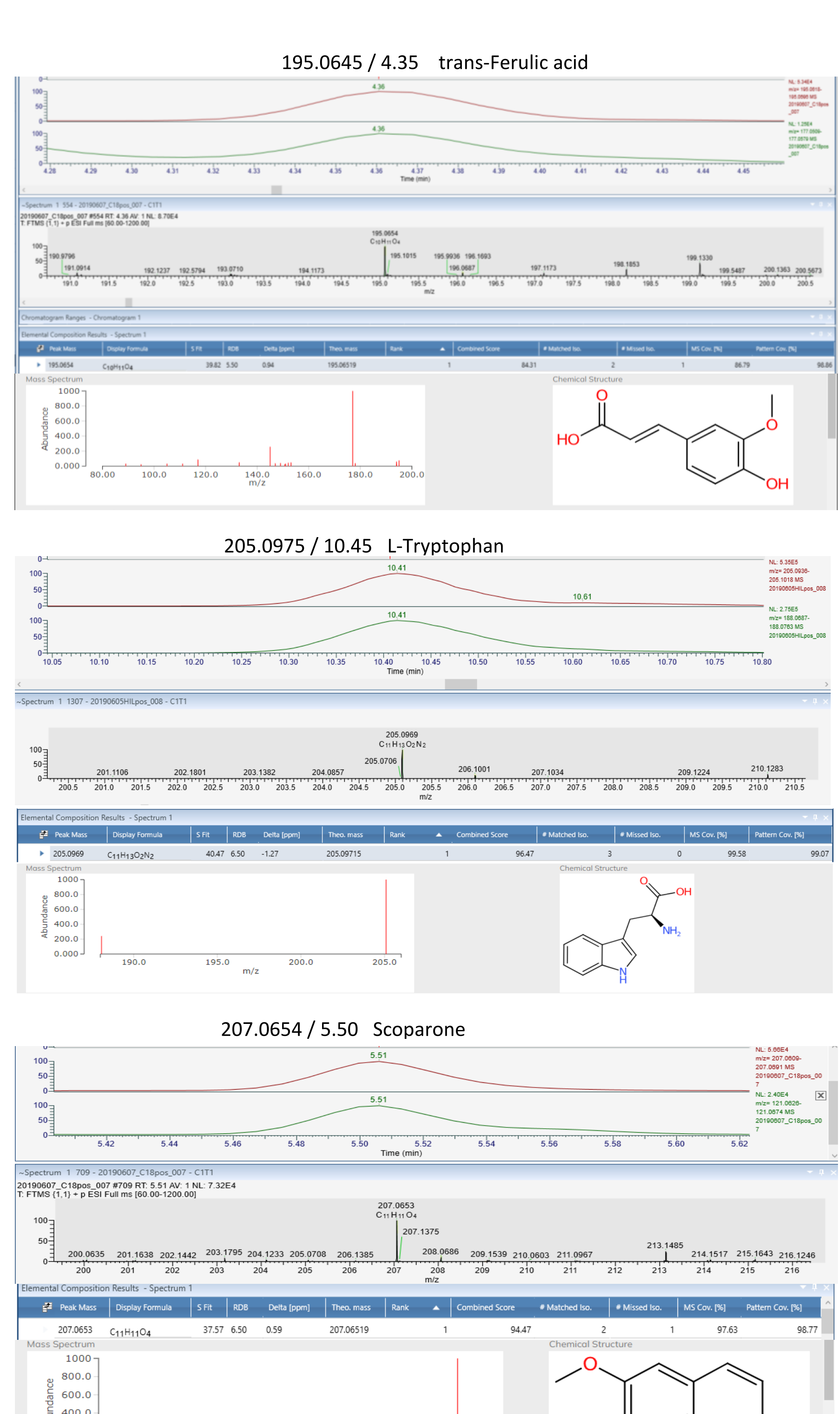
183.065 / 5.02 Homovanillic



191.0705 / 3.94 7-methoxy-4-methylcoumarin

193.0502 / 4.50 7-Hydroxycoumarin 6-Methoxy-7-hydroxycoumarin

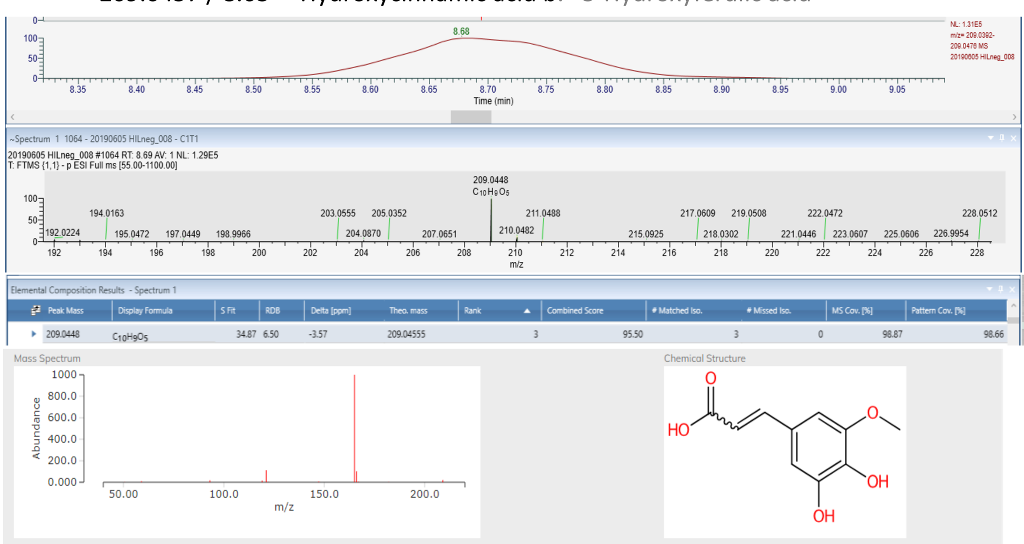




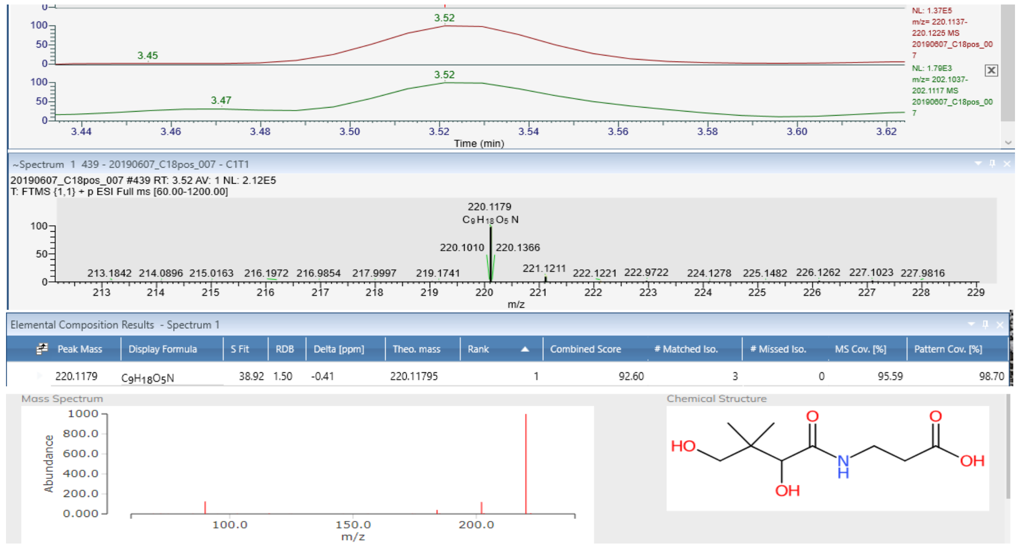
195.0645 / 4.35 trans-Ferulic acid
Mass Spectrum
Chemical Structure
HO
O
OH
205.0975 / 10.45 L-Tryptophan
Time (min)
Elemental Composition Results - Spectrum 1
Peak Mass
Display Formula
S Fit
RDB
Delta [ppm]
Theo. mass
Rank
Combined Score
Matched Iso.
Missed Iso.
MS Cov. [%]
Pattern Cov. [%]
205.0969
40.47
6.50
-1.27
205.09715
1
96.47
3
0
99.58
99.07
Mass Spectrum
Abundance
m/z
Chemical Structure
OH
NH2
207.0654 / 5.50 Scoparone
~Spectrum 1 709 - 20190607_C18pos_007 - C1T1
20190607_C18pos_007 #709 RT: 5.51 AV: 1 NL: 7.32E4
T: FTMS (1,1) + p ESI Full ms [60.00-1200.00]
207.0653
37.57
6.50
0.59
207.06519
1
94.47
2
1
97.63
98.77
Mass Spectrum
Abundance
m/z
Chemical Structure
O

209.0298 / 14.54 Carbohydrate Galactaric acid

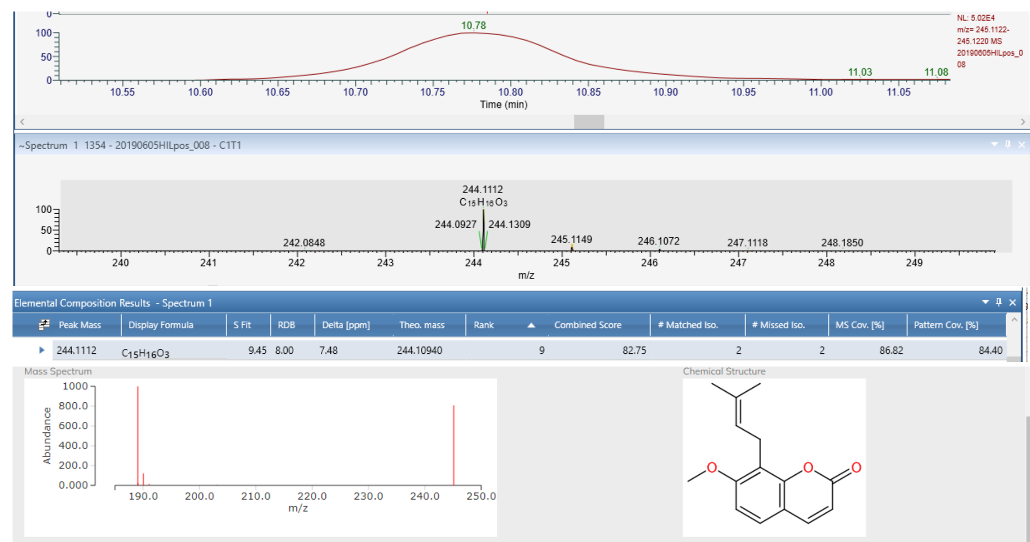
209.0457 / 8.63 Hydroxycinnamic acid b. 5-Hydroxyferulic acid



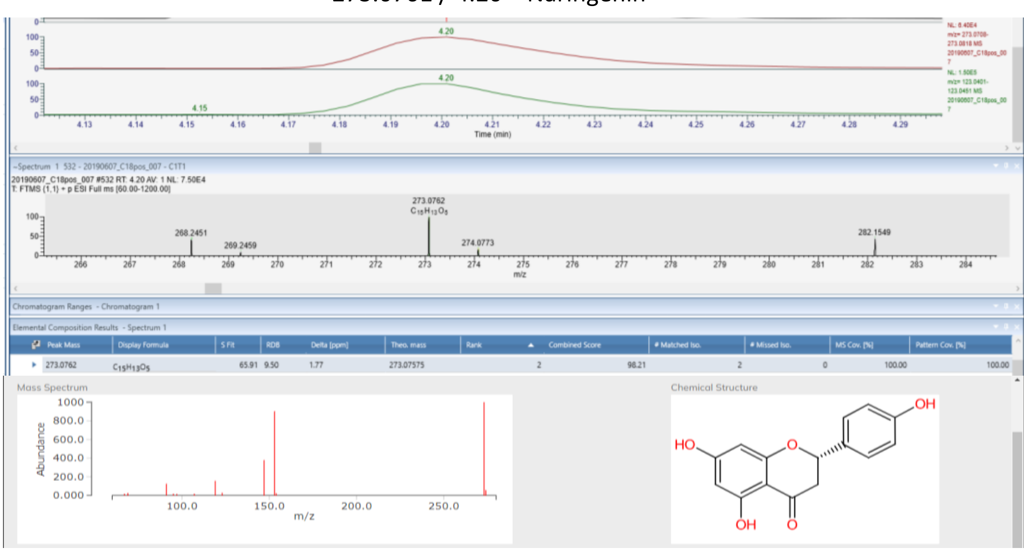
220.119 / 3.52 Pantothenic Acid

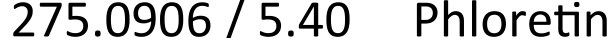


245.114 / 10.83 Coumarin a. Osthol

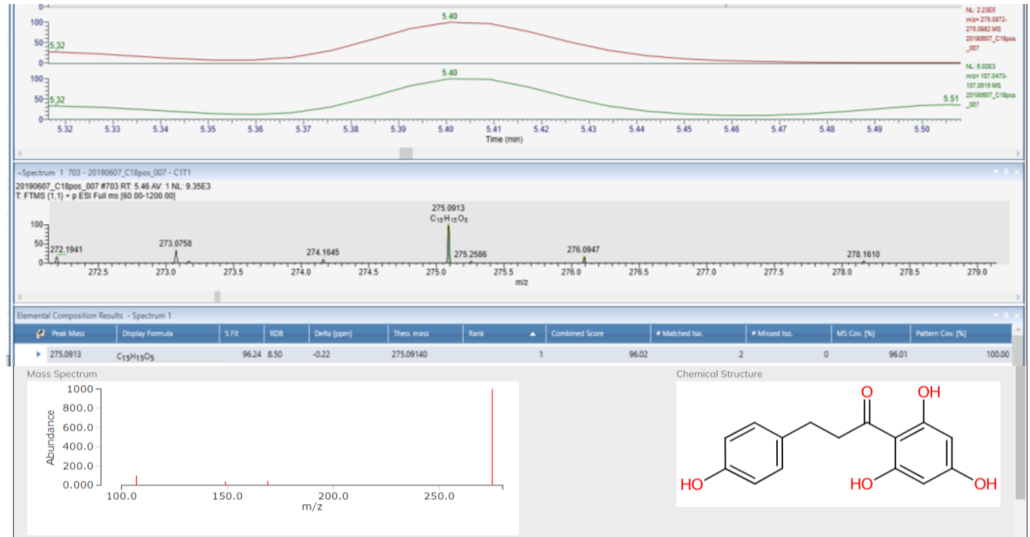


273.0761 / 4.20 Naringenin





275.0906 / 5.40 Phloretin



287.0549 / 5.10 Flavone a. Luteolin

NL: 1.23E6 m/z= 287.0499-287.0613 MS 20190607_C18pos_007

~Spectrum 1 659 - 20190607_C18pos_007 - C1T1

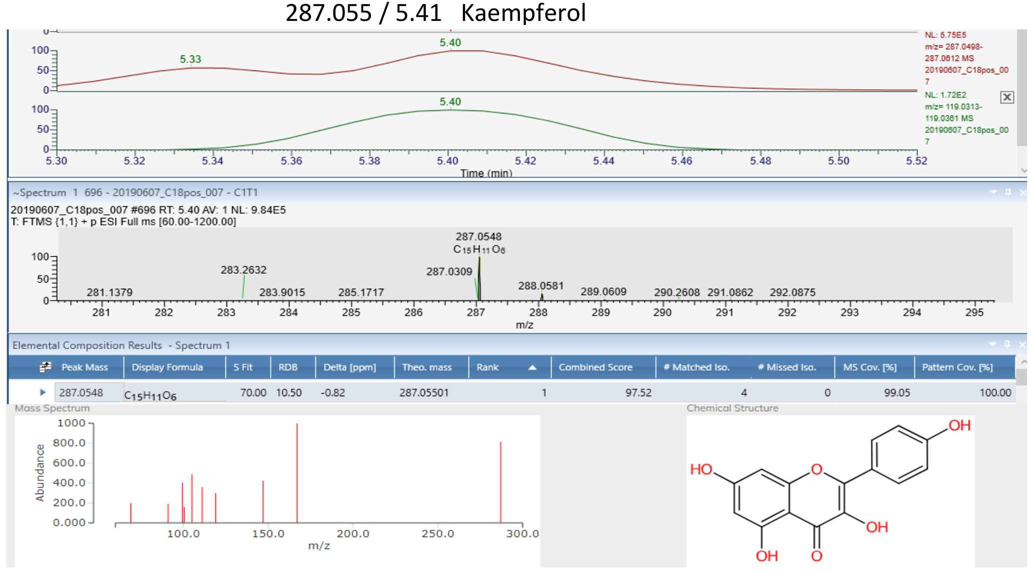
20190607_C18pos_007 #659 RT: 5.10 AV: 1 NL: 1.37E6
T: FTMS {1,1} + p ESI Full ms [60.00-1200.00]

Elemental Composition Results - Spectrum 1

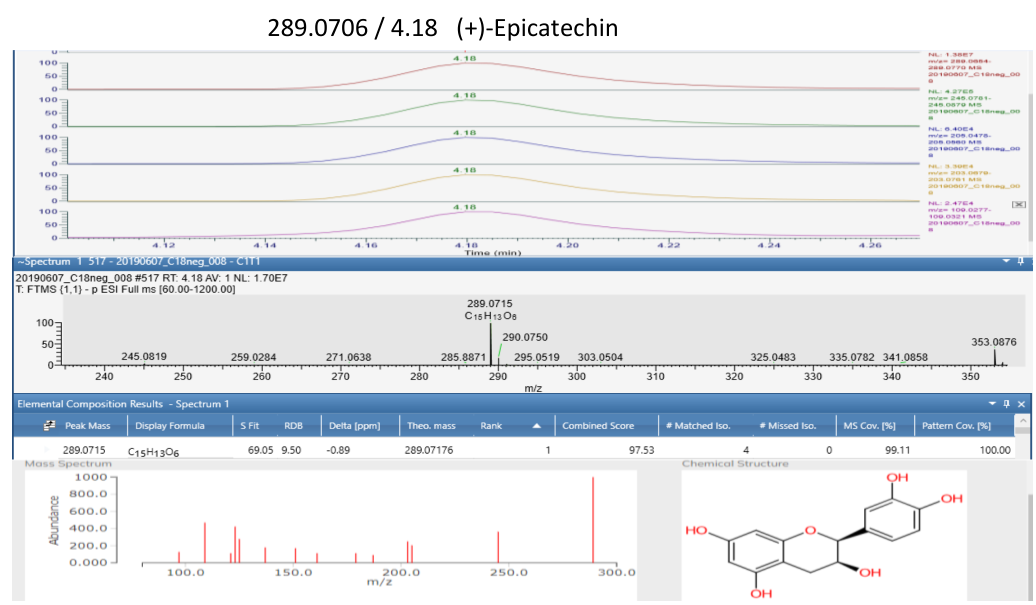
Peak Mass	Display Formula	S Fit	RDB	Delta [ppm]	Theo. mass	Rank	Combined Score	# Matched Iso.	# Missed Iso.	MS Cov. [%]	Pattern Cov. [%]
287.0549	$C_{15}H_{11}O_6$	42.79	10.50	-0.40	287.05501	4	94.87	4	0	97.77	97.93

Mass Spectrum — Chemical Structure

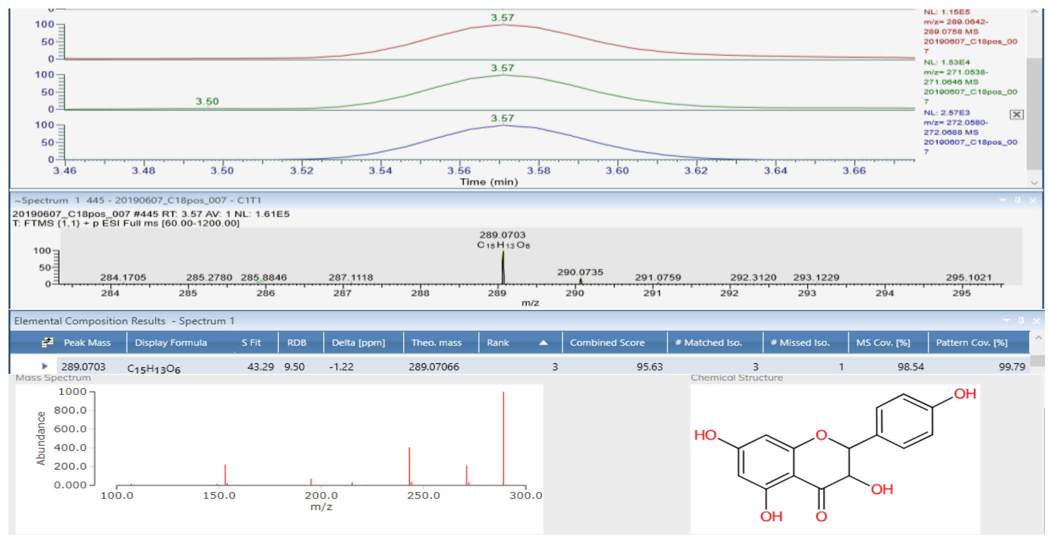
287.055 / 5.41 Kaempferol



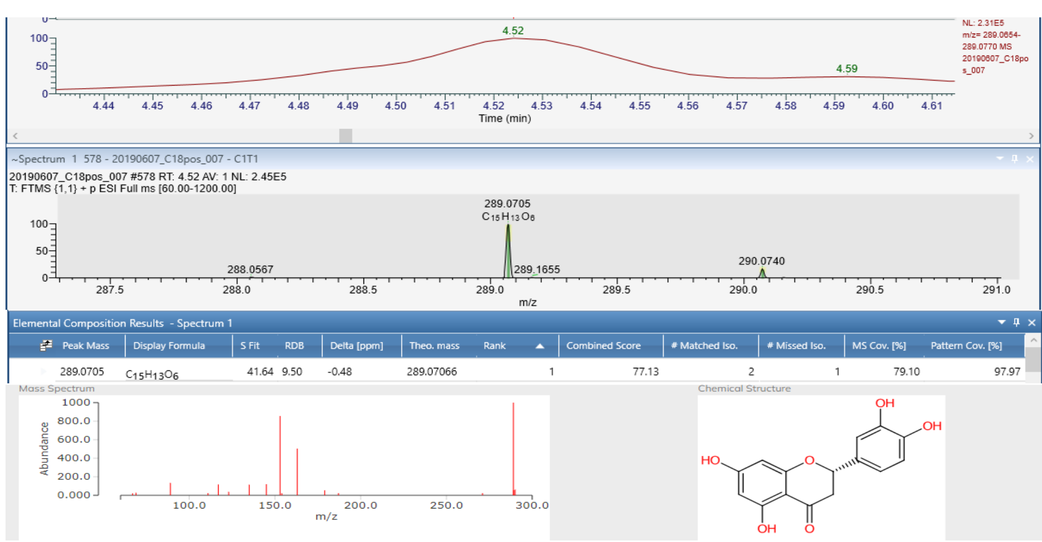
289.0706 / 4.18 (+)-Epicatechin



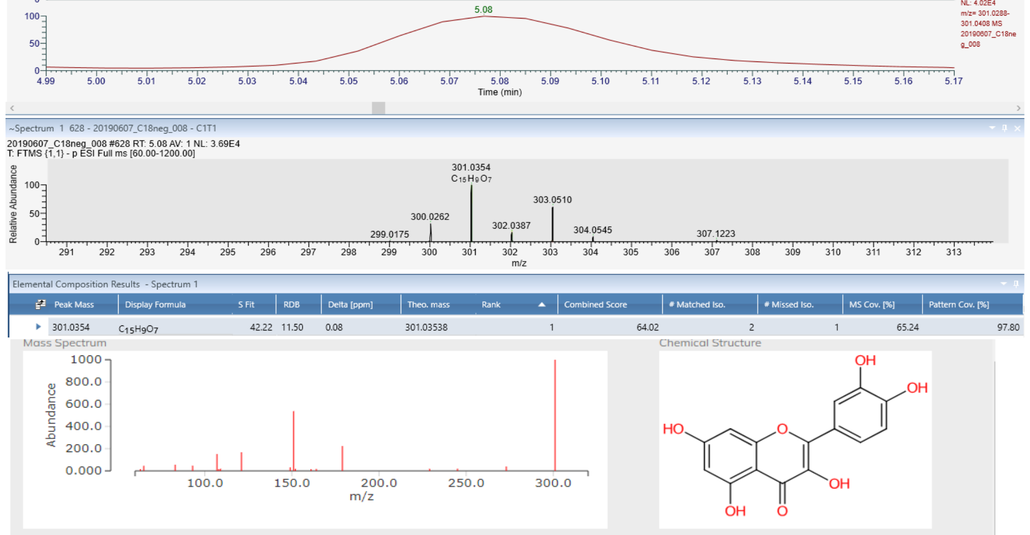
289.0713 3.58 Dihydrokaempferol



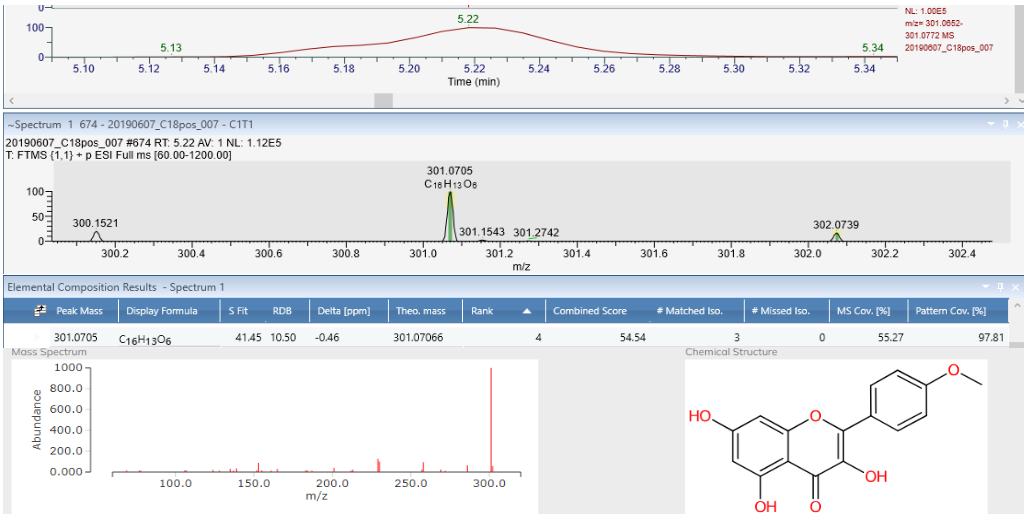
289.0713 4.52 Flavan b. Eriodictyol



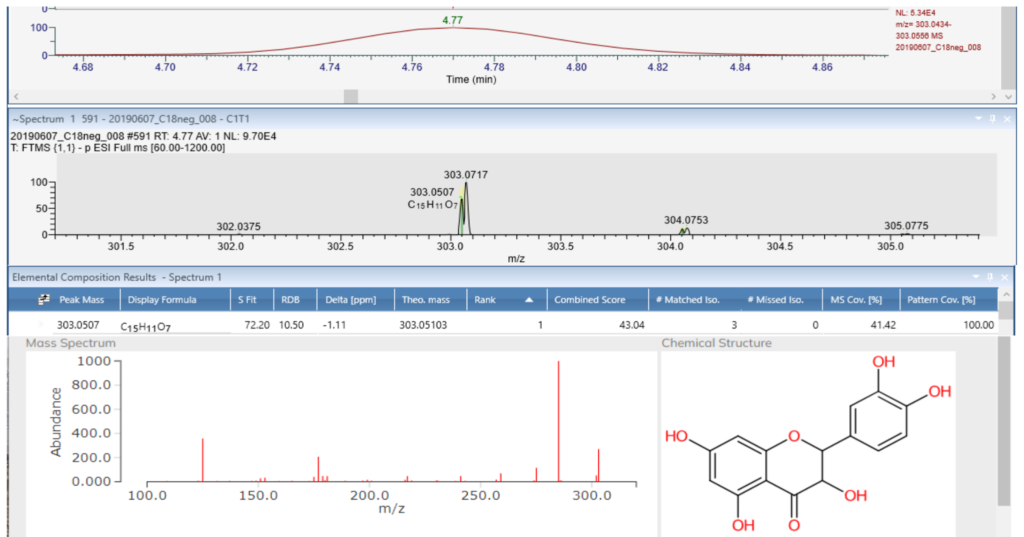
301.0355 / 5.91 Flavonol c. Quercetin



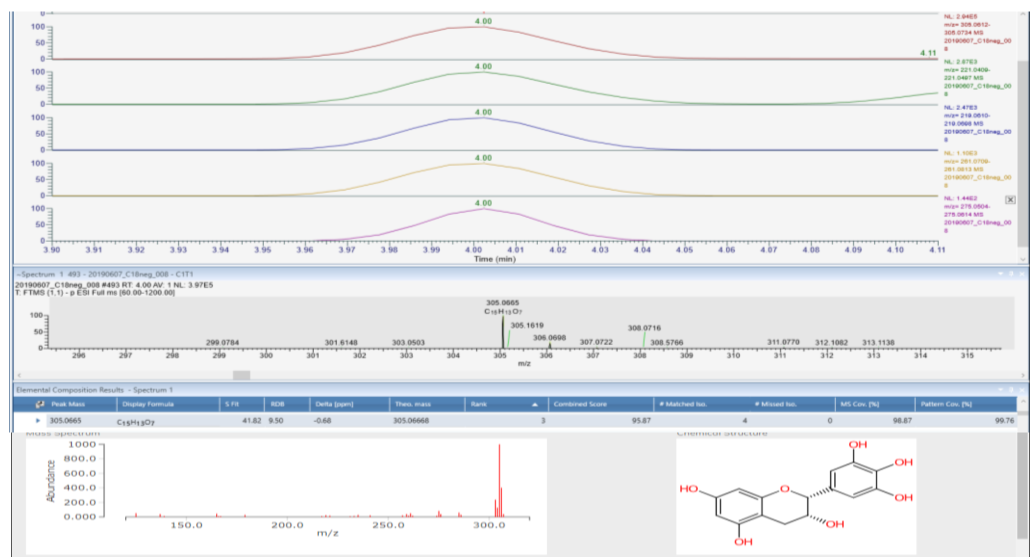
301.0709 / 5.22 Flavonol b. Kaempferide



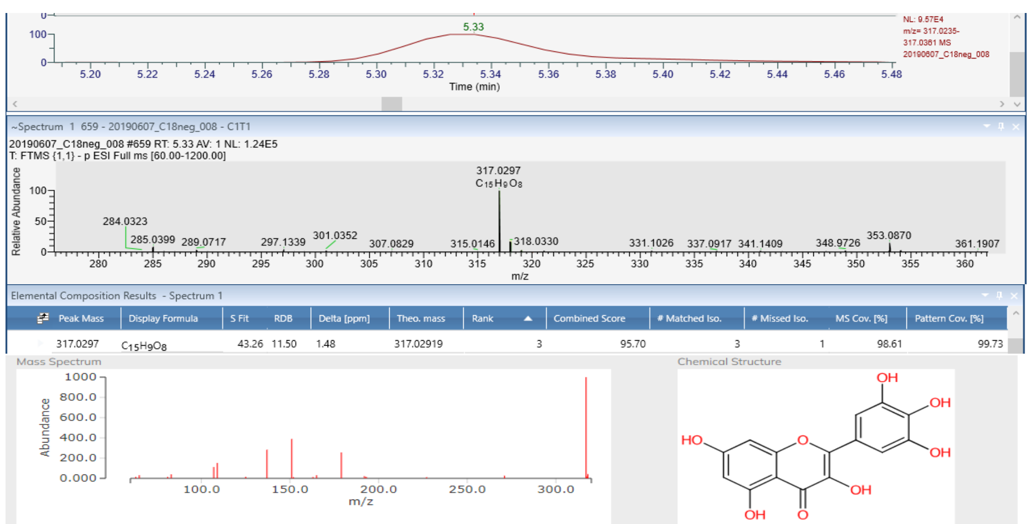
303.0524 / 4.77 Flavanonol Dihydroquercetin



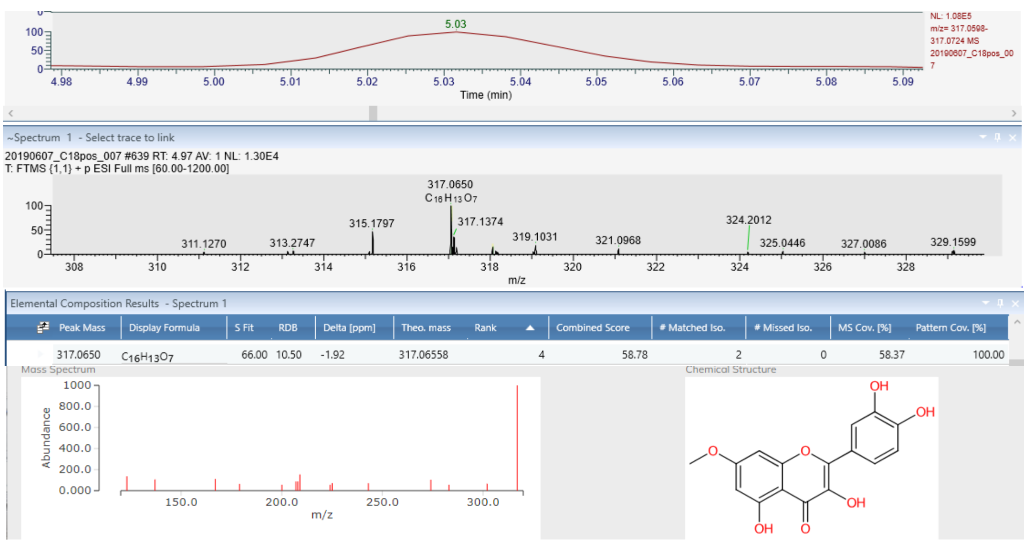
305.0651 / 4.00 Epigallocatechin



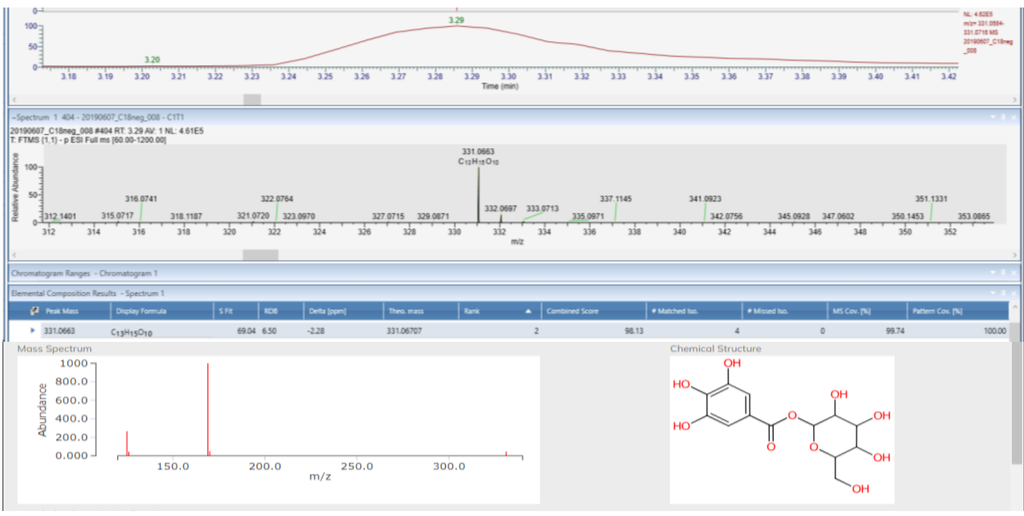
317.0304 / 5.34 Flavonol d. Myricetin



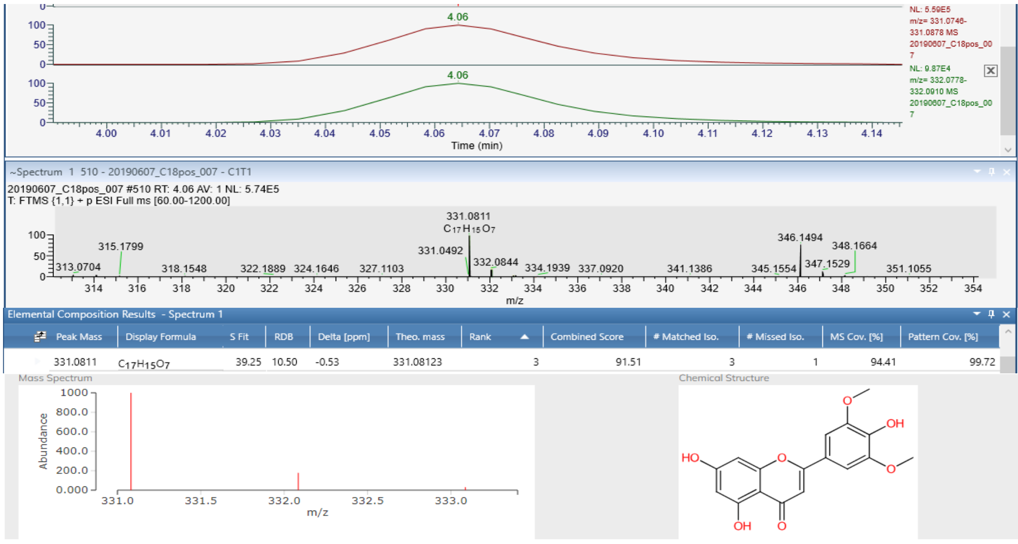
317.0647 / 5.03 Flavonol a. Quercetin 7-methyl ether



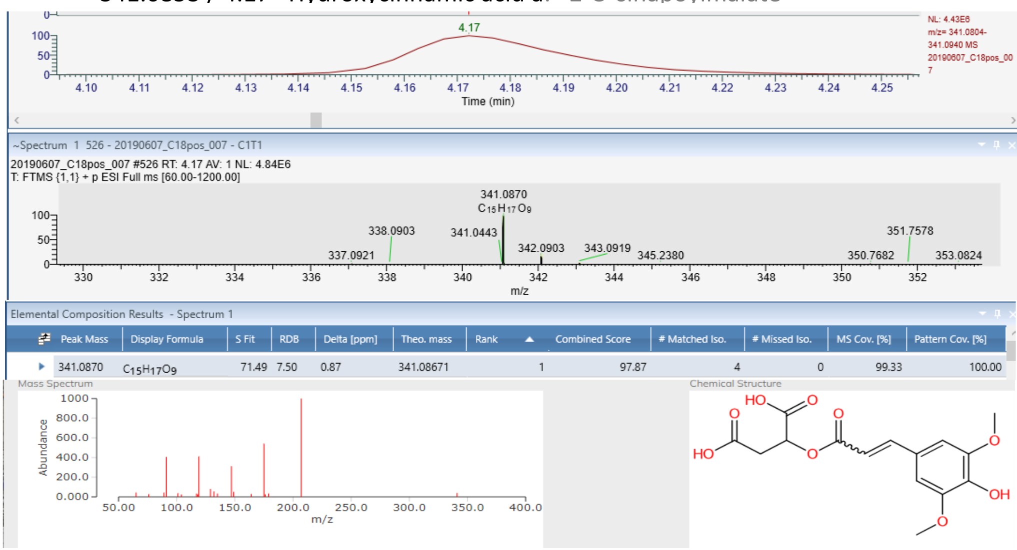
331.0671 / 3.30 Gallic acid hexoside



331.0829 / 4.06 Flavone b. Tricin

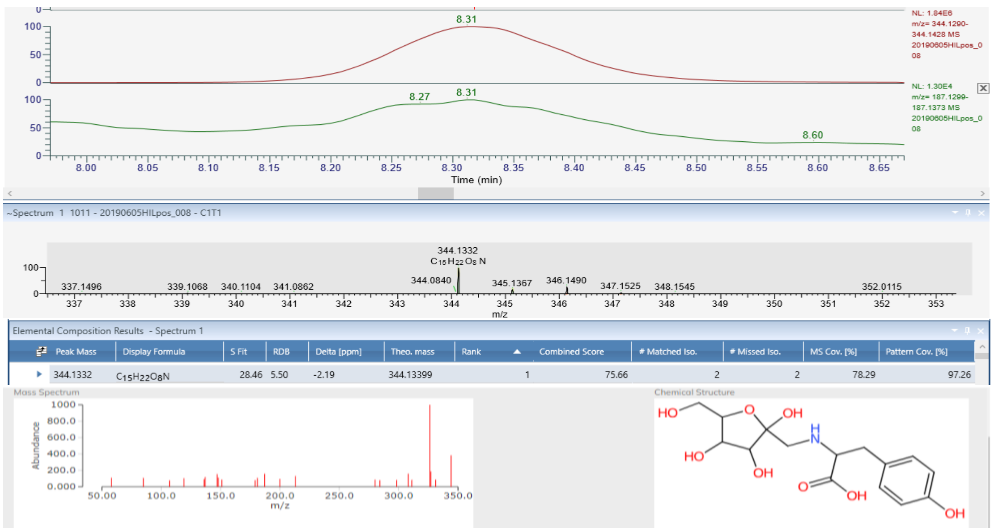


341.0853 / 4.17 Hydroxycinnamic acid a. 2-O-sinapoylmalate



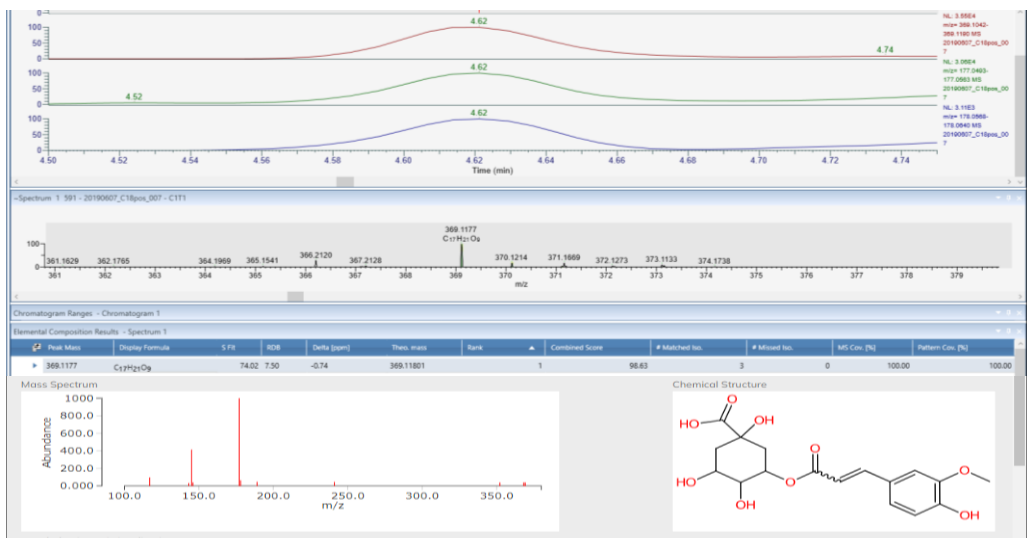
341.1401 / 5.03 Flavan a. 8-Prenylnaringenin

344.1349 / 8.33 N-Fructosyl tyrosine

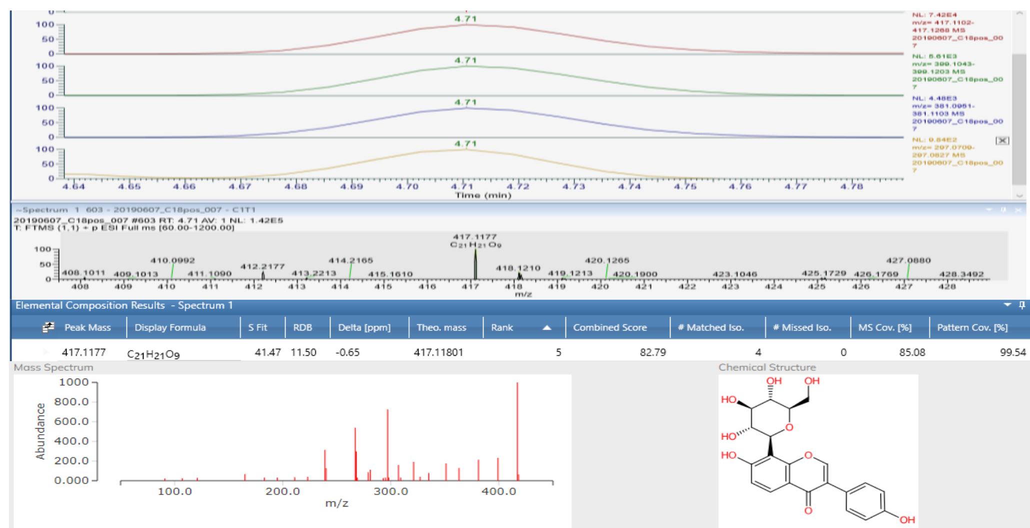


353.0871 / 3.82 Chlorogenic acid

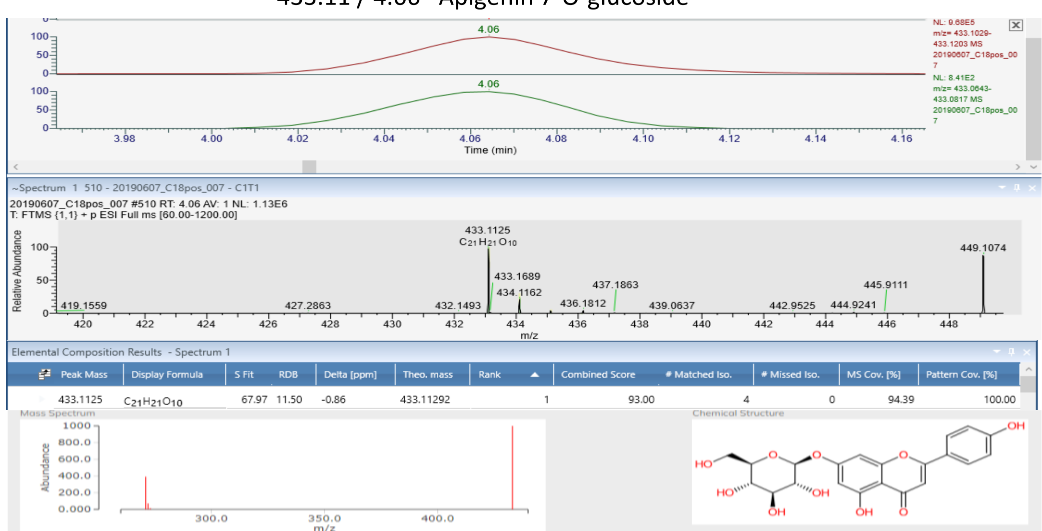
369.1191 / 4.60 Feruloyl quinic acid



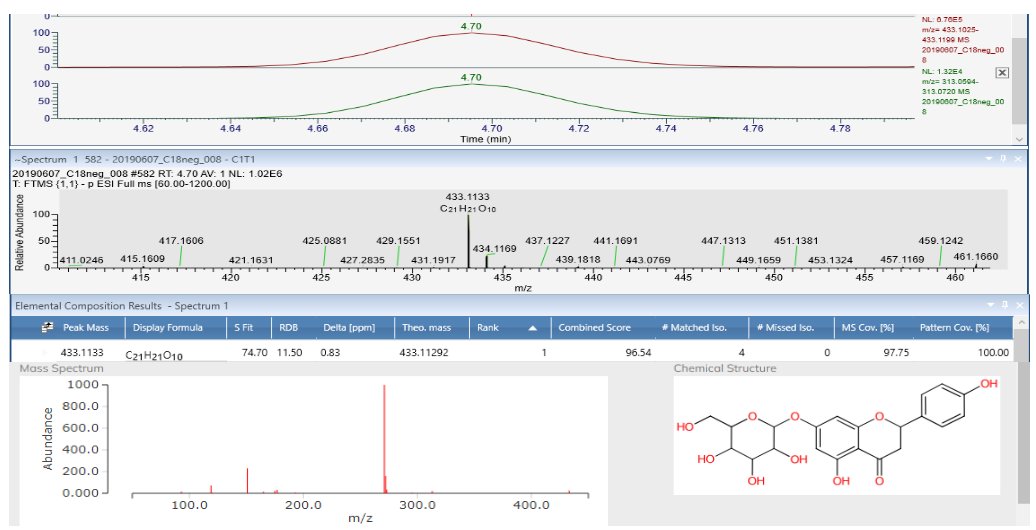
417.1185 / 4.71 Puerarin



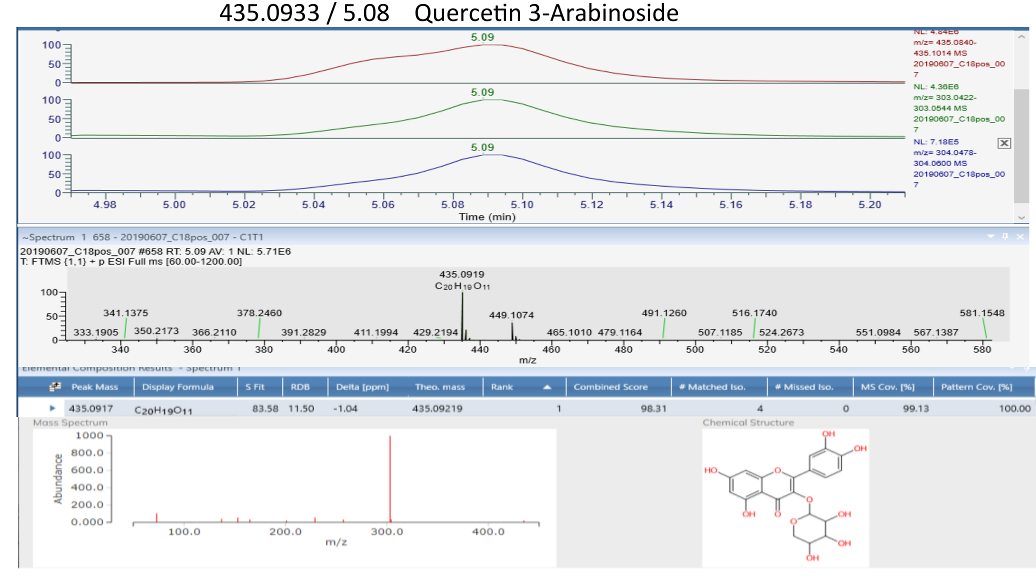
433.11 / 4.06 Apigenin 7-O-glucoside



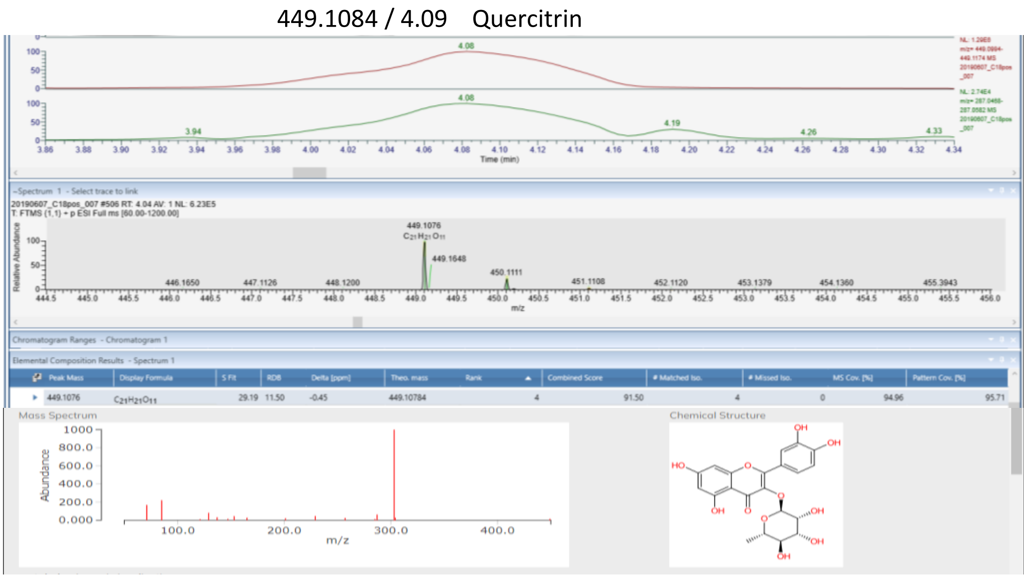
433.1124 / 4.69 Naringenin 7-O-glucoside



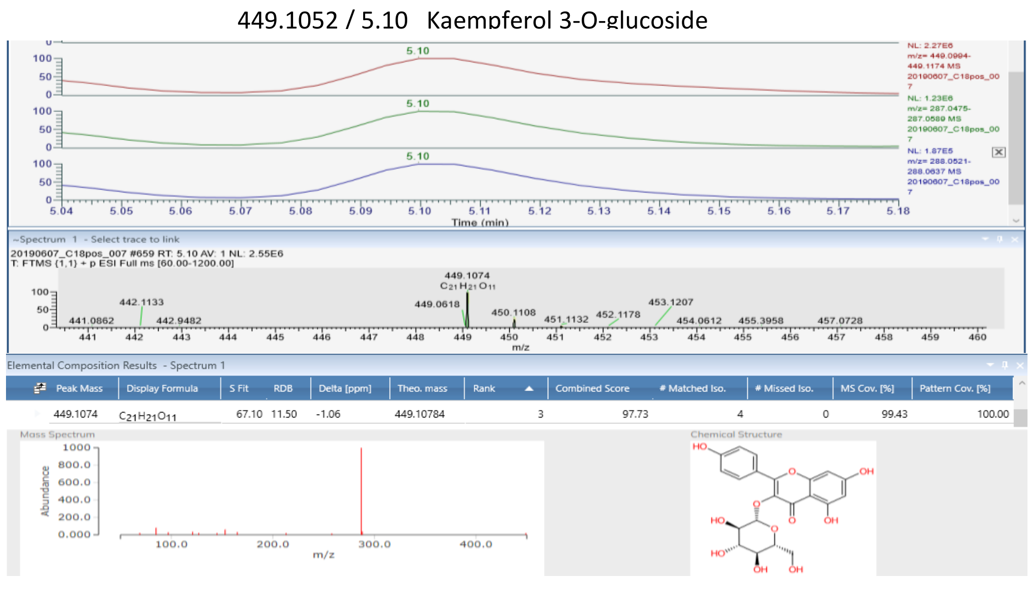
435.0933 / 5.08 Quercetin 3-Arabinoside



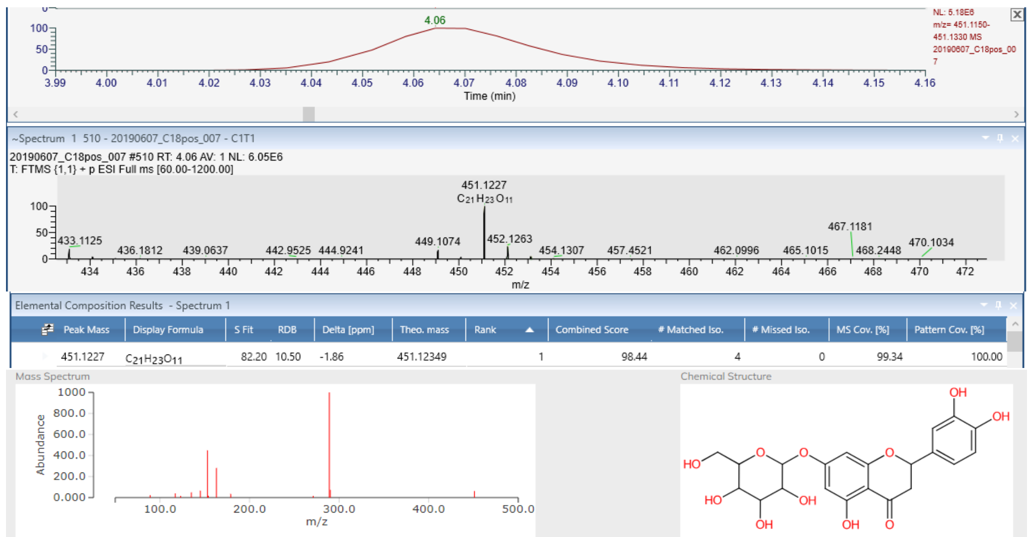
449.1084 / 4.09 Quercitrin



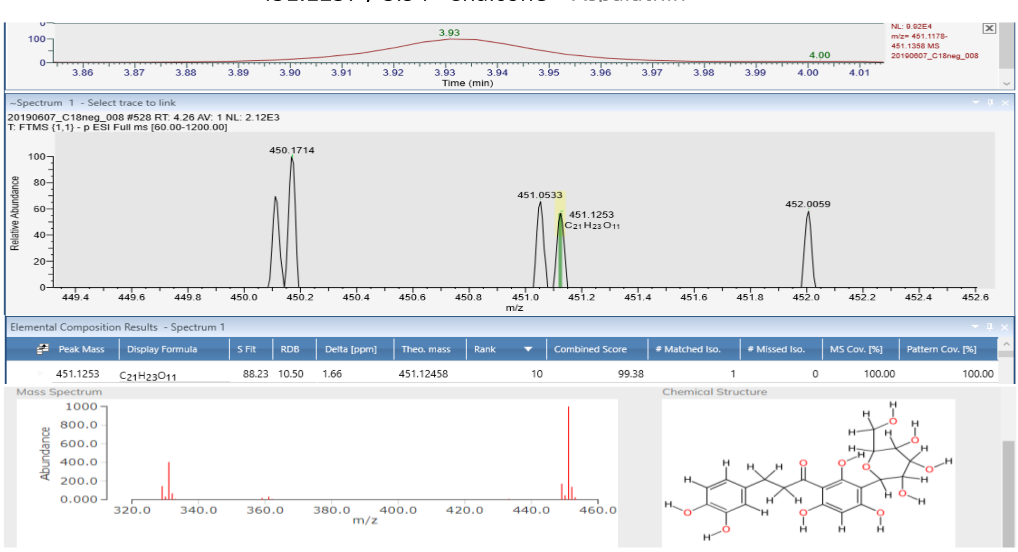
449.1052 / 5.10 Kaempferol 3-O-glucoside



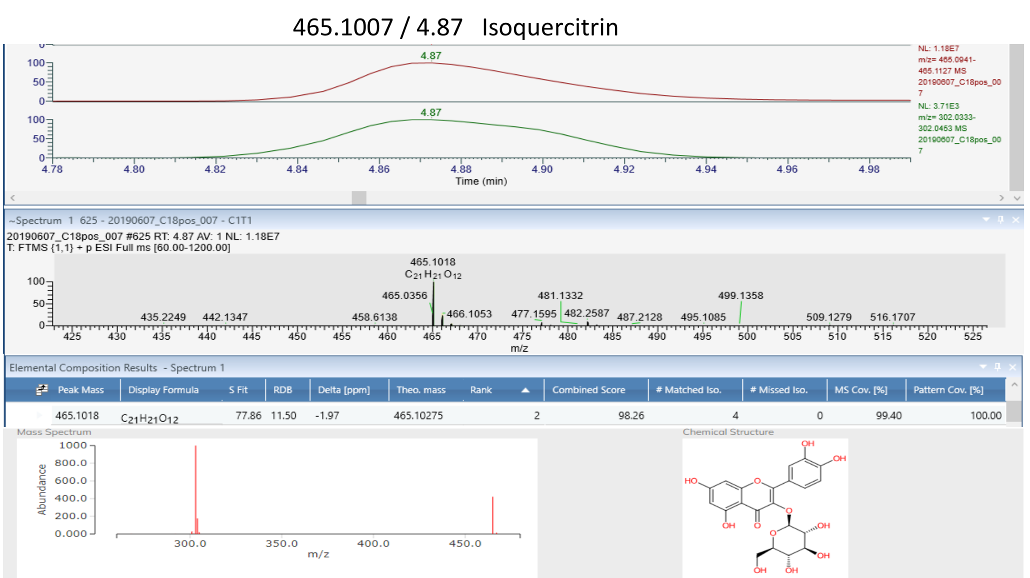
451.1225 / 4.06 Flavonone Eriodictyol 7-O-glucoside



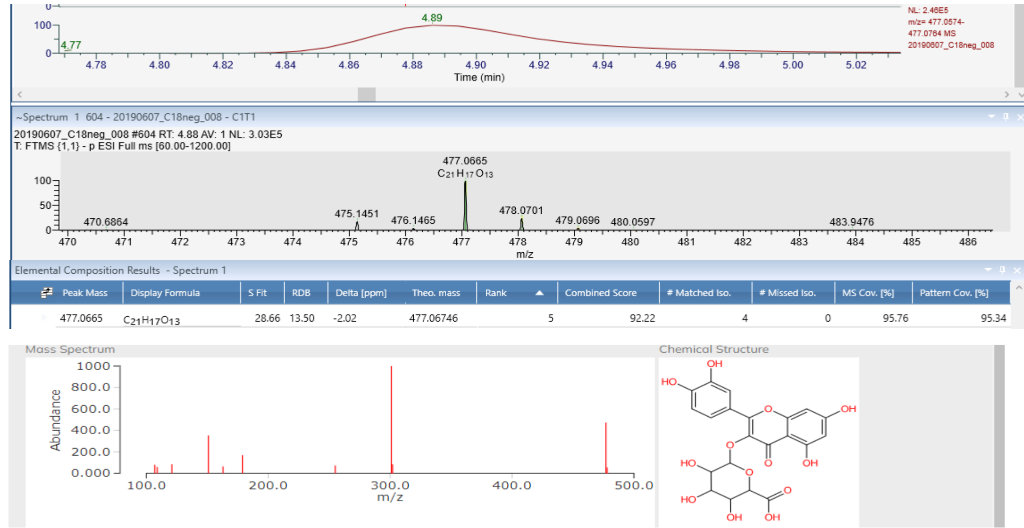
451.1257 / 3.94 Chalcone Aspalathin



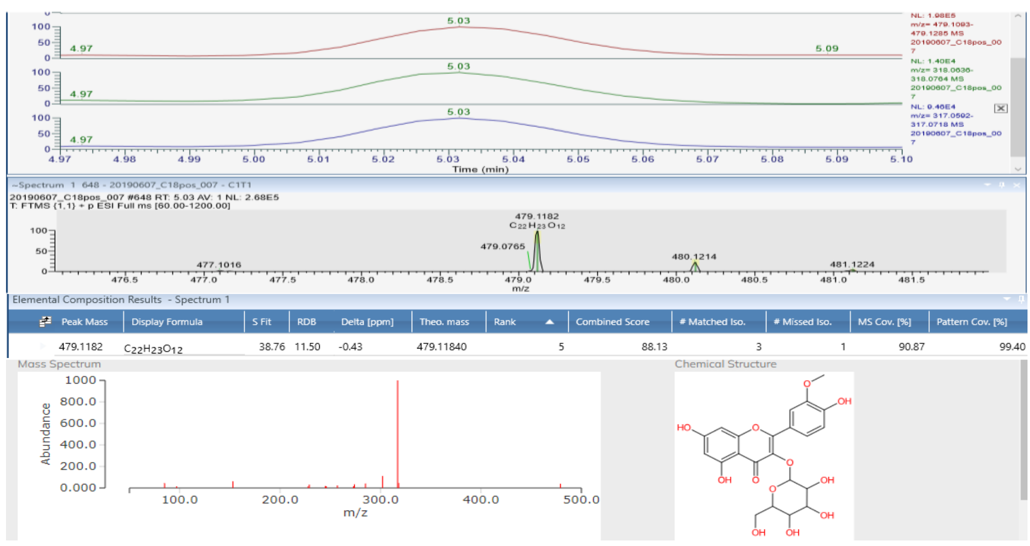
465.1007 / 4.87 Isoquercitrin



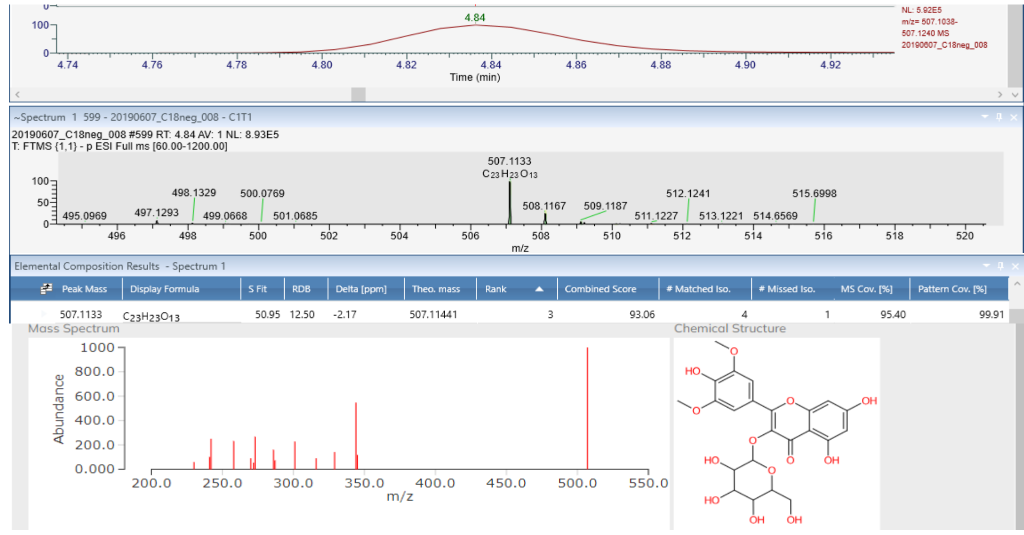
477.069 / 4.89 Flavonoid-3-O-glucuronide Quercetin 3-0-Glucuronide



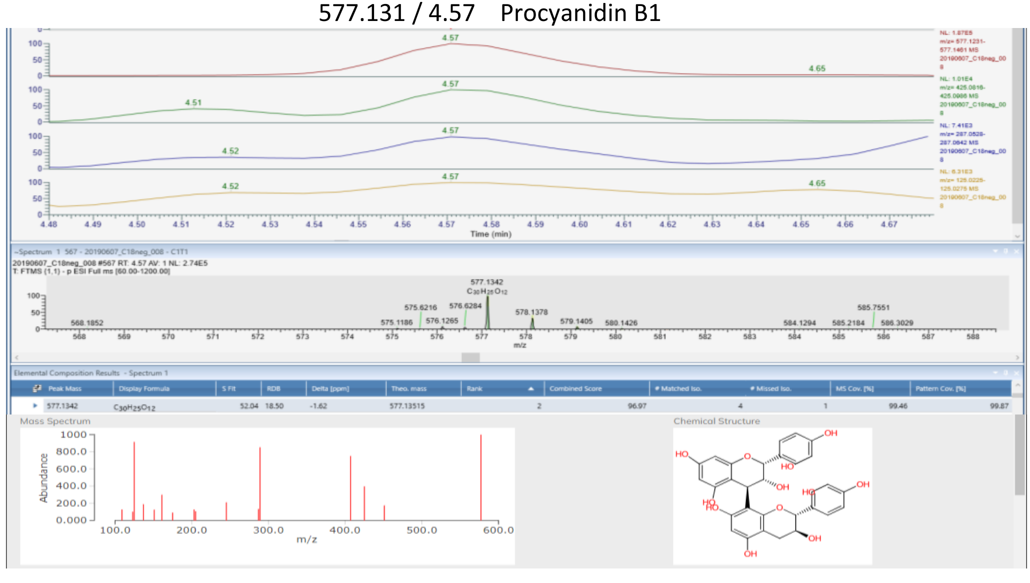
479.1189 / 5.03 Isorhamnetin 3-O-glucoside



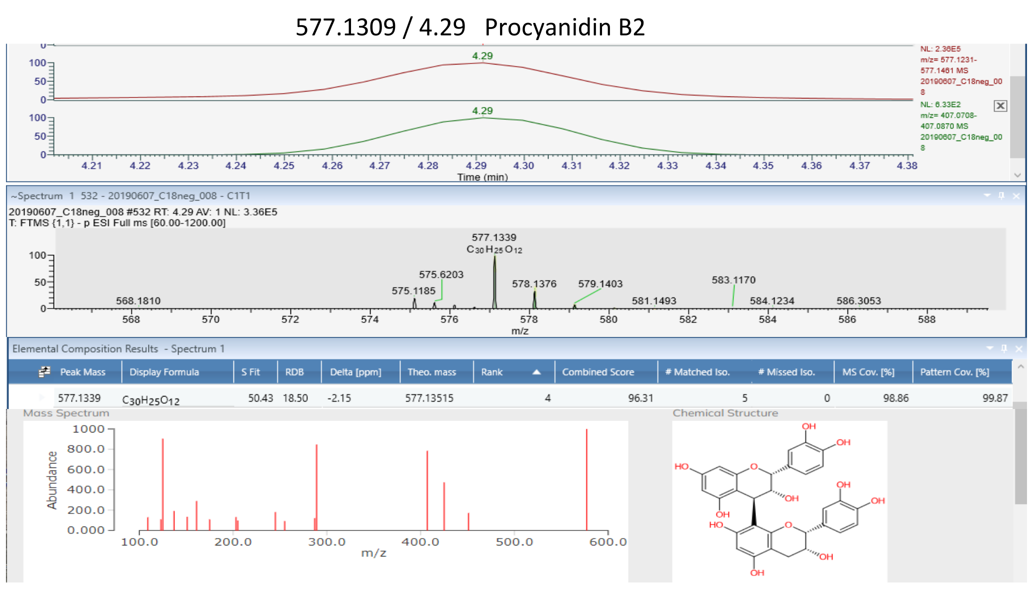
507.111 / 4.84 Flavonoid-3-O-glycoside Syringetin 3-O-glucoside



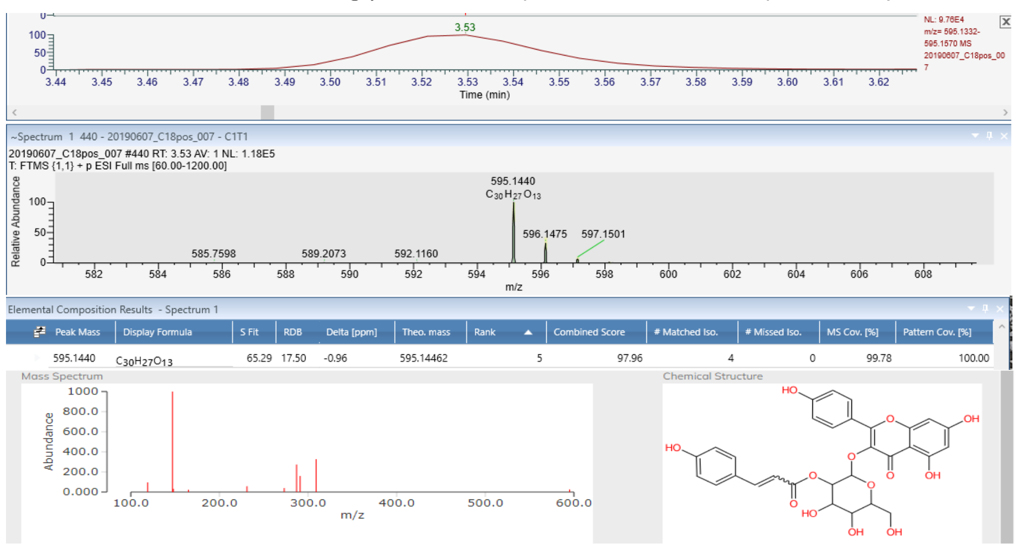
577.131 / 4.57 Procyanidin B1



577.1309 / 4.29 Procyanidin B2



595.145 / 3.53 Flavonoid glycoside Kaempferol-3-Glucoside-2''-p-coumaroyl

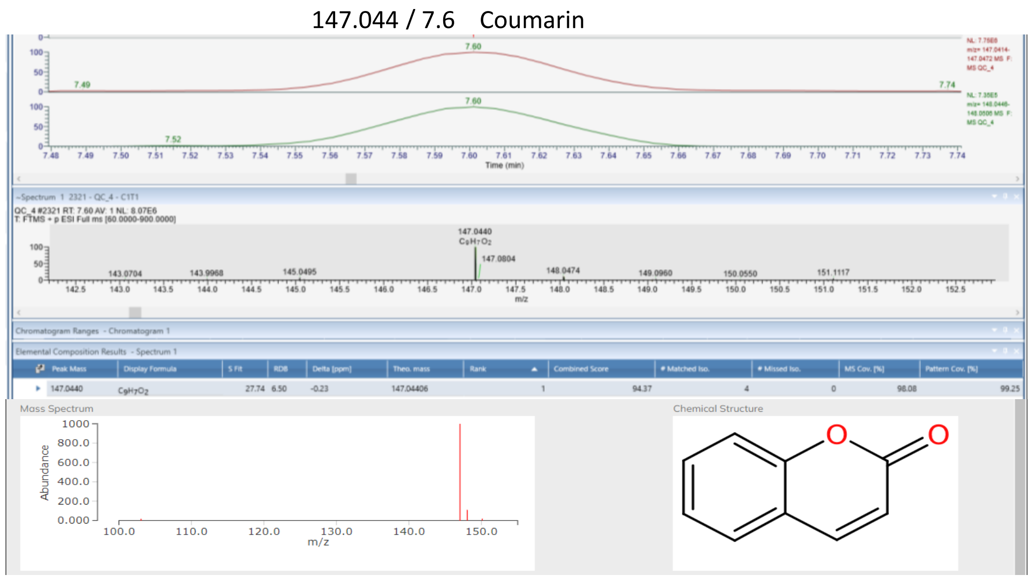


Appendix B:

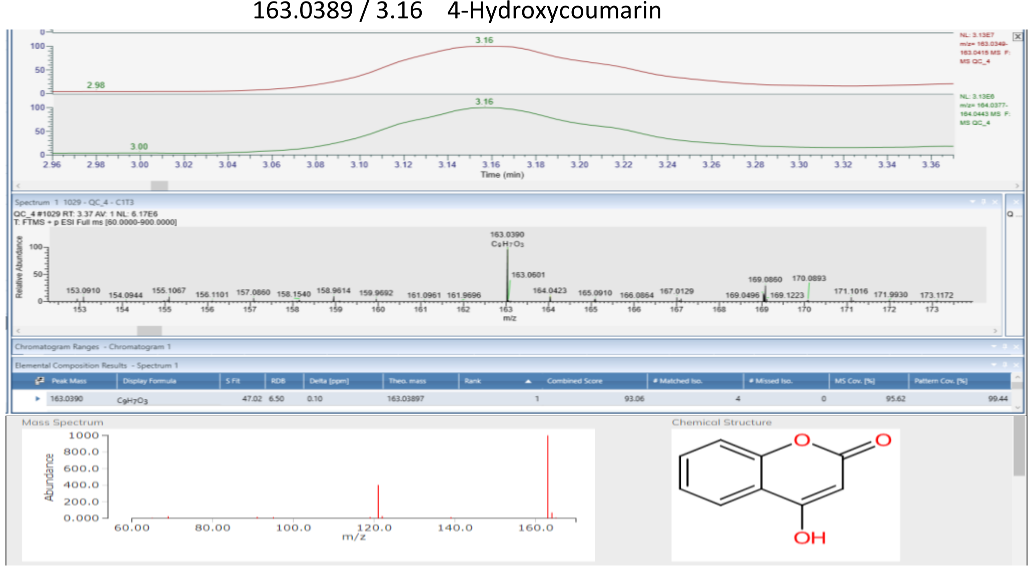
Chapter 4, Annotated compound confirmations.

All annotation confirmation data composes of screen images from 1: ThermoFisher — Excalibur “Freestyle”. In the top chromatogram is retention time (rt.). The spectrum beneath provides the m/z value and below that the chemical formula and mass error (Delta [ppm]). 2: The bottom field shows the mass spectral graphic in the online “MassBank” mass spectral library from which parent and fragment ion *m/z* abundance values have been derived and the chemical structure is also displayed. Compounds are ordered by ascending *m/z* value. Zooming in on each will reveal finer detail.

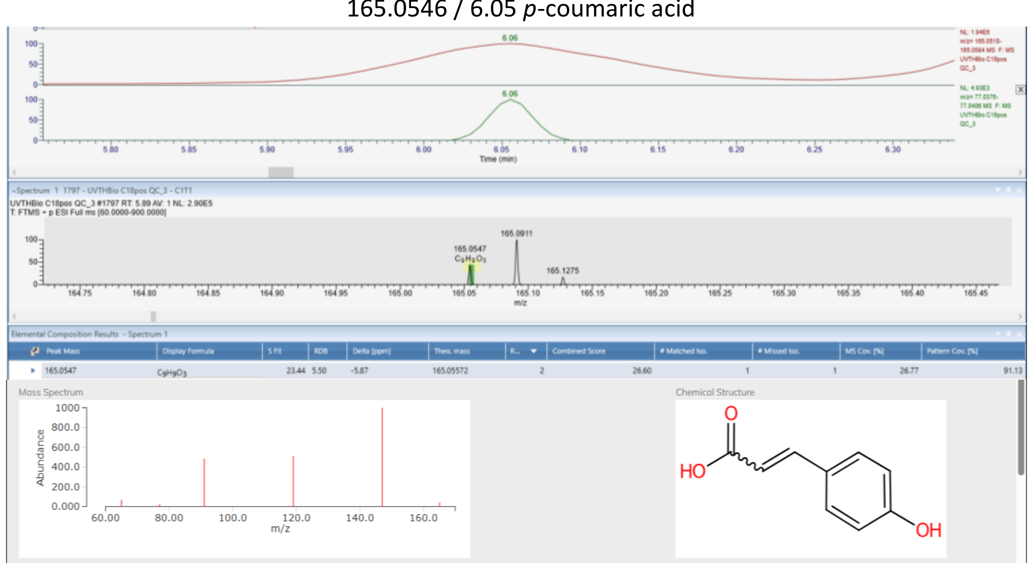
147.044 / 7.6 Coumarin



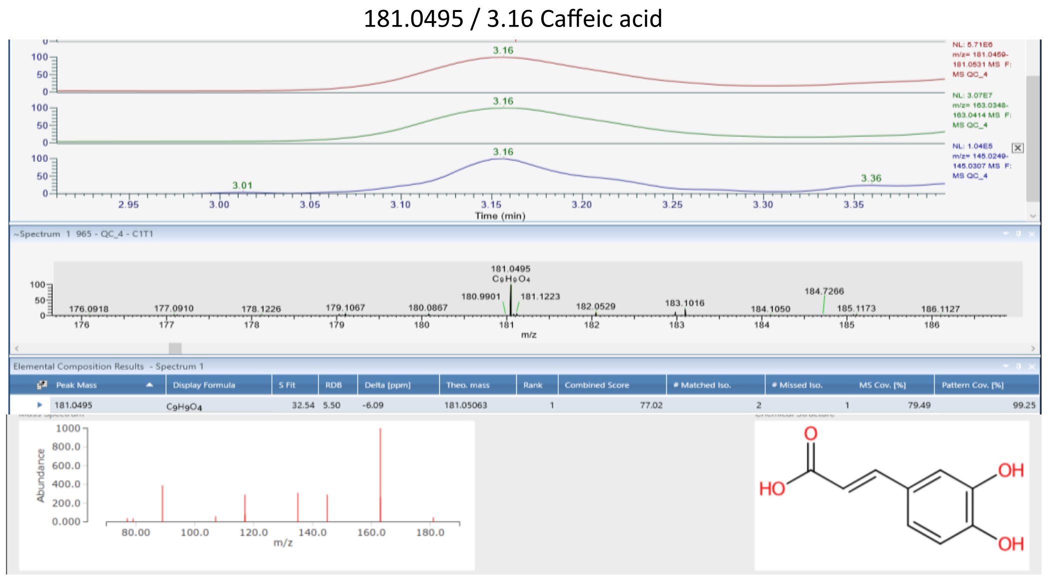
163.0389 / 3.16 4-Hydroxycoumarin



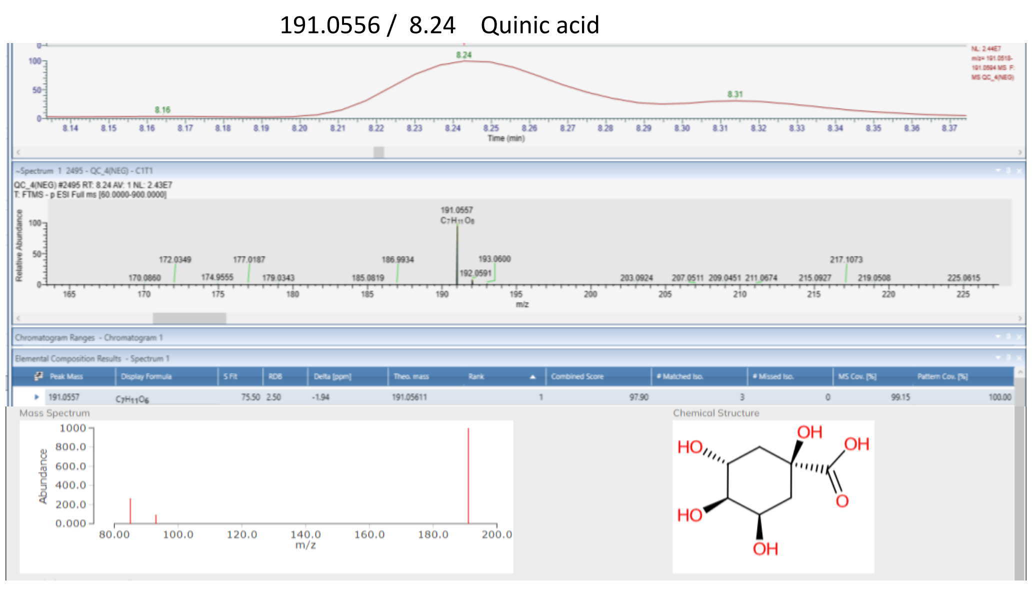
165.0546 / 6.05 *p*-coumaric acid



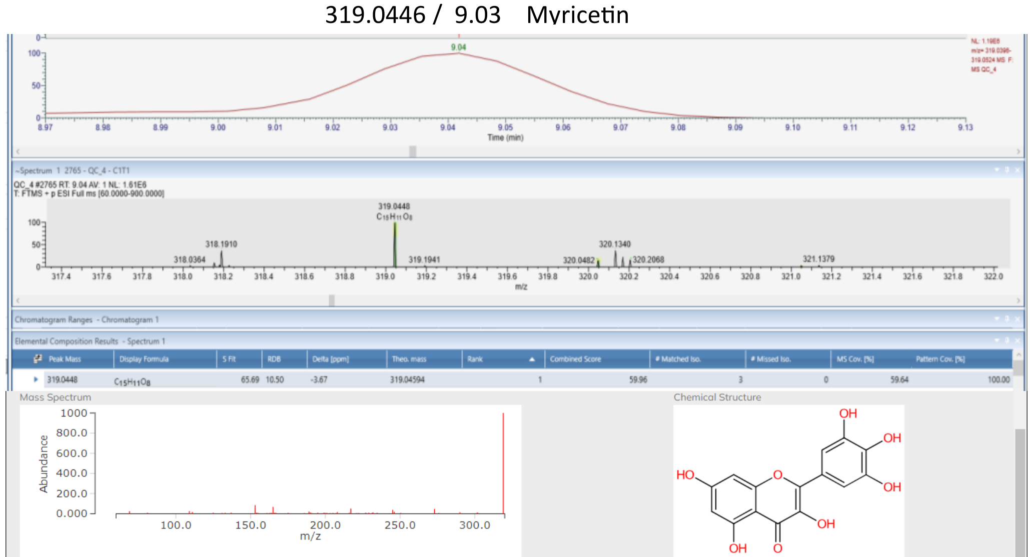
181.0495 / 3.16 Caffeic acid



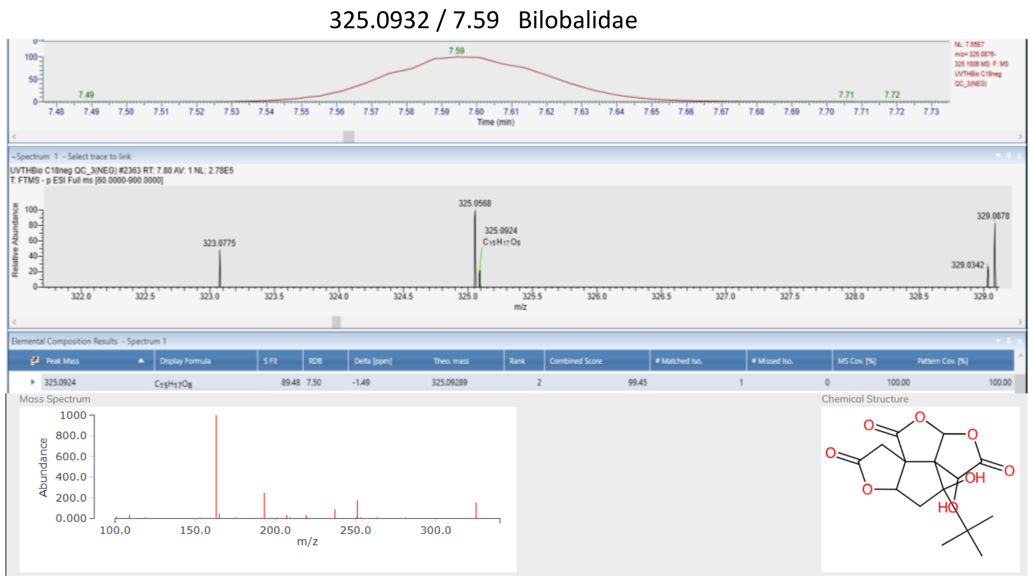
191.0556 / 8.24 Quinic acid



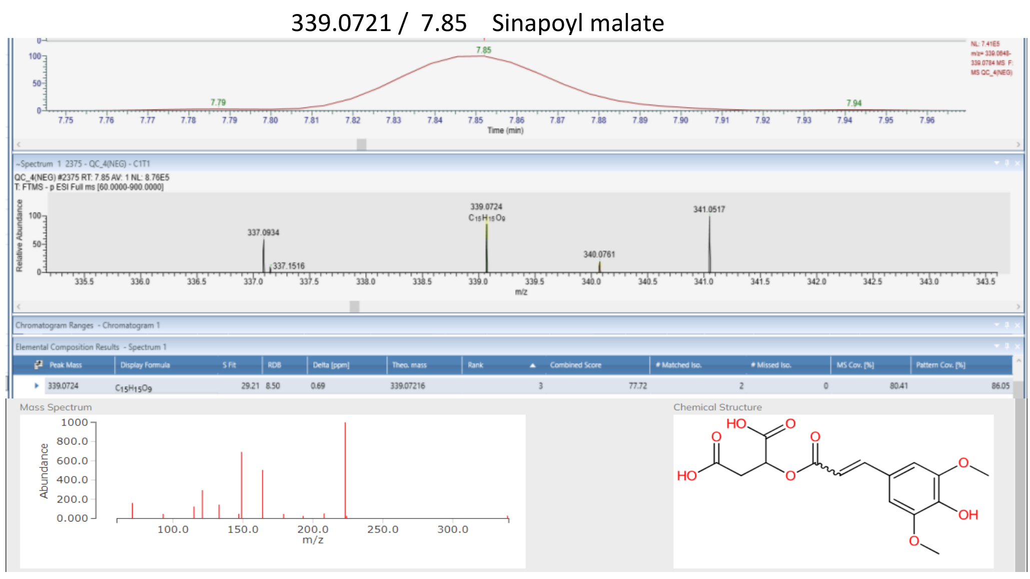
319.0446 / 9.03 Myricetin



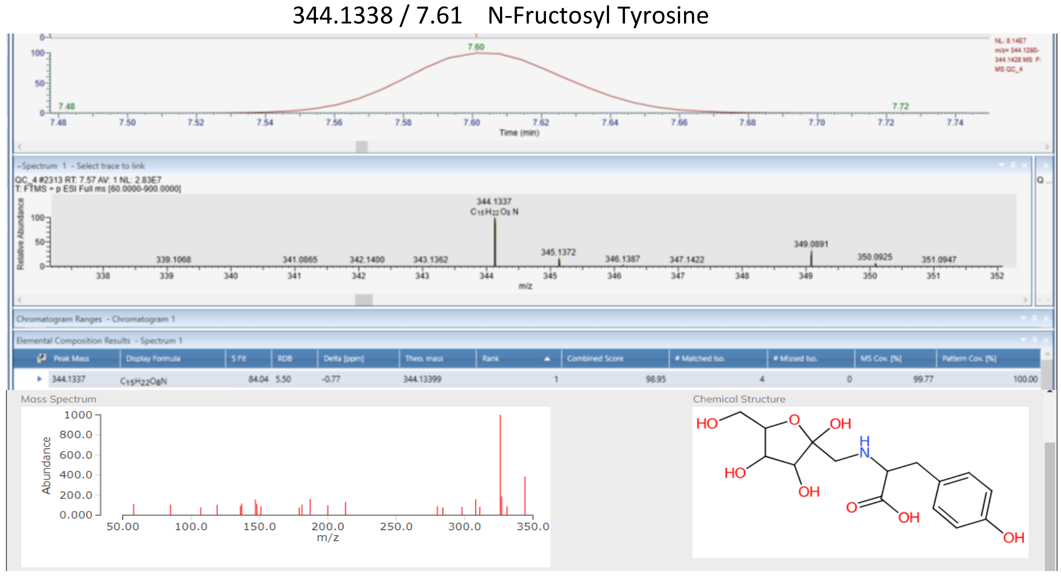
325.0932 / 7.59 Bilobalidae



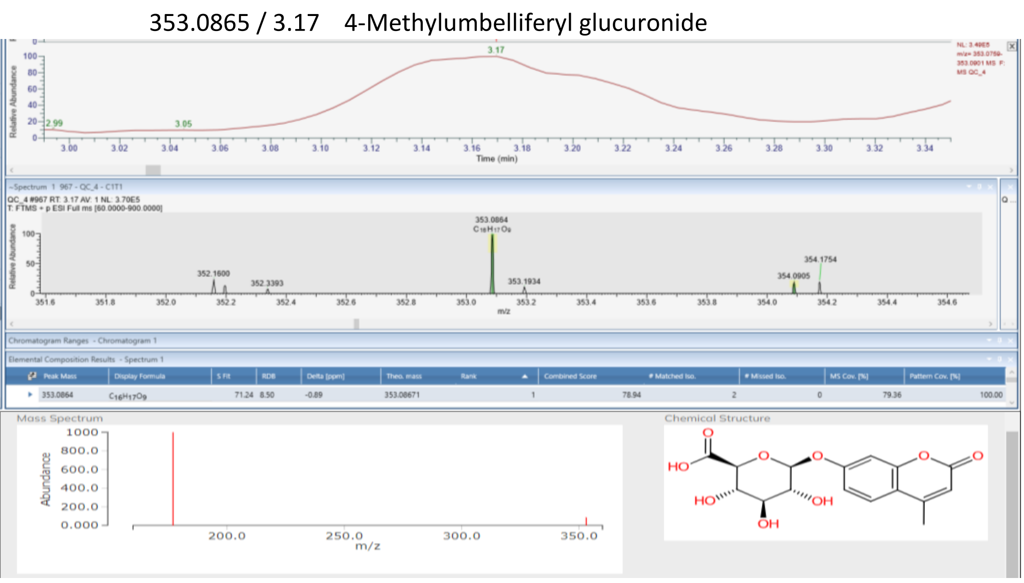
339.0721 / 7.85 Sinapoyl malate



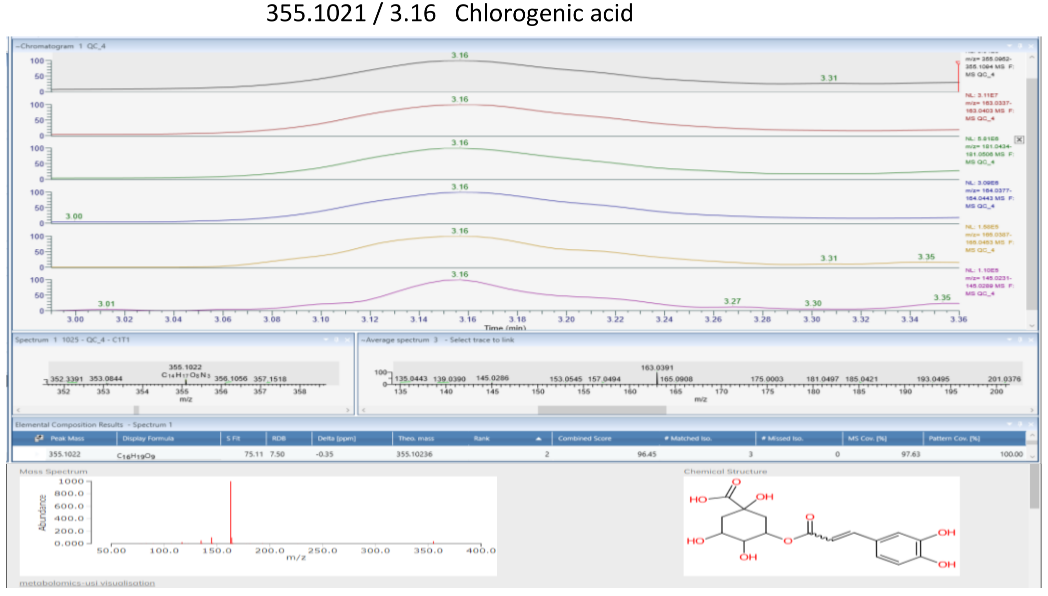
344.1338 / 7.61 N-Fructosyl Tyrosine



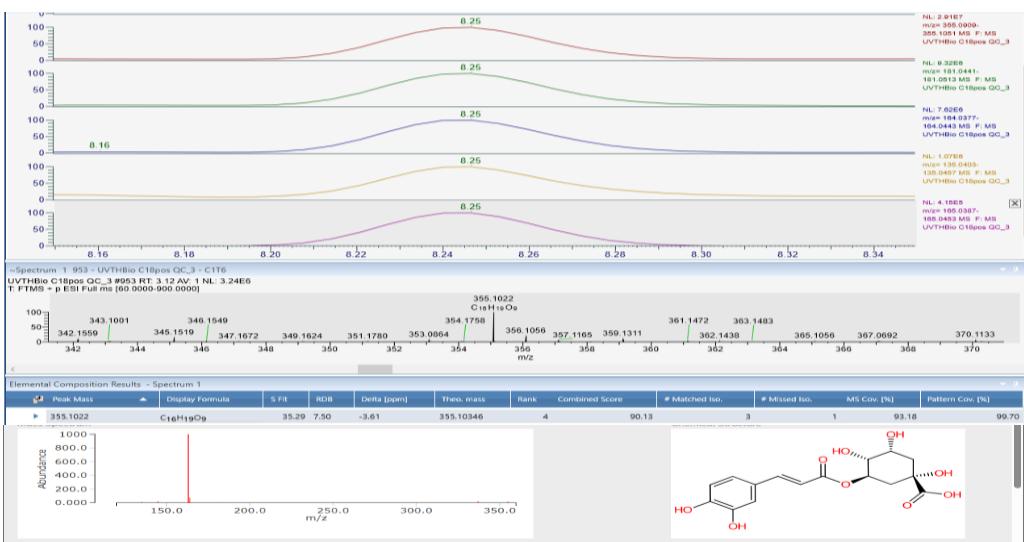
353.0865 / 3.17 4-Methylumbelliferyl glucuronide



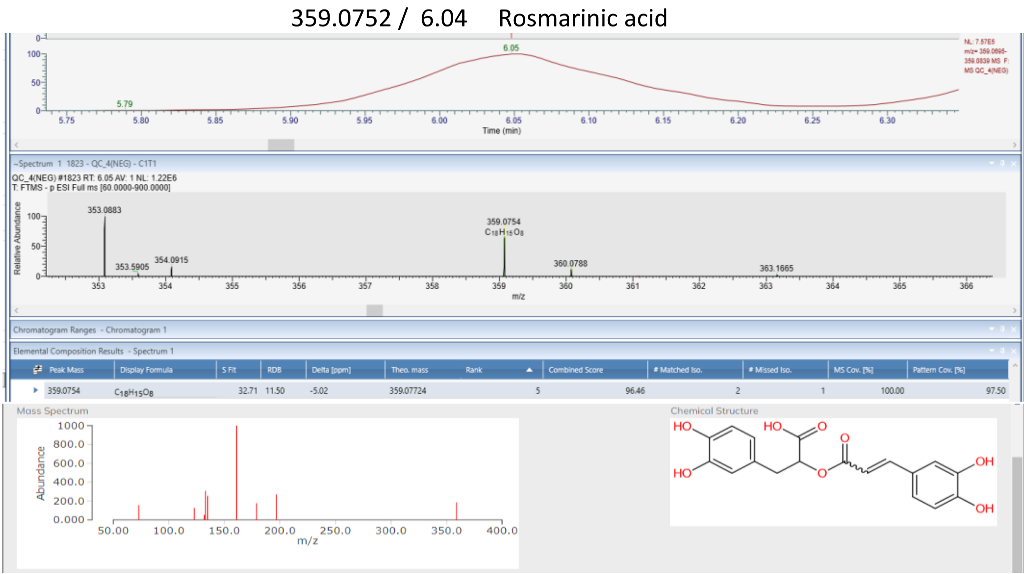
355.1021 / 3.16 Chlorogenic acid



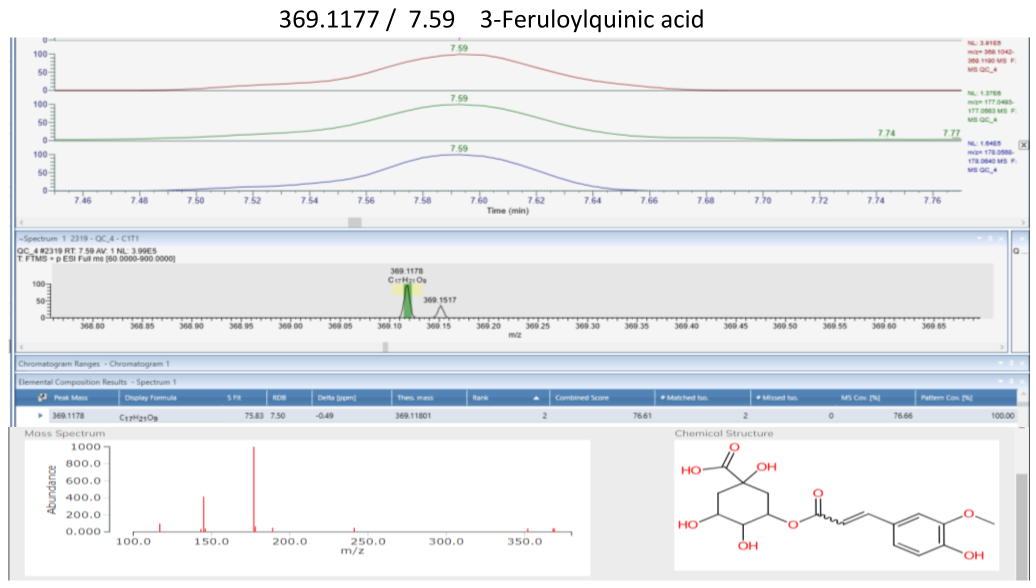
355.1021 / 8.25 Caffeoylquinic acid



359.0752 / 6.04 Rosmarinic acid



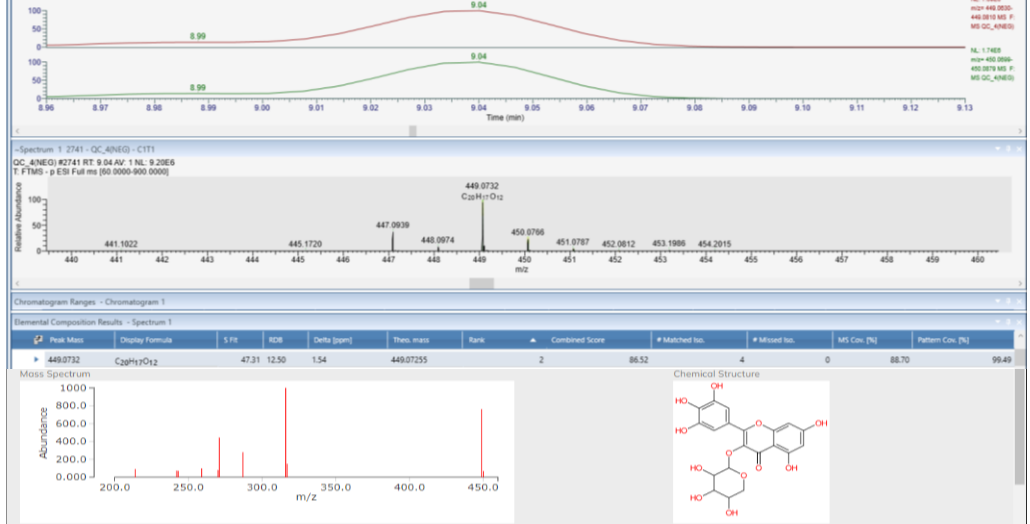
369.1177 / 7.59 3-Feruloylquinic acid



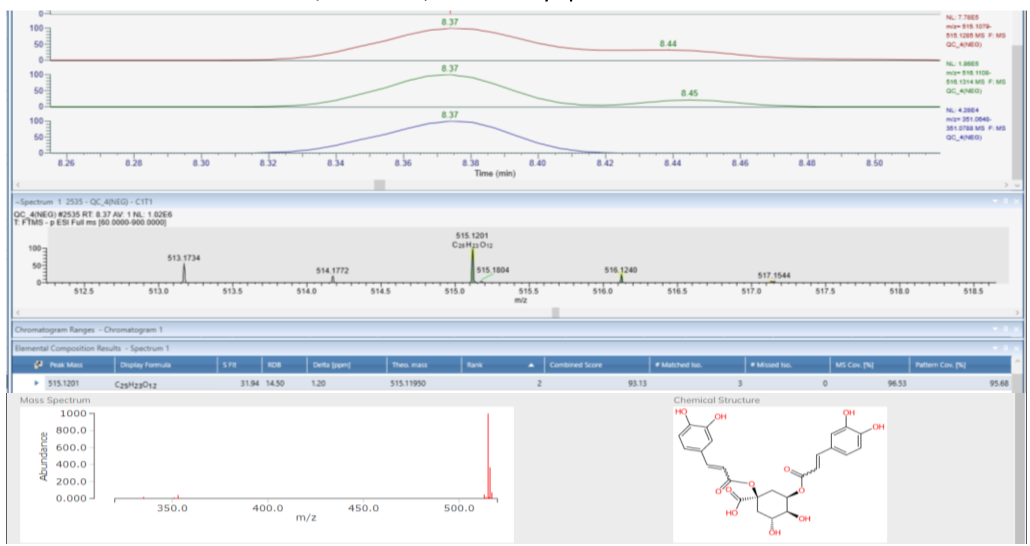
389.1246 / 9.78   3,4',5-Trihydroxystilbene-3-beta-D-glucoside

Peak Mass	Display Formula	S Fit	RDB	Delta [ppm]	Theo. mass	Rank	Combined Score	# Matched Iso.	# Missed Iso.	MS Cov. [%]	Pattern Cov. [%]
389.1247	$C_{20}H_{21}O_8$	38.96	10.50	1.37	389.12419	2	79.57	2	1	81.83	97.17

449.0733 / 9.04   Myricetin-3-Xyloside



515.12 / 8.37   1,5-Dicaffeoylquinic acid

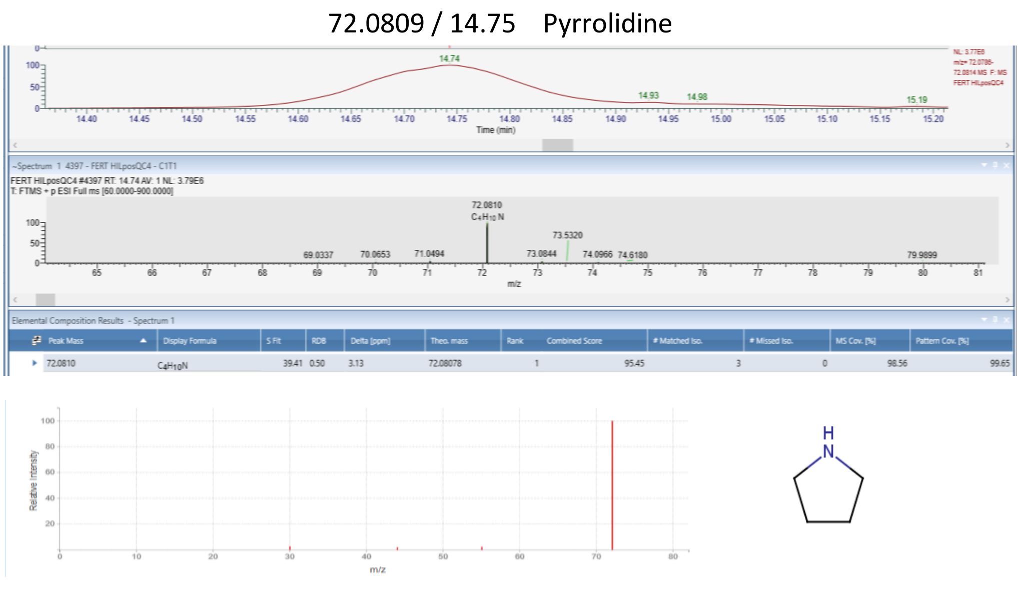


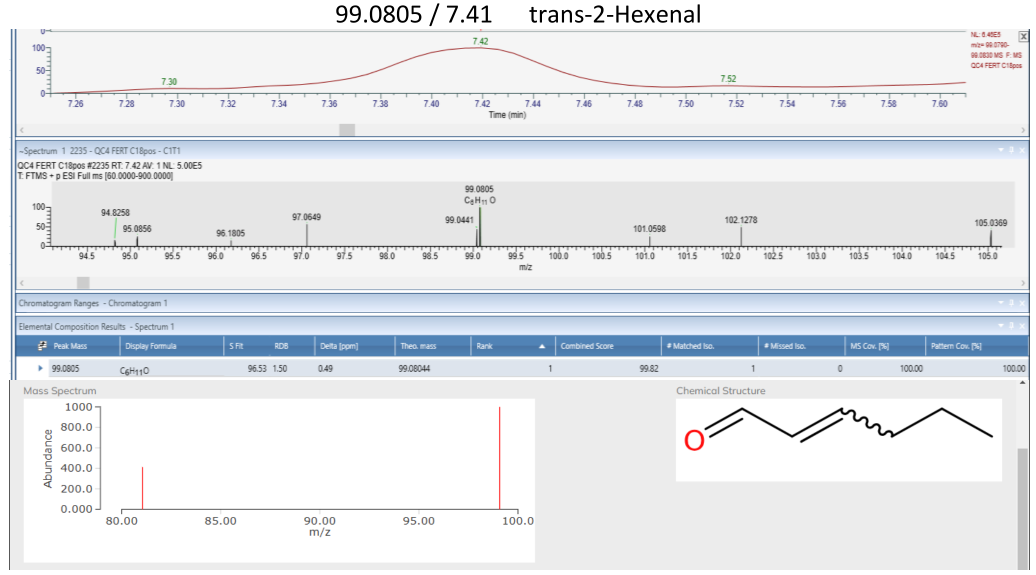
595.1444 / 1.87 Kaempferol-3-Glucoside-2''-p-coumaroyl

Appendix C:

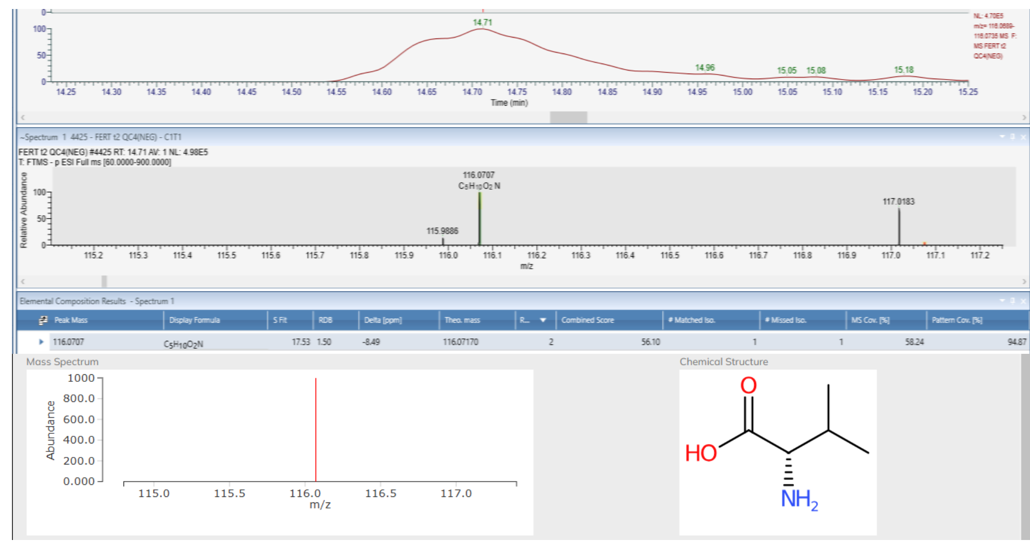
Chapter 5, Annotated compound confirmations.

All annotation confirmation data composes of screen images from 1: ThermoFisher — Excalibur “Freestyle”. In the top chromatogram is retention time (rt.). The spectrum beneath provides the m/z value and below that the chemical formula and mass error (Delta [ppm]). 2: The bottom field shows the mass spectral graphic in the online “MassBank” mass spectral library from which parent and fragment ion *m/z* abundance values have been derived and the chemical structure is also displayed. Compounds are ordered by ascending *m/z* value. Zooming in on each will reveal finer detail.

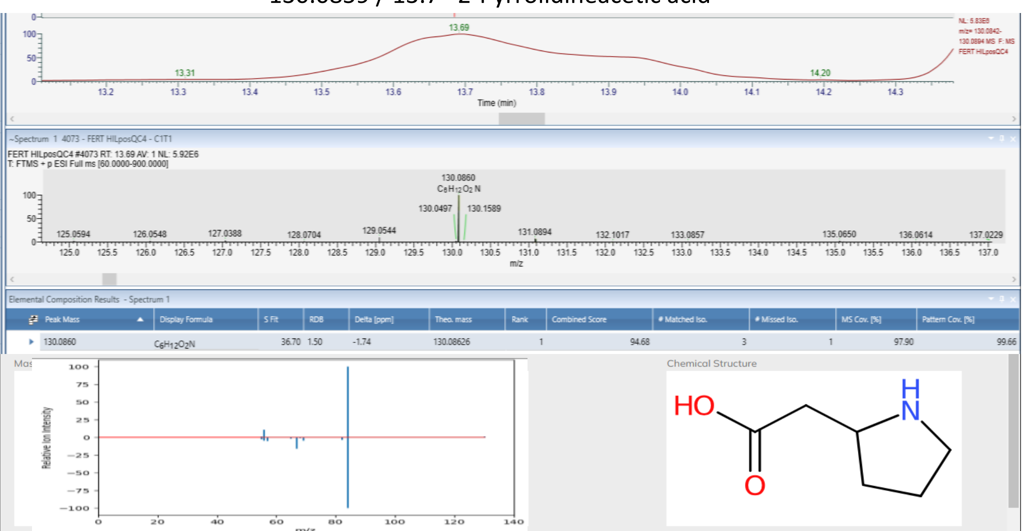




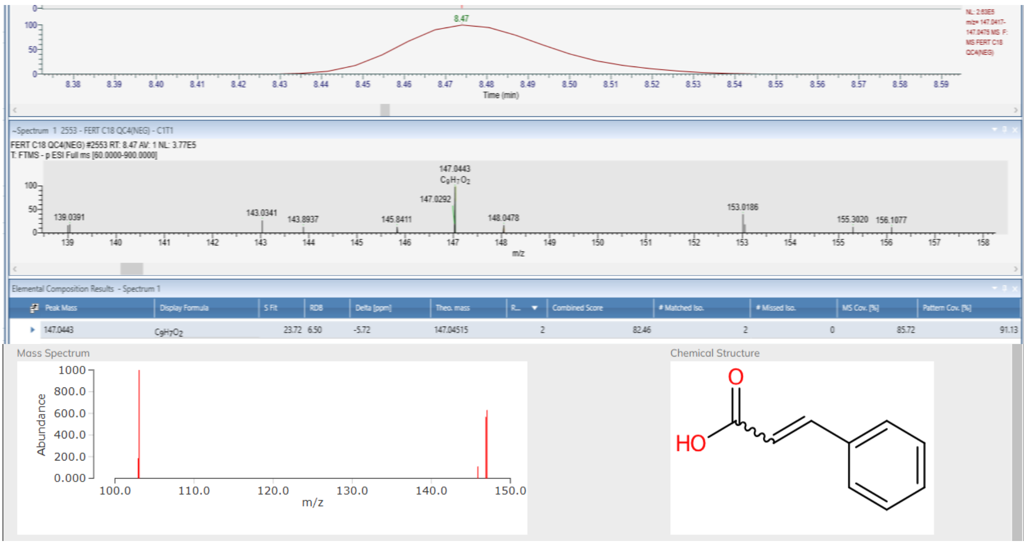
116.0706 / 14.72   L-Valine

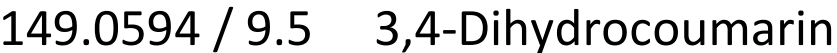


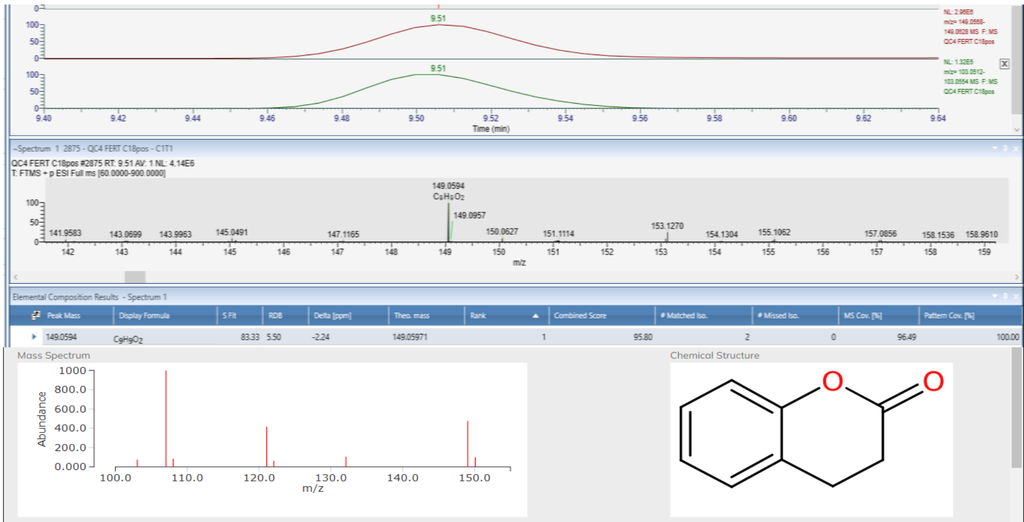
130.0859 / 13.7   2-Pyrrolidineacetic acid



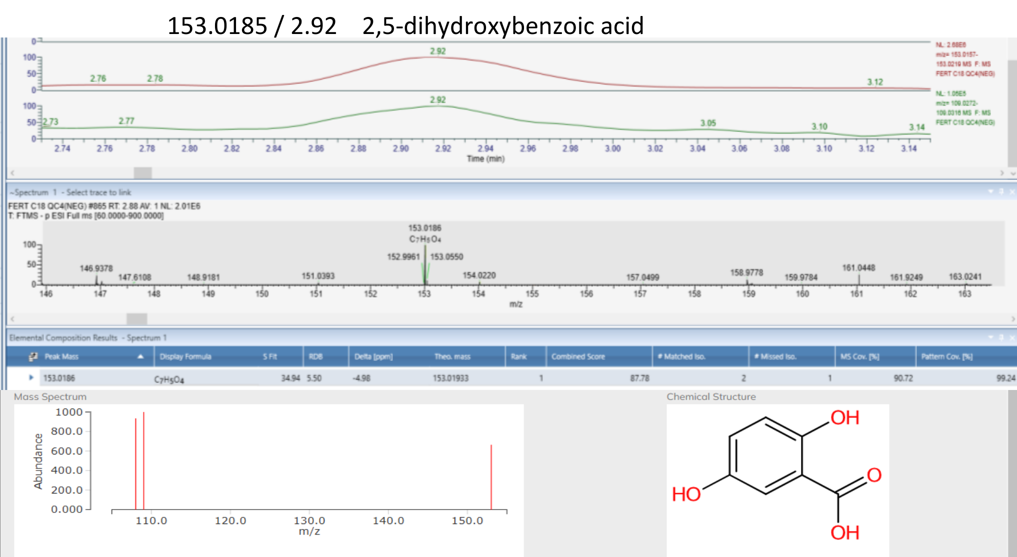
147.0443 / 8.46   trans-Cinnamic acid



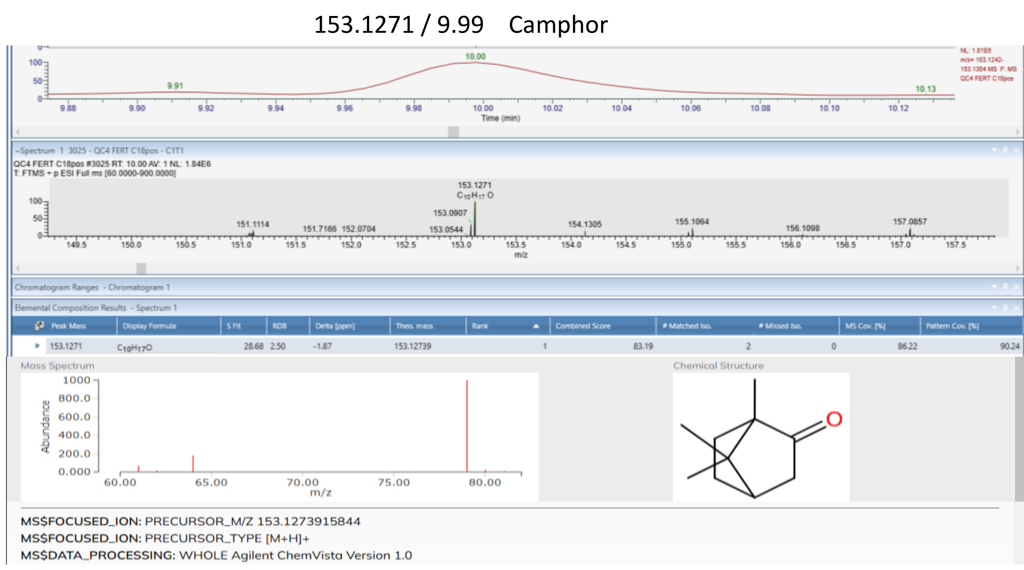




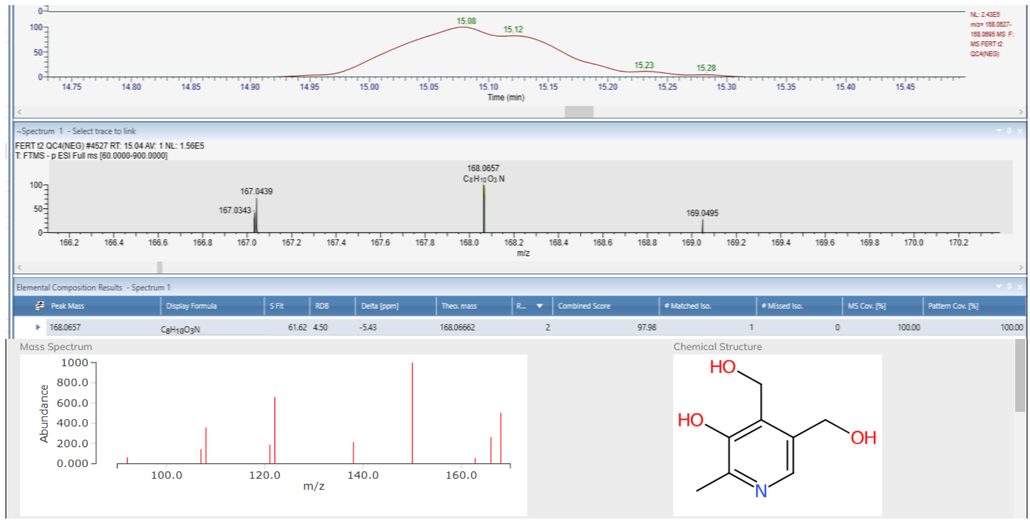
153.0185 / 2.92 2,5-dihydroxybenzoic acid



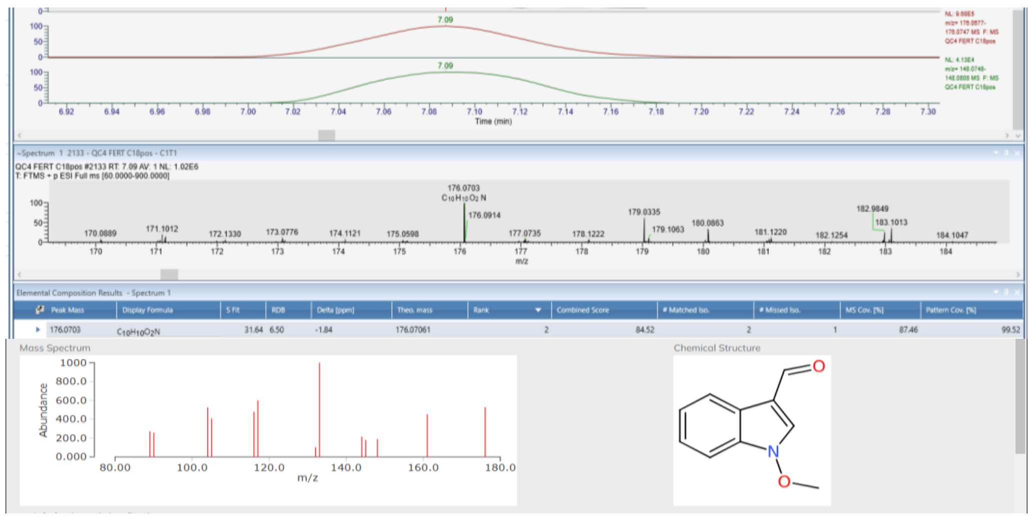
153.1271 / 9.99 Camphor



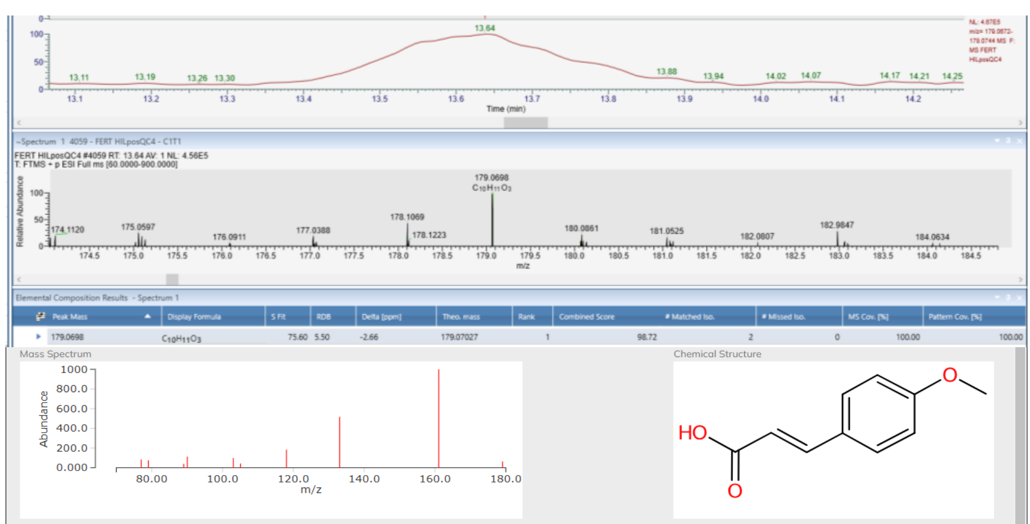
168.0656 / 15.09 Pyridoxine



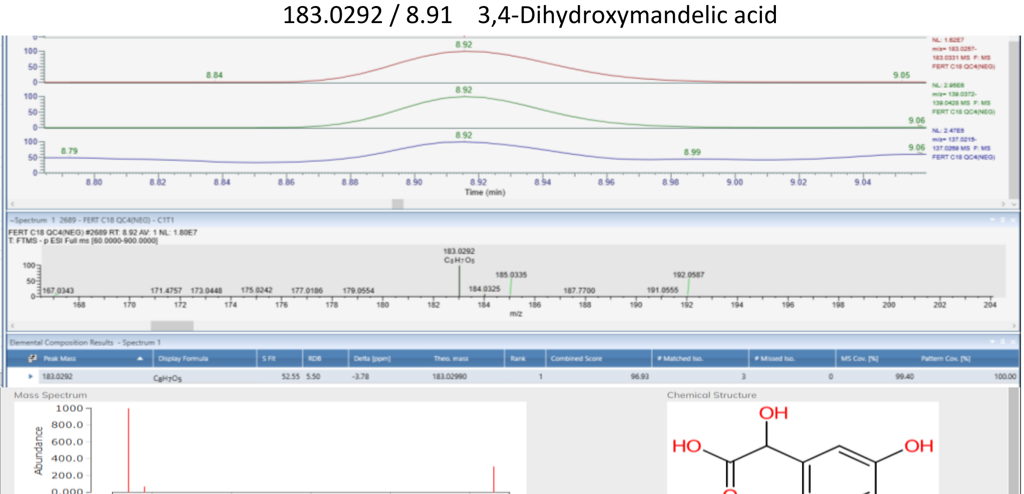
176.0703 / 7.09 1-Methoxy-3-formylindole



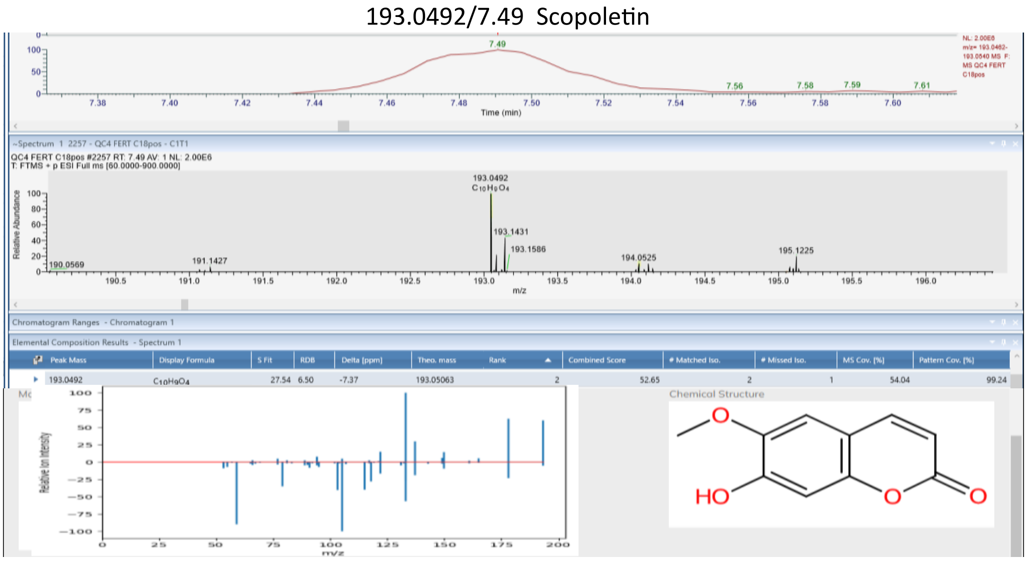
179.0697 / 13.61 4-Methoxycinnamic acid



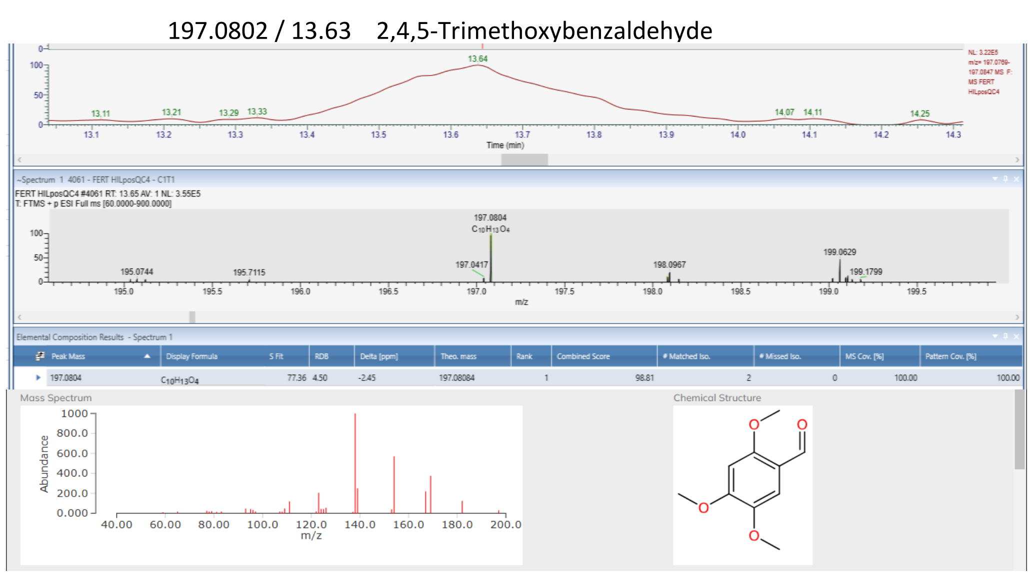
183.0292 / 8.91 3,4-Dihydroxymandelic acid



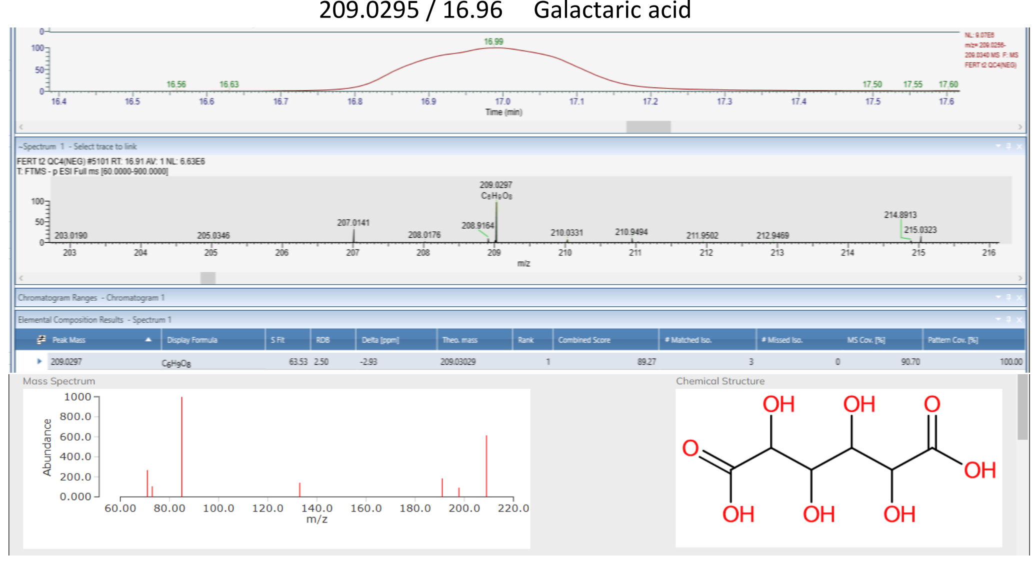
193.0492/7.49 Scopoletin



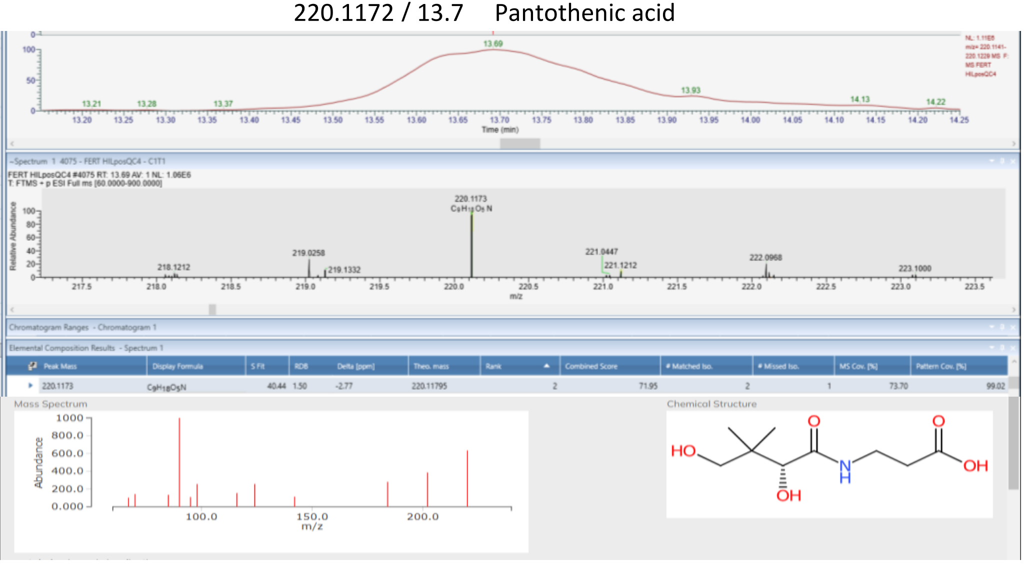
197.0802 / 13.63 2,4,5-Trimethoxybenzaldehyde



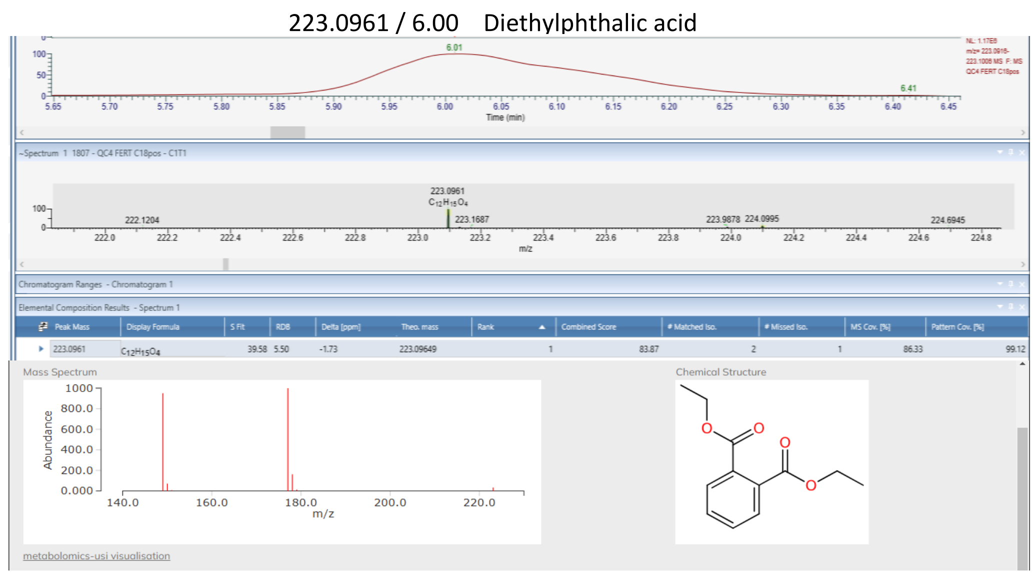
209.0295 / 16.96 Galactaric acid



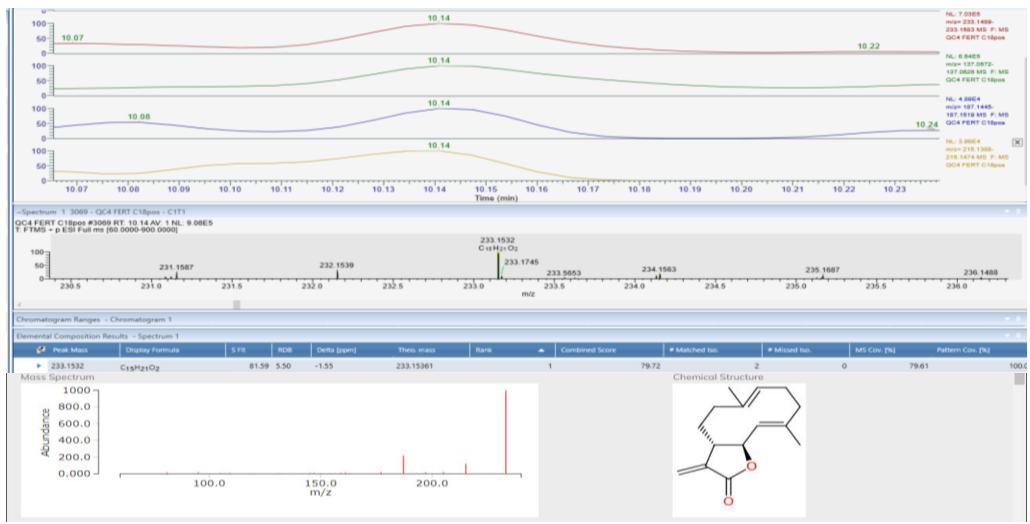
220.1172 / 13.7 Pantothenic acid



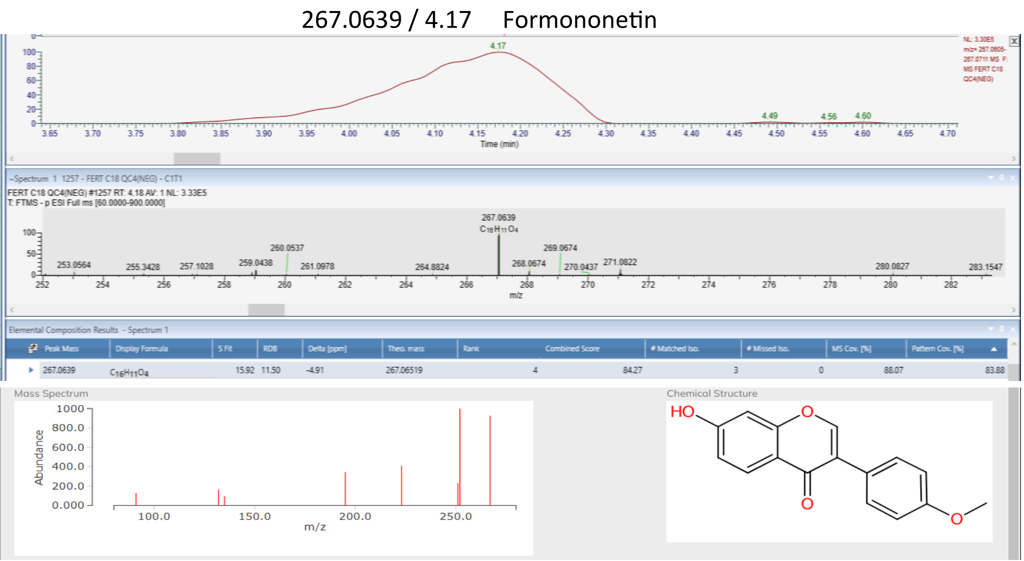
223.0961 / 6.00 Diethylphthalic acid



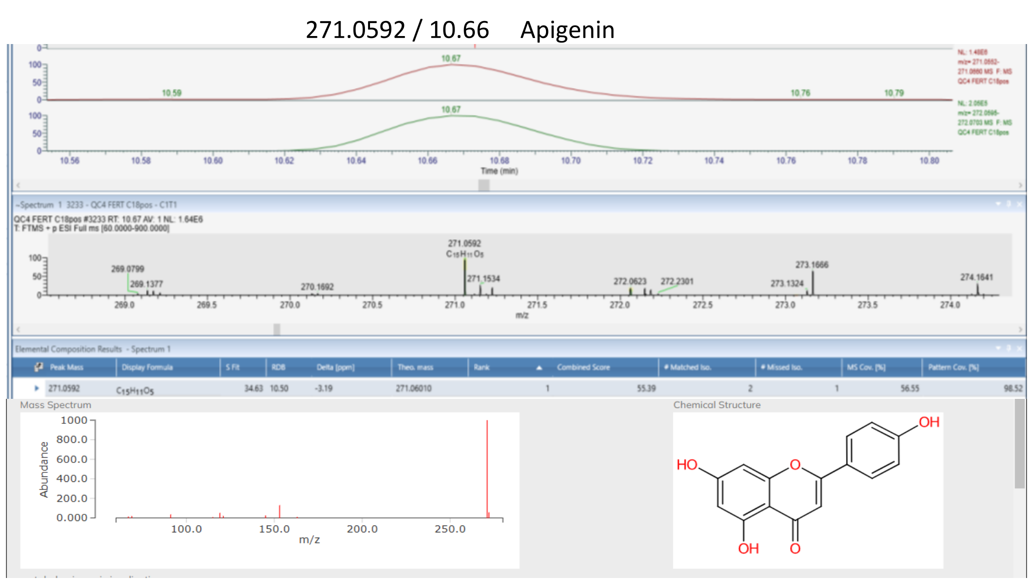
233.1531 / 10.14 Costunolide



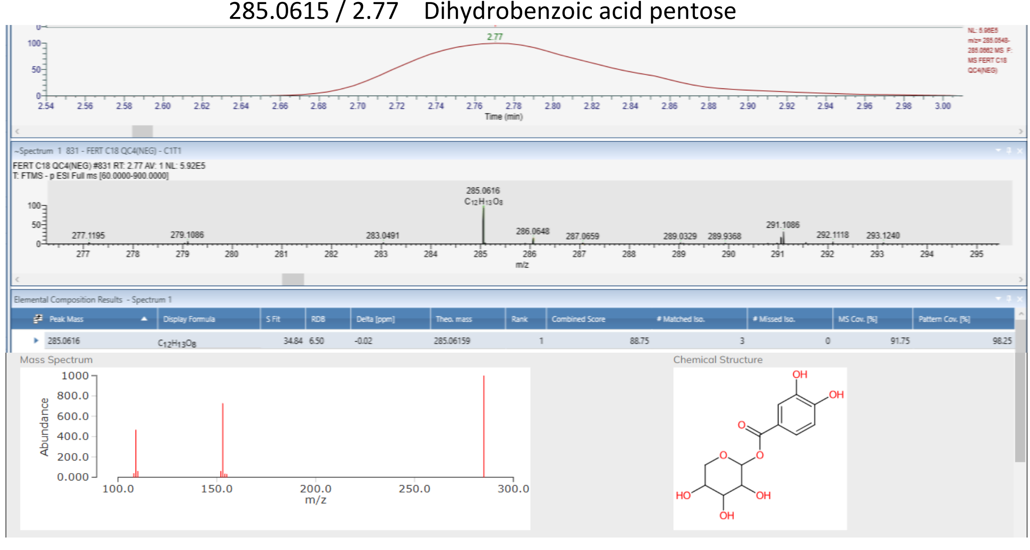
267.0639 / 4.17 Formononetin



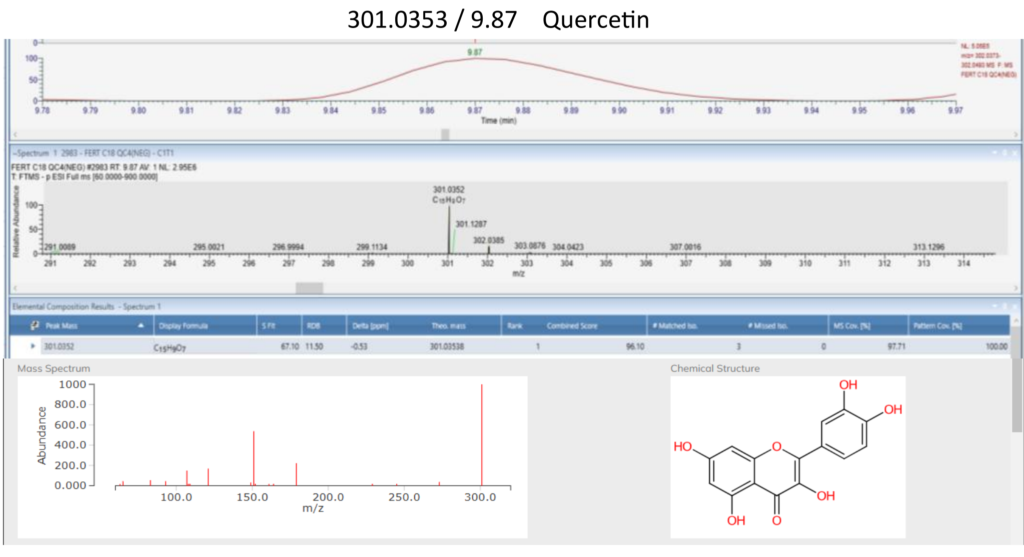
271.0592 / 10.66 Apigenin



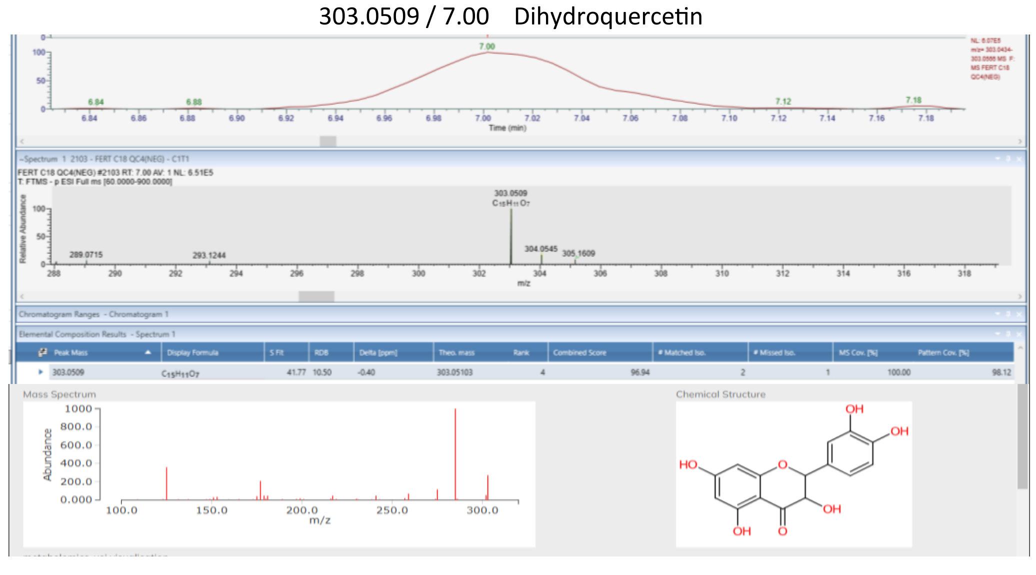
285.0615 / 2.77 Dihydrobenzoic acid pentose



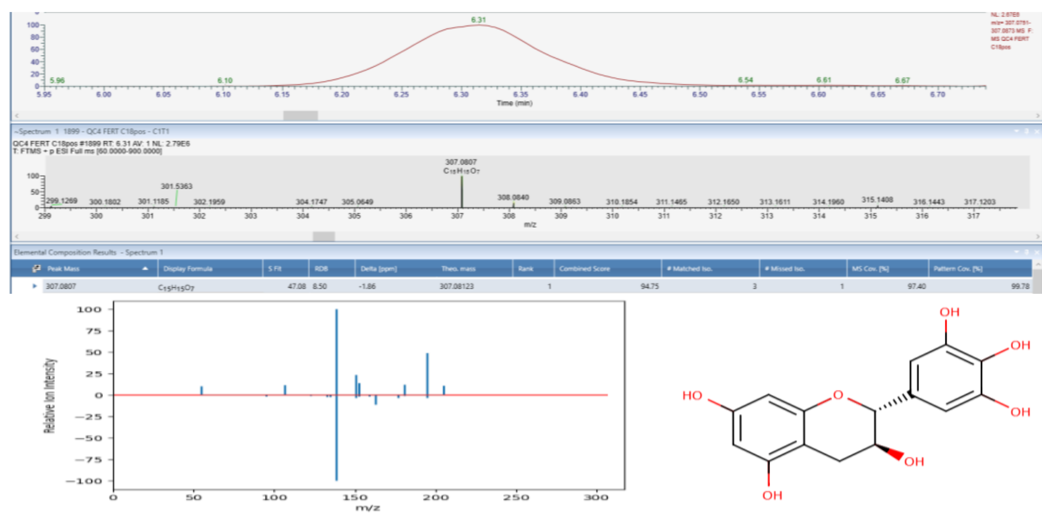
301.0353 / 9.87 Quercetin



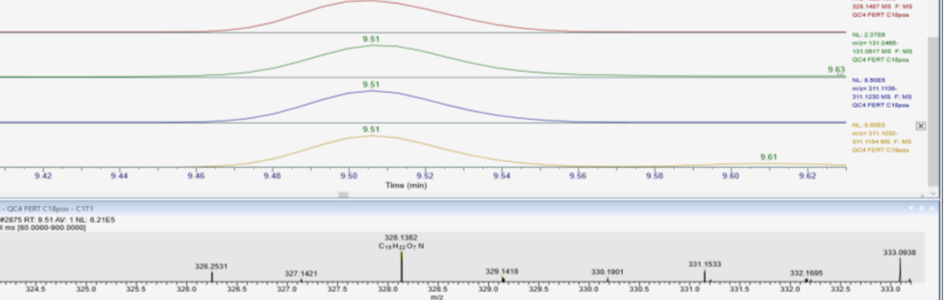
303.0509 / 7.00 Dihydroquercetin

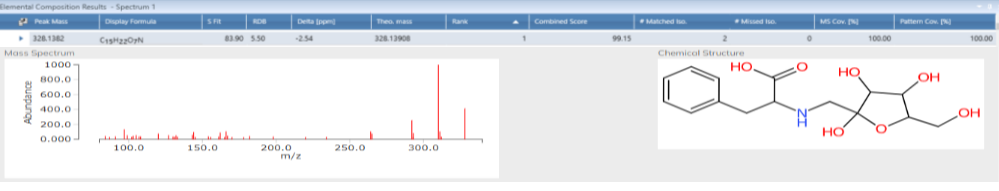


307.0807 / 6.30 (+)-Gallocatechin

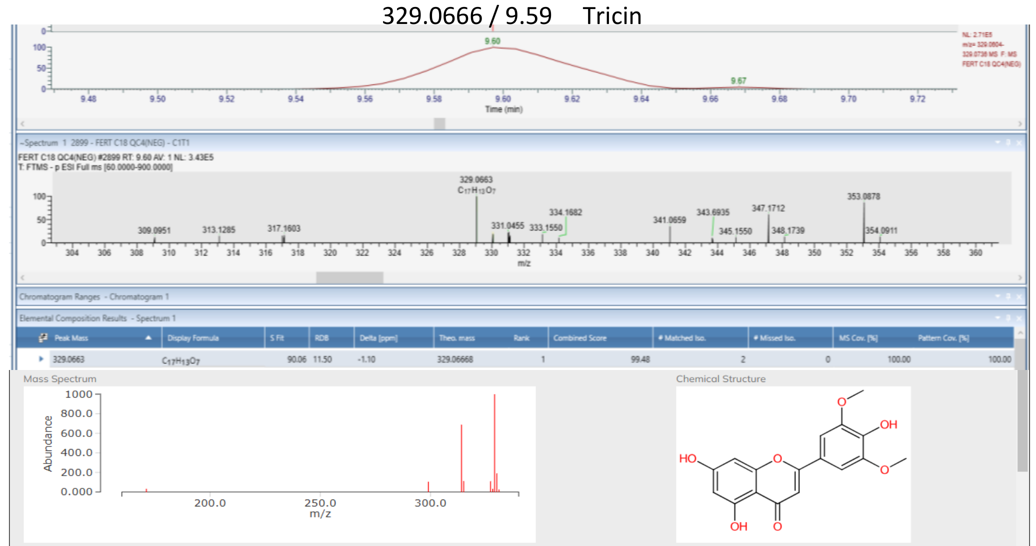


328.1383 / 9.5 N-Fructosyl phenylalanine

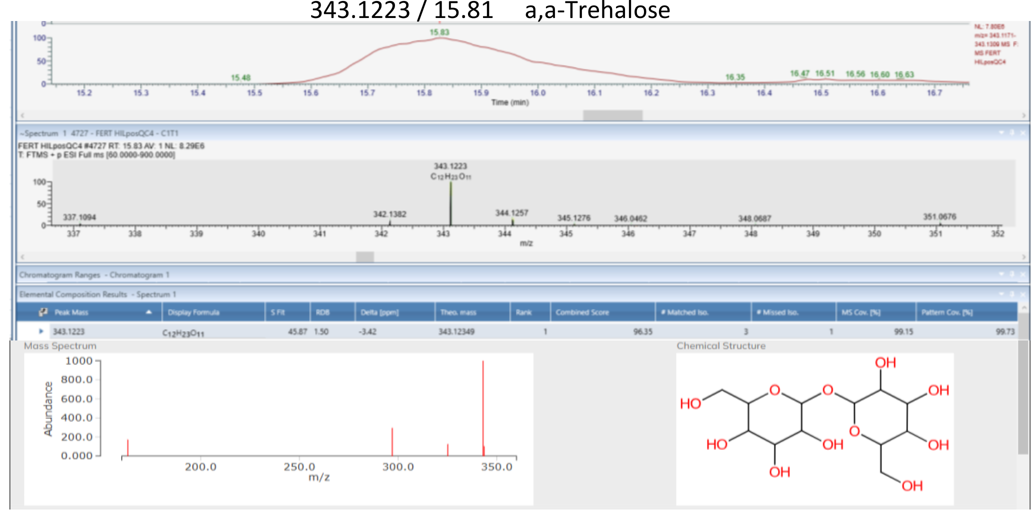




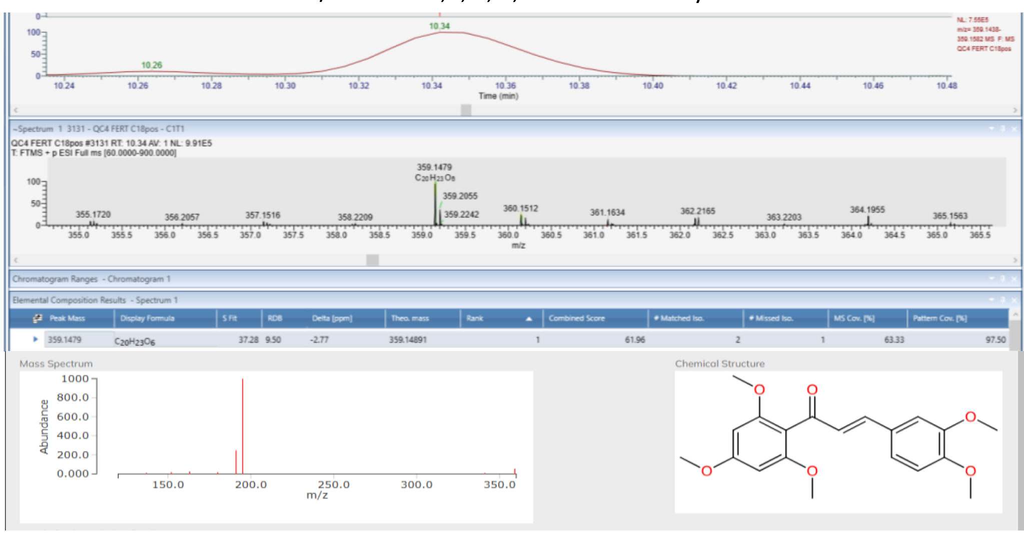
329.0666 / 9.59 Tricin



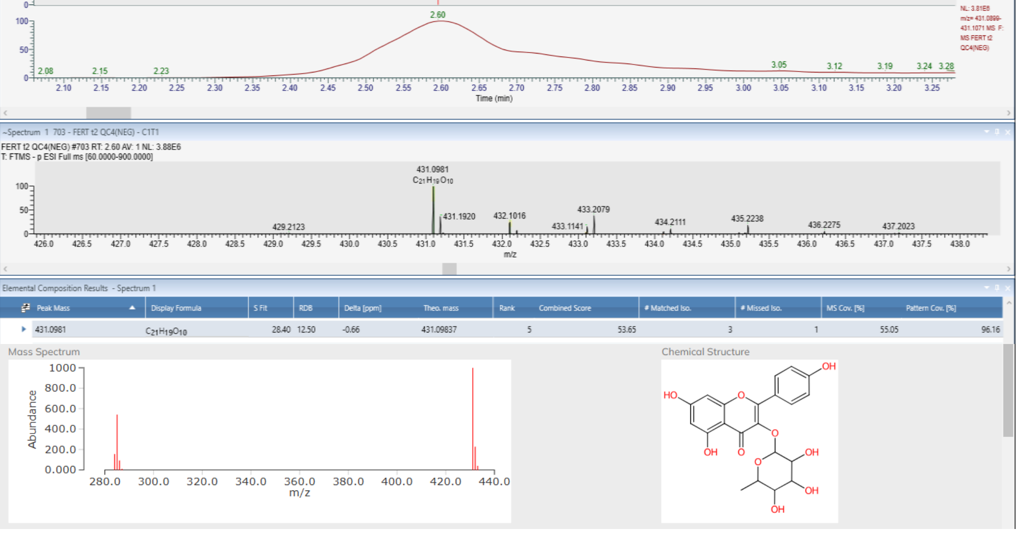
343.1223 / 15.81 a,a-Trehalose

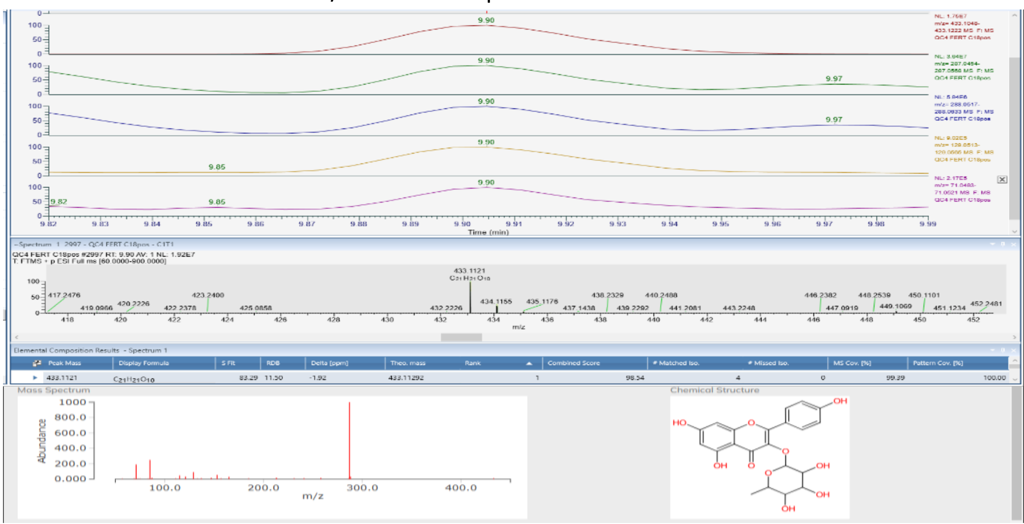


359.1481 / 10.34 3,4,2',4',6'-Pentamethoxychalcone

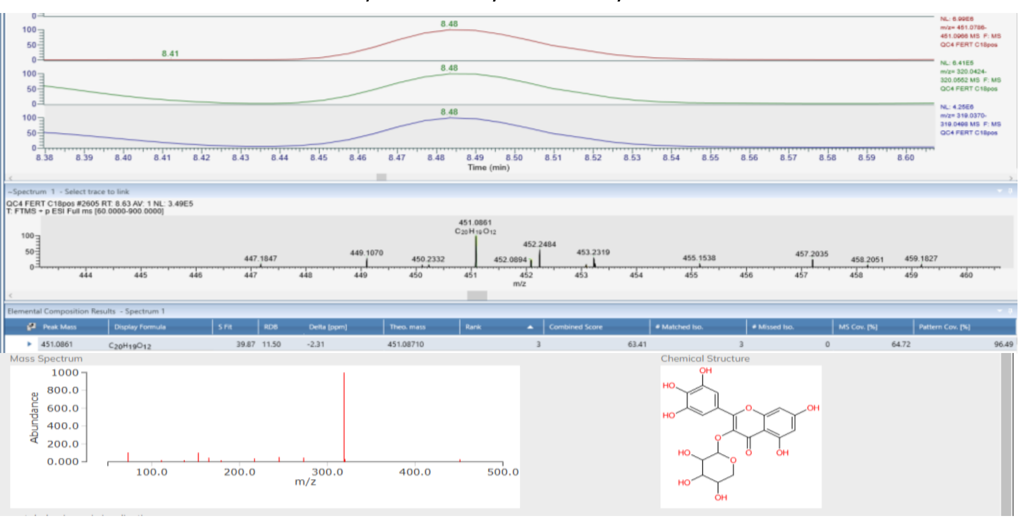


431.0978 / 2.51 Apigenin 7-O-glucoside

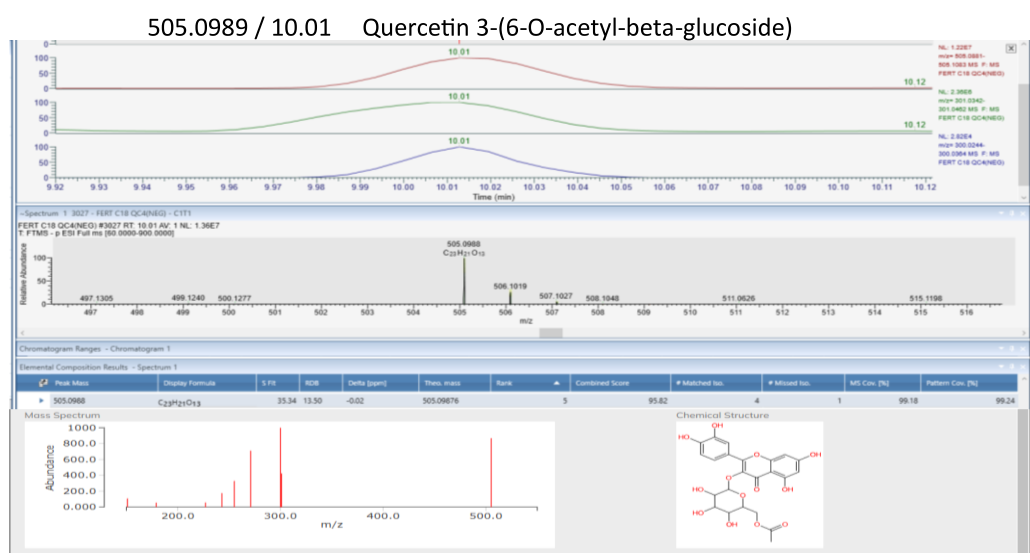




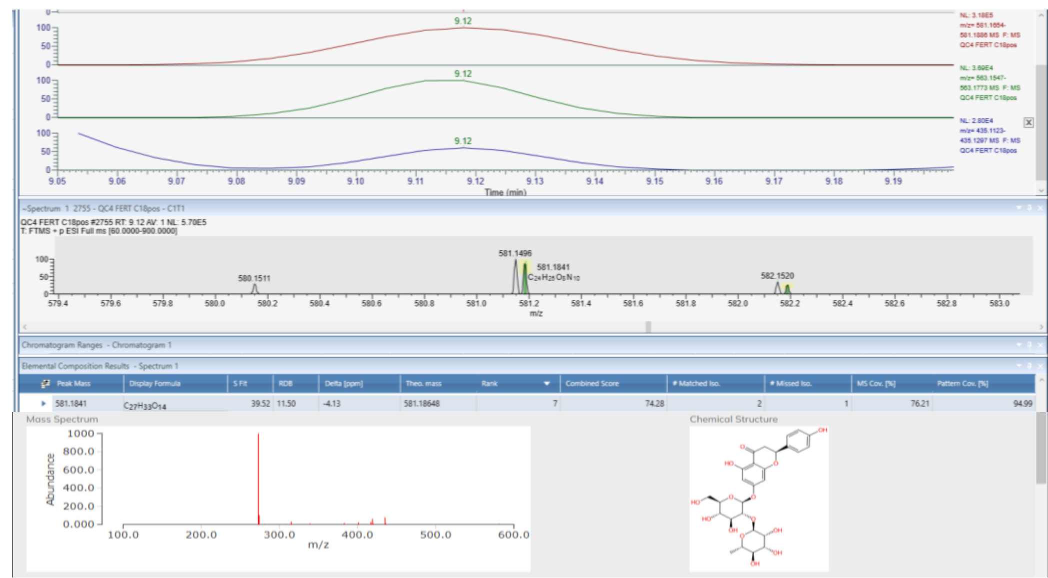
451.0862 / 8.49 Myricetin-3-Xyloside



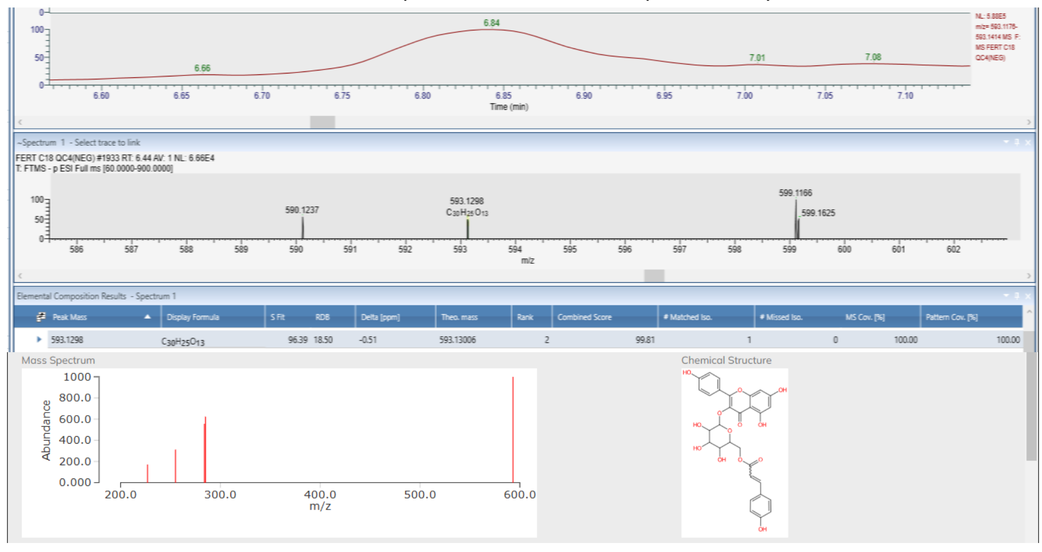
505.0989 / 10.01 Quercetin 3-(6-O-acetyl-beta-glucoside)



581.1849 / 9.11 Naringin



593.1303 / 6.84 Kaempferol-3-Glucoside-2''-p-coumaroyl



Appendix D:

Statements of contribution.

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Paul Barrett
Name and title of main supervisor:	Assoc. Prof. Maria Minor
In which chapter is the manuscript/published work?	Chapter 2

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

DPB, conceived all questions and objectives of this review and thesis programme. Carried out literature review and interpreted findings. Primary author of the manuscript. SVF, advised on background information regarding the heather biocontrol programme and revised the manuscript. AKS, advised on metabolomic technology capability, limitations and interpretation of technical information and revised the manuscript. RG advised on concepts of weed biocontrol and potential applications of metabolomics and revised the manuscript. ACM, advised on concept, interpretation and organisation of the review, and revised the manuscript.

Please select one of the following three options:

(●) **The manuscript/published work is published or in press**

Please provide the full reference of the research output:

Barrett, D. P., Fowler, S. V., Subbaraj, A. K., Groenteman, R., & Clavijo-McCormick, A. (2021). Metabolomic analysis of host plant biochemistry could improve the effectiveness and safety of classical weed biocontrol. Biological control, 104663.

( ) **The manuscript is currently under review for publication**

Please provide the name of the journal:

( ) **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:	[signature]	Main supervisor's signature:	[signature]

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Student name:	Paul Barrett
Name and title of main supervisor:	Assoc. Prof. Maria Minor
In which chapter is the manuscript/published work?	Chapter 3

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

DPB, conceived all questions and objectives of the investigation. Conducted all sampling, processing, statistical analysis, interpretation and annotation of data. Primary author of the manuscript. AKS, co-supervisor ran UHPLC-MS analyses, advised on metabolomic data analysis. RJP, assisted with field site selection and access in Scotland UK. Facilitated hosting and access to laboratory facilities at James Hutton Institute. PP. provided field site information and historical data on heather biocontrol program. ACM, co-supervisor, principal investigator, secured funding. Advised on design and interpretation of investigations, supported fieldwork. All coauthors revised the manuscript.

Please select one of the following three options:

(●) **The manuscript/published work is published or in press**

Please provide the full reference of the research output:

Barrett, D. P., Subbaraj, A. K., Pakeman, R. J., Peterson, P., & McCormick, A. C. (2024). Metabolomics reveals altered biochemical phenotype of an invasive plant with potential to impair its biocontrol agent's establishment and effectiveness. Scientific reports, 14(1), 27150.

( ) **The manuscript is currently under review for publication**

Please provide the name of the journal:

( ) **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

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Student name:	Paul Barrett
Name and title of main supervisor:	Assoc. Prof. Maria Minor
In which chapter is the manuscript/published work?	Chapter 4

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

DPB, conceived all questions and objectives of the investigation. Conducted all experiments, sampling, processing, metabolomic analysis, interpretation and annotation of data. Primary author of the manuscript. AKS, advised on UHPLC-MS techniques and metabolomic analysis. JJW advised on technical aspects of ultra-violet trials and measurements. PP advised on heather beetle rearing and bio-assay experiments. DJL ran all samples using UHPLC-MS. MM, advised on experimental design, analysis and data interpretation. ACM secured funding. Advised on concept, design and interpretation of investigations, revised the manuscript. All coauthors revised the manuscript.

Please select one of the following three options:

○ **The manuscript/published work is published or in press**

Please provide the full reference of the research output:

◉ **The manuscript is currently under review for publication**

Please provide the name of the journal:
Journal of Chemical Ecology

○ **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

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Student name:	Paul Barrett
Name and title of main supervisor:	Assoc. Prof. Maria Minor
In which chapter is the manuscript/published work?	Chapter 5

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

DPB, conceived all questions and objectives of the investigation. Conducted field trials, sampling, sample processing, analysis, annotation and interpretation of data. Primary author of the manuscript. MM, advised on all aspects of experimental design, analysis and interpretation of data. PP advised on experimental design, provided historical data, and assisted with field trials. SVF, advised on heather nutrient status and prior experimental data. AKS, advised on metabolomic data analysis. DJL ran all samples using UHPLC-MS. ACM, secured funding and principal investigator. Advised on concept, design and interpretation of investigations. All coauthors revised the manuscript.

Please select one of the following three options:

- ○ **The manuscript/published work is published or in press**
  Please provide the full reference of the research output:
- ○ **The manuscript is currently under review for publication**
  Please provide the name of the journal:
- ◉ **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:	[signature]	Main supervisor's signature:	[signature]

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