University of Reading

Rocket Science: Phytochemical, Postharvest, Shelf-life & Sensory Attributes Of Rocket Species

Submitted for the qualification of Doctor of Philosophy in Food & Nutritional Science

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Table of Contents

Abstract

Rocket species are increasing in popularity with consumers, and in the last ten years scientific interest has also increased due to the potential health benefits of consuming leaves. They are known for pungent and bitter taste components, and the chemical compounds largely responsible for these sensations are also health beneficial. These compounds are called isothiocyanates (ITCs), and they are ubiquitous in the plant family *Brassicaceae*. Precursor compounds called glucosinolates (GSLs) are converted to ITCs via the action of myrosinase enzyme.

This thesis presents data relating to numerous aspects of rocket species, such as differences in GSLs and ITCs. Other phytochemical constituents (flavonols, volatile organic chemicals (VOCs), free amino acids, free sugars, polyatomic ion content, and organic acids) are explored to determine their impacts on human sensory perceptions and consumer acceptance.

The data presented highlight significant differences between 'wild' accessions of rocket and commercially available varieties, in terms of flavonol and GSL content and sensory attributes. There is great potential to develop underutilised genetic resources in breeding programs, and through collaboration with a breeding company (Elsoms Seeds Ltd., Spalding, UK) and a commercial salad supplier (Bakkavör Group Ltd., Spalding, UK), several accessions were selected for detailed analyses. Analysis of VOC profiles further demonstrated the differences between the selected cultivars, and by combining these data with sensory and consumer studies, it was observed that the diversity of phytochemical components fundamentally underpins taste, flavour, and consumer acceptance.

The same accessions of rocket were also tested under commercial growth, processing and storage conditions. It was hypothesised that this would negatively impact GSL and ITC content of leaves, but in fact increased concentrations up to five

fold from the point of harvest in all accessions analysed. We also observed a previously undocumented link between GSL, ITC and free amino acid content with bacterial load.

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Luke Bell

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Thanks to all my friends and family for their constant support, love, understanding, and patience over the last four years. Thanks to the staff of the University of Reading, Elsoms Seeds and Bakkavör for all their help and resources. Special thanks go to Carol, Lisa and Jose for all their time, support and guidance.

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List of Abbreviations

A – Appearance

AA – Amino Acid

AE – Aftereffects

AFLP – Amplified Fragment Length Polymorphism

AHC – Agglomerative Hierarchical Cluster

ANOVA – Analysis Of Variance

BBSRC – Biotechnology & Biological Sciences Research Council

C – Cluster

C – Compound

CE – Capillary Electrophoresis

CGN – Centre for Genetic Resources in the Netherlands

D0 – Day 0 of shelf life

D2 – Day 2 of shelf life

D5 – Day 5 of shelf life

D7 – Day 7 of shelf life

D9 – Day 9 of shelf life

DAD – Diode Array Detector

DCM – Dichloromethane

DMB – Dimeric 4-mercaptobutyl glucosinolate

DUD – Display Until Date

ESI – Electro-Spray Injection

F – Flavour

GC – Gas Chromatography

GSL – Glucosinolate

GST – Glutathione S-transferase

H - Harvest

HPLC – High Performance Liquid Chromatography

HSD – Honest Significant Difference

IC – Ion Chromatograph

IDG – Isorhamnetin-3,4'-diglucoside

IG – Isorhamnetin-3-glucoside

IPK – Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben

ISSR – Inter-Simple Sequence Repeat

ITC – Isothiocyanate

JA – Jasmonic Acid

KDG – Kaempferol-3,4'-diglucoside

KG – Kaempferol-3-glucoside

KGG – Kaempferol-3-diglucoside-7-glucoside

KSG – Kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside

KTP – Knowledge Transfer Partnership

LC – Liquid Chromatography

M – Myricetin

MF – Mouthfeel

MRD – Maximum Recovery Diluent

MS – Mass Spectrometry

ND – Not detected

NGS – Next Generation Sequencing

NSP – Nitrile Specifier Protein

O - Odour

OA – Organic Acid

PC – Principal Component

PCA – Principal Component Analysis

PH – Pre Harvest

PI – Polyatomic Ion

PR – Pre Wash

PROP – Propylthiouracil

PT – Post Transport

PVDF – Low Protein Binding Durapore Polvinylidene Fluoride

PW – Post Wash

QC – Quercetin-3,4'-diglucoside-3'-(6-caffeoyl-glucoside)

QG – Quercetin-3-glucoside

QS – Quercetin-3,4'-diglucoside-3'-(6-sinapoyl-glucoside)

QTG – Quercetin-3,3,4'-triglucoside

QTL – Quantitative Trait Loci

QUAFETY – European Food Quality & Safety

RAPD – Randomly Amplified Polymorphic DNA

RT-PCR – Real Time Polymerase Chain Reaction

SA – Salicylic Acid

SNP – Single Nucleotide Polymorphism

SR – Salad Rocket

SRAP – Sequence Related Amplified Polymorphism

T – Taste

TD-GC-TOF-MS – Thermal Desorption Gas Chromatography Time-Of-Flight Mass Spectrometry

TPC – Total Plate Count

UPLC – Ultra Performance Liquid Chromatography

VOC – Volatile Organic Compound

WR – Wild Rocket

CHAPTER 1: Introduction

1.1. Project Background, PhD Aims & Objectives

The origin of this project began in 2009 as a Knowledge Transfer Partnership (KTP) between Elsoms Seeds Ltd. (Spalding, Lincolnshire) and the University of Warwick HRI (now known as the Warwick Crop Centre). Sue Kennedy (Head of Vegetable Plant Breeding at Elsoms), Prof. David Pink, Dr. Paul Hand (Harper Adams University; both formerly University of Warwick), Dr. Guy Barker (University of Warwick) and Dr. Graham Teakle (University of Warwick Crop Centre) supervised the project. Elsoms was established in 1844 and has sold commercial seed for much of its history. Although predominantly a UK based wholesale seed merchant for European seed companies, such as Bejo Zaden, Gautier Semences and Florimond Desprez, Elsoms also has several of its own advanced breeding programs. The company has produced F_1 hybrid varieties of swede, parsnip and purple-sprouting broccoli with a dedicated breeding and research team. Elsoms specializes in high-quality plant breeding of niche crops, and initiated the KTP with the aim of expanding the company portfolio to include salads and herbs.

The role given to the KTP Associate (the author) was divided into two parts: developing and introducing molecular markers to a parsnip male-sterile breeding program, and to initiate new breeding programs in crops with commercial potential. The first of these breeding programs to be initiated was in rocket species, as Elsoms Seeds had identified a potential 'gap' in the breeding market.

Most cultivars of rocket species are considered to be lacking in breeding quality; i.e. 'varieties' are commonly produced from open-pollinated populations, and lack the uniformity and robustness of true varieties that are developed through intensive inbreeding, selection and cross-pollination (Bell & Wagstaff, 2014). Any

improvement that might be made through conventional breeding in traits such as germination, uniformity of colour and leaf shape, and disease resistance, would have added market value (Goldman, 2014; Hall, Jobling, & Rogers, 2012b; Stein & Roerink, 2015), and develop a market share for Elsoms where previously they had none. Initially, improvements in morphological traits and uniformity were the objectives of the new breeding program.

There are two predominant cultivated rocket species across the globe, “salad” rocket (*Eruca sativa*) and “wild” rocket (*Diplotaxis tenuifolia*; Hall et al., 2012b). There are numerous other related species which can fall under these labels, such as *Eruca vesicaria, Diplotaxis erucoides, Diplotaxis harra, Diplotaxis simplex* and *Diplotaxis muralis* (D’Antuono, Elementi, & Neri, 2008), as well as *Bunias orientalis* (“Turkish” rocket). As will be explained in Chapter 2, these species all have similar morphological characteristics, though *E. sativa* is by far the most diverse in this sense (Egea-Gilabert, Fernandez, Migliaro, Martinez-Sanchez, & Vicente, 2009). Leaves range from large, rounded types that almost resemble lettuce or spinach, to small, ‘skeletal’ and serrated leaf types that are most commonly associated with *D. tenuifolia* (Hall, Jobling, & Rogers, 2015). It is this overlap in characteristics that has often led to confusion with retailers, and indeed within the scientific literature, as to which-is-which. It is quite common for example, for *E. sativa* to be grown and marketed as “wild” rocket due to the similar traits of some cultivars. There is even debate amongst taxonomists as to whether there are in fact two distinct species of “salad” rocket (*E. sativa* and *E. vesicaria*) or whether they are sub-species (Bell & Wagstaff, 2014). The only definitive way of telling the genera and species apart is by chromosome counting, or scrutinizing the flower morphology (which growers and producers would probably never observe due to short cropping cycles; Hall et al. 2015).

These points might seem trivial and academic, but could have potential commercial implications that Elsoms Seeds are keen to take advantage of. By far the most cultivated species in the UK and Europe is *D. tenuifolia*, and its distinctive, deeply lobed shape has become the one that most consumers are familiar with (Lokke, Seefeldt, & Edelenbos, 2012). Plants also have robust leaves that are conducive to industrial processing, as they tend not to tear or break easily. The species usually has a deep green colour (though this varies according to light exposure; Jin et al., 2009), which consumers find appealing and "fresh" (Lokke et al. 2012). The species does, however, have numerous drawbacks. Plants are typically very slow to germinate and grow to marketable size (Hall et al. 2012b), often taking between 36 to 99 days to reach harvestable size depending on the season (Hall, Jobling, & Rogers, 2012a). Producers grow "wild" rocket in high densities to encourage upward growth to improve yields (Bennett et al., 2013; Lovegrove et al., 2015) which also makes harvesting easier. But plants sometimes become stressed through competition, causing red-purple leaf discoloration (Bell & Wagstaff, 2014). The close proximity of plants also reduces airflow around leaves, and coupled with high levels of humidity, can lead to downy and powdery mildew infestations (Gilardi, Gullino, & Garibaldi, 2012). If fungal infection is severe, this can make entire fields, glasshouses and polytunnels of "wild" rocket unsaleable, as leaves turn pale or yellow.

While *E. sativa* shares some of these issues, it has an important distinction in that it germinates and establishes much more quickly than *Diplotaxis* species; usually between 26 to 68 days, depending on the season (Hall et al. 2012a). *D. tenuifolia* is also often anecdotally touted as being 'hotter' than *E. sativa*, however there is no quantitative evidence to support this claim. The fact that some *E. sativa* cultivars have similar leaf and phytochemical characteristics to *D. tenuifolia* led the project

collaborators to postulate that a “salad” rocket could be effectively sold and marketed as “wild” rocket, but with added benefits of improved germination and a shorter crop cycle.

With these goals and ideas in mind, accession material was sourced from European germplasm collections. This was initially aided by a European project known as ‘GenRes’ (European Commission, Action 001 AGRI GEN RES No. 870/2004), with the objective being to catalogue and characterise traits of minor leafy vegetables, such as rocket. The expressed aim was to make information available to breeders about such underutilised cultivars. Numerous commercial varieties were also collected from packet seed to act as a comparison to gene bank accessions, and to potentially utilise in breeding selections. Collections at the Genetic Resources Unit at the University of Warwick, the Centre for Genetic Resources in the Netherlands (CGN) and the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben (IPK) were assessed, and nearly 250 distinct accessions/cultivars/varieties of rocket were collected for the initial screening. The majority of those collected were of *Eruca* species, and this was partly the reason why they became the predominant focus of the Elsoms breeding program. For an unknown reason, *Diplotaxis* accessions were relatively few in germplasm collections despite their commercial prevalence, and those that were listed often had depleted seed reserves or were under embargo.

Initial screening began to eliminate material with undesirable characteristics, such as early bolting, flowering, and poor germination. After this process, around 100 accessions remained, but a substantial proportion of *Diplotaxis* accessions were de-selected, predominantly due to poor germination. It was also at this point that the potential for *E. sativa* crop development became apparent, as there were numerous novel characteristics within the germplasm material. It was theorized that not only

could varieties be bred to fill the current market requirements, but also to create new ones with unique leaf characteristics.

It was at this point in the project in 2010 that collaboration began with Dr. Lorraine Shaw of Bakkavor Group Ltd. Bakkavor is an international food manufacturing company specializing in prepared foods. Although Icelandic-owned, the company operates predominantly in the UK, which is its largest market. The Group operates 32 manufacturing facilities in the UK, each with a different food preparation focus. Sites in Spalding and Bourne in Lincolnshire, for example, deal predominantly with ready meals and bagged salads; products in which rocket features heavily. Bakkavor were therefore interested in the breeding material that was being produced at Elsoms and any future commercial potential, and began assisting in breeding selections with a producer/processor's perspective. This close association became essential in developing breeding lines with specific, novel foci in mind.

The KTP project ended in 2011 and succeeded in establishing new breeding programs for both "salad" and "wild" rocket types. It was around this time that discussions began with Dr. Carol Wagstaff (University of Reading) into beginning a PhD project focusing on the phytochemical diversity and sensory properties of rocket. Through the breeding selections conducted each year, it was common practice to taste leaves while in the field/glasshouse and inform selections on this basis, as well as a visual one. It became apparent that, even between closely related sister breeding lines, taste, flavour and aroma could vary dramatically, and that there was a wealth of phytochemical and sensory variability to be selected for/against. The University of Reading Food & Nutritional Sciences department is world renowned for research into phytochemical compounds, sensory science, and consumer studies. With facilities and expertise otherwise inaccessible, Elsoms and Bakkavor initiated a

BBSRC iCASE Award with The University of Reading, which is presented in this thesis. The overall aim of the PhD was to elucidate rocket phytochemical and sensory diversity in order to develop new breeding 'markers' for the industrial collaborators.

By the start of the PhD project, 19 of the original 250 accessions were still present within the Elsoms *Eruca* breeding population. The total number of lines this encompassed exceeded 300, with many of the lines having been diversified greatly into sister populations via single seed descent. These 19 original lines supplied the large amount of morphological variation seen in the breeding population, and so it was decided that this project 'go back' to the germplasm collections and re-source the material to determine if the same was true for phytochemical and sensory diversity. Our objective was to characterise each accession and begin the process of selecting accessions for their chemical traits (initially glucosinolate and flavonol concentrations). The result of this screening is presented in Chapter 3, where the 19 accessions and numerous commercial comparators were assessed using LC-MS/MS. A similar characterization was performed on headspace volatile organic compounds (VOCs) by TD-GC-TOF-MS, to determine if these varied significantly between accessions. Our objective was to study VOCs produced by leaf damage over time in a simulated shelf-life environment, and to observe what effects (if any) there might be on composition and relative abundances. This was especially of interest for glucosinolate-myrosinase reaction products such as isothiocyanates. Results of this series of experiments are presented in Chapter 4.

We aimed to establish how phytochemical diversity affects sensory perception and consumer acceptance of rocket. As rocket contains many similar glucosinolate and flavonol compounds to *Brassica* species (Cartea, Francisco, Soengas, & Velasco, 2011; Lelario, Bianco, Bufo, & Cataldi, 2012), a sensory and consumer

study was conducted with the objective of establishing if similar effects relating to sensory attributes (Beck, Jensen, Bjoern, & Kidmose, 2014; Hansen, Laustsen, Olsen, Poll, & Sorensen, 1997) were applicable to rocket. Firstly, the sensory attributes of rocket were determined utilising a trained sensory panel (presented in Chapter 5). Secondly, a consumer trial was conducted to establish which attributes people like and/or dislike according to their bitter taste receptor genotype (TAS2R38; Dinehart, Hayes, Bartoshuk, Lanier, & Duffy, 2006) and the phytochemical constituents of the accessions presented to them (presented in Chapter 6).

The final aim of the project was to test accessions in a 'real world' industrial supply chain. We wanted to demonstrate objectively the effects of commercial cultivation, harvesting, processing and storage on phytochemical constituents of rocket, and how this might affect their nutritional intake. These data are presented in Chapter 7, where a full-scale supply chain was utilised to test our hypotheses, and for the first time, demonstrate its effects on glucosinolate, isothiocyanate, amino acid and sugar content of leaves.

In the final chapter of this thesis (Chapter 8), the future aims and objectives of subsequent projects will be discussed in detail, and will summarize the key outputs of this PhD. Briefly, one such output has been the initiation of a project to obtain the full genetic sequence of three recombinant inbred lines of *E. sativa* – a world first to the author's knowledge. These data will be utilised to link the genetic composition of *E. sativa* with phytochemical, sensory and consumer data. Coupled with our existing knowledge, the aim is to generate new, nutritionally and sensorially superior breeding lines. These will be made available to our collaborators, and in turn, will allow for further breeding programs to produce new commercial varieties.

1.2. Description of Rocket Species

1.2.1. Eruca Species

E. sativa is sometimes referred to as “cultivated” rocket, “annual” rocket, “true” rocket, arugula, roquette, or “white pepper” (Garg & Sharma, 2014). This species is also sometimes synonymously referred to as *E. vesicaria* subsp. *sativa* (Pasini, Verardo, Cerretani, Caboni, & D’Antuono 2011), but the exact taxonomic classification has yet to be properly agreed as rigorous genetic studies are absent from the literature.

The *Eruca* genus is sometimes quoted as having a greater genetic diversity due to its supposed monospecific nature (Hall et al. 2012a). This is debatable, as there are currently five species recognised by the Med-Checklist (an online inventory of vascular plants of circum-Mediterranean countries). These include the two aforementioned species, as well as *Eruca loncholoma, Eruca pinnatifida* and *Eruca setulosa*. Until a comprehensive genomic survey is conducted, the ambiguity surrounding the diversity of the species will remain.

E. sativa is a diploid organism containing 11 pairs of chromosomes (Table 1.1, 2*n* = 22; Nothnagel, Budahn, Schrader, & Klocke, 2012). The species is a preferential out-breeder, with varying levels of self-incompatibility between cultivars. This can be overcome to a degree by performing bud-pollinations by hand and reducing the ambient temperature during flowering. Crosses with *Diplotaxis* species have been attempted with no viable results, but somatic hybrids have been produced with *Brassica oleracea*, with the purpose of introducing cytoplasmic male sterility into the species (Nothnagel et al. 2012).

The mitochondrial genome of *E. sativa* has been sequenced (Wang et al. 2014), however this did little to resolve the number of species present within the genus; only determine that it is more related to *B. oleracea* than *Raphanus sativus*

(radish) and *Arabidopsis thaliana*. As will be discussed in Chapter 2, the assertion that *E. sativa* is more highly domesticated than "wild" rocket (*D. tenuifolia*) has no supporting evidence (Bell & Wagstaff, 2014). One study by Egea-Gilabert, Fernández, Migliaro, Martínez-Sánchez, & Vicente (2009) looked at the genetic diversity between *E. vesicaria* and *D. tenuifolia* for agronomic traits and found a large amount of diversity within each species. The analysis was however very limited, as it

Table 1.1. Rocket species names, observed chromosome ploidy counts, and native areas according to Med-Checklist (Greuter, Burdet, & Long, eds. 2008) and Eschmann-Grupe, Hurka, & Neuffer (2003).

Species name	Chromosome ploidy	Geographical origin
Eruca spp.		
Eruca sativa	2n = 22	Algeria, Turkey, Spain, Bulgaria, France, Greece, Cyprus, Israel, Jordan, Italy, Libya, Lebanon, Syria, Portugal, Morocco, Malta, Ukraine, Iran, India, Pakistan
Eruca vesicaria		Algeria, Spain, Morocco
Eruca loncholoma		Algeria, Tunisia
Eruca pinnatifida		Algeria, Spain, Morocco, Tunisia
Eruca setulosa		Algeria, Morocco
Diplotaxis spp.		
Diplotaxis erucoides	2n = 14	Belgium, France, Canary Islands, Romania, Spain, Italy, Algeria, Egypt, Israel, Jordan, Serbia, Lebanon, Syria, Morocco, Malta, Tunisia
Diplotaxis harra	2n = 26	Egypt, Algeria, Spain, Israel, Jordan, Libya, Lebanon, Syria, Morocco, Italy, Tunisia
Diplotaxis harra ssp. *crassifolia*		Italy, Spain, Algeria, Morocco, Tunisia
Diplotaxis siettiana	2n = 16	Spain
Diplotaxis ibicensis		
Diplotaxis brevisiliqua		Morocco
Diplotaxis catholica	2n = 18	Spain, Portugal, Morocco
Diplotaxis viminea	2n = 20	Germany, Greece, Algeria, Turkey, Spain, Bulgaria, Cyprus, Egypt, France, Spain, Israel, Jordan, Italy, Serbia, Lebanon, Syria, Portugal, Morocco, Malta, Ukraine
Diplotaxis siifoila		Morocco, Algeria, Spain, Portugal
Diplotaxis tenuifolia	2n = 22	Switzerland, Germany, Netherlands, Austria, Italy, Hungary, Italy, France, Romania, Spain, Turkey, Albania, Bulgaria, Serbia, Lebanon, Syria, Portugal, Morocco, Malta, Ukraine
Diplotaxis cretacea		Russia
Diplotaxis simplex		Algeria, Egypt, Libya, Tunisia
Diplotaxis muralis	4n = 42	Germany, Greece, Spain, Austria, Italy, Belgium, Denmark, Algeria, Albania, Bulgaria, France, Serbia, Libya, Morocco, Malta, Ukraine, Tunisia, Turkey

only included nine ISSR primers which cannot be linked to any traits measured due to the absence of publically available genomic sequence data.

Regardless of the speciation status of the *Eruca* genus, *E. sativa* is a member of the *Brassicaceae* family of plants, and is noted for its fast growing nature, and the hotness and pepperiness of its leaves (Pasini et al. 2011). This is reflected in the latin name, which originates from “uro” or “urere”, which translates to “burn” in English. In the current botanical classification of rocket species, *E. sativa* is thought to be descended from the *Brassica rapa* / *oleracea* lineage in the subtribe *Brassicinae*, along with the genera *Brassica*, *Diplotaxis* (see next section), and *Erucastrum* (Hall et al. 2012b).

Historically *E. sativa* has been grown in the countries and regions surrounding the Mediterranean Sea and its use can be traced back to Roman times. A common use for the plant in such times was not in fact food, but as an aphrodesiac, and is regularly referenced in ancient texts for such properties (Hall et al. 2012b). There is no scientific evidence to support this property of leaves however.

Its natural ecological distribution covers southern Europe, north Africa, Iran, India and Pakistan, and is traditionally grown as a winter crop in dry areas. It has evolved a fast-growing and efficient root system and is capable of withstanding severe drought conditions. This property makes it an important traditional food source in arid areas (Garg & Sharma, 2014). Due to its weedy and hardy nature, the species has become naturalised on every continent, with the obvious exception of Antarctica.

In western countries, it is now most commonly used as a salad or garnish (Jin et al. 2009). Leaves are sold in both processed and fresh markets (Hall, Jobling, & Rogers, 2013) and the popularity with consumers is increasing significantly year-on-year (Dr. Lorraine Shaw, Bakkavor, personal communication, 2016). This means that

the crop is gaining significant economic importance in baby leaf salad markets (Hall et al. 2012a). In India and Pakistan *Eruca* species are also used widely as oliseed, forage and fodder crops. Roots, flowers and seeds are all consumed, in a similar fashion to how mustard species are in the west (Garg & Sharma, 2014).

Due to its various uses and the non-distinction between cultivated rocket species, it is difficult to determine how much is produced and consumed globally. In the UK, recent figures have indicated that the bagged rocket salad market is worth approximately £43 million (March 2015 – February 2016), which was an increase of 3.9% compared to the previous year. It is estimated that 39.9 million bags of rocket were consumed during the same period, with shipped volumes increasing by 2.4% from the previous year (Dr. Lorraine Shaw, Bakkavor, personal communication, 2016).

1.2.2. Diplotaxis Species

Diplotaxis species are synonymously refferred to as rocket, as with the other species highlighted in the previous section. *D. tenuifolia* can be more specifically referred to as “perennial wall rocket” and is the predominant species cultivated in this genus (Hall et al. 2012a). It is similarly known for its peppery and hot flavours (Pasini et al. 2011), and is arguably the most important species economically due to the prevalence of its commercial growth in Europe, North America and Australia (Hall et al. 2012a).

As with *Eruca*, *Diplotaxis* species are also native to the countries surrounding the Mediterranean Sea, as well as India, Pakistan, Cape Verde and Nepal (Hall et al. 2012b). *D. harra* and *D. simplex* are common in Tunisia for example, where traditionally, plants have been used for medicinal purposes because of reported antimicrobial properties, as well as for general food consumption (Falleh et al. 2013).

Unlike *Eruca* species, there is greater consensus about the diversity of the *Diplotaxis* genus. It is agreed that it is polyphyletic, but that morphology is an inadequate means of determining evolutionary relatedness (Arias & Pires, 2012). *Diplotaxis* species are diploid, with one exception; *D. muralis* is thought to be an amphiploid of *D. tenuifolia* and *D. viminea*, as it is the only known tertrapoid species within the genus (Eschmann-Grupe, Hurka, & Neuffer, 2003).

D. tenuifolia contains 11 pairs of chromosomes (2*n* = 22) like *E. sativa*, but no progeny have ever resulted from crosses. *D. erucoides* has the smallest chromosome ploidy of the genus, and it is speculated that it may represent an ancestral species because of the continuous series of haploid chromosome numbers, ranging from seven to 13 (Martín & Sánchez-Yélamo, 2000). See Table 1.1 for a summary of rocket species chromosome ploidy counts.

Within-species diversity is not well established, with some authors arguing for large amounts of genetic variation (Hall et al. 2012a), but again, this is not substantiated by much evidence. To date it has only been inferred from differences between morphological and phytochemical traits, and limited RAPD marker analyses.

In terms of growth habit, *D. tenuifolia* is much slower to establish and grow than *E. sativa*. The species *D. erucoides* however comprises attributes of both these species, having the early vigour of *E. sativa* and the distinctive lobed leaf shape of *D. tenuifolia* making it an attractive choice for breeders to utilise in the production of new varieties.

1.3. Glucosinolate Biosynthesis & Metabolism

Glucosinolates are specialised plant defense compounds produced by all members of the *Brassicaceae* (Stauber et al. 2012). The specific GSLs produced by

rocket species will be discussed in Chapter 2, but a brief background to biosynthesis and metabolism will be presented here.

The majority of research into GSL metabolism has been conducted in plant species such as *A. thaliana* (Ishida, Hara, Fukino, Kakizaki, & Morimitsu, 2014) and *Brassica oleracea* (Tian, Xu, Liu, Xie, & Pan, 2016), with very little specific research conducted in rocket species. Rocket typically contains aliphatic GSLs, but also smaller concentrations of indolic and aromatic GSLs.

The major pathways identified in aliphatic GSL biosynthesis (in *A. thaliana*) to-date are presented in Figure 1.1. MYB transcription factors control the complete GSL biosynthetic pathway, and also influence primary and sulfate metabolic pathways. Differing transcript levels associated with MYB genes has been shown to affect indole GSL accumulation and the related metabolism products when plants are under pathogen stress (Frerigmann, et al. 2016).

Aliphatic GSLs are synthesised from the amino acid methionine, and indolic GSLs predominantly from tryptophan (Kim & Jander, 2007). The gene *BoGSL-PRO* in *B. oleracea* converts methionine into dihomomethionine and a process of chain-elongation begins. This is further regulated by other genes such as *BoGSL-ELONG*, and determines the length of the carbon side-chain (e.g. propyl, butyl, pentyl, etc.). Other genes, such as *BoGSL-ALK,* further modify the R-group of the GSL molecule later in the synthesis pathway, and determine its final configuration (Ishida, Hara, Fukino, Kakizaki, & Morimitsu, 2014).

Levels of GSL biosynthesis are regulated by plant defense signaling compounds, such as salicylic acid (SA), ethylene and jasmonic acid (JA). The synergistic or antagonistic crosstalk between these three compounds determines the relative expression of genes, such as *CYP79B2, CYP79B3, CYP79F1* and *CYP79F2*. These genes also regulate the GSL biosynthesis pathway and determine

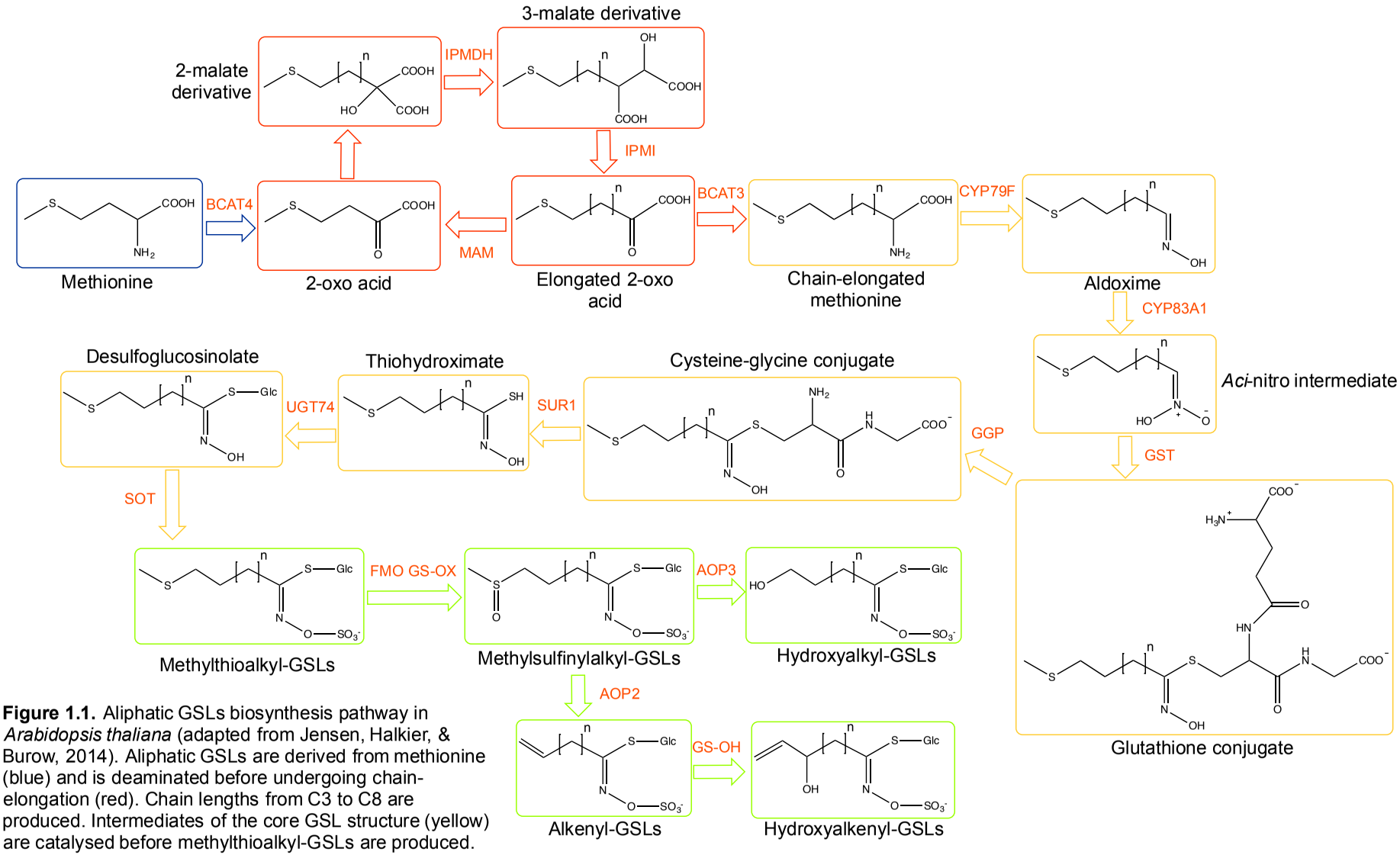

Figure 1.1. Aliphatic GSLs biosynthesis pathway in *Arabidopsis thaliana* (adapted from Jensen, Halkier, & Burow, 2014). Aliphatic GSLs are derived from methionine (blue) and is deaminated before undergoing chain-elongation (red). Chain lengths from C3 to C8 are produced. Intermediates of the core GSL structure (yellow) are catalysed before methylthioalkyl-GSLs are produced. GSL groups (green) are accumulated by the tissues of plants. Red text = enzyme group; n = variable number of methyl groups.

the overall GSL profile of tissues, influencing the ratios between aliphatic and indolic GSLs (Mikkelsen et al. 2003). The level to which these and other biosynthetic genes are expressed depends greatly on the stimuli that initiate, which can be both biotic and abiotic in nature. Factors that have been shown to influence GSL profiles and concentrations include: plant age, light intensity, fungal infection, wounding, insect damage, temperature, and the growing season, to name but a few (Chen & Andreasson, 2001; Cartea, Velasco, Obregón, Padilla, & de Haro, 2008; Kim, Durrett, Last, & Jander, 2004). The relationship with primary sulfur metabolism is also important for GSL production, as two to three sulfur atoms are required per aliphatic GSL molecule (Jensen, Halkier, & Burow, 2014).

Many species of insect have evolved a tolerance to the volatile compounds produced by the so-called 'mustard oil bomb' – the chemical reaction that converts GSLs into ITCs, nitriles and various other defensive compounds. Insects such as *Pires rapae* larvae have evolved their own nitrile specifier protein (NSP) to metabolise aromatic and aliphatic nitriles, for example, which can then be excreted harmlessly. The consumption of GSLs and the production of metabolic degradation products such as cyanide have also been proposed as being nutritious to the insects consuming them. Far from being harmful, they could provide a potentially valuable source of nitrogen for amino acid synthesis (Stauber et al. 2012).

1.4. Health Effects of Glucosinolate Hydrolysis Products

1.4.1. Reported Adverse Health Benefits

From the late 1960s to the mid-1990s, much of the focus on glucosinolates and the associated hydrolysis products was in relation to adverse health effects. Much of the concern surrounded goitrogenic compounds, which are produced from the GSLs epiprogoitrin and progoitrin. The oxazolidine-2-thiones and thiocyanate

compounds produced by the hydrolysis of these GSLs interfere with thyroid metabolism and induce a condition known as goiter. In the presence of nitrate they also undergo nitrosation reactions, and the products of which are thought to have negative health consequences (Bones & Rossiter, 2006).

High doses of GSL-derived nitriles have also been shown to be toxic (Chiang, Pusateri, & Leitz, 1998). The compounds under study typically elicited conflicting results, showing tumor promotion in some papers, and prevention in others (Suzuki, Ohnishi-Kameyama, Saskai, Murata, & Yoshida, 2006).

Most of the adverse effects reported come not from human, but animal studies. It is likely that due to the diverse nature of the human diet, ingested quantities of *Brassica* vegetables are not enough to reach toxic levels as with herbivores. Instead, at relatively low levels the compounds are beneficial to humans and enhance cellular defenses against cancer and other diseases (Angelino et al. 2015).

1.4.2. Reported Human Health Benefits

From the late 1990s onwards, the beneficial health effects of GSLs and ITCs was more widely recognised, and breeding goals in crops were modified to maximise the potential health benefits to the consumer. Breeding strategies are now being adapted to increase phytochemical concentrations within crops, and breeders are recognising that getting consumers to eat more vegetables is not a realistic goal (Kopsell, Barickman, Sams, & McElroy, 2007). By increasing the nutritional density of crops, people would only need to maintain a reasonable intake of vegetables to gain improved health benefits.

Much of the reported health effects are attributed to the hydrolysis products of GSLs, such as glucoraphanin, glucoerucin and glucobrassicin (Vaughn & Berhow,

2005). The ITC and indole products (sulforaphane, erucin and indole-3-carbinol, respectively) have shown strong anti-carcinogenic effects in cell and animal studies (Sun, Liu, Zhao, Yan, & Wang, 2011), but as will be discussed in the next section, these studies are somewhat limited in their applicability to humans and day-to-day consumption. Other more limited and less well understood health-related effects associated with these compounds include: bactericidal effects on food-borne pathogens (van Eylen et al. 2009), reductions in the development of cardiovascular problems, and ultra-violet light protection in the skin (Schouten et al. 2009).

Much of the evidence for anti-carcinogenic effects of ITC/indole consumption comes from epidemiological studies analysing dietary patterns of *Brassica* vegetable consumption and incidences of cancer. These studies highlight an important overall trend, but do not elucidate the mechanisms responsible for the observed effects (Angelino et al. 2015).

Genetic studies on humans have identified several genes that play a role in ITC metabolism, and in turn infer the health benefits an individual will get from consuming *Brassicaceae* vegetables. Glutathione S-transferase (GST) loci and the associated polymorphisms of the GSTM1, GSTT1 and GSTP genotypes greatly impact the relative protective effects of ITCs that an individual will receive. The interactions between vegetable, gut microflora and human genetics are not yet fully understood, and are likely to be extremely complex (Traka & Mithen, 2009).

A study by Bogaards, Verhagen, & Willems (1994) demonstrated that after human males consumed 300g of Brussels sprouts per day, that there was a significant increase in GST products in the blood compared to those on a GSL-free diet. While indicative of an underlying metabolic mechanism for ITC degradation, few people would be willing or able to consume such large quantities of Brussels sprouts

on a daily basis. The impracticality of some studies in the 'real world' often detracts from the importance of the mechanistic findings.

1.4.3. Limitations of in vitro & in vivo Animal Studies

Hundreds of papers have been published analysing the *in vitro* effects of GSL hydrolysis products on cancer cell lines and model animals. These studies have looked at various effects on cancer cells, such as cell proliferation, tumorigenesis, apoptosis, mutation, detoxification and cell survival rates. Some studies focus on individual GSL compounds, whereas others look at the effects of whole extracts, usually in the form of a juice to which cancer cells are exposed and challenged.

Smith, Lund, Clarke, Bennett, & Johnson (2005) conducted a study exposing human colorectal carcinoma cells (HT29) to Brussels sprout juice. The study demonstrated a significant inhibition of cell proliferation, and determined that several GSL hydrolysis products possibly contributed to this effect. In another study of healthy skin biopsies, broccoli sprout juice was added to measure changes in NAP(P)H:Quinone Oxidoreductase (NQO1, a phase II detoxification enzyme; Dinkova-Kostova et al. 2007). Results showed a 1.5-fold increase in NQO1 after one exposure to the juice, and a 4.5-fold increase after three compared to placebo controls. While these studies clearly demonstrate an effect on cancer and healthy cell lines is present, the translation of the results into a living human is not straightforward.

The concentrations to which cells are exposed in studies such as these are far in excess of what would be possible in a living human. There are numerous factors affecting the *in vivo* assimilation of ITCs and other compounds, such as the level of cooking (see next section) and the ability of the human to absorb, metabolise and excrete such compounds. The numerous reducing factors involved in these

processes ultimately means that any ingested compounds would often be lower than threshold doses established by such *in vitro* studies. They also often lack rigorous investigation in determining the molecular mechanisms responsible for the observed effects. Whole juice extracts from *Brassica* vegetables do not take into account the effects of other compounds present, such as flavonoids and carotenoids, which have also been reported to have anti-carcinogenic and beneficial health effects (Kopsell et al. 2007).

That ITCs/indoles have beneficial effects to humans is not in question, as a large volume of diverse studies now supports this hypothesis (Traka & Mithen, 2009). The specific modes of action, the relative effects of some compounds compared to others, and the effect the regularity of *Brassicaceae* consumption has on human health, are not well understood however.

1.4.4. Effects of Cooking on Health Beneficial Compounds

Rocket species are unusual for *Brassicaceae* vegetables because they do not require cooking in order to be consumed by humans. One of the main drawbacks of cooking and consuming GSL-rich plants is that the availability of the health-beneficial compounds is often significantly reduced depending on the duration of cooking and the temperature used (Rungapamestry, Duncan, Fuller, & Ratcliffe, 2007), as myrosinase enzymes are usually denatured at high temperatures (van Eylen et al. 2009). The consumption of raw *Brassica* vegetables over cooked has been advocated in the literature (Hayes, Kelleher, & Eggleston, 2008) but this would not be well accepted by consumers due to the toughness of some vegetables, and the strong sulfurous and bitter tastes common in uncooked *Brassica* species.

Some bacteria found within the human gut are known to possess myrosinase enzymes. They act as a potential means by which humans can ingest ITCs, even if

cooking has inactivated plant myrosinase. It has been speculated that such bacteria play a vital role in mediating the health benefits of GSLs, but the degree to which this occurs is unclear and requires extensive study (Angelino et al. 2015; Traka & Mithen, 2009). See Figure 1.2 for the proposed mode of action by gut bacteria and the subsequent metabolism and mechanisms for health effects by ITCs.

Broccoli is a crop that has seen great interest because it contains high concentrations of glucoraphanin and its ITC sulforaphane. Varieties of broccoli have been bred specifically for enhanced glucoraphanin concentration, and to overcome

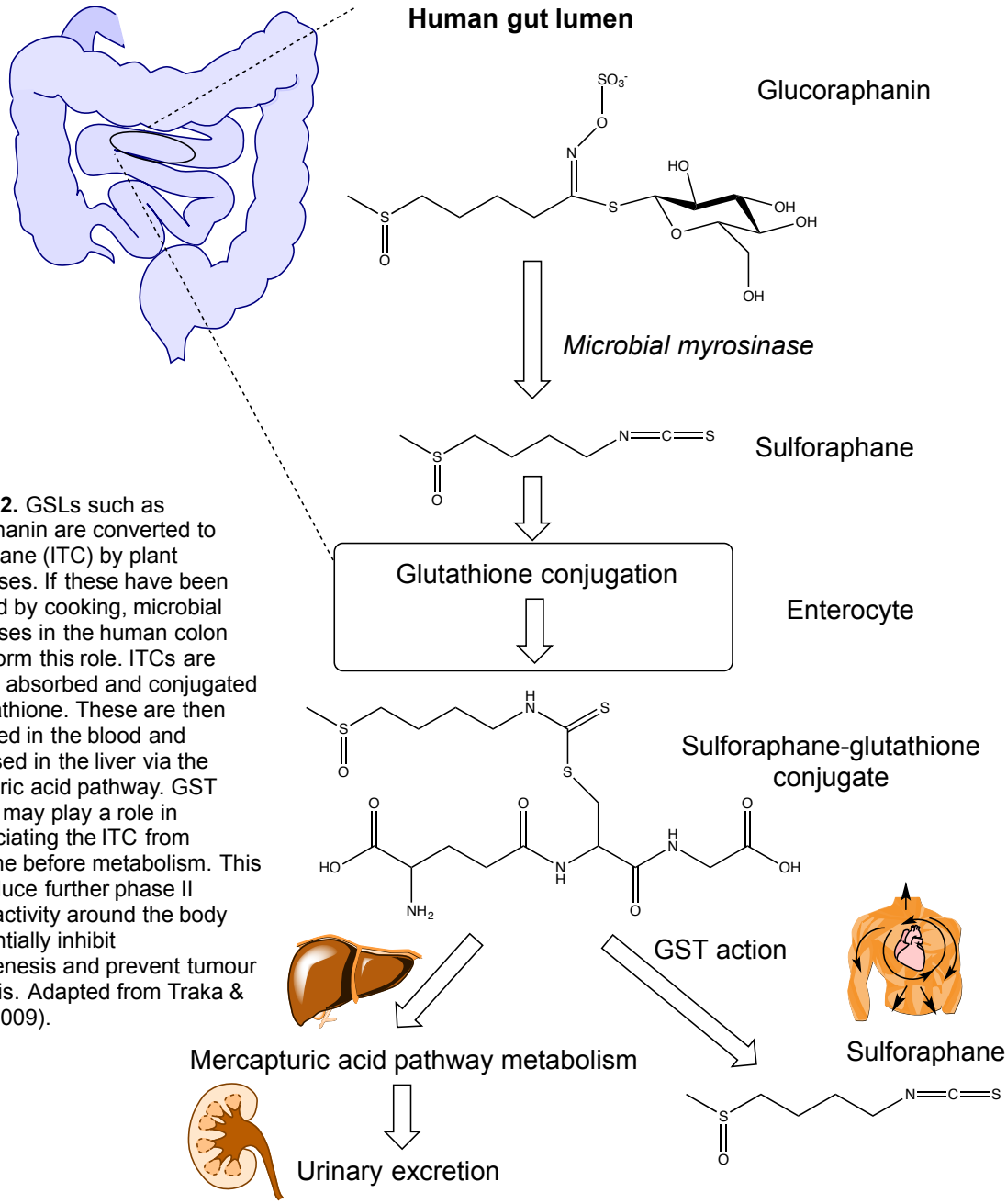

Figure 1.2. GSLs such as glucoraphanin are converted to sulforaphane (ITC) by plant myrosinases. If these have been denatured by cooking, microbial myrosinases in the human colon may perform this role. ITCs are passively absorbed and conjugated with glutathione. These are then transported in the blood and metabolised in the liver via the mercapturic acid pathway. GST enzymes may play a role in dissassociating the ITC from glutathione before metabolism. This would induce further phase II enzyme activity around the body and potentially inhibit carcinogenesis and prevent tumour metastasis. Adapted from Traka & Mithen (2009).

the negative effects associated with cooking (Traka & Mithen, 2009). Rocket species are similarly an ideal plant to work with in this respect, as modification of GSL profiles through breeding could potentially provide an important source of anti-carcinogenic compounds to the human diet that does not require cooking. That being said, the effects of the commercial supply chain on GSL/ITC concentrations, myrosinase activity and potential health benefits are poorly understood (Verkerk & Dekker, 2004).

1.5. Literature Critique of Rocket Salad Research

1.5.1. General

Within the scientific literature several studies have been conducted in relation to phytochemical diversity of rocket species (Jin et al., 2009; Martinez-Sanchez, Llorach, Gil, & Ferreres, 2007; Pasini, Verardo, Caboni, & D'Antuono, 2012; Villatoro-Pulido et al., 2013) and have provided a foundation for this project. Very few studies have been conducted on sensory aspects however (D'Antuono et al. 2009; Lokke et al. 2012; Pasini et al. 2011), and none on consumer responses. No published work (to the author's knowledge) has previously explored the supply chain of rocket in a working industrial setting, and the genomic sequence of multiple *E. sativa* inbred lines has never been obtained.

There are three key underlying aspects of the study of rocket crops that have been identified in the course of this project that show inconsistencies and require improvement. These are briefly presented here, and have acted as an 'undercurrent' for the reasoning and experimental choices made in each of the chapters/papers of this thesis. Many of the assumptions that have been previously made within the literature will be assessed and challenged.

1.5.2. Experimental Aims & Designs

As a general overview of rocket research to-date, it can be said that there has been little cohesion, continuity or direction. Richard Bennett and colleagues published several papers in the previous decade (Bennett et al., 2002; Bennett, Rosa, Mellon, & Kroon, 2006; Bennett, Carvalho, Mellon, Eagles, & Rosa, 2007), which laid an important foundation in identifying the core GSL components and diversity of rocket. The same can be said of Martinez-Sanchez and colleagues (Martinez-Sanchez, Gil-Izquierdo, Gil, & Ferreres, 2008; Martinez-Sanchez et al. 2007) in identifying polyglycosylated flavonols in rocket leaves.

Several groups have published works relating to LC-MS/MS analysis of rocket glucosinolate (GSLs), but the experimental designs of some experiments have been significantly flawed in the author's opinion (Chun, Arasu, Lim, & Kim, 2013; Kim & Ishii, 2006; Pasini et al. 2012; Villatoro-Pulido et al., 2013). Many publications have failed to take into account the changing GSL profile of plants over the course of their life cycle, and fewer still have sampled at an appropriate commercially relevant growth stage. This has made consensus on exact GSL composition difficult, and is therefore of limited use to breeders. Similarly, the number of accessions studied is usually small, and the characterisation of lines across environments and research groups is largely non-existent. These issues are perhaps more a symptom of the fact that there are comparably so few studies in rocket compared to high value and mainstream *Brassica* crops like broccoli.

1.5.3. Glucosinolate Extraction Methods

Methods of extraction of GSLs have also been inconsistent between research groups. While results presented for desulfo and 'crude' GSL extracts of rocket do not differ wildly, there are arguments to be made for and against each method (Ishida,

Kakizaki, Ohara, & Morimitsu, 2011; Wathelet, Iori, Leoni, Quinsac, & Palmieri, 2004). Research groups have tended to favor the desulfation method in the past, which utilises Sephadex anion exchange columns and sulfatase enzyme to remove GSLs from crude samples. It is argued that the desulphation method of extraction improves chromatographic resolution due to the removal of the sulphate group, which therefore decreases the polarity of the molecules. Crude extracts are becoming more prevalent in the literature however, as it is much less time consuming (Ares, Nozal, Bernal, & Bernal, 2015), and seems to produce equally robust results. This paper has favored the crude method of extraction and detection because of its less laborious nature.

1.5.4. Acknowledgement Of Dimeric Glucosinolates

The final area of importance is the acknowledgement of the unique disulfide GSLs found in rocket species and their possible independent organoleptic properties. Since the confirmation of the existence of 4-mercaptobutyl-GSL (glucosativin), the presence of dimeric GSLs have often been dismissed as products of extraction, and discounted as being naturally produced (Bennett et al., 2002). An excellent paper by Cataldi, Rubino, Lelario, & Bufo (2007) convincingly demonstrated that this was not the case, and that dimeric-4-mercaptobutyl-GSL (DMB) and diglucothiobeinin are in fact naturally occurring within the leaves of rocket species. Despite this significant result, very few research papers have since cited or acknowledged this key outcome. Several subsequent papers disregarded detections of monomeric glucosativin on the basis of previous speculations and assumptions, and potentially missed important details in the variability of GSL profiles between cultivars (Bennett et al., 2007; Chun et al. 2013; D'Antuono et al. 2008; Pasini et al. 2012; Villatoro-Pulido et al., 2013).

Some of the aforementioned papers actually cite Cataldi et al. (2007), yet still did not properly quantify the monomer and dimer separately.

The importance of the Cataldi et al. (2007) paper and its findings is supported by many of the results presented in this thesis; particularly in Chapter 3, where concentrations of monomeric and dimeric glucosativin and their respective ratios differ significantly between accessions. There are instances where only the monomer form is present, indicating a potential genetic component to their synthesis. In other instances, relative amounts of each form are higher/lower in different accessions. Both of these outcomes further cast doubt on the hypothesis that the dimer is an extraction artifact. The abundance of each respective form also appears to be affected by growth stage and industrial processing (Chapter 7), and has implications for sensory (Chapter 5) and consumer acceptance (Chapter 6).

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CHAPTER 2: Glucosinolates, myrosinase hydrolysis products and flavonols found in rocket (*Eruca sativa* and *Diplotaxis tenuifolia*)

2.1. Introduction To Paper (as published in the *Journal of Agricultural & Food Chemistry,* 2014, Vol. 62, Issue 20)

At the start of this project, the main compounds of interest in rocket species were GSLs and flavonols. These two classes of phytochemical have numerous and important implications for potential health benefits. This is of particular interest to plant breeders and producers such as the industrial collaborators, as there is the potential for improving upon current cultivars by utilising underused genetic resources.

Several research papers have highlighted the key constituents of GSL and flavonol profiles in rocket, but upon starting experimental procedures in this project, it became clear that the data needed to identify compounds in rocket by mass spectrometry was fragmentary within the literature. For glucosinolates in broccoli, for example, finding ion data is relatively straightforward, as this crop and its GSLs have been well studied for several decades. Rocket however contains GSLs that are less well characterised, and often have poor identification parameters associated with them. It was therefore a substantial challenge to amass reliable ion data from the literature without conducting extensive reading and cross-comparisons between studies.

The goals of the review paper presented in this chapter were therefore: 1) to provide a collated view of rocket phytochemical research from the perspective of plant breeding and crop improvement; 2) provide a useable, referenced resource of all GSL and flavonol compounds detected in rocket, with corresponding primary ions and fragmentation ions; and 3) highlight factors in the industrial supply chain that

may have implications for GSL and flavonol concentrations found in leaves. This has given a specific and focused view of rocket species, rather than relying on comparisons with related species such as broccoli and watercress. While there are many similarities with such crops, rocket has quite distinct and separate usages within cuisine and people's diets. As is shown in this and later chapters, even the two predominant rocket species, *Diplotaxis* and *Eruca*, have subtle differences in terms of morphology, cultivation and phytochemical composition.

2.2.Introduction

In recent years, several species of minor leafy-crops have risen to prominence as potentially important commercial and edible species. One example is rocket, which has quickly gained popularity in the Western diet. Originally found as an obscure crop in Mediterranean and Middle-Eastern countries, rocket has become popular largely due to the pungent aromas and tastes associated with it. Glucosinolates (GSLs)/isothiocyanates (ITCs) and flavonols derived from many species (Chaudhary et al., 2012; Gross, Dalebout, Grubb, & Abel, 2000; Jongen, 1996; Vinson, Dabbagh, Serry, & Jang, 1995) have been shown to infer significant protection against cancer and heart disease (Clarke, Dashwood, & Ho, 2008; Hayes, Kelleher, & Eggleston, 2008; Herr & Buechler, 2010; Melchini & Traka, 2010; Pappa et al., 2006; Vinson, Dabbagh, Serry, & Jang, 1995; Yang et al., 2002; Zhang, 2004). In Western countries, diets are generally lacking in fruits and vegetables. Despite government initiatives (such as the "5-a-day" campaign in the UK and USA), these diseases are increasingly leading to premature deaths (Casagrande, Wang, Anderson, & Gary, 2007). Plant breeders aim to maximise levels of such beneficial compounds, but with little genomic information about rocket species presently available, this is a formidable task. This review will give an overview of research in

rocket, an outbreeding crop, and how breeders and processors can utilise it to enhance beneficial compounds.

2.3. Rocket species

Rocket (also known as arugula, rucola and roquette) is a leafy vegetable crop that has gained substantial popularity across the world, particularly over the last fifteen years (D'Antuono, Elementi, & Neri, 2009; Hall, Jobling, & Rogers, 2012; Koukounaras, Siomos, & Sfakiotakis, 2007; Lamy et al., 2008). Two main species are predominantly farmed as salad crops; these are *Eruca sativa* ('salad' or 'cultivated' rocket; sometimes referred to as *Eruca vesicaria* subsp. *sativa*) and *Diplotaxis tenuifolia* ('wild' rocket). Both species share a peppery taste and aroma that is very distinctive (Pasini, Verardo, Cerretani, Caboni, & D'Antuono, 2011). They have been reported to contain high levels of vitamin C, GSLs, flavonols and phenolics (Bennett et al., 2002; Bennett, Carvalho, Mellon, Eagles, & Rosa, 2007; Bennett, Rosa, Mellon, & Kroon, 2006; Cataldi, Rubino, Lelario, & Bufo, 2007; Chun, Arasu, Lim & Kim, 2013; Kim & Ishii, 2007; Martinez-Sanchez, Gil-Izquierdo, Gil, & Ferreres, 2008; Martinez-Sanchez, Llorach, Gil, & Ferreres, 2007). These are all known to have both anti-oxidant and anti-cancer properties, and are also implicated in lowering the risk of cardiovascular and cognitive disease. For excellent information on these beneficial effects and their underlying causes, see Drewnowski & Gomez-Carneros (2000), Keum, Jeong, & Kong (2004), D'Antuono, Elementi, & Neri (2008), Egea-Gilabert, Fernandez, Migliaro, Martinez-Sanchez, & Vicente (2009), Degl'Innoocenti, Pardossi, Tattini, & Guidi (2008), Bjorkman et.al (2011) and Jeffery et.al (2003).

2.4. Taxonomy and domestication

A distinction should be made that both *Eruca* and *Diplotaxis* species have overlapping characteristics, and that one can be easily mistaken for the other by the untrained eye, and/or before a certain level of maturity has been reached (D'Antuono et al. 2008). It is also arguable that *D. tenuifolia* is the least 'wild' of the two species even though the common name is 'wild rocket'. It is featured and favored in commercial products and breeding programs, and is likely to be more domesticated than *Eruca* species as a result. *Diplotaxis* varieties are generally uniform phenotypically, with *Eruca* varieties being more diverse in this respect (Bennett et al., 2006). No direct genomic evidence has been presented in the literature to suggest one species is any more or less genetically variable than the other. Variability in GSL data seems to support the hypothesis that *Diplotaxis* species are more 'wild' (Pasini, Verardo, Caboni, & D'Antuono, 2012), though it is not conclusive, as only a relatively small number of cultivars have been tested. This is a point that needs clarification through research and extensive breeding, as neither species can be considered fully domesticated (Egea-Gilabert et al. 2009). For example, germination rates are variable, reproductive organs are typically small, seedpods shatter and disperse freely (rather than staying on the plant), and physical defenses such as leaf hairs are still present in many commercial varieties (Gepts, 2010).

2.5. Phytochemicals in *Eruca sativa* and *Diplotaxis tenuifolia*: types and structures

2.5.1. Glucosinolates

GSLs are β-thioglucoside *N*-hydrosulphates that are responsible for the sharp and bitter-tasting flavors found in cruciferous vegetables (Rungapamestry, Duncan, Fuller, & Ratcliffe, 2007; Velasco, Cartea, Gonzalez, Vilar, & Ordas, 2007). In

combination with the enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147), GSLs are hydrolyzed to create isothiocyanates, nitriles, thiocyanates, epithionitriles, indoles, oxazolidine-2-thiones, cyanopithioalkanes, ascorbigens, goitrogens and epithioalkanes (Baik et al., 2003; Bones & Rossiter, 2006; Grubb & Abel, 2006; Hecht, 1999; Jia et al., 2009; Jirovetz, Smith, & Buchbauer, 2002; Matusheski & Jeffery, 2001; McNaughton & Marks, 2003; Rangkadilok et al., 2002a; Taiz & Zeiger, 2006; Yan & Chen, 2007; Yuan, Sun, Yuan, & Wang, 2009; Zhang, Talalay, Cho, & Posner, 1992); see Figure 2.1. Many of these hydrolysis products

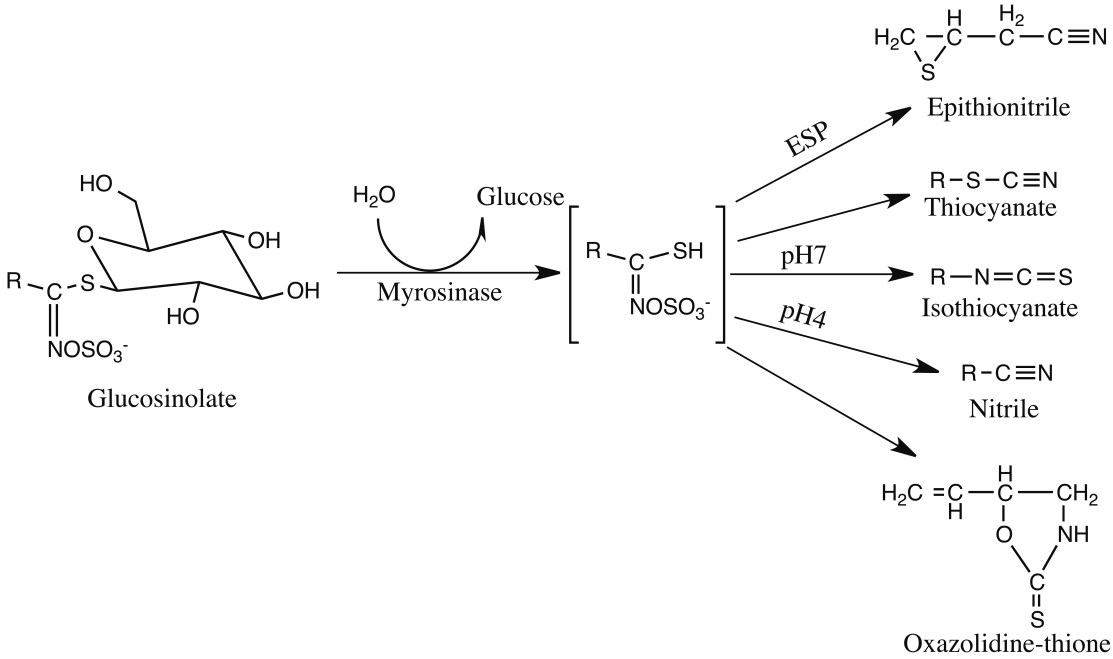


Figure 2.1. The glucosinolate-myrosinase reaction and some of the subsequent compounds produced under different conditions, such as pH and the influence of epithiospecifier proteins (ESP) (Adapted from Zhang, 2004, and Hall, McCallum, Prescott, & Mithen; 2001).

have antibacterial, antifungal and insect repellant effects (Halkier & Gershenzon, 2006; Jeffries, 1990; Mithen & Campos, 1996; Newman, Hanscom, & Kerfoot, 1992; Newman, Kerfoot, & Hanscom, 1990; Ostrofsky & Zettler, 1986). GSLs and ITCs are being increasingly used as 'biofumigants' to suppress soil borne pathogens, nematodes and weeds. Some of the volatile products have the opposite effect of attracting species that can tolerate high GSL concentrations, such as types of

ovipositing insect (Brown & Morra, 1995; Vaughn, Isbell, Weisleder, & Berhow, 2005).

The conditions under which hydrolysis of GSLs occurs will affect the respective proportions of the chemicals produced; pH, iron ions, thiol ions, temperature and hydration play a particularly prominent role in this process *in vivo* (Foo et al., 2000). The separation of GSLs in specialist 'S-cells' from myrosinase in myrosin cells means that the two components only come into contact upon tissue disruption; for example when damaged via chewing or digestion (Andreasson, Jorgensen, Hoglund, Rask, & Meijer, 2001; Chen & Andreasson, 2001; Fenwick & Heaney, 1983; Getahun & Chung, 1999; Hoglund, Lenman, Falk, & Rask, 1991; Husebye, Chadchawan, Winge, Thangstad, & Bones, 2002; Kliebenstein, Kroymann, & Mitchell-Olds, 2005; Song & Thornalley, 2007; Talalay & Fahey, 2001; Tripathi & Mishra, 2007; Verkerk, Dekker, & Jongen, 2001). It is the biological activity of the ITC hydrolysis products in humans that are of most interest in rocket (Halkier & Gershenzon, 2006). GSLs can be hydrolyzed within the intestinal tract by gut microflora that are known to have specific myrosinase activity (Fahey, Zhang, & Talalay, 1997; Heaney & Fenwick, 1980; Rabot, Nugonbaudon, Raibaud, & Szylit, 1993; Shapiro, Fahey, Wade, Stephenson, & Talalay, 1998), but the efficacy of their action is not yet well determined.

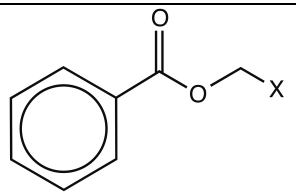
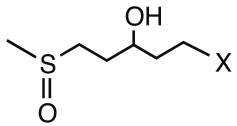
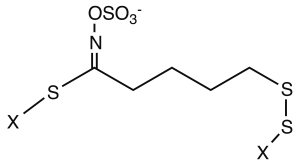
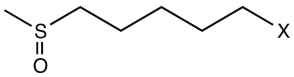
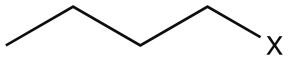
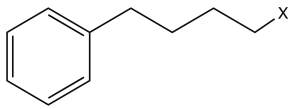
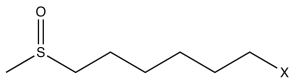
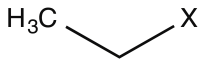
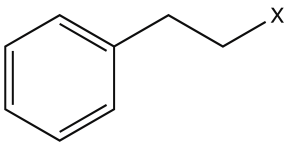
GSL concentrations can vary and change over time depending on environmental conditions and stress (Herr & Buechler, 2010). Other factors affecting GSL profiles include the plant age, organ type, developmental stage, ambient air temperature, level of water stress, photoperiod, agronomic practice, degree of wounding, and geographical origin of the variety/species (Agerbirk, Olsen, & Nielsen, 2001; Ahuja, de Vos, Bones, & Hall, 2010; Bartlet, Kiddle, Williams, & Wallsgrove, 1999; Coogan, Wills, & Nguyen, 2001; Hasegawa, Yamada, Kosemura, Yamamura,

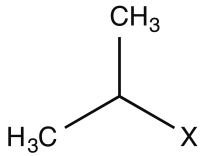
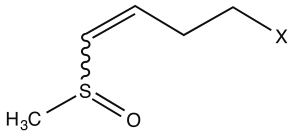
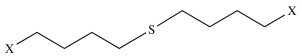
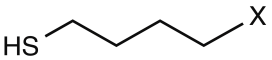
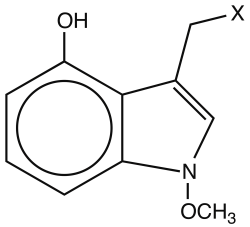
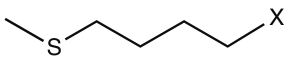
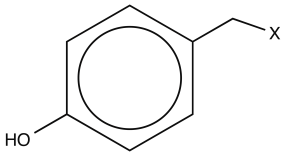
& Hasegawa, 2000; Kushad et al., 1999; Rangkadilok et al., 2002b; E. Rosa & Heaney, 1996). These can often affect the profiles of *all* phytonutrients contained within tissue, not just GSLs (Jin et al., 2009), and they are all factors that plant breeders aim to mitigate through development of genetically advanced and uniform breeding lines.

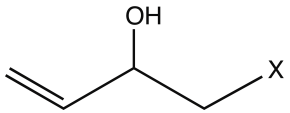
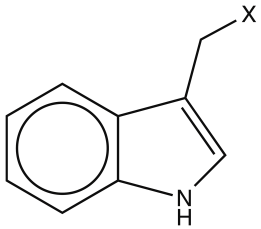
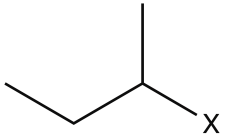
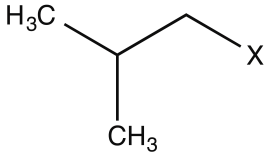
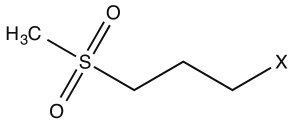
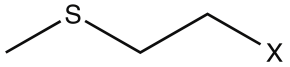
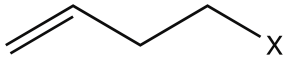
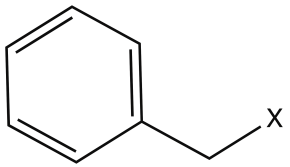
GSLs and the ITC derivatives have been an integral part of the human diet for millennia because of the presence of them in the family *Brassicaceae* (Cartea, Francisco, Soengas, & Velasco, 2011; Chen & Andreasson, 2001; Conaway, Yang, & Chung, 2002; Fahey, Zalcmann, & Talalay, 2001; Holst & Williamson, 2004; Rose, Won, Ong, & Whiteman, 2005; Stoewsand, 1995; Talalay & Fahey, 2001; Tripathi & Mishra, 2007; Zhang & Talalay, 1994). GSLs are evolutionarily recent secondary metabolic products having arisen 10-15 million years ago (Wheat et al., 2007; Windsor et al., 2005), acting to prevent pathogen attack and dissuade herbivory. They are known in only a few angiosperm families of the order *Brassicales*, which includes the *Brassicaceae* (Charron, Saxton, & Sams, 2005; Clarke, 2010; Mithen, Dekker, Verkerk, Rabot, & Johnson, 2000; Rodman, Karol, Price, & Sytsma, 1996; Rosa, 1997; Schouten et al., 2009; Schreiner, 2005; Verkerk et al., 2009; Wittstock & Halkier, 2002), and of which *Eruca* and *Diplotaxis* are members.

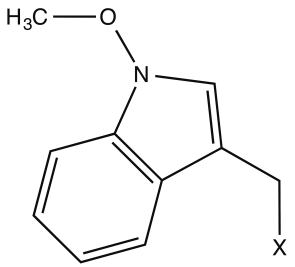
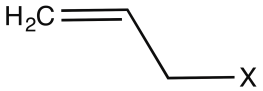
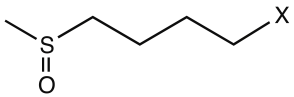
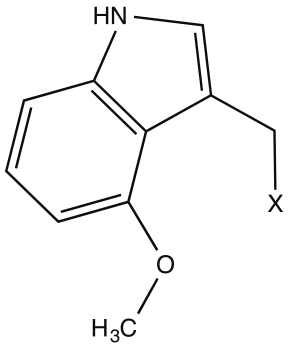
A study by Pasini et al. (2012) of 37 rocket accessions (*Diplotaxis* and *Eruca*) showed that GSL profiles were all very similar, regardless of the species. In total, twelve GSL compounds were found across all accessions; Table 2.1 illustrates all known GSL compounds identified to-date in rocket. These include 4-mercaptobutyl GSL (glucosativin) (Bennett et al., 2002), 4-methylthiobutyl GSL (glucoerucin) (Graser, Schneider, Oldham, & Gershenzon, 2000), and 4-methylsulfinylbutyl GSL (glucoraphanin) (D'Antuono et al. 2008), which constitute the three most abundant GSLs in rocket.

Table 2.1. Intact Glucosinolates Identified Within Leaves Of Rocket, *Eruca* and *Diplotaxis* Species, By LC-MS (Negative Ion Mode)

R-group	Common name	R-group structure [x]	Mass parent ion	MS^2 spectrum ions (signature ions in bold)	Reference
2-(benzoyloxy) ethyl	-		466	386	Pasini et al. (2012)
3-hydroxy-5-(methylsulfinyl) pentyl	-		482	403	
4-(β-D-glucopyranosyldisulfanyl) butyl	Diglucothiobeinin		600	521	Pasini et al. (2012); Lelario, et al. (2012)
5-(methylsulfinyl) pentyl	Glucoalyssin		450	371	
N-butyl	Dihydrogluconapin		374	294	
4-phenylbutyl	Glucoamoracin		450	371	D'Antuono et al. (2008)
7-(methylsulfinyl) heptyl	Glucoibarin		446	414	
Ethyl	Glucolepiidin		346	266	
2-phenylethyl	Gluconasturtiin		422	343	

1-methylethyl	Glucoputranjivin		360	280	D'Antuono et al. (2008)
4-(methylsulfinyl)-3-butenyl	Glucoraphenin		434	354	
Dimeric 4-mercaptobutyl	DMB		811	731, 569, **405**	Bennett et al. (2002); Pasini et al. (2012); Lelario et al. (2012)
4-mercaptobutyl	Glucosativin		406	**259**, 209, 194, 138 97, 96	
4-hydroxy-3-indolymethyl	4-Hydroxyglucobrassicin		463	383, **285**, 275, 267, **259**, 240	Pasini et al. (2012); Rochfort et al. (2008)
4-(methylthio) butyl	Glucoerucin		420	340, 291, 275, **259**, 242, 227, 195, 178, 163	
4-hydroxybenzyl	Glucosinalbin		424	344, 291, 275, 261, **259**, 246, 231, 228, 182	

(R,S)-2-hydroxy-3-butenyl	Progoitrin/epiprogoitrin		388	332, 308, 301, 275, 259, 210, 195, 136	
3-indolymethyl	Glucobrassicin		447	275, **259**, 251, 205	Pasini et al. (2012); Rochfort et al. (2008); Botting et al. (2002)
1-methylpropyl	Glucocochlearin		374	294	Fahey et al. (1994); Lelario et al. (2012)
2-methylbutyl	Glucojiaputin		388	308	
5-(methylsulfonyl) pentyl	Glucoerysihienin		466	386	D'Antuono et al. (2008); Pasini et al. (2012)
3-(methylthio) propyl	Glucoiberverin		406	326, 275, **259**, 228, 145	Fahey et al. (2001); Rochfort et al. (2008)
3-butenyl	Gluconapin		372	292, 275, **259**, 227, 195, 194, 176	
Benzyl	Glucotropaeolin		408	328, 275, **259**, 241, 230, 212, 195, 166	D'Antuono et al. (2008); Rochfort et al. (2008)

1-methoxyindol-3-ylmethyl	Neoglucobrassicin		477	447, **466**, 284, **259**	
2-propenyl	Sinigrin		358	278, 275, **259**, 227, 195, 180, 162	
4-(methylsulfinyl) butyl	Glucoraphanin		436	**372**, 291, **259**, 97, 96	Bennett et al. 2002; Pasini et al. (2012); Rochfort et al. (2008); Botting et al. (2002)
4-methoxy-3-indolymethyl	4-Methoxyglucobrassicin		477	291, 275, **259**, 235, 227, 195	Rochfort et al. (2008); Kim & Ishii (2006)

[x] = standard GSL molecule according to IUPAC nomenclature

Table 2.2. List Of Flavonol Compounds Identified In Leaves Of *Eruca* And *Diplotaxis* Species, By LC-MS (Negative Ion Mode).

Flavonol compound [a]	*Eruca* [p]	*Diplotaxis* [p]	Mass parent ion	MS^2 spectrum ions (signature ion in bold)	Reference
I 3,4'-diGlc	✓	✓	639	**477**	Martinez-Sanchez et al. (2008); Pasini et al. (2012)
I 3-Glc	✓		477	-	
K 3-(2-Sinp-Glc)-4'-Glc	✓		817	-	
K 3,4'-diGlc	✓	✓	609	-	
K 3-Glc	✓		447	**285**	
Q 3-Glc	✓		463	301	
K 3-diGlc-7-Glc	✓		771	609	Pasini et al. (2012)
K 3-Sinp-triGlc-7-Glc	✓		1139	977, 771, 609, 429	
Q 3,4'-diGlc-3'-(6-Caf-Glc)		✓	949	787, **625**, 463, 301	
M	✓		317	151	Villatoro-Pulido et al. (2013)
Q	✓		301	151	
R	✓		609	300	
Q 3-(2-Caf-Glc)-3'-(6-Sinp-Glc)-4'-Glc		✓	1155	993, 831, 787, 669, **625**, 463, 301	Martinez-Sanchez et al. (2008); Martinez-Sanchez et al. (2007)
Q 3-(2-Mcaf-Glc)-3'-(6-Sinp-Glc)-4'-Glc		✓	1185	1023, 817, 669, 655	
Q 3-(2-Fer-Glc)-3'-(6-Fer-Glc)-4'-Glc		✓	1139	977, 639, 463	Martinez-

Q 3-(2-Fer-Glc)-3'-(6-Sinp-Glc)-4'-Glc	✓	1169	1007, 831, 669, 639, 463, 301	Sanchez et al. (2008); Martinez-Sanchez et al. (2007); Pasini et al. (2012)
Q 3-(2-Sinp-Glc)-3'-(6-Sinp-Glc)-4'-Glc	✓	1199	1037, 831, 669, 463, 301	
Q 3,3',4-triGlc	✓	787	625, 463, 301	
Q 3,4'-diGlc-3'-(6-Fer-Glc)	✓	963	801, 639, 463, 301	
Q 3,4'-diGlc-3'-(6-Mcaf-Glc)	✓	979	817, 655, 463, 301	
Q 3,4'-diGlc-3'-(6-*p*.Coum-Glc)	✓	933	771, 609, 463, 301	
Q 3,4'-diGlc-3'-(6-Sinap-Glc)	✓	993	831, 669, 463, 301	

[a] = Abbreviations: Caf, caffeyol; Mcaf, methoxycaffeyol; *p*.Coum, *p*-coumaroyl; Fer, feruloyl; Sinp, sinapoyl; Glc, glucoside; Q, quercetin; K, kaempferol; I, isorhamnetin; M, myricetin; R, rutin

[p] = ✓ compound positively identified in species

2.5.2. Flavonols

Flavonols are diphenylpropanes (C_6–C_3–C_6) (Arabbi, Genovese, & Lajolo, 2004) and are another important group of chemicals found within rocket species. Flavonols in rocket are found with sugar conjugates, and typically occur in relatively large quantities (Podsedek, 2007). The aglycones found (such as quercetin and kaempferol) are glycosylated and acylated, which in turn affects their biological properties (Martinez-Sanchez et al. 2008). A study by Martínez-Sánchez et al. (2008) identified over 50 different flavonol compounds across four different species. Watercress, mizuna and two species of rocket were all found to accumulate very different compounds within their leaves, and in varying quantities. Wild rocket showed high levels of quercetin-3,3',4-triglucosyl (43.5 mg per $100g^{-1}$ fw) and salad rocket had mostly kaempferol-3,4'-diglucosyl (97.8mg per $100g^{-1}$ fw). The group also showed a correlation between quercetin derivatives and high antioxidant activity, despite the significant variations seen between species.

Studies conducted on rocket tissues have identified significant concentrations of polyglycosylated flavonols. The core aglycones of these are kaempferol, quercetin and isorhamnetin (Arabbi et al., 2004); Table 2.2 provides an up-to-date list of all flavonol compounds identified in rocket to-date. Martinez-Sanchez et al. (2008) showed that *Eruca* species accumulate kaempferol derivatives, whereas *D. tenuifolia* accumulates predominantly quercetin instead, meaning that the two chemicals could be used as an identification marker between the two species (Cartea, Velasco, Obregon, Padilla, & de Haro, 2008). Isorhamnetin aglycones are common to both species but typically in much lower concentrations (Pasini et al. 2012). The specific aglycones also infer varying degrees of anti-oxidant activity. For example, quercetin derivatives have a higher activity than kaempferol and isorhamnetin. The differences in structure (the arrangement of hydroxyl groups and glycosylation) affect anti-

oxidant activity by allowing the molecules to act as hydrogen/electron donors, single oxygen scavengers, or as reducing agents (Salah et al., 1995).

2.6. Phytochemicals and the relationship with quality: taste and aroma

It is thought that the presence of glucosativin, glucoerucin and their hydrolysis products within rocket leaves is what gives them a characteristic flavor (Bones & Rossiter, 2006). Many of the health beneficial GSLs and ITCs are thought to be responsible for strong tastes that *some* consumers find repellant (Hansen, Laustsen, Olsen, Poll, & Sorensen, 1997). It seems that to many people, these compounds contribute very little to a pleasurable eating experience and are actively avoided (Holst & Williamson, 2004). Conversely however, some people *do* prefer these strong tastes and aromas, and will actively seek to consume rocket when it is available. Growers in Italy often prefer the subsequent cuts because of the more intense tastes and aromas that are produced (Martinez-Sanchez et al. 2008) and some will even 'sacrifice' the first cut in favour of the subsequent leaf growth. This highlights a divide between consumers that may be indicative of underlying genotype(s) for taste perception and preference.

The breeding process in rocket varieties to-date has effectively made the species 'milder' in taste when compared to plants that grow naturally in the wild. Whether this has been intentional or as a result of selecting for other unrelated traits (such as leaf morphology) is debatable. Some recent commercial varieties have been bred for a 'hotter' taste, such as '*Wildfire*', by Tozer Seeds (Surrey, UK).

A study by Pasini et al. (2011) demonstrated how breeding for sensory traits could be achieved, by highlighting which glucosinolates contributed to specific taste and aroma elements in rocket. It was found that progoitrin/epiprogoitrin is responsible for bitter taste attributes, despite being only a minor component of the overall GSL

profile of rocket (4.3-11.4% of total GSL concentration). The perceived pungency of leaves was positively related to the overall GSL content of accessions, and the levels of glucoraphanin negatively contributed to the typical 'rocket' flavour. The study also highlighted an important difference between rocket and other *Brassica* sensory studies (Schonhof, Krumbein, & Bruckner, 2004), in that bitterness was perceived as a favorable characteristic according to panelists. The flavonol compound kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside also significantly and positively contributed to flavor attributes in *Eruca* accessions. This would indicate that GSL compounds are not totally responsible for flavor in rocket. The study itself stopped short of saying how or if the information obtained would be used in breeding programs, but with study into rocket flavor components, milder (and/or stronger) varieties could be bred more efficiently once the responsible compounds are properly identified (Drewnowski & Gomez-Carneros, 2000).

2.7. Health promoting properties of glucosinolate-myrosinase products and flavonols of rocket

2.7.1. Isothiocyanates

ITC hydrolysis products have been identified in rocket (Jirovetz et al. 2002), such as 4-(methylthio)butyl ITC (erucin) (Cerny, Taube, & Battaglia, 1996; Iori, Bernardi, Gueyrard, Rollin, & Polmieri, 1999) which is known to show anti-proliferative activity in human lung carcinoma A549 cells, hepatoma (HepG2) cells, colon cancer cells, prostate cancer cell lines (PC3, BPH-1 and LnCap) and leukemia cells (Melchini et al., 2009). Erucin is a structurally reduced analog of sulforaphane, (which is predominantly found in broccoli) and has shown promising anti-cancer properties *in vitro* (e.g. anti-proliferation of human erytroleukemic K562 cells; Leoni et al., 1997). Research into the chemopreventative and anti-genotoxic nature of ITCs

has shown promising results (Wu, Zhou, & Xu, 2009; see Figure 2.2). Other studies involving chemically induced genotoxicity have shown very strong anti-genotoxic effects of *E. sativa* extracts (Lamy et al., 2008) which is in agreement with other

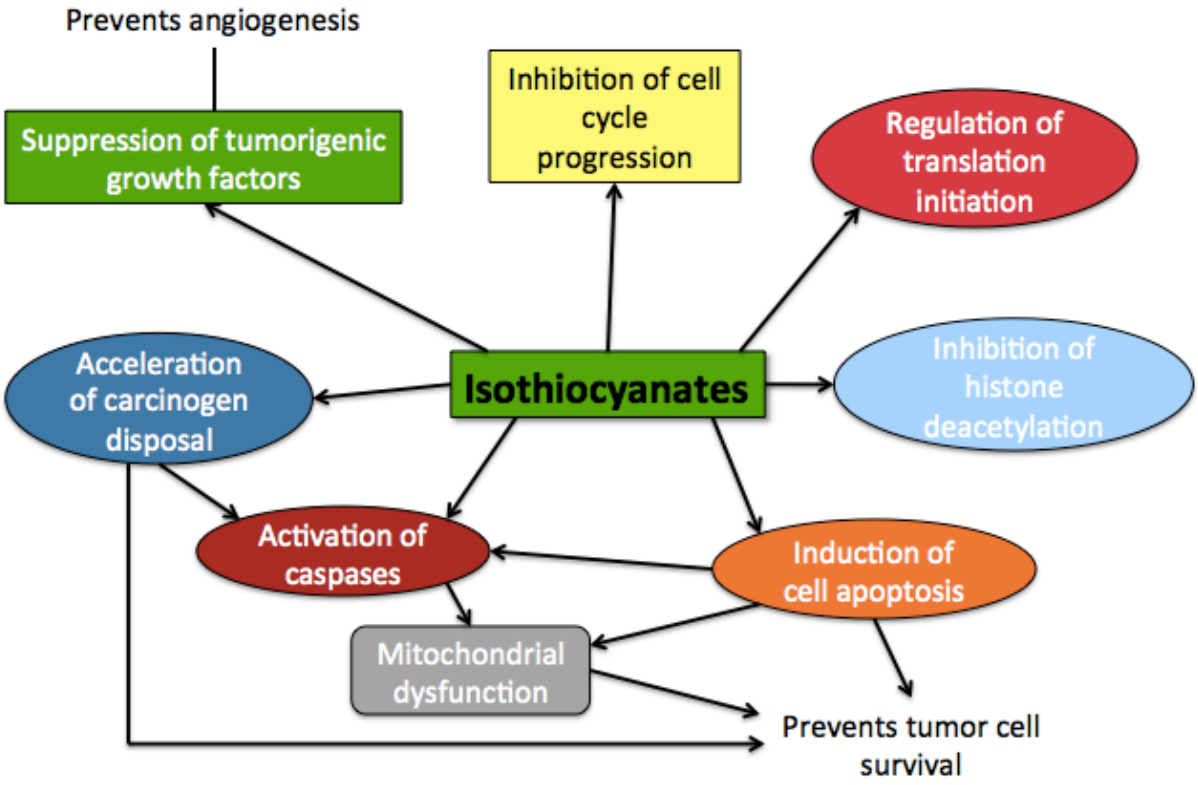


Figure 2.2. Pathways of documented ITC action in tumorigenic cells. See Wu, Zhou, & Xu, (2009) for a detailed review of the roles ITCs play in cancer prevention.

Brassicaceae studies (Kassie et al., 2002; Zhu & Loft, 2003). Identifying specific cultivars of rocket with elevated levels of erucin and glucoraphanin would be an important first-step in developing superior varieties from a human nutrition standpoint.

The results of GSL/ITC research prompted an investment in broccoli breeding in the last decade. A similar concerted effort could be made for rocket which contains similar compounds, and which are potentially just as efficacious in humans (Alqasoumi, Ai-Sohaibani, Ai-Howiriny, Al-Yahya, & Rafatullah, 2009). Erucin for example, has been shown to have very similar, and even superior, biological activity

to sulforaphane (Hanlon, Coldham, Sauer, & Ioannides, 2009). One paper has specifically demonstrated that the concentrations of rocket ingested in an average daily diet is significant enough to infer a cancer preventative effect (Lamy et al., 2008). The metabolism of ITCs in humans via the mercapturic acid pathway has been investigated. ITCs are conjugated with glutathione and degraded by N-acetylation, initiating an increase of phase II detoxification enzymes; see Figure 2.3 for detailed pathway breakdown of erucin (Wu, Zhou, & Xu, 2009).

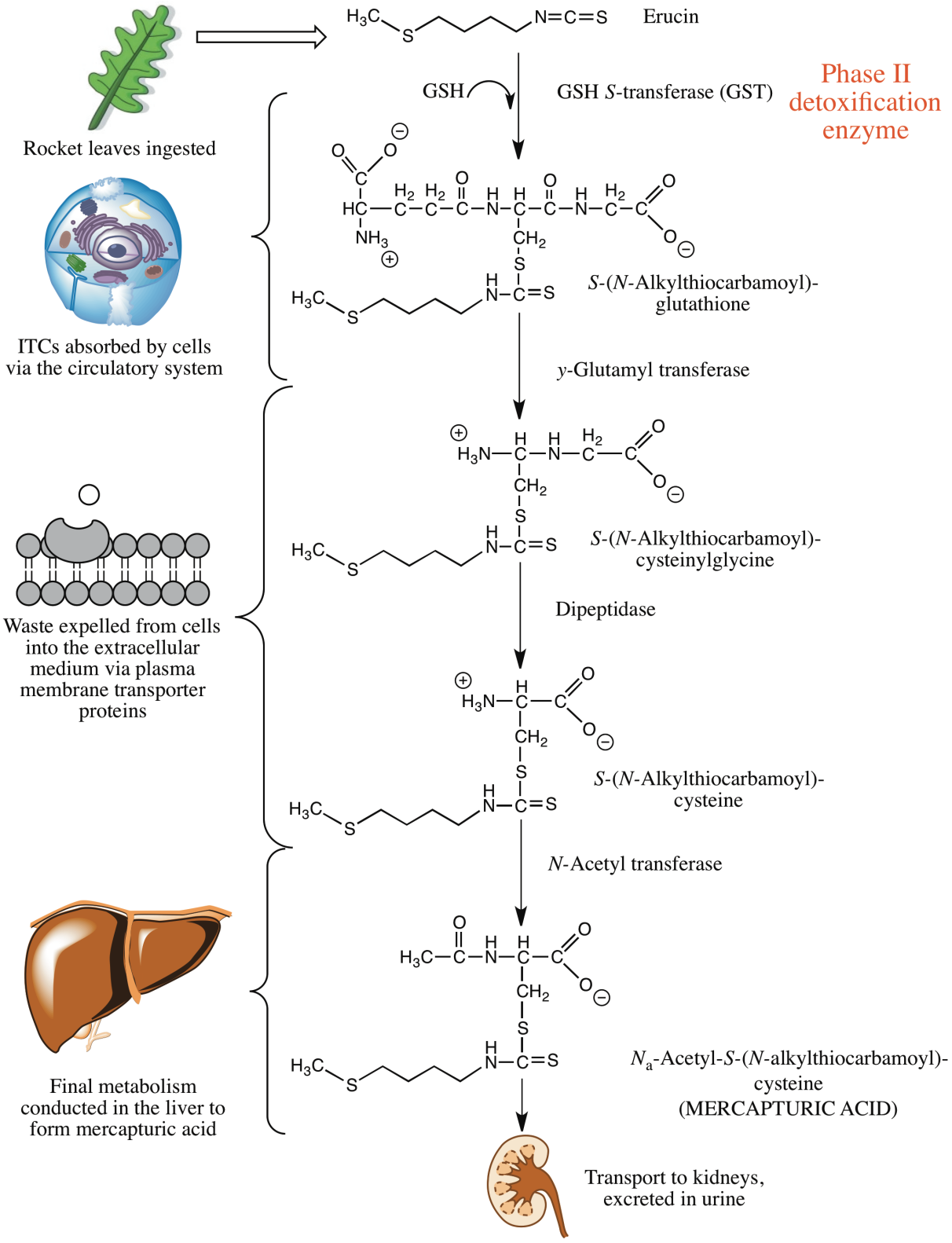


Figure 2.3. The mercapturic acid pathway of ITC metabolism in the human body. After ingestion of rocket leaves glucoerucin is hydrolyzed by myrosinase to form erucin. This is released and absorbed in the ileum, where it is transported in the blood to cells around the body. ITCs initiate Phase II detoxification enzymes in this pathway, and are known to aid in cancer prevention. (Adapted from Wu, Zhou, & Xu, 2009).

2.7.2. Nitriles

Along with ITCs, nitriles are the most abundant bioactive compounds produced by GSL hydrolysis (Alqasoumi et al. 2009). The hydrolysis of glucoraphanin for example, yields predominantly sulforaphane and sulforaphane nitrile. The ratio in which the two are formed depends greatly upon the environmental conditions and the plant cultivar that is used (Hanlon, Poynton, Coldham, Sauer, & Ioannides, 2009). A low pH medium tends towards the formation of nitriles, whereas high pH forms ITC (Cole, 1976; Matusheski et al., 2001). The presence of thiol and iron ions favors nitriles, and high temperature and hydration produce more ITCs (Macleod & Rossiter, 1986; Uda, Kurata, & Arakawa, 1986). This can have substantial consequences for any potential health benefits that might be inferred from eating rocket (Matusheski et al., 2001). The nitrile form is approximately three orders of magnitude less efficacious than the ITC in inducing quinone reductase (phase II enzyme), and thus infers a reduced enzymatic and anticarcinogenic response. Nitriles also compete with ITCs in this induction, and reduce potential positive effects further (Matusheski & Jeffery, 2001). As the ratio of these compounds may depend on plant variety, care must be taken in rocket breeding when selecting plants for GSL content, as this may not be reflective of the bioactives produced in subsequent hydrolysis reactions (Tookey & Wolff, 1970). Other underlying genetic factors may influence which degradation pathway is taken.

2.7.3. Indoles

Indoles are the predominant autolysis product of indole glucosinolates such as glucobrassicin, as their ITC counterparts are unstable (Fahey et al. 2001). Glucobrassicin has been detected as a minor GSL in rocket species (Pasini et al. 2012), and the predominant indole species produced is indole-3-carbinol. This

compound is known to be cancer-preventative (Cashman et al., 1999; Graham, 1983), particularly in reproductive organs *in vitro* and *in vivo*. A condensation product of indole-3-carbinol, 3,3'-diindolymethane, is also responsible for beneficial physiological effects. Both compounds have been shown to reduce cell proliferation in breast, prostate, cervical and colon cancer cell lines. They also show distinct differences from ITCs such as sulforaphane (Bonnesen, Eggleston, & Hayes, 2001), and inhibition of tumor development in the stomach, breast, uterus, tongue and liver of rodents (Bradlow, Michnovicz, Telang, & Osborne, 1991; Bresnick, Birt, Wolterman, Wheeler, & Markin, 1990; Kim et al., 1994, 1997; Kojima, Tanaka, & Mori, 1994; Tanaka et al., 1990; Tanaka, Kojima, Morishita, & Mori, 1992; Wattenberg & Loub, 1978). Experiments in rodents have shown an increase in drug-metabolizing enzymes in the stomach, liver and small intestines of individuals consuming both ITCs and indoles. This is suggestive of enhanced detoxification phase II enzymes (such as quinone reductase, glutathione reductase and glutathione transferase; Tanaka et al., 1990), and a mechanism by which these phytochemicals infer chemopreventative effects (Kim et al., 1994; Staack, Kingston, Wallig, & Jeffery, 1998).

Typically indoles inhibit cell proliferation through cytostatic mechanisms, whereas ITCs induce cytotoxicity within cell lines (at above 12.5 μM concentrations), which ultimately leads to increased apoptosis (Verhoeven, Verhagen, Goldbohm, van den Brandt, & van Poppel, 1997; Wattenberg, 1985). This indicates that both types of compound could act and be effective at different stages of cancer development (Pappa et al., 2006). Indoles have been shown to induce programmed cell death in prostate, breast and osteocarcinoma cell lines (Kuang & Chen, 2004) and G_1 cell cycle arrest in breast and prostate cancer cell lines (Cover et al., 1998; Sarkar & Li, 2004). It is these cytostatic effects on cell proliferation that has been suggested as

the mechanism responsible for the lack of apoptosis effects in indoles (Ge, Fares, & Yannai, 1999).

Using information on GSL content in rocket, the ITC and indole effects can be potentially maximized in new varieties, and be of a greater benefit to human health when considered in tandem, rather than separately (Bonnesen et al. 2001).

2.7.4. Oxazolidine-2-thiones & goitrogens

The hydrolysis of β-hydroxy-alkyl GSL compounds (e.g. progoitrin; a minor GSL in rocket) can produce oxazolidine-2-thiones such as goitrin (5-vinyloxazolidine-2-thione) (Cover et al., 1998; Greer, 1962; Grubb, Gross, Chen, & Abel, 2002; Lijang, Iori, & Thornalley, 2006; Sarkar & Li, 2004; Wink, 2010; Zhao, Tang, & Ding, 2007). It is these compounds that are largely attributed to the thyroid condition of goiter in mammals (Ghawi, Methven, & Niranjan, 2013), but the action of microflora in the gut is thought to mediate the problems associated with high oxazolidine-2-thione intake (Higdon, Delage, Williams, & Dashwood, 2007; Mcdanell, Mclean, Hanley, Heaney, & Fenwick, 1988). That being said, oxazolidine-2-thiones interfere with thyroxine synthesis (Dewick, 2009) and are therefore likely to have an adverse biological effect regardless of gut microflora action or bodily iodine status (Jongen, 1996). A study by Nishie & Daxenbilcher (1980) showed that these compounds are not teratogenic or embryotoxic however.

These molecules contribute significantly to the bitter taste of rocket that some people perceive quite strongly (Dewick, 2009). The detection of these compounds may be mediated in a similar genetic fashion as PROP (propylthiouracil), for example (Fenwick & Griffiths, 1981; Nishie & Daxenbichler, 1980). By using phytochemical data in rocket breeding programs these oxazolidine-2-thione components could be reduced, potentially improving consumer acceptance (depending on the target

consumer) and avoiding any possible adverse health effects associated with over-consumption.

2.7.5. Ascorbigens

Ascorbigens are formed via the reaction of indole-3-carbinol and 3,3'-diindolymethane with ascorbic acid in the stomach during myrosinase-catalyzed degradation of indoly-3-methyl glucosinolates (Fenwick, Griffiths, & Heaney, 1983; Lewis & Fenwick, 1987). In this manner it is thought that ascorbigens have a role in cancer-modulation (Buskov et al., 2000) via quinone reductase induction (Zhu & Loft, 2003). As has been highlighted previously, this has important implications for breeding for plant varieties with enhanced chemopreventative effects.

2.7.6. Epithioalkanes

Epithioalkanes are formed as part of the myrosinase reaction with GSLs at low pH with epithiospecifier protein and ferrous ions. These GSLs typically have a side-chain with a double bond, such as sinigrin (Hrncirik, Valusek, & Velisek, 2001; Preobrazhenskaya, Bukhman, Korolev, & Efimov, 1993). It is uncertain whether these compounds produce any significant bioactive effect in humans, but the ratio in which they are produced alongside ITCs, nitriles and indoles may impact on these compounds' efficacy as anti-carcinogens.

2.7.7. Flavonols

The antioxidant and anti-inflammatory function of flavonols in the human diet are well known and include protecting the colonic epithelium from free radical damage (Harborne & Williams, 2000; Hollman & Katan, 1997, 1999; Olsson, Veit, Weissenbock, & Bornman, 1998). They can induce the up-regulation of enzymes

(such as cytochrome P450), that may lead to a decreased risk of cancer, cardiovascular disease, immune dysfunction, atherosclerosis and chronic inflammation (Kroon et al., 2004; Manach, Scalbert, Morand, Remesy, & Jimenez, 2004).

2.8. Factors affecting phytochemical content

2.8.1. Breeding and cultivation

Rocket has been consistently shown to be a good dietary source for flavonols, GSLs and anti-oxidants. However, there can be large differences between plants of the same germplasm accession due to a combination of genetic and environmental variability. This is probably due to the outbreeding nature of the species (Cartea et al. 2008) and a lack of overall uniformity in varieties. Commercial varieties cannot be considered truly domesticated because of this tendency for outcrossing, and the susceptibility of plants to inbreeding depression (a loss of genetic variability due to repeated self-pollination or crossing with a closely related individual). Development of advanced open-pollinating breeding lines (lines that are allowed to cross-pollinate freely in a population of selected individuals), or even F_1 hybrids (superior varieties produced by crossing distinctly different, elite inbred lines), could potentially minimise such variation.

Throughout the food chain there are many aspects that can have an adverse effect on GSL levels within leaves (Figure 2.4). These include the cultivar choice,

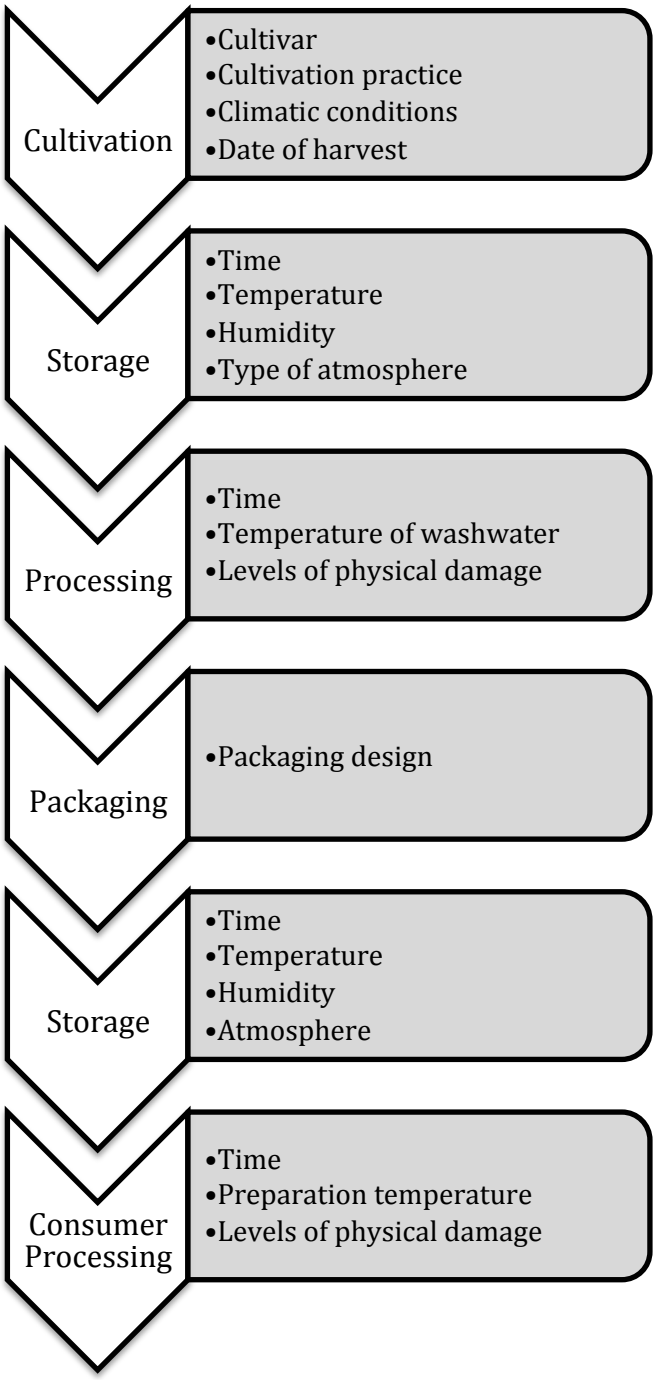


Figure 2.4. Factors and conditions within the commercial supply chain that affect GSL and flavonol levels within rocket leaves.

cultivation practice, climatic conditions, photoperiod, sulphur and nitrogen availability, harvest date, time spent in storage, the temperature of wash water, levels of physical damage to leaves, packaging atmosphere and food preparation methods (Aires, Rosa, & Carvalho, 2006; Bjorkman et al., 2011; Degl'Innoocenti et al. 2008; Engelen-Eigles, Holden, Cohen, & Gardner, 2006; Jeffery et al., 2003; Palaniswamy, McAvoy, & Bible, 1997; Palaniswamy, McAvoy, Bible, Singha, & Hill, 1995).

2.8.2. Harvesting

Rocket species have the ability to re-grow their leaves repeatedly after cutting, which allows for several harvests to take place under optimal conditions (Martinez-Sanchez et al. 2008). In parts of southern Italy, it is not unheard of for up to seven harvests to occur from a single planting. This has obvious cost-saving benefits for growers, but multiple harvests also induce stress responses in rocket that may be detrimental to the flavor and aesthetics of the crop. Stress drives up the production of secondary metabolites such as GSLs and anthocyanins, which will produce very strong, bitter tastes. There are other detrimental effects of multiple harvests; leaves become progressively smaller and more 'skeletal' in appearance with each cutting, for example. High anthocyanin levels also affect the color of leaves, turning them an undesirable pink, purple or red. Color has been found to be one of the most important characteristics consumers look for in rocket (A Koukounaras, Siomos, & Sfakiotakis, 2010), and so the loss of fresh appearance can ultimately lead to rejection of crops by supermarkets and processors.

2.8.3. Industrial and culinary processing

There are five main influences that have been identified in affecting GSL levels during processing (Verkerk et al., 2009). These are the action of myrosinase hydrolysis, myrosinase inactivation, the lysis and leaching of GSLs into wash-water, thermal degradation of GSLs, and the loss of ascorbic acid, iron and other enzyme co-factors. Myrosinase inactivation and thermal degradation of GSLs is probably less of an issue in rocket species, as the leaves are not typically cooked. The leaves are not ordinarily frozen, and so freeze-thaw hydrolysis is not likely to be a major factor either. Other factors almost certainly play a significant role in GSL and phytochemical

loss in rocket. Verkerk et al. (2009) highlighted four key areas that affect GSL levels before reaching the end consumer. These are:

1. The variety / cultivar used
2. Storage and packaging (post-harvest, post-processing & in shops/supermarkets)
3. Industrial processing
4. Consumer preparation methods

If each of these areas can be mitigated through breeding superior varieties, consumers will receive an end product that is of higher nutritive quality and thus provide increased health benefits.

2.8.4. Post harvest storage

Studies on both *Diplotaxis* and *Eruca* species have been conducted to determine the effects of post harvest storage conditions on chlorophyll content and respiration rates (Koukounaras et al. 2007). Both species of rocket have been found to have high respiration rates (Martinez-Sanchez et al. 2008) leading to rapidly impaired visual quality, such as stem browning, tissue yellowing and general decay (Koukounaras, Siomos, & Sfakiotakis, 2006). Provided initial GSL loss can be mitigated through breeding, ITC formation has been shown to increase over nitrile formation during the storage period (Howard, Jeffery, Wallig, & Klein, 1997).

Time, temperature, humidity and atmospheric conditions are all optimised for specific crops within the logistics chain, but these factors are often only designed to prevent visual degradation and not phytochemical breakdown (Schouten et al., 2009). Getting producers, packagers and transporters to change their current practices in order to better preserve the health-promoting compounds in rocket would be a difficult task. Treatments and storage conditions are often integrated parts of

protocols and procedures, and changing these would require significant testing on a commercial scale.

2.9. New selection tools for breeders

2.9.1. Phytochemical selection

It should not be forgotten that some GSLs and their breakdown products are thought to be toxic, and even carcinogenic, at high concentrations (Kim et al., 1997). Breeders and researchers should be mindful that more of a certain compound does not necessarily mean 'better' (Kassie et al., 1996). Humans seem to be able to tolerate GSLs much better than pigs, rats and rabbits for example; but overconsumption of these compounds may have serious health consequences (Tripathi & Mishra, 2007) as high dose-effect relationships are as yet unknown in humans (Verkerk et al., 2009). Few papers in GSL research (regardless of species) have acknowledged the potential for plant breeders to utilise HPLC/UPLC/LC-MS/GC-MS methods within breeding programs to 'monitor' and select plants for their phytochemical content in this manner. These techniques would provide valuable information on breeding lines relatively rapidly, especially for GSL and flavonol breeding (Rochfort, Trenerry, Imsic, Panozzo, & Jones, 2008). It is not common practice to select rocket plants based on their phytochemical profile at present, but as interest in these compounds increases it will be necessary for breeders to modify their selection criteria and information sources in order to remain competitive in the salad vegetable market (Verkerk et al., 2009). This has been achieved with '*Beneforte'* broccoli (Seminis Vegetable Seeds; subsidiary of Monsanto Company, St. Louis, Missouri, USA; www.beneforte.com) for example. It has also been indicated in hybrid varieties of *Brassica* that ITC/nitrile ratios can be selected for (Faulkner, Mithen, & Williamson, 1998).

2.9.2. Genetic resources and Marker Assisted Breeding

European initiatives (such as the EU GENRES project 'Leafy vegetables germplasm, stimulating use'; http://documents.plant.wur.nl/cgn/pgr/leafyveg/) have included rocket species within their remit, indicating the rising prominence of the species, and the desire for more work to be conducted on them. The germplasm accessions stored in gene banks are a valuable genetic resource for breeders to take advantage of (Xu, 2010). The accessions contained within these collections are highly variable and have unique visual and sensory characteristics that could be introgressed into breeding lines relatively easily (Bozokalfa, Yagmur, Ilbi, Esiyok, & Kavak, 2009).

Genetic information about rocket within the published literature is very scarce. Some molecular marker techniques such as Random Amplification of Polymorphic DNA (RAPD), Inter-Simple Sequence Repeats (ISSR) and Amplified Fragment Length Polymorphisms (AFLP) have been used to analyse morphological characteristics of *Eruca vesicaria* (Egea-Gilabert et al. 2009). ISSR and AFLP are relatively robust for screening variable populations and discriminating between cultivars (Xu, 2010) but RAPDs are notoriously unreliable and suffer from a lack of reproducibility and resolution. Perhaps one of the most underutilised marker types is SRAP (Sequence Related Amplified Polymorphism). The forward and reverse primers are designed to target arbitrary GC and AT rich sequences of the genome respectively, and are therefore more likely to anneal to active genomic regions (Li & Quiros, 2001). This could be of use in understudied crops such as rocket, as it provides a simple, repeatable and reliable way of screening large populations.

These techniques are now for the most part however, obsolete in advanced molecular plant breeding, as NGS (Next Generation Sequencing) and SNP (Single

Nucleotide Polymorphism)/QTL (Quantitative Trait Loci) analyses are far more specific, reliable and cost-effective. SNPs are the most abundant marker type within genomes, and their high density is ideal for studying specific regions in detail (Baird et al., 2008). NGS techniques are now relatively affordable, even for relatively small companies. They are widely available in academic institutions, but many companies are bypassing these in favor of dedicated private commercial services (Glenn, 2011) or are developing their own in-house facilities. The inability of some research institutions to provide adequate customer service, cost-effectiveness, data storage, and results on time is jeopardising how much knowledge is in the public domain. Increasingly, both large and small breeding companies are collaborating privately and advancing techniques far beyond those found in academic institutions. Future work by institutes in advanced genomics, sequencing and genotyping is likely to be obsolete in some cases because private research is already finding new innovations, e.g. for data storage and bioinformatics. Because private companies have no obligation to share their knowledge, many of these advances may be unobserved by the mainstream scientific community. Institutes and Universities need to do more to attract business from industry in order to keep up with the pace of private advances in this area.

Transcriptome sequences are now (generally) adequate for breeders to use and make huge advances in only a few years. Linkage mapping and QTL analyses can be conducted on desktop computers, making integration into breeding companies relatively straightforward from an IT point of view, even if the actual sequencing and genotyping are outsourced. Again, this may typically be to private companies providing a dedicated service. The availability of software licenses and advanced training courses from private companies also means plant breeders do not

necessarily need the expertise found in Universities and research institutes in order to attain their goals.

2.10. Summary

Of all the research papers concerning rocket species and their phytochemistry, none have directly addressed how information could be used within a working breeding population. Often it is explained or postulated purely as theory rather than actual practice, or only given a cursory mention. Only very rarely is a plant breeding program reflective of theory, due to the large number of environmental factors affecting plant growth, development and reproduction. The progressive selection of rocket plants through conventional/molecular breeding would be a valuable tool for the research community as well as providing an excellent incentive for breeding companies to fund research. The actual monitoring and quantification of GSL/flavonol levels through successive generations (i.e. not just one as has been the case with most studies) would not only validate the heritability of such traits in rocket, but would also provide a 'roadmap' for how other minor crops might be developed for commercial use.

Attention must be paid to the phytochemical content of varieties within breeding populations of rocket. By focusing solely on morphological traits, important phytochemical genotypes may be inadvertently lost from populations; this could be said of all *Brassicaceae* species, not just rocket. The balance of glucosinolate-myrosinase degradation products does seem to have a genetic component to it and so could be selected for also. Utilising genetic resources, the falling costs of sequencing and bioinformatics can produce nutritively superior varieties of rocket in the near future. Plant breeding typically takes longer than the average research project allows for, even with the use of advanced genomic selection methods. This is

a situation that could be remedied by long-term industrial collaboration and sponsorship by plant breeding firms.

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CHAPTER 3: Identification and quantification of glucosinolate and flavonol compounds in rocket salad (*Eruca sativa, Eruca vesicaria* and *Diplotaxis tenuifolia*) by LC-MS: highlighting the potential for improving nutritional value of rocket crops

3.1. Introduction To Paper (as published in *Food Chemistry,* 2015*,* Vol. 172)

With the aim of addressing some of the issues highlighted in the previous chapter, a comprehensive screening of GSLs and flavonols was conducted using LC-MS/MS. By examining the profiles and concentrations found in both germplasm and commercial accessions of rocket salad, the range of compound concentrations would be elucidated in greater detail, and enable a measure of how different the two are in this respect. The ion data gathered in the review paper (Chapter 2) was utilised to give a rigorous analysis of the compounds that may, or may not, be present within leaves.

An intention of this research paper was to develop a relatively simple extraction and analysis method that could be easily replicated. A relatively complex and laborious GSL desulphation method was attempted initially to isolate GSLs from other phytochemicals. For our purposes, this method proved too time consuming, costly, and did not produce viable results. A crude methanol extraction method was therefore adopted, which solved each of these problems. It also allowed both GSLs and flavonols to be extracted simultaneously, and reduced running costs, as a DAD was used to look for both types of compound at two wavelengths. This method of extraction and analysis has been very successful, and is now utilised by several groups within the Food & Nutritional Sciences department at the University of Reading.

A comment that has been made subsequent to the publication of this paper relates to the quantification of glucosativin and DMB separately, as mentioned in Chapter 1. Many people are familiar with the paper published by Bennett et al. (2002) identifying glucosativin as the predominant GSL of rocket. In that study a dimer was observed (DMB) and was deemed to be a product of the extraction method, rather than an independent plant metabolite. Several papers have based their quantification of glucosativin on this basis, despite the fact that it has been experimentally shown to be false. An elegant experiment conducted by Cataldi, Rubino, Lelario and Bufo (2007) demonstrated that DMB is in fact highly likely to be naturally occurring. Unfortunately, some researchers are not as aware of this experiment as the Bennett et al. (2002) paper, and so the disagreement with our method and results sometimes persists.

The paper in this chapter also challenges the experimental design choices of previous studies. As will be demonstrated, the types of conditions under which plants are grown and the time at which leaves are harvested varies markedly between experiments. This was done, not to be confrontational or disparaging to others' work, but to rather provoke an awareness of how such choices could impact results. This in turn may influence how the results are interpreted, and could therefore lead to false conclusions when relating data to industrial produce, for example.

A final objective of this paper was to identify germplasm accessions with substantially different flavonol and GSL profiles. The number of accessions utilised were too numerous to continue with into future experiments (such as those presented in Chapters 4-7), and a means with which to 'narrow down' the study samples was determined. The accessions chosen and the reasoning behind the choices will be explained in Chapter 4.

3.2. Introduction

The groups of crops collectively known as rocket (or arugula, rucola, roquette) are all members of the *Brassicaceae* family, and are native to the areas surrounding the Mediterranean Sea (Martinez-Sanchez, Marin, Llorach, Ferreres, & Gil, 2006). Rocket crops belong to two genera, *Eruca* and *Diplotaxis*, and are increasingly important in the salad vegetable market (Pasini, Verardo, Cerretani, Caboni, & D'Antuono, 2011). The species are now grown commercially all over the world in countries as diverse as the USA, UK, Italy, Spain, Morocco, Israel, India and Australia (Bozokalfa, Esiyok, & Yagmur, 2011).

Previous studies have highlighted rocket as a rich source of glucosinolate (GSL) compounds (Kim, Jin, & Ishii, 2004). Virtually all other members of the *Brassicaceae* contain GSLs as secondary metabolites that act as part of plant defense mechanisms (Schranz, Manzaneda, Windsor, Clauss, & Mitchell-Olds, 2009). GSLs and their hydrolysis products have also been implicated in giving rocket its characteristic pungent aromas and flavours (Bennett et al., 2002) and volatiles (such as isothiocyanates (ITCs) and indoles) have been consistently linked with anticarcinogenic activity in mammalian tissues (Lynn, Collins, Fuller, Hillman, & Ratcliffe, 2006).

Both *Eruca* and *Diplotaxis* species contain similar profiles of GSLs within the leaf tissue, the most prominent of which are glucosativin (4-mercaptobutyl-GSL), glucoerucin (4-(methylthio)butyl-GSL) and glucoraphanin (4-(methylsulfinyl)butyl-GSL). Glucosativin and glucoerucin breakdown products are thought to contribute most to pungency and flavour in rocket (Pasini, Verardo, Caboni, & D'Antuono, 2012). Numerous other GSLs have also been identified within rocket tissue, for example diglucothiobeinin (4-(β-D-glucopyranosyldisulfanyl)butyl-GSL; Kim et al.,

2007), 4-hydroxyglucobrassicin (4-hydroxy-3-indolymethyl-GSL; Cataldi et al. 2007) and 4-methoxyglucobrassicin (4-methoxy-3-indolymethyl-GSL; Kim & Ishii, 2006).

Rocket species also contain large concentrations of polyglycosylated flavonol compounds, which are known to infer numerous beneficial health effects in humans and other animals. Particularly of note are their effects on the gastrointestinal tract and in cardiovascular health (Bjorkman et al., 2011; Traka & Mithen, 2011). Several studies in rocket have identified and quantified polyglycosylated flavonols, which belong to three core aglycones: isorhamnetin, kaempferol and quercetin (Bennett, Rosa, Mellon, & Kroon, 2006).

Prolonged intake of *Brassicaceae* vegetables and leaves has a demonstrably beneficial impact on human health (D'Antuono, Elementi, & Neri, 2009); however much of the world's population do not consume enough of them to have these benefits, as is highlighted in several studies (Casagrande, Wang, Anderson, & Gary, 2007). Therefore, instead of only promoting increased consumption of leafy vegetables such as rocket, we propose increasing the nutritional quality and phytochemical density of varieties by using advanced screening and plant breeding methods, whilst still maintaining the sensory and visual acceptance of the consumer. This has already been achieved in broccoli with the production of varieties such as *Beneforté* which accumulates high concentrations of glucoraphanin (Traka et al., 2013).

In this study we draw a comparison between commercial rocket varieties available for public consumption and underutilised genetic resources. Nineteen gene bank accessions of *Eruca sativa* and sixteen commercial varieties (comprising *Eruca sativa*, *Eruca vesicaria* and *Diplotaxis tenuifolia*) were evaluated for GSL and polyglycosylated flavonol composition under controlled environment conditions. We hypothesise that through selective breeding for morphological traits in rocket, many

important health promoting phytochemical traits may have been lost in commercial varieties, and that by breeding from underutilised accessions, nutritionally superior varieties can be produced. We also hypothesise that controlled environment growing conditions minimizes the effects of environmental stress on rocket plants, and provides a platform for comparable results between research groups and repeat experiments. We also call on other groups to consider plant maturity and time of harvest as an important factor in determining the usefulness of data to breeders and growers.

3.3. Materials and methods

3.3.1. Plant material

Rocket accessions were selected from three European gene banks based upon information provided by Elsoms Seeds Ltd. (Spalding, Lincolnshire, UK). In total 19 were sourced; 2 from the Centre for Genetic Resources in the Netherlands (CGN, Wageningen, The Netherlands), 12 from the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK, Gatersleben, Germany), and 5 from the University of Warwick Crop Centre Genetic Resources Unit (Wellesbourne, UK; formerly Warwick HRI). A further 16 commercial varieties were collected: 13 were independently sourced from retailers, 1 provided by Elsoms Seeds Ltd., and 2 from Bakkavor Group Ltd. (Bourne, Lincolnshire, UK).

Three biological replicates of each accession/variety were germinated under controlled environmental conditions (in Saxcil growth cabinets) after being sown in a random sequence. Long-day lighting was used (16h light, 8h dark) at an intensity of 200 μmol m^{-2} s^{-1} (equivalent to 10,800 Lux of sunlight). Daytime temperatures were set at 20°C and nighttime temperatures at 14°C. Seedlings were grown for ten days in seedling trays and then transplanted to larger trays; four plants of each replicate

were grown on. Plants were grown for another twenty days and then leaves from the four plants were harvested together. Sampling for each plant took approximately one minute from the cutting of the leaves at the petiole, to being placed in zip-loc freezer bags on dry ice inside a polystyrene container (with lid). For health and safety reasons it was decided that liquid nitrogen would not be used in this process.

Thirty days was chosen as the optimum point of harvest as it reflects the typical number of days commercial growers grow their crop after sowing. Bags were placed in a -80°C freezer immediately after harvest and transport was completed (<30 minutes). Samples were freeze-dried in batches for three days (in a Vertis Bench-top Series). Leaves from each rep were ground into a fine powder using a combination of pestle and mortar and miniature coffee grinder (De'Longhi KG49, Treviso, Italy).

3.3.2. Reagents and chemicals

All solvents and chemicals used were of LC-MS grade and obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated.

3.3.3. Glucosinolate/flavonol extraction

The following method was adapted from Pasini et al. (2012) and Jin et al. (2009). Three experimental replicates of each biological rep were prepared as follows: 40mg of ground rocket powder was heated in a dry-block at 75°C for 2 minutes, as suggested by Pasini et al. (2012), as a precautionary measure to inactivate as much myrosinase enzyme as possible before liquid extraction. 1ml of preheated 70% (v/v) methanol (70°C) was then added to each sample and placed in a water bath for 20 minutes at 70°C. Samples were then centrifuged for 5 minutes (6,000 rpm, 18°C) to collect loose material into a pellet. The supernatant was then

taken and put into fresh Eppendorf tubes. The volume was adjusted to 1ml with 70% (v/v) methanol and frozen at -80°C until analysis by LC-MS.

3.3.4. LC-MS^2 Analysis

Immediately before LC-MS analysis each sample was filtered using 0.25 μm filter discs with a low protein binding Durapore polvinylidene fluoride (PVDF) membrane (Millex; EMD Millipore, Billerica, MA, USA) and diluted with 9 ml of HPLC-grade water. Samples were run in a random order with QC samples (Dunn, Wilson, Nicholls, & Broadhurst, 2012). An external reference standard of sinigrin hydrate was also prepared for quantification of GSL compounds, and isorhamnetin for flavonol compounds. Preparation was as follows: A 12 mM solution was prepared in 70% methanol. A dilution series of concentrations was prepared as an external calibration curve with HPLC-grade water (200ng μl, 150ng μl, 100ng μl, 56ng μl, 42ng μl, 28ng μl, 14ng μl and 5.6ng μl; sinigrin correlation coefficient: $y = 12.496x - 15.012$; $r^2 = 0.993$, isorhamnetin correlation coefficient: $y = 0.3205x - 5.3833$, $r^2 = 0.921$). Standard response factors were used in the calculation of GSL concentration where available (Wathelet, Iori, Leoni, Quinsac, & Palmieri, 2004). Where such data could not be found for intact GSLs, response factors were assumed to be 1.00 (Lewis & Fenwick, 1987).

LC-MS analysis was performed in the negative ion mode on an Agilent 1200 Series LC system equipped with a binary pump, degasser, autosampler, thermostat, column heater, photodiode array detector and Agilent 1100 Series LC/MSD mass trap spectrometer. Separation of samples was achieved on a Zorbax SB C18 column (2.1 x 100mm; 1.8μm; Agilent, Santa Clara, CA, USA) with precolumn filter. Both GSLs and flavonols were separated in the same sample during a 40-minute chromatographic run. Mobile phases consisted of ammonium formate (0.1%) and

acetonitrile with an isocratic gradient of 95% and 5% respectively at a flow rate of 0.3 ml/min, with a column temperature of 30°C. 5 μl of sample was injected.

MS analysis settings were as follows: ESI was carried out at atmospheric pressure in negative ion mode (scan range *m/z* 50 to 1050 Da). Nebulizer pressure was set at 50psi, gas-drying temperature at 350°C, and capillary voltage at 2,000V. Compounds were identified using their nominal mass and characteristic fragment ions, and by comparing data with those published in the literature (see Tables 3.1 & 3.2). GSLs were quantified at a wavelength of 229 nm, and flavonols at 330 nm. All data were analysed using Bruker Daltonics software.

3.3.5. Statistical analysis

The results reported are the averages of three biological replicates and three separately extracted technical replicates (*n* = 9). Processed GSL and flavonol data were analysed with ANOVA and Tukey's HSD test, and principal component analysis (PCA) was performed in XL Stat (Addinsoft, New York City, New York, USA).

3.4. Results and discussions

3.4.1. Glucosinolate identification and concentration

Table 3.1 lists all of the GSL compounds identified across all rocket samples, including systematic names, common names and the identifying ions. Unlike previous studies, the GSL profiles of each rocket accession were markedly different in some cases. See Table 3.3 for a comparison of results with similar, previous studies. Total average GSL concentration ranged from 3.1 $mg.g^{-1}$ DW ('*Buzz*') to 11.6 $mg.g^{-1}$ DW (SR10). Both of these accessions are *Eruca sativa*, indicating the large degree of variability between accessions of this species, both commercial and

Table 3.1. Identification of intact glucosinolates of *Eruca* and *Diplotaxis* varieties and accessions

Common name	R-group	$[M-H]^-$ *m/z*	MS^2	References
4-hydroxyglucobrassicin	4-hydroxy-3-indolylmethyl	463	381	Pasini et al., 2012; Rochfort et al., 2008
Glucotropaeolin	Benzyl	408	328, 259, 195	D'Antuono et al., 2008; Rochfort et al., 2008
Glucolepiidin	Ethyl	346	266	D'Antuono et al., 2008
Glucoraphanin	4-(methylsulfinyl)-butyl	436	371, 194	Bennett et al., 2002; Botting et al., 2002; Pasini et al., 2012; Rochfort et al., 2008
Glucoiberverin	3-(methylthio)-propyl	406	325, 274, 258, 227	Fahey et al., 2001; Rochfort et al., 2008
Glucosativin	4-mercaptobutyl	406	258, 209, 194, 138	Bennett et al., 2002; Lelario et al., 2012; Pasini et al., 2012
DMB	Dimeric-4-mercaptobutyl	811	731, 405, 258, 207	
Glucoalyssin	5-(methylsulfinyl)-pentyl	450	371	Lelario et al., 2012; Pasini et al., 2012
Glucoerucin	4-(methylthio)-butyl	420	339, 274, 258, 241, 194	Pasini et al., 2012; Rochfort et al., 2008
Glucoraphenin	4-methylsulfinyl-3-butenyl	434	354	D'Antuono et al., 2008
Diglucothiobeinin	4-(β-D-glucopyranosyldisulfanyl)-butyl	600	-	Lelario et al., 2012; Pasini et al., 2012
Glucoibarin	7-(methylsulfinyl)-heptyl	494	415	D'Antuono et al., 2008

Table 3.2. Identification of flavonol of *Eruca* and *Diplotaxis* varieties and accessions

Common name	$[M-H]^-$ *m/z*	MS^2	References
Myricetin	317	151	Villatoro-Pulido et al., 2013
Kaempferol-3-glucoside (Astragalin)	447	285	
Quercetin-3-glucoside (Isoquercetrin)	463	301	
Isorhamnetin-3-glucoside	477	357, 314, 285, 151	Martinez-Sanchez et al., 2008; Pasini et al., 2012
Kaempferol-3,4'-diglucoside	609	447, 285	
Isorhamnetin-3,4'-diglucoside	639	477	
Kaempferol-3-diglucoside-7-glucoside	771	609	Pasini et al., 2012
Quercetin-3,3,4'-triglucoside	787	625, 463, 301	Martinez-Sanchez et al., 2008; 2007; Pasini et al., 2012
Kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside	817	729, 685, 653, 447, 285	Martinez-Sanchez et al., 2008; Pasini et al., 2012
Quercetin-3,4'-diglucoside-3'-(6-caffeoyl-glucoside)	949	787, 625, 463, 301	Pasini et al., 2012
Quercetin-3,4'-diglucoside-3'-(6-sinapoyl-glucoside)	993	831, 669, 463, 301	Martinez-Sanchez et al., 2008; 2007; Pasini et al., 2012

Table 3.3. Concentration ranges reported in $mg.g^{-1}$ DW (conversion of $\mu mol.g^{-1}$ DW of sinigrin hydrate) and days growth after sowing when plants were harvested

Glucosinolate	Bennett et al., 2007 *ce*	Chun et al., 2013 *h*	Jin et al., 2009 *ce*	Kim & Ishii, 2006 *h*	Pasini et al., 2012 *f*	Villatoro-Pulido et al., 2013 *f*	Bell et al., 2014 *ce*
	~7 days	**69 days**	**56 days**	**49 days**	**?**	**>56 days**	**30 days**
Glucoerucin	0.0 – 12.7	0.3 – 2.2	0.0 – ~1.5	1.3	0.2 – 0.5	0.1 – 1.8	0.0 – 1.6
Glucoraphanin	0.2 – 2.7	0.4 – 1.7	0.0 – ~1.0	0.5	0.2 – 1.3	1.6 – 6.5	0.0 – 0.9
Glucoiberverin	ND	ND	0.0 – ~2.0	ND	ND	0.1 – 0.3	0.0 – 0.1
Glucosativin	0.2 – 14.5	ND	2.0 – ~7.0	ND	ND	3.2 – 4.6	0.2 – 9.1
4-hydroxyglucobrassicin	ND	ND	ND	ND	<0.1 – 0.1	0.1	0.0 – 0.1
Diglucothiobeinin	ND	ND	0.0 – ~0.5	0.3	0.1	ND	0.0 – 0.2
DMB	ND	1.5 – 7.7	ND	2.3	0.2 – 0.7	ND	0.0 – 7.1
Glucoalyssin	ND	ND	ND	ND	<0.1 – 0.1	ND	0.0 – 0.1

ND = not detected

ce = controlled environment

f = field environment

h = hydroponic environment

germplasm. The lowest average accumulation for *Diplotaxis* was '*Wild Tirizia'* with 4.4 $mg.g^{-1}$ DW and the highest was 10.4 $mg.g^{-1}$ DW, ('*Wild Grazia'*).

For glucosativin both the monomeric and the dimeric forms were identified and quantified separately and concentrations of both forms varied significantly between accessions. On average 91.3% of the total GSL concentration was made up of glucosativin/DMB. This is much higher than the proportions presented in previous studies where values of around 60% have been generally given (Pasini et al. 2012).

Other GSL compounds such as glucoraphanin and glucoerucin were not detected in all accessions. Again, previous studies have highlighted the prevalence of these compounds, but we found them to be relatively minor. Concentrations ranged from nil to 0.9 $mg.g^{-1}$ DW ('*Wild Grazia'*) for glucoraphanin and nil to 1.6 $mg.g^{-1}$ DW (SR16) for glucoerucin. Several other GSLs were quantified, and in some cases these were as high as the more generally accepted 'major' GSLs of rocket in concentration. The other compounds were: 4-hydroxyglucobrassicin, glucotropaeolin, glucolepiidin, glucoiberverin, glucoalyssin, glucoraphenin, diglucothiobeinin and glucoibarin. None of these GSLs discriminated between species.

In general, the total concentrations detected were similar to those found in other studies. In some of these, plants were grown in field conditions and therefore subject to many different environmental stresses and inconsistencies. It is widely known that both GSLs and flavonols increase in concentration as plants become stressed (Rohr, Ulrichs, Mucha-Pelzer, & Mewis, 2006). With this in mind it is somewhat unusual that the concentrations reported here were not lower, as stress was minimal in comparison to field conditions. Studies conducted in outdoor conditions are not directly comparable for this reason. Field conditions and climate vary greatly between growing regions and GSL proportions may change due to these variables. Our study represents GSL and flavonol accumulation in rocket varieties

and species under conditions that can be easily replicated using controlled environment apparatus. This allows the basic genetic differences in GSL profile to be observed, rather than the differences between how accessions respond to their normal, field-based growing environment. A trial of five gene bank accessions used in this study have been grown under field conditions and will be analysed using identical LC/MS methods to determine the effects the outdoor environment has on GSL and flavonol profiles.

Table 3.3 summarizes the range of concentrations of some GSLs previously reported in comparison with our own data. The types of growing method employed vary, as do the number of days growth before harvest. This makes comparing and contrasting between studies difficult and could potentially lead to erroneous conclusions. The details of these varying factors are discussed in the concluding section.

3.4.2. Flavonol identification and concentration

Table 3.2 lists all identified flavonol compounds detected across all samples, including systematic names and identifying ions. In total eleven flavonol compounds were positively identified.

Myricetin was detected in relatively few accessions, but predominantly in *Eruca*. Previously this flavonol has not been identified in *Diplotaxis* species (to the authors' knowledge), however, in this study it was detected in the commercial variety '*Wild Grazia'*.

Kaempferol glucosides kaempferol-3-glucoside (Astragalin) and kaempferol-3-diglucoside-7-glucoside have only been previously reported in *Eruca* species, but were additionally detected in two *Diplotaxis* varieties in our study ('*Wild Grazia'* and WR2). The ion fragments present in Table 3.2 confirmed their presence in these two

commercial varieties. Kaempferol-3,4'-diglucoside was detected in both genera as reported by Pasini et al. (2012) and Martinez-Sanchez, Llorach, Gil, & Ferreres (2007). The only kaempferol glucoside that was exclusive to *Eruca* species was kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside.

A similar situation was observed for quercetin glucosides. Quercetin-3-glucoside (Isoquercetrin) has only been previously reported in *Eruca* species, however it was also detected in one commercial accession of *Diplotaxis* ('*Wild Grazia'*). The converse was also found with quercetin-3,3,4'-triglucoside, quercetin-3,4'diglucoside-3'-(6-caffeoyl-glucoside) and quercetin-3,4'diglucoside-3'-(6-sinapoyl-glucoside), which have only previously been reported in *Diplotaxis*. These were detected in several *Eruca* accessions, as well as in *Diplotaxis*. Quercetin-3,3,4'-triglucoside showed the correct *m/z* 787 mass and secondary ions, and quercetin-3,4'diglucoside-3'-(6-caffeoyl-glucoside) was determined by the presence of a characteristic 625 fragment. Quercetin-3,4'-diglucoside-3'-(6-sinapoyl-glucoside) was determined by primary *m/z* 993 ion and corresponding secondary fragment ions (Table 3.2).

Two isorhamnetin glucosides were detected in our analysis; isorhamnetin-3-glucoside and isorhamnetin-3,4'-diglucoside. The latter compound was detected in both *Eruca* and *Diplotaxis* accessions, as has been reported in other studies (Martinez-Sanchez, Gil-Izquierdo, Gil, & Ferreres, 2008). Isorhamnetin-3-glucoside has only been previously reported in *Eruca*, but was also detected in seven *Diplotaxis* accessions.

The concentration of each identified flavonol glucoside is presented in Table 3.5. As a general, overall observation, it can be said that *Diplotaxis* accessions have greater concentrations of quercetin flavonol compounds than *Eruca*, and the converse could be said for kaempferol. However using this as a broad, sweeping

Table 3.4

Total GSL concentration and relative amounts of each compound (± standard error) in rocket accessions. Differing letters in the same column indicate a significant difference (P = ≤0.05). Italics denote commercial varieties. Results are expressed as mg.g^{-1} DW of sinigrin hydrate.

Accession name	Source	Species	4-hydroxyglucobrassicin	Glucotrapaeolin	Glucolepidin	Glucoraphanin	Glucoiberverin	Glucosativin	DMB	Glucoalyssin	Glucoerucin	Glucoraphenin	Diglucothiobeinin	Glucoibarin	Average Total GSL (mg.g^{-1} DW)
Apollo	Fothergills	Es	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.5±0.2^{a}	2.9±0.7^{a-e}	<0.1±<0.1^{a}	ND^{a}	0.1±0.1^{a}	ND^{a}	ND^{a}	3.6±0.7^{a}
Buzz	Fothergills	Es	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	1.3±0.4^{ab}	1.7±0.3^{a-c}	ND^{a}	0.2±0.2^{a}	ND^{a}	ND^{a}	ND^{a}	3.1±0.6^{a}
SR1	CGN	Es	ND^{a}	ND^{a}	ND^{a}	0.5±0.1^{a}	ND^{a}	6.0±0.5^{d-g}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	<0.1±<0.1^{a}	ND^{a}	6.5±0.5^{ab}
SR2	CGN	Es	ND^{a}	ND^{a}	ND^{a}	0.4±0.1^{a}	<0.1±<0.1^{a}	3.5±0.5^{a-e}	2.3±0.4^{a-d}	ND^{a}	0.3±0.1^{a}	<0.1±0.1^{a}	ND^{a}	0.1±0.1^{ab}	6.6±0.6^{ab}
SR3	Elsoms Seeds Ltd.	Es	ND^{a}	0.1±0.1^{a-c}	ND^{a}	0.3±0.1^{a}	<0.1±<0.1^{a}	2.7±0.2^{a-e}	1.2±0.3^{ab}	ND^{a}	0.6±0.2^{ab}	ND^{a}	ND^{a}	ND^{a}	4.9±0.6^{a}
SR4	IPK	Es	ND^{a}	ND^{a}	ND^{a}	0.3±0.1^{a}	ND^{a}	6.4±1.3^{e-g}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	<0.1±<0.1^{a}	ND^{a}	6.7±1.4^{a-c}
SR5	IPK	Es	0.1±0.1^{a}	ND^{a}	ND^{a}	0.2±0.1^{a}	ND^{a}	7.7±0.8^{fg}	3.3±0.3^{b-f}	<0.1±<0.1^{a}	0.1±0.1^{a}	ND^{a}	ND^{a}	<0.1±<0.1^{ab}	11.5±0.9^{c}
SR6	IPK	Es	ND^{a}	ND^{a}	ND^{a}	0.6±0.4^{a}	0.1±<0.1^{a}	3.5±0.2^{a-e}	4.4±0.4^{d-g}	<0.1±<0.1^{a}	1.3±0.3^{ab}	0.2±0.2^{a}	ND^{a}	ND^{a}	10.0±1.1^{bc}
SR7	IPK	Es	ND^{a}	ND^{a}	ND^{a}	0.3±0.2^{a}	ND^{a}	4.8±0.6^{b-f}	2.7±0.6^{a-e}	ND^{a}	0.1±0.1^{a}	ND^{a}	ND^{a}	ND^{a}	7.9±1.0^{a-c}
SR8	IPK	Es	ND^{a}	ND^{a}	ND^{a}	0.1±0.1^{a}	ND^{a}	3.3±1.1^{a-e}	1.7±0.4^{a-c}	ND^{a}	0.2±0.2^{a}	ND^{a}	ND^{a}	ND^{a}	5.3±1.8^{ab}
SR9	IPK	Es	ND^{a}	ND^{a}	ND^{a}	0.6±0.3^{a}	ND^{a}	5.8±0.7^{d-g}	2.5±1.0^{a-e}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	8.9±1.0^{a-c}
SR10	IPK	Es	ND^{a}	ND^{a}	ND^{a}	0.4±0.3^{a}	ND^{a}	9.1±1.8^{g}	1.4±0.6^{a-c}	ND^{a}	0.7±0.4^{ab}	ND^{a}	ND^{a}	ND^{a}	11.6±2.1^{c}
SR11	IPK	Es	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	5.4±0.6^{c-g}	0.1±0.1^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	5.6±0.7^{ab}
SR12	IPK	Es	ND^{a}	ND^{a}	ND^{a}	0.3±0.2^{a}	0.1±<0.1^{a}	5.7±0.7^{d-g}	1.9±0.4^{a-c}	ND^{a}	0.5±0.3^{a}	ND^{a}	ND^{a}	ND^{a}	8.4±0.8^{a-c}
SR13	IPK	Es	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	5.1±0.5^{b-f}	3.1±0.6^{a-f}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	8.2±0.6^{a-c}
SR14	IPK	Es	ND^{a}	<0.1±0.1^{ab}	ND^{a}	ND^{a}	<0.1±<0.1^{a}	5.1±0.4^{c-f}	2.2±0.5^{a-d}	<0.1±<0.1^{a}	0.1±0.1^{a}	ND^{a}	ND^{a}	ND^{a}	7.5±0.7^{a-c}
SR15	IPK	Es	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	3.2±1.1^{a-e}	2.5±0.5^{a-e}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	5.7±1.5^{ab}
SR16	HRIGRU	Es	ND^{a}	ND^{a}	ND^{a}	0.4±0.1^{a}	ND^{a}	6.0±0.9^{d-g}	0.7±0.3^{ab}	ND^{a}	1.6±0.7^{b}	ND^{a}	ND^{a}	ND^{a}	8.7±1.2^{a-c}
SR17	HRIGRU	Es	ND^{a}	ND^{a}	ND^{a}	0.8±0.1^{a}	ND^{a}	3.6±0.5^{a-e}	2.4±0.6^{a-e}	ND^{a}	0.5±0.1^{ab}	ND^{a}	ND^{a}	ND^{a}	7.3±0.9^{a-c}

SR18	HRIGRU	Es	ND^{a}	ND^{a}	ND^{a}	0.4±0.2^{a}	ND^{a}	5.8±1.1^{d-g}	ND^{a}	ND^{a}	0.2±0.2^{a}	ND^{a}	ND^{a}	ND^{a}	6.4±1.3^{ab}
SR19	HRIGRU	Es	0.1±0.1^{a}	ND^{a}	ND^{a}	0.3±0.1^{a}	ND^{a}	3.4±0.5^{a-e}	2.2±0.6^{a-d}	0.1±0.1^{a}	ND^{a}	ND^{a}	0.2±0.1^{b}	ND^{a}	6.3±0.8^{ab}
SR20	HRIGRU	Es	ND^{a}	ND^{a}	ND^{a}	0.2±0.1^{a}	ND^{a}	4.3±1.4^{a-f}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	4.5±1.4^{a}
Pegasus	Tozer Seed	Es	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.7±0.5^{a}	3.1±0.9^{a-f}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.1±0.1^{ab}	3.9±1.3^{a}
Runway	Fothergills	Es	ND^{a}	ND^{a}	0.1±0.1^{a}	ND^{a}	ND^{a}	0.2±0.2^{a}	3.3±0.5^{b-f}	0.1±0.1^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	3.6±0.5^{a}
Sky	Tozer Seed	Es	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	2.0±0.5^{a-d}	3.7±0.5^{b-g}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	5.7±0.9^{ab}
Sweet Oakleaf	Tozer Seed	Ev	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	1.0±0.4^{ab}	2.0±0.7^{a-d}	ND^{a}	ND^{a}	<0.1±<0.1^{a}	ND^{a}	0.1±0.1^{ab}	3.3±1.0^{a}
Unwins	Unwins	Ev	ND^{a}	ND^{a}	ND^{a}	0.1±0.1^{a}	ND^{a}	1.6±0.7^{a-c}	7.1±1.3^{g}	0.1±0.1^{a}	ND^{a}	0.1±0.1^{a}	ND^{a}	0.1±0.1^{ab}	9.0±1.9^{a-c}
Unwins Organic	Unwins	Ev	ND^{a}	ND^{a}	0.1±0.1^{b}	0.1±0.1^{a}	ND^{a}	0.9±0.2^{a}	4.3±0.5^{c-g}	<0.1±<0.1^{a}	0.4±0.2^{a}	ND^{a}	ND^{a}	0.1±0.1^{ab}	5.9±0.6^{ab}
Olivetta	Fothergills	Dt	ND^{a}	0.2±0.2^{bc}	ND^{a}	ND^{a}	ND^{a}	1.4±0.7^{a-c}	4.9±1.1^{d-g}	ND^{a}	ND^{a}	<0.1±<0.1^{a}	ND^{a}	ND^{a}	6.6±1.6^{ab}
WR1	Bakkavor	Dt	0.1±0.1^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	1.7±0.7^{a-c}	3.8±0.9^{b-g}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.3±0.1^{b}	5.9±1.5^{ab}
Voyager	Tozer Seed	Dt	ND^{a}	ND^{a}	<0.1±<0.1^{a}	<0.1±<0.1^{a}	0.1±0.1^{a}	4.8±0.7^{b-f}	3.2±0.5^{b-f}	ND^{a}	0.1±0.1^{a}	ND^{a}	ND^{a}	0.2±0.1^{b}	8.5±0.5^{a-c}
Wild Grazia	Fothergills	Dt	ND^{a}	0.1±0.1^{a-c}	ND^{a}	0.9±0.6^{a}	ND^{a}	3.6±0.7^{a-e}	5.6±1.0^{e-g}	ND^{a}	0.3±0.3^{a}	ND^{a}	ND^{a}	ND^{a}	10.4±2.1^{bc}
Wild Tirizia	Fothergills	Dt	ND^{a}	ND^{a}	ND^{a}	0.3±0.2^{a}	ND^{a}	1.4±0.5^{a-c}	2.6±0.4^{a-e}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.1±0.1^{ab}	4.4±0.8^{a}
Wildfire	Tozer	Dt	ND^{a}	0.3±0.2^{c}	ND^{a}	ND^{a}	ND^{a}	1.6±0.8^{a-c}	7.0±0.9^{fg}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.1±0.1^{ab}	9.0±1.6^{a-c}
WR2	Bakkavor	Dt	ND^{a}	ND^{a}	0.2±0.2^{b}	ND^{a}	ND^{a}	2.6±1.3^{a-e}	5.5±1.4^{d-g}	ND^{a}	0.8±0.7^{ab}	ND^{a}	ND^{a}	0.2±0.1^{b}	9.3±3.3^{a-c}

ND = not detected

1

Table 3.5 Total flavonol concentration and relative amounts of each compound (± standard error) in rocket accessions. Differing letters in the same column indicate a significant difference ($P = \leq 0.05$). Italics denote commercial varieties. Results expressed as $g.kg^{-1}$ DW of isorhamnetin.

Accession	Myricetin	Kaempferol-3-glucoside	Quercetin-3-glucoside	Isorhamnetin-3-glucoside	Kaempferol-3,4'-diglucoside	Isorhamnetin-3,4'-diglucoside	Kaempferol-3-diglucoside-7-glucoside	Quercetin-3,3,4'-triglucoside	Kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside	Quercetin-3,4'diglucoside-3'-(6-caffeoyl-glucoside)	Quercetin-3,4'diglucoside-3'-(6-sinapoyl-glucoside)	Average total flavonol ($g.kg^{-1}$ DW)
Apollo	$<0.1\pm<0.1^{ab}$	$0.4\pm0.1^{a-c}$	ND^{a}	$<0.1\pm<0.1^{ab}$	$0.6\pm0.1^{a-d}$	0.2 ± 0.1^{a}	0.4 ± 0.1^{ab}	ND^{a}	$0.4\pm0.1^{a-c}$	0.1 ± 0.1^{ab}	ND^{a}	$2.5\pm0.4^{a-c}$
Buzz	$<0.1\pm<0.1^{a-c}$	0.2 ± 0.1^{ab}	0.1 ± 0.1^{a}	0.2 ± 0.1^{ab}	$0.5\pm0.1^{a-d}$	$0.5\pm0.1^{a-c}$	0.3 ± 0.1^{ab}	0.1 ± 0.1^{a}	ND^{a}	0.5 ± 0.1^{ab}	ND^{a}	2.6 ± 0.3^{bc}
SR1	ND^{a}	$0.1\pm<0.1^{a}$	ND^{a}	$0.1\pm<0.1^{ab}$	$0.4\pm0.1^{a-c}$	0.2 ± 0.1^{ab}	ND^{a}	$<0.1\pm<0.1^{a}$	ND^{a}	ND^{a}	ND^{a}	0.9 ± 0.2^{ab}
SR2	$<0.1\pm<0.1^{ab}$	$0.1\pm<0.1^{a}$	$<0.1\pm<0.1^{a}$	$0.1\pm<0.1^{ab}$	$0.5\pm0.1^{a-c}$	$0.5\pm0.1^{a-c}$	0.2 ± 0.1^{ab}	0.2 ± 0.1^{ab}	0.2 ± 0.1^{ab}	0.4 ± 0.1^{ab}	ND^{a}	2.5 ± 0.5^{bc}
SR3	$<0.1\pm<0.1^{ab}$	$0.1\pm<0.1^{a}$	ND^{a}	$0.1\pm<0.1^{ab}$	$0.4\pm0.1^{a-c}$	0.2 ± 0.1^{a}	$<0.1\pm<0.1^{a}$	$0.1\pm<0.1^{a}$	0.1 ± 0.1^{ab}	0.2 ± 0.1^{ab}	ND^{a}	1.3 ± 0.2^{ab}
SR4	ND^{a}	$0.2\pm<0.1^{a}$	$<0.1\pm<0.1^{a}$	$0.2\pm<0.1^{ab}$	$0.5\pm0.2^{a-d}$	0.3 ± 0.2^{ab}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	1.2 ± 0.4^{ab}
SR5	ND^{a}	$0.3\pm<0.1^{ab}$	ND^{a}	0.2 ± 0.1^{ab}	$0.5\pm0.1^{a-c}$	0.4 ± 0.1^{ab}	$<0.1\pm<0.1^{a}$	$<0.1\pm<0.1^{a}$	0.1 ± 0.1^{ab}	0.3 ± 0.1^{ab}	0.1 ± 0.1^{ab}	$1.9\pm0.3^{a-c}$
SR6	ND^{a}	0.4 ± 0.1^{ab}	ND^{a}	0.2 ± 0.1^{ab}	1.1 ± 0.1^{e}	0.6 ± 0.1^{bc}	0.2 ± 0.1^{ab}	$<0.1\pm<0.1^{a}$	$0.1\pm<0.1^{a}$	0.4 ± 0.1^{ab}	ND^{a}	3.2 ± 0.4^{c}
SR7	ND^{a}	0.2 ± 0.1^{a}	ND^{a}	ND^{a}	$0.3\pm0.1^{a-c}$	$<0.1\pm<0.1^{a}$	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.5 ± 0.1^{a}
SR8	ND^{a}	0.8 ± 0.2^{c}	ND^{a}	ND^{a}	1.0 ± 0.1^{de}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	$1.8\pm0.3^{a-c}$
SR9	ND^{a}	0.4 ± 0.1^{ab}	$<0.1\pm0.1^{a}$	$<0.1\pm<0.1^{ab}$	1.0 ± 0.3^{de}	1.0 ± 0.4^{c}	ND^{a}	$0.1\pm<0.1^{a}$	$<0.1\pm<0.1^{a}$	ND^{a}	ND^{a}	$2.5\pm0.7^{a-c}$
SR10	ND^{a}	$0.2\pm<0.1^{a}$	$<0.1\pm<0.1^{a}$	0.2 ± 0.1^{ab}	$0.4\pm0.1^{a-c}$	0.2 ± 0.1^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	1.0 ± 0.2^{ab}
SR11	ND^{a}	$0.1\pm<0.1^{a}$	ND^{a}	ND^{a}	$0.4\pm<0.1^{a-c}$	0.2 ± 0.1^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.7 ± 0.1^{a}
SR12	ND^{a}	0.2 ± 0.1^{a}	ND^{a}	0.2 ± 0.1^{ab}	$0.6\pm0.1^{a-d}$	0.3 ± 0.1^{ab}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	$0.2\pm0.1^{a-c}$	0.3 ± 0.1^{ab}	ND^{a}	$2.3\pm0.5^{a-c}$
SR13	ND^{a}	$0.1\pm<0.1^{a}$	ND^{a}	$<0.1\pm<0.1^{ab}$	$0.6\pm<0.1^{a-d}$	0.2 ± 0.1^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.8 ± 0.1^{ab}
SR14	ND^{a}	0.3 ± 0.1^{ab}	ND^{a}	$0.1\pm<0.1^{ab}$	$0.6\pm0.1^{a-d}$	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	ND^{a}	0.1 ± 0.1^{ab}	0.3 ± 0.1^{ab}	ND^{a}	$1.7\pm0.3^{a-c}$

SR15	ND^{a}	0.2±<0.1^{a}	ND^{a}	ND^{a}	0.7±0.1^{b-e}	<0.1±0.1^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	1.0±0.1^{ab}
SR16	ND^{a}	0.1±<0.1^{a}	<0.1±<0.1^{a}	0.1±<0.1^{ab}	0.3±0.1^{a-c}	0.2±0.1^{ab}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.9±0.2^{ab}
SR17	ND^{a}	0.1±<0.1^{a}	ND^{a}	ND^{a}	0.4±0.1^{a-c}	0.2±0.1^{ab}	ND^{a}	ND^{a}	ND^{a}	<0.1±<0.1^{a}	ND^{a}	0.7±0.2^{a}
SR18	ND^{a}	0.2±0.1^{ab}	<0.1±<0.1^{a}	0.2±<0.1^{ab}	0.4±0.2^{a-c}	0.2±0.2^{ab}	ND^{a}	<0.1±<0.1^{a}	ND^{a}	ND^{a}	ND^{a}	1.1±0.4^{ab}
SR19	ND^{a}	0.2±0.1^{a}	ND^{a}	ND^{a}	0.3±0.1^{a-c}	0.4±0.1^{ab}	0.1±0.1^{a}	<0.1±<0.1^{a}	<0.1±<0.1^{a}	0.2±0.1^{ab}	ND^{a}	1.4±0.3^{ab}
SR20	ND^{a}	0.2±<0.1^{a}	0.1±0.1^{a}	0.1±<0.1^{ab}	0.4±0.1^{a-c}	0.3±0.1^{ab}	ND^{a}	<0.1±<0.1^{a}	ND^{a}	ND^{a}	ND^{a}	1.1±0.2^{ab}
Pegasus	ND^{a}	0.6±0.3^{bc}	ND^{a}	0.4±0.3^{a-c}	0.3±0.1^{a-c}	<0.1±0.1^{a}	0.5±0.3^{b}	ND^{a}	0.2±<0.1^{a-c}	0.2±0.1^{ab}	ND^{a}	2.7±1.0^{bc}
Runway	<0.1±<0.1^{ab}	0.3±0.1^{ab}	ND^{a}	0.4±0.1^{a-c}	0.6±0.2^{a-d}	0.6±0.1^{a-c}	0.2±0.1^{ab}	<0.1±<0.1^{a}	0.1±0.1^{ab}	0.6±0.2^{b}	0.3±0.1^{b-d}	3.1±1.0^{bc}
Sky	0.1±<0.1^{c}	0.2±<0.1^{ab}	ND^{a}	0.1±0.1^{ab}	0.6±0.1^{a-d}	0.4±0.1^{a-c}	0.2±0.1^{ab}	0.4±0.2^{b}	0.3±0.2^{a-c}	0.2±0.1^{ab}	ND^{a}	2.6±0.5^{bc}
Sweet Oakleaf	0.2±0.1	0.2±0.1	<0.1±<0.1^{a}	<0.1±<0.1^{ab}	0.4±<0.1^{a-c}	0.3±0.1^{ab}	0.1±0.1^{a}	0.2±0.1^{ab}	ND^{a}	0.4±0.1^{ab}	ND^{a}	1.7±0.2^{a-c}
Unwins	<0.1±<0.1^{bc}	0.5±0.1	ND^{a}	0.3±0.1^{ab}	0.8±0.1^{c-e}	0.2±0.1^{ab}	0.4±0.1^{ab}	ND^{a}	0.6±0.3^{c}	0.6±0.2^{b}	ND^{a}	3.8±0.5^{c}
Unwins Organic	<0.1±<0.1^{ab}	0.3±0.1^{ab}	ND^{a}	0.1±0.1^{ab}	0.6±0.1^{a-d}	<0.1±<0.1^{a}	0.3±0.1^{ab}	ND^{a}	0.4±0.2^{bc}	0.4±0.1^{ab}	0.1±0.1^{ab}	2.5±0.2^{a-c}
Olivetta	ND^{a}	ND^{a}	ND^{a}	0.4±0.3^{a-c}	<0.1±<0.1^{a}	0.4±0.2^{ab}	ND^{a}	0.2±<0.1^{ab}	ND^{a}	ND^{a}	0.1±0.1^{a-c}	0.9±0.5^{ab}
WR1	ND^{a}	ND^{a}	ND^{a}	0.3±0.2^{ab}	ND^{a}	0.1±<0.1^{a}	ND^{a}	0.2±<0.1^{ab}	ND^{a}	<0.1±<0.1^{a}	0.4±0.1^{cd}	0.6±0.2^{a}
Voyager	ND^{a}	ND^{a}	ND^{a}	0.3±0.2^{ab}	ND^{a}	0.1±<0.1^{a}	ND^{a}	0.2±<0.1^{ab}	ND^{a}	<0.1±<0.1^{a}	0.4±0.1^{d}	0.7±0.4^{a}
Wild Grazia	<0.1±<0.1^{ab}	0.1±0.1^{a}	0.1±0.1^{a}	0.6±0.2^{bc}	0.1±0.1^{a}	0.2±0.1^{ab}	0.1±0.1^{a}	0.2±<0.1^{ab}	ND^{a}	0.1±0.1^{ab}	0.7±0.1^{e}	1.6±0.6^{a-c}
Wild Tirizia	ND^{a}	ND^{a}	ND^{a}	0.4±0.1^{a-c}	ND^{a}	0.1±<0.1^{a}	ND^{a}	0.2±<0.1^{ab}	ND^{a}	0.1±<0.1^{a}	0.2±0.1^{a-d}	0.7±0.1^{ab}
Wildfire	ND^{a}	ND^{a}	ND^{a}	1.0±0.4^{c}	ND^{a}	0.1±<0.1^{a}	ND^{a}	0.2±<0.1^{ab}	ND^{a}	0.1±0.1^{ab}	0.1±0.1^{a-d}	1.5±0.4^{a-c}
WR2	ND^{a}	0.1±0.1	ND^{a}	0.4±0.1^{a-c}	0.2±0.2^{ab}	0.1±0.1^{a}	0.2±0.2^{ab}	0.1±<0.1^{a}	ND^{a}	0.1±<0.1^{a}	0.4±0.1^{de}	1.1±0.4^{ab}

ND = not detected

view to classify the two genera would be a mistake. Our results clearly show the cross genera presence of substantial concentrations of different flavonols that are by no means exclusive to one or the other. Indeed the two species may still be in the process of evolutionary divergence as far as phytochemical content is concerned. Total average flavonol content ranged from 0.5 $g.kg^{-1}$ DW (SR7) to 3.8 $g.kg^{-1}$ DW (‘*Unwins’*) in *Eruca* samples, and from 0.6 $g.kg^{-1}$ DW (WR1) to 1.6 $g.kg^{-1}$ DW (‘*Wild Grazia’*) in *Diplotaxis*.

In agreement with Pasini et al. (2012) and Martinez-Sanchez et al. (2007), kaempferol-3,4’-diglucoside was the most common kaempferol glucoside detected. Isorhamnetin-3-glucoside concentrations ranged from nil to 1.0 $g.kg^{-1}$ DW (‘*Wildfire’*), and isorhamnetin-3,4’-diglucoside similarly ranged from nil to 1.0 $g.kg^{-1}$ DW (SR10). Interestingly, flavonol concentrations were generally higher for commercial varieties than gene bank accessions. This may reflect inadvertent selection on the part of breeders when traits such as taste and flavour are considered.

Our results are roughly 20% of the concentrations that have been previously reported for rocket (Pasini et al. 2012). The controlled, unstressed growth environment used in our experiment may explain this. Jin et al. (2009) previously reported that flavonol concentrations are significantly affected by different light intensities. The outdoor equivalent to the light intensities used in our experiment would be akin to shade illuminated by an entire, clear blue sky at midday. Using this as a comparative scenario, the plants in this experiment experienced no direct sunlight stress conditions (equivalent to >2,000 $\mu mol\ m^{-2}s^{-1}$). Our method therefore offers a representation of unstressed conditions for rocket flavonol accumulation, as outdoor light intensities can vary greatly according to the growing region, climate and time of year.

3.4.3. Glucosinolate composition and profiles

The profiles of all rocket accessions tested were broadly similar in terms of composition. No GSLs were detected that discriminated between the different species or commercial/gene bank accessions, and the dominance of glucosativin and DMB on GSL content broadly rendered differentiation between samples difficult. PCA analyses (not presented) showed data extremely skewed in the direction of glucosativin. Although some accessions such as SR5 contained relatively rare (for rocket species) GSLs such as 4-hydroxyglucobrassicin and glucoibarin, these concentrations were not significant enough to discriminate on a PCA scores plot due to this dominance.

3.4.4. Flavonol composition and profiles

Flavonol composition was markedly different from GSL composition. Figure 3.1 shows the scores and loadings plot of a PCA, where PCs 1 and 2 accounted for 55.79% of the observed variation. The scores plot shows a clear differentiation between *Diplotaxis* and *Eruca* with the two genera forming two distinct clusters. When compared with the loadings plot, it is clear that this divide is largely due to differences in kaempferol-3,4'-diglucoside and kaempferol-3-glucoside concentration in *Eruca*, and the tendency for *Diplotaxis* to accumulate quercetin and isorhamnetin glucosides in greater amounts.

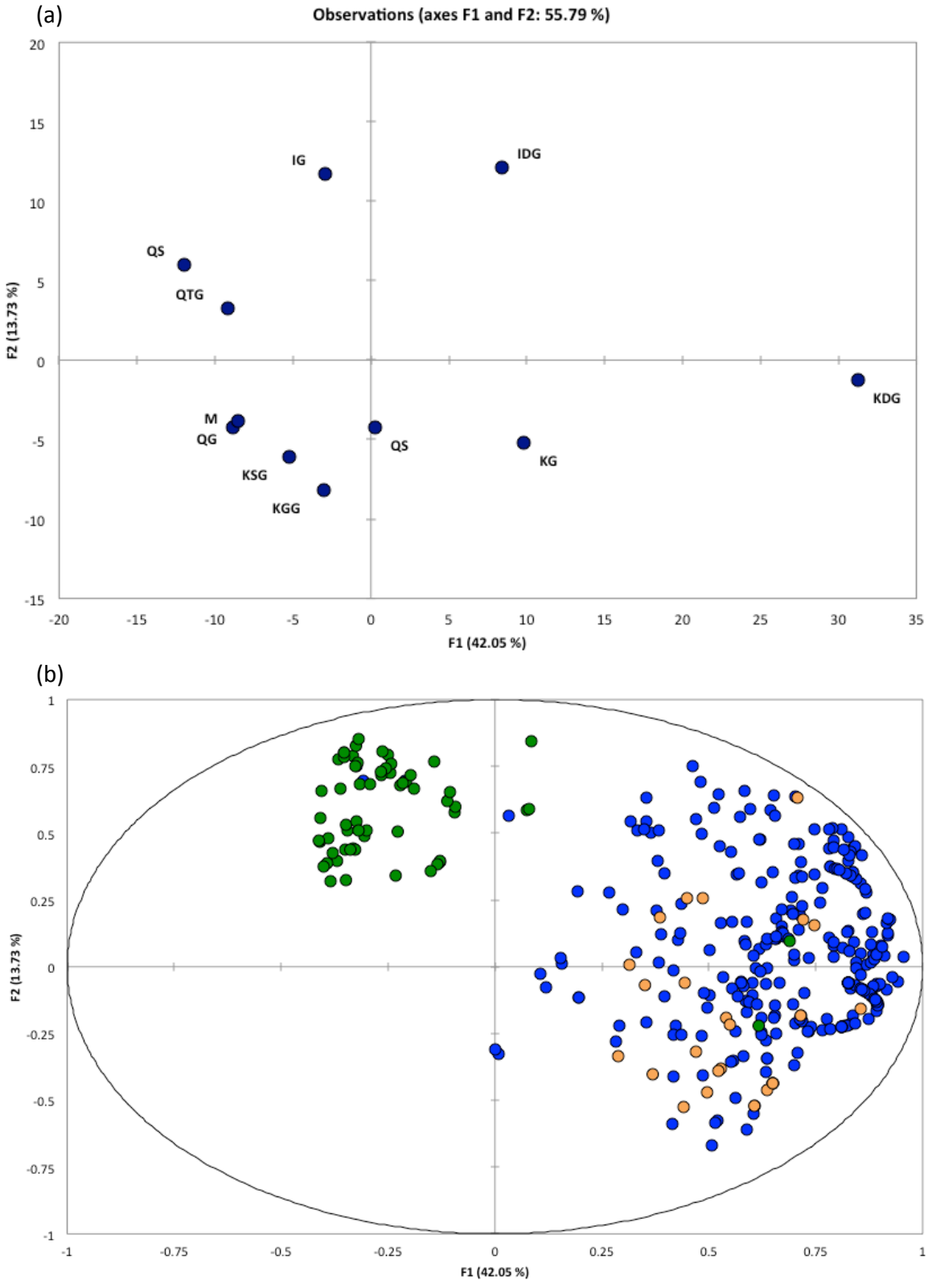

Figure 3.1. (a) PCA loadings plot of flavonol compounds detected by LC-MS analysis. Abbreviations: M, myricetin; KG, kaempferol-3-glucoside; QG, quercetin-3-glucoside; IG, isorhamnetin-3-glucoside; KDG, kaempferol-3,4'-diglucoside; IDG, isorhamnetin-3,4'-diglucoside; KGG, kaempferol-3-diglucoside-7-glucoside; QTG, quercetin-3,3,4'-triglucoside; KSG, kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside; QC, quercetin-3,4'-diglucoside-3'-(6-caffeoyl-glucoside); QS, quercetin-3,4'-diglucoside-3'-(6-sinapoyl-glucoside). (b) PCA scores plot for individual LC-MS samples tested and their relative distributions in relation to the loadings plot of flavonol composition. Green = *Diplotaxis tenuifolia*; Blue = *Eruca sativa*; Orange = *Eruca vesicaria*.

3.5. Conclusions

3.5.1. Effects of growing conditions on GSL concentrations

This study has highlighted phytochemical accumulation for rocket varieties and accessions grown under controlled conditions. This is in contrast to field conditions that often stress plants and create phytochemical profiles reflective of fluctuating environmental stresses such as light intensity, temperature, pests and diseases. These studies, while undoubtedly valuable to rocket salad research, are not always directly comparable with other growing regions and climatic backgrounds. It has been demonstrated in this study that under controlled conditions, and therefore due to genetic regulation rather than environmental response, that rocket predominantly accumulates glucosativin, and that virtually all other glucosinolates detected were minor by comparison. There was significant variability in these accumulations between varieties, providing scope for plant breeders to select varieties based on their baseline accumulations of health-beneficial precursors such as glucoraphanin and glucoerucin. This can also be said of flavonol compounds detected in rocket. Significant variability was detected between accessions, and high accumulators may be a valuable genetic resource for breeders. By determining the baseline accumulations of phytochemicals in this manner, varieties can then be tested in a field environment to ascertain any differences that could affect commercial production.

3.5.2. Effects of time-of-harvest and plant maturity on GSL concentrations

Several previous studies have made mention of using phytochemical screening as a means of selecting accessions to introduce into breeding programs. In almost every instance however, the experimental design of these studies was flawed by the fact that time-of-harvest was either much too early or much too late

relative to the commercial average. Not only does this make comparing results between studies more difficult, it also ignores the fact that phytochemical concentration and profiles change as plants grow (Fernandes, de Pinho, Valentao, Pereira, & Andrade, 2009). If researchers wish to make their data as useful to breeding programs as possible, the phytochemical profile must be determined at the point of commercial harvest, as this is when concentrations will be at their most useful in a “real-world” commercial setting. Plant breeders and food processors will not be interested in the phytochemical content of seedlings or of plants that have bolted or flowered (unless they provide products for a very niche market), as their customers will not eat the product at these points.

Table 3.3 features the number of days each of the mentioned studies grew rocket plants before harvesting. Regardless of growing conditions, the number generally chosen seems arbitrary. It is generally quoted within the literature that rocket is harvested anywhere between 30 and 60 days (Martínez-Sánchez, Allende, Cortes-Galera, & Gil, 2008), however in reality it is more like between 25 and 35 days. Bolting and flowering in rocket varieties is highly variable, but in general, most will reach this stage before 45 days of growth. This is why in our study 30 days was chosen as the point of harvest, and was determined in consultation with commercial partners who grow rocket on a large scale, in the UK, Italy and Portugal.

Bennett, Carvalho, Mellon, Eagles, & Rosa (2007) harvested seedlings at the point where the cotyledons were fully expanded, which is typically around seven days of growth. This is not however the point at which growers will harvest their crop (unless it is marketed as a ‘microleaf’ product), and although GSL concentrations are likely to be higher in young leaves, this is not necessarily reflective of what the end consumer will receive. Conversely, the other studies all harvested at or after forty-nine days (with the exception of Pasini et al., 2012) where no point of harvest time

was given). While still theoretically within the commercial harvest window, it is unlikely that growers would wait this long to harvest a crop, as the demand for rocket is so high. Chun, Arasu, Lim, & Kim (2013) stated that their work was part of a breeding program to determine varieties with high concentrations of health promoting GSLs. However, the point of harvest was at 69 days, which is well beyond commercial viability. Indeed it is stated that plants were of a height of up to 46cm when harvest occurred. From this it is clear that plants had begun flowering (or at the very least bolting), and as such, the GSL profile is likely to have altered substantially from the marketable stage of plant growth.

If researchers and breeders wish to effectively breed new varieties with enhanced phytochemical content, the consumer end-point and supply-chain must be considered in the experimental design. Selecting plants with high GSL concentrations at cotyledon and flowering stage will not necessarily be the same plants with the highest concentrations at the marketable stage.

3.5.3. Effects of genetics

Research into the underlying genetic mechanisms for GSL regulation has shown that MYB transcription factors are responsible. In *Arabidopsis thaliana* it has been shown that the HAG2/MYB76 and HAG3/MYB29 transcription factors are responsible for the biosynthesis of aliphatic GSLs and the down-regulation of indolic GSL biosynthesis (Gigolashvili, Engqvist, Yatusevich, Müller, & Flügge, 2008). This would seem to indicate that *Brassicaceae* plants are capable of adapting their GSL profile to different environmental stimuli. Very little specific research has been conducted in rocket in this regard, but it is likely that the species share analogous genes and transcription factors with both *A. thaliana* and *Brassica* crops. With detailed study into these mechanisms, it is possible that breeders could select plants

based on sets of genes, to specify responses to different environments. In this way, health beneficial GSLs could be enhanced, and less desirable ones minimized or removed entirely. This could also apply to flavonols, which are also known to be regulated by MYB transcription factors (Stracke et al., 2007).

3.5.4. Commercial vs. Gene bank accessions

Our hypothesis that some phytochemical constituents have been lost through breeding does not appear to be wholly accurate. While some gene bank accessions showed very high concentrations, others showed the exact opposite. The same can be said for the commercial varieties, as some were very poor accumulators of health beneficial compounds, but others contained high concentrations. It seems that while gene banks are a valuable resource for beneficial phytochemical traits, not all accessions are worth breeding from. Breeders must therefore screen as large a number of accessions as possible in order to pick out the very best material. The 'super broccoli' variety '*Beneforté'* was bred in a similar fashion to this, by utilising hybridization with wild relatives. Broccoli accumulates predominantly glucoraphanin within floret tissue, and through selective breeding a threefold increase in yield was achieved (variety 1639; ~11.1 $mg.g^{-1}$ DW) (Traka et al., 2013). Although rocket does not contain such inherently high concentrations, being only a small plant by comparison, there is no reason why similar concerted efforts could not enhance accumulations of glucoraphanin or other GSLs for the purposes of benefitting the consumer. It also has the added benefit that it does not need to be cooked before eating. This eliminates myrosinase thermal degradation and maximizes the production of health-beneficial volatiles such as indoles and ITCs.

Both genera showed significant variation in terms of the overall presence and absence of different phytochemicals. Several flavonols have been detected in each

species that have not been previously documented. This inherent variability between cultivars provides breeders and food producers with the opportunity to create products that are specific to the tastes and preferences of consumers. That being said, concentrations within accession groups and commercial varieties were highly variable in our study. More high quality breeding is needed to improve uniformity in this respect. The data produced in this study will be used actively in the production of new varieties of superior nutritional and sensory quality, in conjunction with industrial partners.

3.5.5. Future work

Despite the increase in rocket research in the last few years, much more study is needed to properly determine the effects of specific stresses on GSL composition and concentration. Here we have shown that concentrations under controlled conditions are generally in agreement with those of studies on field and hydroponic grown rocket. Flavonol concentration varied substantially however, and was likely due to controlled environment lighting conditions. Future work in our research group aims to compare field-grown material to the results presented here in order to properly determine which phytochemicals are affected by outdoor stresses, such as high light, high temperature, restricted water availability and increased growing density.

Researchers and breeders may need to consider more carefully the producer, supply chain, and end consumer when selecting material for breeding programs. Furthermore, much more work is needed to properly understand the degradation products of GSLs, and the underlying genetics responsible for which volatiles are produced by myrosinase interaction, in what proportions, and what effects this may have for human health.

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CHAPTER 4: Use of TD-GC-TOF-MS to assess volatile composition during post-harvest storage in seven accessions of rocket salad (*Eruca sativa*).

4.1. Introduction To Paper (as published in *Food Chemistry,* 2016, Vol. 194)

Based upon the results presented in the previous chapter, the number of germplasm accessions to be studied was narrowed to six, plus one commercial comparator. These accessions were chosen on the basis of their varying GSL and flavonol profiles and compositions. The selection was not wholly based on the LC-MS/MS results however, as other factors such as seed availability had to be considered. As limited stocks of seed are kept within gene banks, it is not always possible to obtain enough for multiple experiments.

Based on these considerations the accessions selected for further study were: SR2, SR5, SR6, SR12, SR14 and SR19. The commercial comparator was SR3, a variety sold by Elsoms Seeds Ltd., but not bred by them. This choice was largely based on the large amount of seed available for this variety.

The experiment presented in this chapter began as method development collaboration with Cardiff University. Dr. Natasha Spadafora, Dr. Carsten Müller and Dr. Hilary Rogers' had purchased thermal-desorption GC-MS apparatus and also had an interest in rocket salad volatiles link with a European Food Quality & Safety project (QUAFETY). The initial experiments involved using different methods of tissue disruption, sampling intervals, and storage temperatures, to determine the optimal conditions for VOC recovery using the apparatus, and how abundances rise/fall over time. This was initially only conducted on supermarket-bought rocket (*D. tenuifolia*) and test sampling was conducted at the University of Reading with portable thermal-desorption tubes. This process of remote sampling is potentially

useful in field or processing settings, as the technique is very basic and requires little apparatus (the method itself will be discussed in this chapter).

After the success of these first experiments, collaboration began in a more formal way, and an experiment was designed to determine how headspace volatiles change over time at a commercially relevant temperature. VOCs could be utilised as 'markers' for leaf quality throughout the supply chain. The experiment would also allow us to assess the genotypic variability of VOC production between accessions over time, and potentially, as a means to make breeding selections in future.

4.2. Introduction

Rocket (or arugula, rucola, roquette) species are of increasing commercial importance across the world. Leaves of the crop are usually sold in mixed salad bags or whole bags, and in some niche markets as gourmet microleaves. The nutritional and sensory quality of leaves throughout the supply chain is of major concern to producers and supermarkets, as they will ultimately be accepted or rejected by the consumer based on these attributes. Much of the current rocket supply chain is designed to preserve predominantly visual and morphological traits of leaves (such as stem browning/yellowing) before they reach the consumer. Very little research has been conducted to determine the phytochemical and volatile organic compound (VOC) losses incurred post-harvest (Verkerk, Dekker & Jongen, 2001).

Rocket varieties are genetically very diverse, and morphological uniformity can be an issue for plant breeders and growers alike. One plant can have very different leaf shapes from another, even within the same variety (Egea-Gilabert et al., 2009). Rocket species are generally preferential outbreeders, making production of uniform breeding lines difficult. This variability has been shown to extend to concentrations of phytochemicals (Bell, Oruna-Concha & Wagstaff, 2015), where significant differences

in glucosinolate (GSL) and flavonols have been detected amongst accessions. An important step in breeding for nutritionally enhanced varieties is determining the effects of the post-harvest supply chain on phytochemicals and the changes in volatile degradation products produced over time. The concentrations and/or relative abundances of both of these, which include isothiocyanates (ITCs), may also vary greatly depending on the levels of physical damage during processing of the leaves. The VOC bouquet is the term used to describe the collection of volatiles within the headspace of a plant or other foodstuff, often giving rise to aromas. These aromas will affect the sensory attributes perceived by the consumer when the product is eaten, and influence re-purchase (Ragaert, Verbeke, Devlieghere & Debevere, 2004). This may have consequences on consumers' nutritional intake, and hence long-term health.

VOCs found in rocket comprise of ITCs, alkanes, aliphatic alcohols, carbonyl compounds, fatty acids, esters, phenols and C_{13}-norisoprenoids (Blazevic & Mastelic, 2008). However, comparison of the relative abundance amongst cultivars has not been established, as earlier studies only utilized one commercially bought, bagged variety, and leaves from a wild population (Jirovetz, Smith & Buchbauer, 2002; Blazevic and Mastelic, 2008). Given the very different sample sources, differences between the two studies may be representative of environmental stresses, as well as genetic variation (Varming et al., 2004). These include exposure to fungal diseases, wounding (pre- and post-harvest), and variations in temperature and humidity during growth and while in controlled environment conditions – all of which can lead to changes in phytochemical content and VOCs produced (Schouten et al., 2009).

In this study rocket salad was grown under controlled environment conditions, thus reducing environmental stress responses, enabling effects of post-harvest storage on VOC profiles to be assessed. Thermal Desorption Gas Chromatography

Time-Of-Flight Mass Spectrometry (TD-GC-TOF-MS) was used to determine changes in VOCs during storage of seven different accessions demonstrating that collection of headspace volatiles onto thermal desorption tubes is a rapid and robust method for assessing post-harvest changes and identifying differences in these changes amongst accessions.

4.3. Materials and methods

4.3.1. Plant material

Six *Eruca sativa* accessions were obtained from European gene banks; four from the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK Gatersleben, Germany), one from the Centre for Genetic Resources in the Netherlands (CGN, Wageningen, The Netherlands) and one from The University of Warwick Crop Centre Genetic Resources Unit (Wellesbourne, UK; formerly Warwick HRI). The Elsoms Seeds variety SR3 was used as a commercial comparator. Accessions have been coded to protect commercially sensitive information.

4.3.2. Growing conditions & simulated shelf life

Each accession was germinated under controlled environmental conditions (Fitotron, Weiss-Technik UK, Loughborough, UK). Long-day lighting was used (16 h light, 8 h dark) at an intensity of 200 μmol m^{-2} s^{-1}. Day temperatures were set at 20°C and night temperatures at 14°C. Seedlings were grown for 10 days in seedling trays and then transplanted to larger trays. Subsequently, plants were grown for another 20 days and then leaves were harvested at 30 days. Leaves were collected in batches of 70 g in three experimental replicates per accession. The amount of leaf material was chosen based on preliminary experiments, where abundant yields of VOCs were obtained.

4.3.3. Sample collection

Whole leaves were placed into a multi-purpose roasting bag (25 cm x 38 cm, TJM Ltd.) and sealed using an elastic band and an Eppendorf tube with the end cut off, which served as a sampling port for the TD tubes (see appendix I for diagram). Leaves were then disrupted manually within the bags for 10 seconds by crushing the leaves between the hands and making a vigorous rubbing motion. Care was taken not to perforate the bags and inadvertently release VOCs. Three replicates were taken for each sample, including three 'blank' samples of atmosphere within empty bags to rule out any possible contaminating VOCs. These were prepared and sampled in an identical fashion, with the exception that no leaves were contained in the bag.

4.3.4 Post-harvest storage simulation

Harvested rocket leaves were stored in a dark, controlled temperature room at 4°C, to simulate industrial storage conditions for 7 days. Bags were only removed from the environment while samples were taken (room temperature, ~22°C).

4.3.5. TD-GC-MS-TOF analysis

All tubes were desorbed using a TD100 thermal desorption system (Markes International Ltd., Llantrisant, Wales, UK) using the following settings for tube desorption: 5 min at 100°C followed by 5 min at 280°C, trap flow of 40 ml/min and trap desorption and transfer: 20°C/s to 300°C, split flow of 20 ml/min into GC (7890A; Agilent Technologies, Inc., Stockport, UK). VOCs were separated over 60 min, 0.32 mm ID, 0.5 µm film thickness Rxi-5ms (Restek) at 2 ml continuous flow helium using the following temperature program: Initial temperature 40°C for 2 min, 5°C/min to

240°C, final hold 5 min. The BenchTOF-dx mass spectrometer (Almsco International, Cincinnati, OH, USA) was operated in EI mode at an ion source temperature of 275°C and a mass range of 35 to 350 *m/z*. A retention time standard (C8-C20, Sigma Aldrich, Gillingham, UK) was prepared by injection of 1µl of the standard mixture directly onto a thermal desorption tube, and analysed under the same conditions as the samples.

Data from GC-MS measurements were processed using MSD ChemStation software (E.02.01.1177; Agilent Technologies, Inc., Stockport, UK) and deconvoluted and integrated with AMDIS (NIST 2011) using a custom retention-indexed mass spectral library. MS spectra from deconvolution were searched against the NIST 2011 library (Mallard, Sparkman & Sparkman, 2008) and only compounds scoring >80% in forward and backward fit were included into the custom library. Putative identifications were based on match of mass spectra (>80%) and retention index (RI +/-15) (Beaulieu & Grimm, 2001).

Compounds abundant in controls or in only one of the three replicates were excluded from statistical analyses. Areas of remaining compounds were normalized to total area of chromatograms prior to averaging within samples.

4.3.6. Statistical analysis

Results from the three biological replicates for each time point were averaged and analysed using XL Stat software (Addinsoft, New York City, NY, USA). ANOVA + Tukey's HSD test (p = <0.05) and Principal Component Analyses (PCA; Pearson n-1) were performed on the data to determine significant differences and correlations of compounds detected over time and on each respective sampling day. Tukey's test was chosen because of its high stringency (compared to Fisher's test, for example) and a reduced possibility of Type I statistical errors.

4.4. Results and Discussion

4.4.1. Composition and functional analysis of Eruca sativa VOC bouquet

We focused on *Eruca sativa* accessions to assess the range of VOCs produced by different genotypes from the same species, and then to determine whether within this narrower genetic range there were sufficient VOC differences to discriminate amongst accessions. A total of 39 compounds were putatively identified by comparison to NIST libraries, and a further three unknown volatile compounds were also detected (see Table 4.1 for compounds, their identification codes, retention indices and CAS numbers). Compounds comprised several classes of aliphatic organic compounds including alcohols, aldehydes, ketones, isothiocyanates and furanones; some of which have been reported in other studies (Jirovetz et al. 2002; Blazevic and Mastelic, 2008). However, to our knowledge, only four of these compounds have been previously reported in *Eruca sativa* (**C8, C14, C20** and **C38**). Fifteen of the compounds have been detected in other members of the *Brassicaceae* family. These include broccoli (*Brassica oleracea* var. *italica*; **C10, C15, C9**; Hansen, Laustsen, Olsen, Poll & Sorensen, 1997), radish (*Raphanus sativus*; **C8, C20, C33, C25**; Blazevic & Mastelic, 2009), kale (*Brassica oleracea* var. *acephala*; **C3, C10, C8, C31, C20, C29**; Fernandes, de Pinho, Valentao, Pereira & Andrade, 2009), oilseed rape (*Brassica napus*; **C20, C6, C7**), Thale cress (*Arabidopsis thaliana*; **C20, C29, C3, C9, C14**; Rohloff & Bones, 2005) and mustard (*Brassica juncea*; **C15, C9, C14, C23**; Zhao, Tang & Ding, 2007).

4.4.2. Relative VOC abundance and differences amongst rocket gene bank accessions over simulated shelf life

4.4.2.1. Across-day variation

The abundance of the 42 VOCs was determined in each of the seven *Eruca sativa* accessions (Table 4.2) over three different storage time-points: day 0 (at harvest) and following +3 and +7 days of storage at a commercially relevant temperature of 4°C. Seven days is the typical time taken from harvest to retail (Favell, 1998), assuming a typical supply chain where rocket is imported by lorry from northern or southern Italy (depending on the season) to the UK. Thirty-one of the 42 compounds detected were significantly different in percentage-abundance amongst rocket accessions, across the three sampling days (ANOVA with post-hoc Tukey's HSD test; see Table 4.2). Figure 4.1 (PCA scores plot) shows how each of the genotypes are arranged spatially according to the volatiles produced on each sampling day. On 'Day 0' there is a large degree of separation along the F1 axis, indicating that genotypic variability is high in terms of the types of volatiles produced and their relative abundance (cluster 1). Sample SR2 and SR12 are the most dissimilar in this respect with varying degrees of similarity with the other five genotypes. This indicates that there may be a high degree of genetic variability and control involved in VOC production in the early stages of shelf life, as environmental variation was minimal during plant growth and sampling. On 'Day 3' and 'Day 7', the distinction between accessions is somewhat reduced, and volatile profiles less variable for each respective accession (cluster 2). The profiles of SR2 on 'Day 3' and 'Day 7' are virtually indistinguishable in Figure 4.1a, and similarly with SR5.

Table 4.1. VOC compounds putatively identified in rocket accessions by TD-GC-MS-TOF with retention indices, CAS registry number, chemical group, and references for identifications in previous studies.

Compound number	Compound name	RI	CAS No.	Chemical group	Reference
C1	(E)-4-oxohex-2-enal*	989	EPA-374042	Aldehyde	Sanda, Zacek, Streinz, Dracinsky & Koutek, 2012
C2	4-isothiocyanato-1-butene*	1021	3386-97-8	Isothiocyanate	Guo, Yang, Wang, Guo & Gu 2014
C3	1-penten-3-ol*	677	616-25-1	Alcohol	Fernandes, de Pinho, Valentao, Pereira & Andrade, 2009
C4	1-penten-3-one*	683	1629-58-9	Ketone	Servili, Selvaggini, Taticchi, Esposto & Montedoro, 2003
C5	2-(1,1-dimethylethyl)-1H-indole*	1582	1805-65-8	Aromatic compound	-
C6	2-methyl-2-butenal*	702	1115-11-3	Aldehyde	Starkenmann, 2003
C7	2-hexenal*	865	505-57-7	Aldehyde	Servili, Selvaggini, Taticchi, Esposto & Montedoro, 2003
C8	(E)-2-hexenal	858	6728-26-3	Aldehyde	Blazevic & Mastelic, 2008
C9	(Z)-2-penten-1-ol*	782	1576-95-0	Alcohol	Rohloff & Bones, 2005; Servili, Selvaggini, Taticchi, Esposto & Montedoro, 2003
C10	(E)-2-pentenal*	760	1576-87-0	Aldehyde	Fernandes, de Pinho, Valentao, Pereira & Andrade, 2009

C11	(E,E)—2,4-hexadienal*	933	142-83-6	Aldehyde	Farag, Ryu, Sumner & Paré, 2006
C12	5-ethyl-2(5H)-furanone*	1003	2407-43-4	Furanone	Buttery & Takeoka, 2004
C13	3-ethyl-1,5-octadiene*	961	EPA-114877	Alkene	Rohloff & Bones, 2005
C14	3-hexen-1-ol	872	3681-71-8	Alcohol	Ruther & Kleier, 2005; Blazevic & Mastelic, 2008, 2009
C15	3-hexenal*	810	4440-65-7	Aldehyde	Tandon, Baldwin & Shewfelt, 2000
C16	(Z)-3-hexenal*	898	6789-80-6	Aldehyde	Buttery & Takeoka, 2004
C17	3-octyne*	921	15232-76-5	Alkyne	-
C18	3-pentanone*	691	96-22-0	Ketone	Servili, Selvaggini, Taticchi, Esposto & Montedoro, 2003
C19	5-methyl-4-hexen-3-one*	1073	13905-10-7	Ketone	-
C20	4-methylpentyl isothiocyanate	1184	17608-07-0	Isothiocyanate	Beevi, Mangamoori, Subathra & Edula, 2010
C21	5-nonanone oxime*	791	14475-42-4	Imine	-
C22	O-methyloxime-butanal*	672	31376-98-4	Imine	-
C23	1-isothiocyanato-3-methyl-butane*	1082	628-03-5	Isothiocyanate	Zhao, Tang & Ding, 2007
C24	Ethylidene-cyclopropane*	564	18631-83-9	Cycloalkane	Bajpai, Rahman, & Kang, 2008
C25	Dimethyl-sulfide*	570	75-18-3	Sulphur compound	Manchali, Chidambara Murthy & Patil, 2012
C26	2-ethyl-furan*	696	3208-16-0	Aromatic	Farag, Ryu, Sumner & Paré, 2006

				compound	
C27	3-methyl-furan*	605	930-27-8	Aromatic compound	-
C28	3-methyl-hexadecane*	1658	6418-43-5	Alkane	Wang, Xing, Chin, Ho & Martin, 2001
C29	*n*-hexyl-isothiocyanate *	1223	4404-45-9	Isothiocyanate	Fernandes, de Pinho, Valentao, Pereira & Andrade, 2009
C30	2-oxo-hexanoic acid methyl ester *	1085	6395-83-1	Ester	-
C31	*n*-pentyl isothiocyanate*	1120	629-12-9	Isothiocyanate	Grob & Matile, 1980
C32	Oxalic acid diallyl ester*	808	EPA-309229	Ester	-
C33	Iberverin*	1355	505-79-3	Isothiocyanate	Munday & Munday, 2004
C34	Propanoic acid anhydride*	1086	123-62-6	Acid anhydride	-
C35	4-methyl-2-(2-methyl-propenyl)-pyridine*	1424	104188-16-1	Pyridine derivative	-
C36	Pyrrolidine-1-dithiocarboxylic acid 2-oxocyclopentyl ester*	1391	147723-50-0	Sulphur aromatic compound	-
C37	3-ethyl-thiophene*	885	1795-01-3	Sulphur aromatic compound	-
C38	Tetrahydrothiophene	826	110-01-0	Sulphur aromatic compound	Blazevic & Mastelic, 2008
C39	[Unknown 2]	693	-	-	-

C40	[Unknown 8]	1129	-	-	-
C41	[Unknown 9]	991	-	-	-
C42	Vinylfuran*	726	1487-18-9	Aromatic compound	-

* = Previously unreported in *Eruca sativa*

Table 4.2. Means of percentage relative abundance with standard error and significant differences (p = <0.05) of volatile compounds detected in the headspace of rocket accessions. Values expressed as percentage of the total volatiles detected per sample.

VOC	Cultivar & day of sampling (% of total volatiles detected)																				
	SR2			SR3			SR5			SR6			SR12			SR14			SR19		
	0	3	7	0	3	7	0	3	7	0	3	7	0	3	7	0	3	7	0	3	7
C1	22.4±0.4^{abc}	30.5±2.7^{bc}	25.6±6.2^{abc}	14.8±2.9^{ab}	49.9±0.9^{c}	22.5±4.9^{abc}	10.5±8.6^{ab}	nd^{a}	1.9±1.0^{ab}	4.7±3.8^{ab}	20.6±2.9^{ab}	17.9±7.3^{ab}	2.5±2.0^{ab}	19.7±4.1^{ab}	3.2±1.5^{ab}	13.4±8.6^{ab}	19.8±4.9^{ab}	9.3±4.1^{ab}	12.3±5.1^{ab}	8.4±1.4^{ab}	3.3±1.9^{ab}
C2	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{b}	nd^{a}	nd^{a}	0.1±<0.1^{c}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
C3	1.2±0.1^{ab}	1.5±0.2^{b}	1.3±0.2^{ab}	1.5±0.3^{b}	0.7±0.3^{ab}	1.4±0.7^{ab}	0.5±0.1^{ab}	nd^{a}	nd^{a}	1.2±0.2^{ab}	0.7±0.1^{ab}	0.5±0.2^{ab}	1.5±0.2^{b}	nd^{a}	nd^{a}	1.3±0.1^{ab}	nd^{a}	nd^{a}	1.2±0.2^{ab}	nd^{a}	nd^{a}
C4	1.9±0.1^{a}	4.7±1.0^{a}	4.2±1.0^{a}	2.0±0.2^{a}	2.6±0.9^{a}	4.3±2.2^{a}	1.3±0.5^{a}	1.0±0.6^{a}	0.9±0.6^{a}	1.4±0.3^{a}	1.8±0.3^{a}	1.8±0.3^{a}	1.8±0.2^{a}	1.2±0.2^{a}	0.6±0.2^{a}	1.9±0.2^{a}	3.0±0.4^{a}	2.7±1.5^{a}	2.2±0.3^{a}	1.8±0.7^{a}	0.5±0.1^{a}
C5	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}
C6	nd^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}
C7	4.3±0.5^{abc}	6.5±1.6^{abc}	4.8±0.5^{abc}	7.7±2.1^{abc}	5.8±0.5^{abc}	4.9±2.2^{abc}	4.7±1.6^{abc}	1.4±1.1^{ab}	1.2±0.9^{ab}	7.3±1.8^{abc}	2.5±0.6^{abc}	2.1±0.8^{abc}	9.9±1.7^{c}	2.4±0.7^{abc}	0.4±0.2^{a}	7.7±2.4^{abc}	2.2±0.8^{abc}	nd^{a}	8.9±0.9^{bc}	1.0±0.5^{ab}	0.3±0.2^{a}
C8	0.7±0.2^{ab}	nd^{a}	0.1±0.1^{a}	3.1±1.6^{ab}	nd^{a}	0.6±0.2^{ab}	0.4±0.1^{a}	nd^{a}	nd^{a}	3.5±1.5^{ab}	0.2±0.1^{a}	0.1±<0.1^{a}	5.5±1.7^{b}	0.4±0.2^{a}	nd^{a}	3.4±1.9^{ab}	nd^{a}	nd^{a}	1.9±1.0^{ab}	nd^{a}	nd^{a}
C9	0.1±<0.1^{ab}	nd^{a}	nd^{a}	0.2±0.1^{bc}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	0.2±0.1^{bc}	nd^{a}	nd^{a}	0.3±0.1^{c}	nd^{a}	nd^{a}	0.2±0.1^{bc}	nd^{a}	nd^{a}	0.2±<0.1^{bc}	nd^{a}	nd^{a}
C10	1.0±<0.1^{ab}	3.0±0.6^{bc}	2.8±0.7^{bc}	1.2±0.2^{abc}	4.0±0.8^{c}	2.1±0.8^{abc}	1.0±0.3^{ab}	0.8±0.6^{ab}	0.6±0.5^{ab}	1.0±0.1^{ab}	1.1±0.1^{ab}	1.1±0.3^{ab}	1.3±0.2^{abc}	0.9±0.1^{ab}	0.4±0.2^{ab}	1.3±0.1^{abc}	1.5±0.7^{abc}	nd^{a}	1.5±0.1^{abc}	1.2±0.4^{abc}	0.4±0.1^{ab}
C11	0.4±0.1^{ab}	nd^{a}	nd^{a}	1.0±0.4^{ab}	nd^{a}	0.2±0.2^{ab}	0.2±0.1^{ab}	nd^{a}	nd^{a}	1.4±0.6^{ab}	nd^{a}	nd^{a}	1.6±0.5^{b}	0.3±0.1^{ab}	nd^{a}	1.1±0.5^{ab}	nd^{a}	nd^{a}	0.8±0.2^{ab}	nd^{a}	nd^{a}
C12	0.2±<0.1^{ab}	nd^{a}	nd^{a}	0.2±0.1^{ab}	nd^{a}	0.1±<0.1^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	0.4±<0.1^{bc}	nd^{a}	nd^{a}	0.4±<0.1^{bc}	nd^{a}	nd^{a}	0.5±0.1^{c}	nd^{a}	nd^{a}	0.4±0.1^{bc}	nd^{a}	nd^{a}
C13	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	0.4±<0.1^{ab}	0.2±0.1^{ab}	<0.1±<0.1^{a}	0.9±0.4^{b}	nd^{a}	nd^{a}	0.4±0.2^{ab}	0.2±0.2^{ab}	nd^{a}	0.1±0.1^{a}	nd^{a}
C14	39.3±5.4^{a}	1.0±2.9^{a}	25.0±2.5^{a}	36.9±14.1^{a}	12.5±4.0^{a}	12.5±3.8^{a}	14.1±1.9^{a}	9.9±5.0^{a}	20.7±14.0^{a}	17.0±1.4^{a}	23.9±7.8^{a}	17.0±6.6^{a}	14.3±1.1^{a}	10.4±3.6^{a}	4.7±2.1^{a}	15.7±1.9^{a}	38.0±5.5^{a}	8.6±4.3^{a}	24.2±5.2^{a}	17.5±3.4^{a}	4.9±3.2^{a}
C15	12.2±2.6^{abc}	4.8±2.8^{abc}	6.8±2.8^{abc}	18.7±5.6^{c}	3.4±1.6^{abc}	12.9±5.4^{abc}	6.9±1.6^{abc}	2.1±1.4^{ab}	1.7±1.2^{ab}	15.3±1.8^{abc}	6.1±1.2^{abc}	5.2±2.0^{abc}	16.5±1.2^{bc}	7.3±2.5^{abc}	2.1±0.5^{ab}	11.7±3.3^{abc}	nd^{a}	nd^{a}	17.4±3.2^{bc}	2.0±1.4^{ab}	nd^{a}
C16	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	0.1±<0.1^{a}	nd^{a}	nd^{a}	1.4±1.1^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
C17	0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	<0.1±<0.1^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	0.1±0.1^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}
C18	0.2±<0.1^{a}	nd^{a}	0.1±<0.1^{a}	0.1±0.1^{a}	nd^{a}	0.1±0.1^{a}	0.1±<0.1^{a}	0.2±0.1^{a}	0.1±<0.1^{a}	0.1±<0.1^{a}	0.2±0.1^{a}	nd^{a}	0.1±<0.1^{a}	0.1±<0.1^{a}	nd^{a}	0.2±0.1^{a}	0.2±0.1^{a}	nd^{a}	0.3±0.1^{a}	nd^{a}	nd^{a}
C19	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	0.9±0.4^{a}	1.2±0.9^{a}	nd^{a}	2.3±1.4^{a}	nd^{a}	nd^{a}	3.3±1.4^{a}	nd^{a}	<0.1±<0.1^{a}	1.8±1.4^{a}	nd^{a}	nd^{a}	2.2±1.8^{a}	nd^{a}	nd^{a}
C20	1.2±0.2^{a}	1.5±0.6^{a}	1.5±0.6^{a}	0.7±0.3^{a}	nd^{a}	0.7±0.2^{a}	4.3±1.0^{a}	3.4±1.6^{a}	0.3±0.1^{a}	1.1±0.3^{a}	1.6±0.5^{a}	0.6±0.2^{a}	1.3±0.2^{a}	1.3±0.4^{a}	0.1±<0.1^{a}	0.8±0.1^{a}	nd^{a}	nd^{a}	2.4±0.5^{a}	4.7±2.4^{a}	0.3±0.2^{a}
C21	0.1±<0.1^{a}	14.3±4.7^{abcd}	11.7±3.5^{a}	0.1±<0.1^{a}	8.7±2.8^{abc}	12.9±9.9^{abcd}	0.2±<0.1^{a}	36.9±7.5^{cd}	27.2±5.7^{abcd}	0.1±<0.1^{a}	11.1±2.8^{abcd}	24.7±10.0^{abcd}	<0.1±<0.1^{a}	15.8±2.5^{abcd}	36.8±2.6^{cd}	0.1±<0.1^{a}	13.3±1.2^{abcd}	23.5±7.1^{abcd}	0.3±<0.1^{ab}	30.4±4.8^{bcd}	40.9±3.5^{d}
C22	nd^{a}	7.6±3.4^{abc}	8.2±4.1^{abc}	nd^{a}	4.2±1.8^{ab}	17.7±9.9^{abc}	0.2±0.1^{a}	27.1±7.6^{abc}	43.3±12.8^{abc}	nd^{a}	9.9±3.3^{abc}	21.3±10.8^{abc}	nd^{a}	25.9±6.4^{abc}	48.2±3.0^{c}	nd^{a}	6.3±3.4^{abc}	46.0±19.4^{bc}	nd^{a}	30.9±0.6^{abc}	48.9±2.6^{c}
C23	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{ab}	nd^{a}	nd^{a}	<0.1±<0.1^{ab}	nd^{a}	nd^{a}	<0.1±<0.1^{ab}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{b}	nd^{a}	nd^{a}
C24	<0.1±<0.1^{ab}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	0.1±<0.1^{ab}	nd^{a}	nd^{a}	0.1±<0.1^{ab}	nd^{a}	nd^{a}	0.1±<0.1^{b}	nd^{a}	nd^{a}	<0.1±<0.1^{ab}	nd^{a}	nd^{a}
C25	nd^{a}	1.1±0.7^{ab}	1.5±0.8^{ab}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	1.8±0.6^{ab}	0.3±0.2^{a}	nd^{a}	0.4±0.2^{a}	1.5±0.8^{ab}	nd^{a}	2.6±0.8^{ab}	2.1±1.3^{ab}	nd^{a}	10.0±5.2^{b}	7.8±2.7^{ab}	nd^{a}	nd^{a}	nd^{a}
C26	1.9±0.3^{abc}	5.0±0.6^{c}	3.3±0.7^{abc}	1.3±0.3^{abc}	4.6±1.4^{bc}	2.1±0.9^{abc}	2.8±0.4^{abc}	1.8±0.8^{abc}	0.7±0.4^{ab}	3.2±0.3^{abc}	2.0±0.3^{abc}	1.5±0.4^{abc}	1.9±0.3^{abc}	1.4±0.3^{abc}	0.6±0.3^{ab}	2.7±0.4^{abc}	2.7±0.7^{abc}	1.9±1.0^{abc}	2.1±0.1^{abc}	1.4±0.5^{abc}	0.4±<0.1^{a}
C27	<0.1±<0.1^{c}	nd^{a}	nd^{a}	<0.1±<0.1^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{b}	nd^{a}	nd^{a}	<0.1±<0.1^{b}	nd^{a}	nd^{a}	<0.1±<0.1^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
C28	nd^{a}	nd^{a}	1.3±0.6^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	0.1±<0.1^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
C29	0.4±0.1^{a}	nd^{a}	nd^{a}	0.2±0.1^{a}	nd^{a}	0.1±0.1^{a}	1.8±0.7^{b}	nd^{a}	nd^{a}	0.4±<0.1^{a}	0.3±0.1^{a}	nd^{a}	0.3±<0.1^{a}	0.4±0.2^{a}	nd^{a}	0.2±0.1^{a}	nd^{a}	nd^{a}	0.2±0.1^{a}	nd^{a}	nd^{a}
C30	10.4±1.4^{cd}	nd^{a}	nd^{a}	7.0±2.9^{abc}	nd^{a}	4.7±2.1^{abc}	6.2±1.1^{abc}	nd^{a}	nd^{a}	10.8±1.3^{cd}	3.8±0.4^{abc}	1.0±0.4^{ab}	12.1±1.6^{cd}	3.7±1.6^{abc}	0.6±0.1^{ab}	16.7±3.5^{d}	nd^{a}	nd^{a}	8.9±1.8^{bcd}	nd^{a}	nd^{a}
C31	nd^{a}	nd^{a}	nd^{a}	0.1±<0.1^{a}	nd^{a}	nd^{a}	0.4±0.2^{b}	nd^{a}	nd^{a}	0.1±<0.1^{a}	nd^{a}	nd^{a}	0.1±<0.1^{ab}	nd^{a}	nd^{a}	0.1±<0.1^{ab}	nd^{a}	nd^{a}	0.1±<0.1^{a}	nd^{a}	nd^{a}
C32	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	3.3±1.4^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
C33	nd^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	0.1±0.1^{b}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}
C34	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	2.2±0.9^{ab}	nd^{a}	nd^{a}	3.9±0.5^{b}	nd^{a}	nd^{a}	3.1±1.5^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
C35	nd^{a}	nd^{a}	nd^{a}	0.1±<0.1^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	0.2±<0.1^{ab}	nd^{a}	nd^{a}	0.5±0.1^{b}	nd^{a}	nd^{a}	0.2±0.1^{ab}	nd^{a}	nd^{a}	0.2±0.1^{ab}	nd^{a}	nd^{a}
C36	1.5±0.9^{a}	7.6±3.3^{a}	nd^{a}	2.4±1.1^{a}	nd^{a}	nd^{a}	41.7±16.8^{b}	11.2±2.9^{a}	0.4±0.2^{a}	15.8±7.0^{ab}	12.1±6.2^{a}	2.9±1.3^{a}	7.4±3.2^{a}	4.8±3.3^{a}	nd^{a}	8.2±2.5^{a}	nd^{a}	nd^{a}	11.2±1.6^{a}	nd^{a}	nd^{a}
C37	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{c}	nd^{a}	nd^{a}	<0.1±<0.1^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
C38	0.3±<0.1^{a}	1.6±0.4^{ab}	1.4±0.2^{ab}	0.2±0.1^{a}	3.0±1.2^{b}	0.1±0.1^{a}	1.2±0.1^{ab}	1.2±0.3^{ab}	0.5±0.2^{ab}	0.4±0.1^{ab}	0.9±0.4^{ab}	0.5±0.2^{ab}	0.2±0.1^{a}	0.4±0.3^{ab}	nd^{a}	0.3±<0.1^{a}	2.3±1.1^{ab}	nd^{a}	0.5±<0.1^{ab}	0.5±0.3^{ab}	0.1±0.1^{a}
C39	nd^{a}	nd^{a}	nd^{a}	0.1±<0.1^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	0.1±<0.1^{a}	nd^{a}	nd^{a}	0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}
C40	0.1±<0.1^{a}	nd^{a}	nd^{a}	0.3±0.1^{a}	nd^{a}	nd^{a}	0.3±0.1^{a}	0.1±<0.1^{a}	nd^{a}	0.3±<0.1^{a}	0.5±0.2^{a}	nd^{a}	0.3±0.1^{a}	nd^{a}	nd^{a}	0.2±0.1^{a}	nd^{a}	nd^{a}	0.3±0.1^{a}	nd^{a}	nd^{a}
C41	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	9.0±0.2^{b}	nd^{a}	nd^{a}	7.7±0.4^{b}	nd^{a}	nd^{a}	6.9±3.0^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
C42	<0.1±<0.1^{a}	0.3±0.1^{ab}	0.3±0.1^{ab}	<0.1±<0.1^{a}	0.7±0.3^{b}	0.1±0.1^{a}	<0.1±<0.1^{a}	nd^{a}	nd^{a}	<0.1±<0.1^{a}	<0.1±<0.1^{a}	<0.1±<0.1^{a}	<0.1±<0.1^{a}	<0.1±<0.1^{a}	<0.1±<0.1^{a}	0.1±<0.1^{a}	0.2±0.1^{ab}	nd^{a}	0.1±<0.1^{a}	<0.1±<0.1^{a}	<0.1±<0.1

nd = not detected

Superscript letters in the same row indicate differing levels of significance for each respective cultivar across all sampling days (ANOVA Tukey's HSD test; (p = ≤0.05))

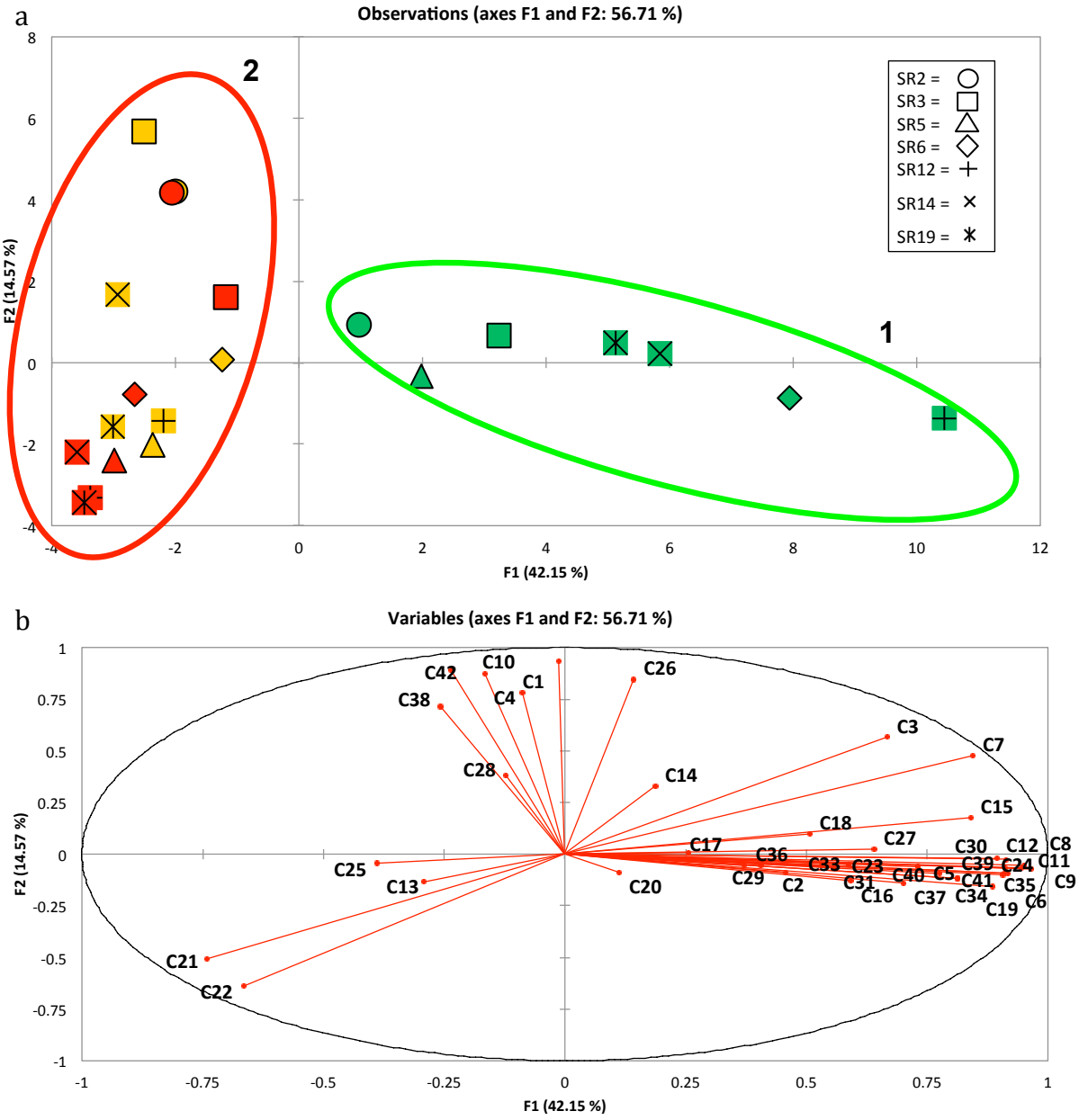

Figure 4.1. Scores plot (a) and loadings plot (b) from Principal Components Analysis of seven accessions of *E. sativa* and the volatile organic compounds identified. Data points were averaged for each accession time point (consisting of three replicates). PC1 vs. PC2 (F1 & F2) accounted for 56.72% of the total variation within data. For compound identities refer to Table 4.1. SR codes refer to each of the 7 accessions used in the experiment (SR2, SR3, SR5, SR6, SR12 SR14 & SR19) with the following colour coding, 0 (green), 3 (orange) and 7 (red) corresponding to the day of sampling. Numbers and circles inset (a) refer to cluster discussed within the text.

4.4.2.2. Within-day variation

4.4.2.2.1. General

A separate ANOVA Tukey's HSD test (p = <0.05) was performed on data from each of the three sampling days. Several significant differences were observed between cultivars on each day. Twelve compounds were significantly different

between accessions on ‘Day 0’, nine on ‘Day 3’ and six on ‘Day 7’. The dwindling abundances of VOCs in the latter shelf-life samples would seem to indicate the depletion of GSLs and other defense related compounds. The most interesting of these are discussed in this section.

4.4.2.2.2. Isothiocyanates

On ‘Day 0’ SR6 and SR5 were the only accessions where 4-isothiocyanato-1-butene (**C2,** both 0.1 ± <0.1%) was detected. 4-methylpentyl ITC (**C20**) was detected in all accessions with SR5 being significantly different (4.3 ± 1.0%) from all others with the exception of SR19 (2.4 ± 0.5%). SR5 contained over six times the abundance of this ITC than SR3.

Hexyl ITC (**C29**) was similarly detected in all accessions (‘Day 0’), with SR5 having the highest abundance (1.8 ± 0.7%) – significantly different in this case from SR19, which had the lowest recorded abundance (0.2 ± 0.1%; nine times less abundant in relative terms). Bell et al. (2015) found that SR5 had high total GSL concentrations (11.5 $mg.g^{-1}$ DW) compared to other *Eruca* and *Diplotaxis* accessions, potentially making it a valuable source of genetic material for breeding programs interested in enhancing GSL/ITC accumulation traits.

4.4.2.2.3. Alcohols

(Z)-2-penten-1-ol (**C9**) was detected in all accessions except SR5, with the highest abundance detected in SR12 (0.3 ± 0.1%; ‘Day 0’) which was significantly different from zero (i.e. significantly different from samples with 0% abundance). Other alcohols, such as 1-penten-3-ol (**C3**) displayed no significant differences on this sampling day. Some of these differences may indicate a genetic component to VOC production, i.e. the types and abundances produced may be under direct

genetic regulation. Alcohols are typically used by plants as a defensive mechanism (Ruther & Kleier, 2005), and often provide the 'cut grass' aroma found in leafy vegetables. Plant defense mechanisms are known to rely on genetic regulation via enzymatic regulation in other species (D'Auria, Pichersky, Schaub, Hansel, & Gershenzon, 2007), and it is reasonable to assume that the same may be true for rocket. D'Auria et al. (2007), showed this to be the case in *Arabidopsis* for production of (Z)-3-hexen-1-yl acetate via a BAHD acetyltransferase enzyme. It is also widely known that nitriles, epithionitriles and thiocyanates are produced enzymatically in *Brassicaceae* through specifier proteins in the GSL-myrosinase reaction (Kuchernig, Backenköhler, Lübbecke, Burow & Wittstock, 2011).

4.4.2.2.4. Sulphur aromatic compounds

3-ethyl-thiophene (**C37**) was detected in only two accessions, SR6 and SR12 (both <0.1 ± <0.1%, 'Day 0') and were significantly different from each other, despite the exceedingly small abundances observed. Tetrahydrothiophene (**C38**) was detected in every accession, but SR5 had the highest abundance (1.2 ± 0.1%, 'Day 0') which was significantly different from the other samples. It is likely that these types of VOCs also contribute to the characteristic pungent/mustard/pepper sensory attributes of rocket, as well as ITCs.

By 'Day 7', pyrrolidine-1-dithiocarboxylic acid 2-oxocyclopentyl ester (**C36**) was detected in only two accessions: SR5 (0.4±0.2%) and SR6 (2.9±1.3%). Tetrahydrothiophene (**C38**) was present in all accessions with the exception of SR12 and SR14.

4.4.2.2.5. Imines

On ‘Day 3’ 5-nonanone-oxime (**C21**) was most abundant in SR5 (36.9 ± 7.5%), significantly higher than in either SR3 or SR6 (8.7 ± 2.8%; 11.1 ± 2.8%). Abundances were generally much higher across all accessions than on ‘Day 0’, though with fewer significant differences between accessions, possibly because of more variation between replicates. SR3 also contained the lowest abundance of *O*-methyloxime-butanal (**C22**) (4.2 ± 1.8%). In contrast, SR19 had the highest abundance (30.9 ± 0.6%) of this compound.

4.4.2.2.6. Aldehydes

A very large range of abundances was observed for (E)-4-oxohex-2-enal (**C1**) amongst accessions. This compound was not observed at all in SR5 on ‘Day 3’, yet accounted for 49.9 ± 0.9% of VOCs produced by SR3 on this sampling day. SR3 also contained significantly higher abundance of (E)-2-pentenal (**C10**) (4.0 ± 0.8%) than SR5 (0.8 ± 0.6%) on ‘Day 3’. In contrast, a narrow range of abundance was observed for 2-hexenal (**C7**); SR19 had the lowest (1.0 ± 0.5%) and SR2 the highest (6.5 ± 1.6%), with both being the only accessions significantly different from each other.

The more exotic aldehyde (E,E)-2,4-hexadienal (**C11**) was only observed in SR12 (0.3 ± 0.1%) on ‘Day 3’. These observations are interesting in contrast with ‘Day 0’, where no significant differences were observed in any of the aldehyde compounds that were present. Despite the apparent differences in the percentage abundances in compound **C1** (ranging from 2.5% to 22.4%), the fact that no significant differences were observed can most likely be explained by the very large standard errors of each accession, and the highly variable nature of VOC production. It might suggest however, that some varieties are more genetically predisposed to

differing rates of lipid oxidation, that are characteristic of plants kept in storage (Varming et al., 2004).

Figures 4.1-4, which display the PCA plots, also show how individual replicates from each accession vary in VOC abundance and profile within each sampling day. In some cases this can be quite marked, illustrating how profiles can be affected by multiple and very small factors, despite efforts to maintain constant experimental conditions. Further independent experiments will allow better elucidation and confirmation of VOC profiles and relative abundances in rocket in terms of within-day and within-cultivar production.

4.4.3. Correlation of VOC abundance with shelf-life time points: PCA

4.4.3.1. General

PCA of VOCs across and within the three time-points revealed significant correlations between accessions and the prevalence of different types of compounds during storage, indicating sizeable variation amongst the seven accessions. All data are represented as sample scores and loadings plots (Figures 4.1-4).

4.4.3.2. Across-day PCA

PC1 vs. PC2 (F1 and F2; Figure 4.1) accounted for 56.72% of the total variation within data and *r*-values became significant at ±0.434 (p = <0.05). 'Day 0' samples formed a distinct, linear cluster (green; Figure 4.1a) spanning the first principal component. In contrast, however 'Day 3' (yellow) and 'Day 7'(red) samples were not separable and both formed a linear cluster along the second principal component (F2).

Table 4.2 illustrates where significant differences between compounds for each accession were observed. When compared with the loadings plot, it can be

seen that a distinct cluster (1) of volatiles are highly correlated with 'Day 0' along the first principal component, and two other separate localisations of VOCs can be seen correlating in the top and bottom left of Figure 4.1b (cluster 2). This suggests that there may be some genotypic differentiation in terms of the volatiles produced on both 'Day 3' and 'Day 7'. Compounds with strong correlations along the first principal component include (Z)-2-penten-1-ol (**C9** *r* = 0.950), (E)-2-hexenal (**C8** *r* = 0.946), (E,E)-2,4-hexadienal (**C11** *r* = 0.964), 2-methyl-2-butanal (**C6** *r* = 0.916), 5-ethyl-2(5H)-furanone (**C12** *r* = 0.915), 4-methyl-2-(2-methyl-1-propenyl)-pyridine (**C35** *r* = 0.907), ethylidene-cyclopropane (**C24** *r* = 0.912) and 5-methyl-4-hexen-3-one (**C19** *r* = 0.886).

Compounds correlating with accessions along the second principal component, and with SR3 and SR2, on 'Day 3' and 'Day 7' consist of vinylfuran (**C42** *r* = 0.874), (E)-4-oxohex-2-enal (**C1** *r* = 0.889) and tetrahydrothiophene (**C38** *r* = 0.716).

The presence of aldehydes within the 'Day 3' and 'Day 7' clusters is consistent with extensive and prolonged lipid degradation over the shelf life period. The exact role of many of these VOCs is unknown, and the exact significance they may have in affecting human sensory attributes when rocket is consumed is similarly not well understood.

4.4.3.3. Within-day PCA

4.4.3.3.1. 'Day 0'

The first two principal components (F1 and F2; Figure 4.2) explained 50.29% of the total variation present within the sample set and correlations between accessions became significant at $r = \pm 0.433$ ($p = <0.05$). Four clusters are apparent in the scores plot (Figure 4.2a), however only one of these clusters contains all three

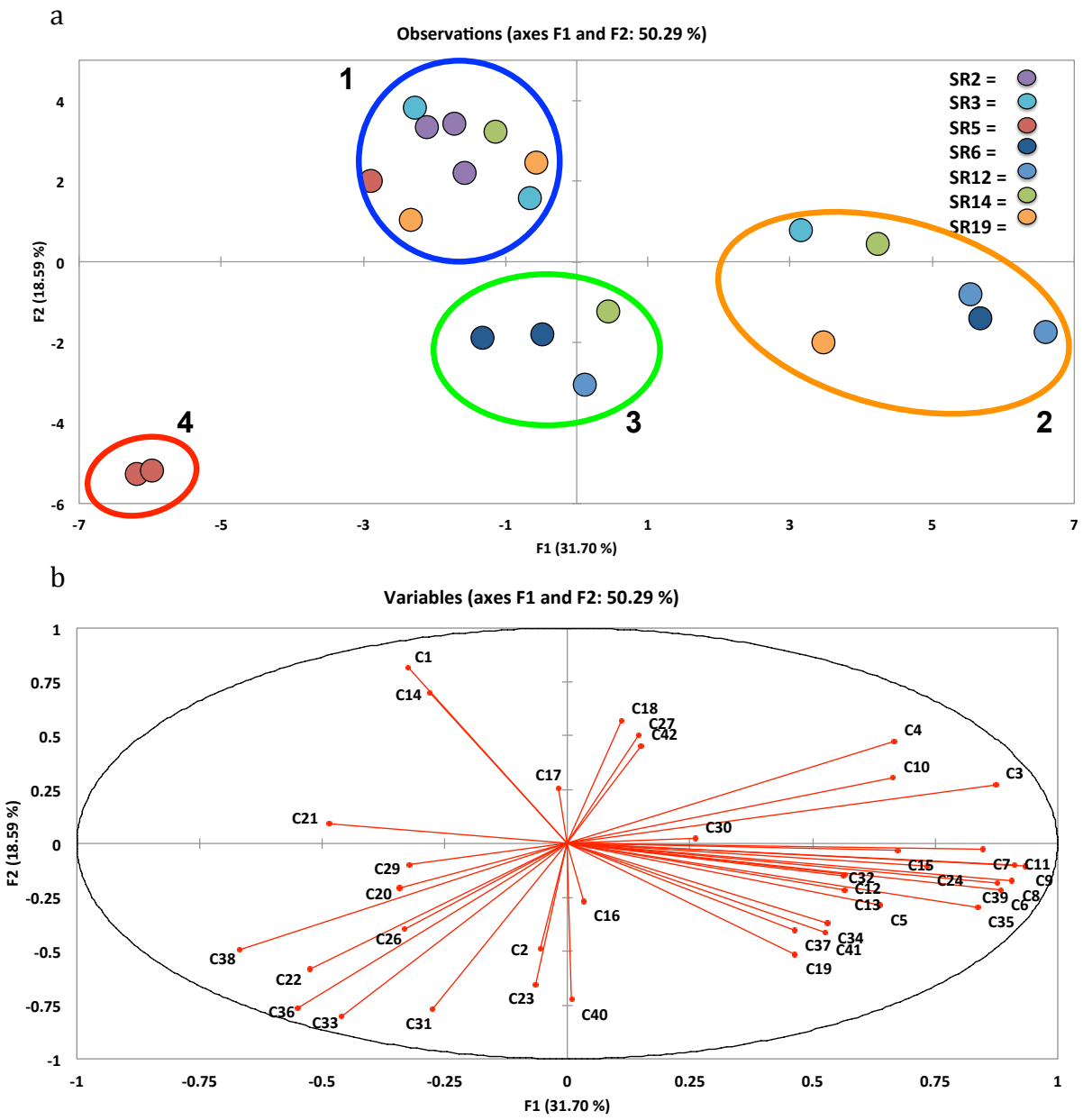

Figure 4.2. Scores plot (a) and loadings plot (b) from Principal Components Analysis of seven accessions of *E. sativa*, and the volatile organic compounds identified on 'Day 0'. Individual replicates were plotted to visualize variation within and between accessions. PC1 vs. PC2 (F1 & F2) accounted for 50.29% of the total variation within data. For compound identities refer to Table 4.1. SR codes refer to each of the seven accessions used in the experiment (SR2, SR3, SR5, SR6, SR12 SR14 & SR19). See inset for accession colour coding. Numbers and circles inset (a) refer to cluster discussed within the text.

respective sample replicates for the accessions (SR2). This spread of individual replicates across clusters indicates that VOC production for different germplasm accessions is inherently variable, even within genotypes.

The cluster located in the top left of the plot (1) contains all three replicates of SR2, indicating a high degree of uniformity in terms of VOC production on the initial sampling day. This cluster also contains two replicates of accessions SR3 and SR19.

It is interesting to note that the commercial accession (SR3) displays more variation than many of the 'wild' germplasm accessions. It would be expected that commercial cultivars would be more uniform than cultivars of open pollinated accessions, as they (in theory) would have had at least had some rudimentary plant breeding before commercial sale. Artificial selections would have been made in a breeding programme to select plants that had desirable characteristics, such as a pungent odour. Throughout successive generations, one would expect the variation associated with such a trait to decrease, but that does not appear to be the case and is perhaps indicative of little concerted breeding to improve varietal uniformity for this trait. When compared with Figure 4.2b, it can be seen that two compounds in particular are correlated with this cluster: (E)-4-oxohex-2-enal (**C1** *r* = 0.816) and 3-hexen-1-ol (**C14** *r* = 0.699). The former of these was highlighted in the previous section as being an abundant compound in accessions, but on sampling 'Day 3' and 'Day 7'.

The second cluster (2) to the extreme right of Figure 4.2a is much less compact, containing two replicates of SR12 and individual replicates of SR3, SR14, SR6 and SR19. Compounds strongly correlated in this position along the principal component in Figure 4.2b are much more numerous and diverse, including (Z)-2-penten-1-ol (**C9** *r* = 0.934), (E,E)-2,4-hexadienal (**C11** *r* = 0.911), (E)-2-hexenal (**C8** *r* = 0.907), 2-methyl-2-butanal (**C6** *r* = 0.884), 4-methyl-2-(2-methyl-1-propenyl)-pyridine (**C35** *r* = 0.837), 2-hexenal (**C7** *r* = 0.847) and ethylidene-cyclopropane (**C24** *r* = 0.733). These compounds are indicative of 'Day 0' VOCs and seem to be produced in most abundance at the initial point of tissue damage, with levels declining or completely disappearing in subsequent sampling days.

The third cluster (3) contains two replicates of SR6 and single replicates of SR14 and SR12. Compounds correlated in this vicinity of the loadings plot are much

more loosely distributed and have no strong correlation with either principal component. The presence of ITC compounds towards the lower section of the analysis plot indicates that production of these compounds is more dominant in certain accessions, or perhaps even individual plants within each accession.

The final cluster (4) consists solely of two replicates of SR5, and is the most extreme within the sample set. As mentioned before, SR5 is a promising cultivar that appears to be very different in many respects to other accessions of *E. sativa*, such as its higher GSL and ITC content.

4.4.3.3.2. 'Day 3'

Figure 4.3 illustrates the PCA for 'Day 3' samples. 46.23% of variation within the data is explained by the analysis, with correlations becoming significant at $r = \pm 0.434$ ($p = <0.05$). Variation between replicates on this sampling day is much reduced, with replicates for each respective accession being much closer together spatially than on 'Day 0'. Clusters are much less well defined, but can be broadly characterized into three groups.

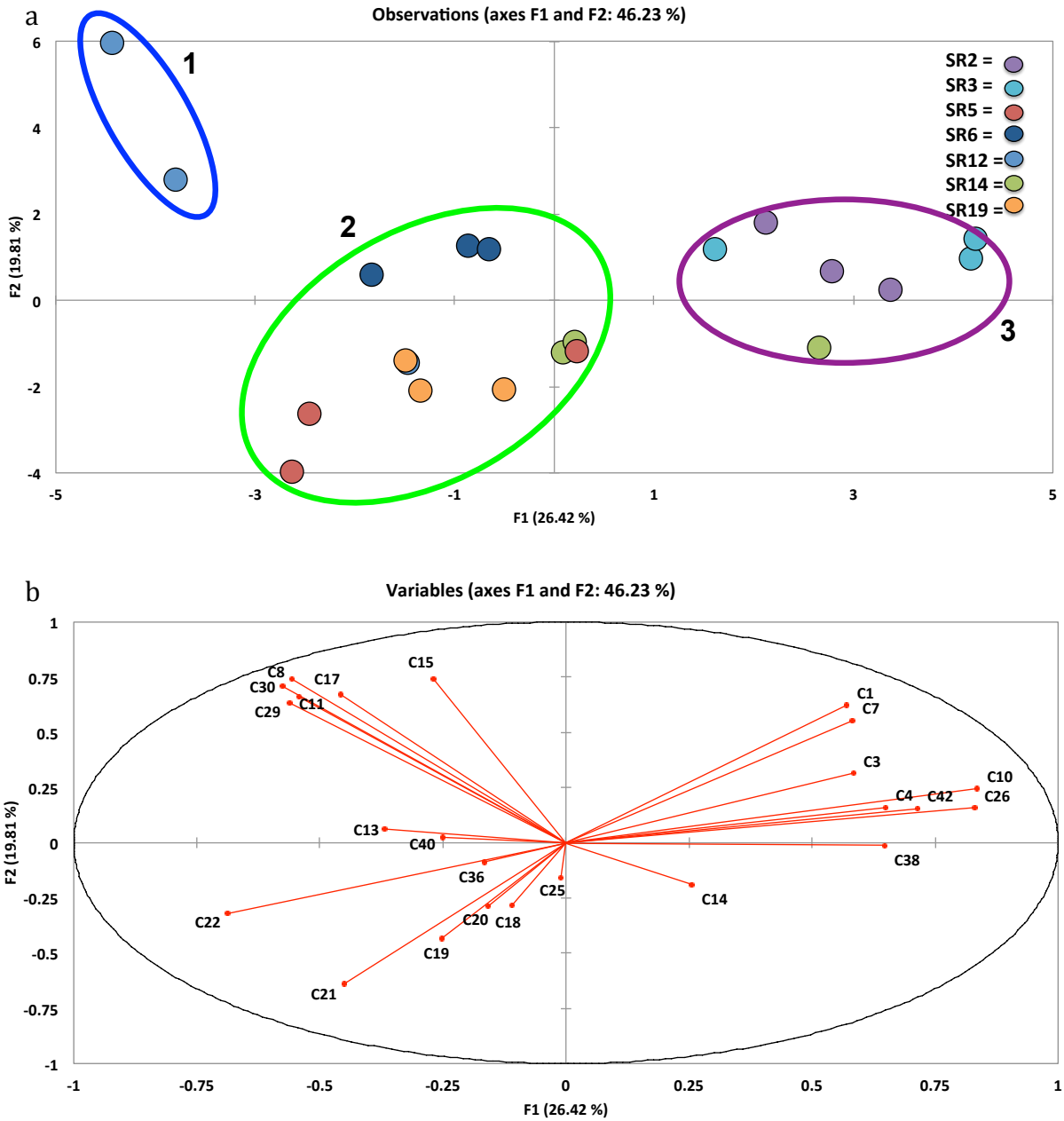

Figure 4.3. Scores plot (a) and loadings plot (b) from Principal Components Analysis of seven accessions of *E. sativa*, and the volatile organic compounds identified on 'Day 3'. Individual replicates were plotted to visualize variation within and between accessions. PC1 vs. PC2 (F1 & F2) accounted for 46.23% of the total variation within data. For compound identities refer to Table 4.1. SR codes refer to each of the seven accessions used in the experiment (SR2, SR3, SR5, SR6, SR12 SR14 & SR19). See inset for accession colour coding. Numbers and circles inset (a) refer to cluster discussed within the text.

The first cluster (1; Figure 4.3a) consists of just two replicates of SR12 which seem to be relative outliers overall (much like SR5 on 'Day 0'). These replicates are generally characterised by the prevalence of a set of VOCs correlated in this same direction: 3-hexenal (**C15** r = 0.741), (E)-2-hexenal (**C8** r = 0.742), 3-octyne (**C17** r = 0.671), and hexyl-ITC (**C29** r = 0.634). Some of these compounds are more

indicative of ‘Day 0’ VOCs, and may indicate a predisposition in this accession for these types of compounds to be produced for a prolonged period of time after the initial production stimuli. This may have implications for the food and agricultural industries, as it implies that beneficial health compounds can be sustained during shelf life by appropriate selection of varieties. The impact of the industrial supply chain on phytochemical content has yet to be properly determined.

The second cluster (2) is central to the plot in Figure 4.3a, stretching towards the lower left. This loose cluster contains all three replicates of SR6, SR5 and SR19, with two replicates of SR14, and a single replicate of SR12. Genotypically, this cluster represents the most diverse range of accessions.

The final cluster to the right side of Figure 4.3a (3) includes all three replicates of SR2 and SR3, and one replicate of SR14. Compounds correlating in this direction include (E)-4-oxohex-2-enal (**C1** r = 0.571), 2-hexenal (**C7** r = 0.582), 1-penten-3-ol (**C3** r = 0.584), (E)-2-pentenal (**C10** r = 0.835), 1-penten-3-one (**C4** r = 0.650), vinylfuran (**C42** r = 0.714), 2-ethyl-furan (**C26** r = 0.830) and tetrahydrothiophene (**C38** r = 0.647). These compounds are indicative of the ‘Day 3’ profile highlighted in the previous section.

4.4.3.3.3. ‘Day 7’

Figure 4.4 displays the PCA plots for ‘Day 7’, where 58.69% of variation was explained by the data, and correlations became significant at $r = \pm 0.434$ ($p = <0.05$). In Figure 4.4a, samples can be broadly divided into two clusters, left and right of the y-axis. Samples on the left are tightly clustered and those to the right are thinly spread along the x-axis. SR3, SR6 and SR5 have replicates contained in both clusters, which is unusual considering that on ‘Day 3’ they were relatively close

together. SR3 is the most extreme example with replicates spread very far apart, indicating increased variability under controlled environmental conditions.

The dense left cluster (1) contains all replicates of SR14, SR12 and SR19, two replicates of SR5 and one replicate of SR3, but contained no significant correlations with any compounds.

The cluster on the right of the plot (2) contains all replicates of SR2, two replicates of SR3 and SR6, and one replicate of SR5. Many of the compounds seem to be skewed on the loadings plot in the direction of the SR3 and SR2 replicates. This suggests that these accessions have maintained a degree of VOC diversity later in the shelf-life period than the other accessions, which may be attributable to differing genetics. Compounds correlated with these samples along the first principal component include: 2-hexenal (**C7** r = 0.920), 3-hexenal (**C15** r = 0.862), (E)-4-oxohex-2-enal (**C1** r = 0.840), 1-penten-3-ol (**C3** r = 0.837), (E)-2-pentenal (**C10** r = 0.791), (E)-2-hexenal (**C8** r = 0.803), 3-octyne (**C17** r = 0.697), 2-ethyl-furan (**C26** r = 0.663). The presence of aldehyde compounds in this group is again indicative of extensive lipid breakdown.

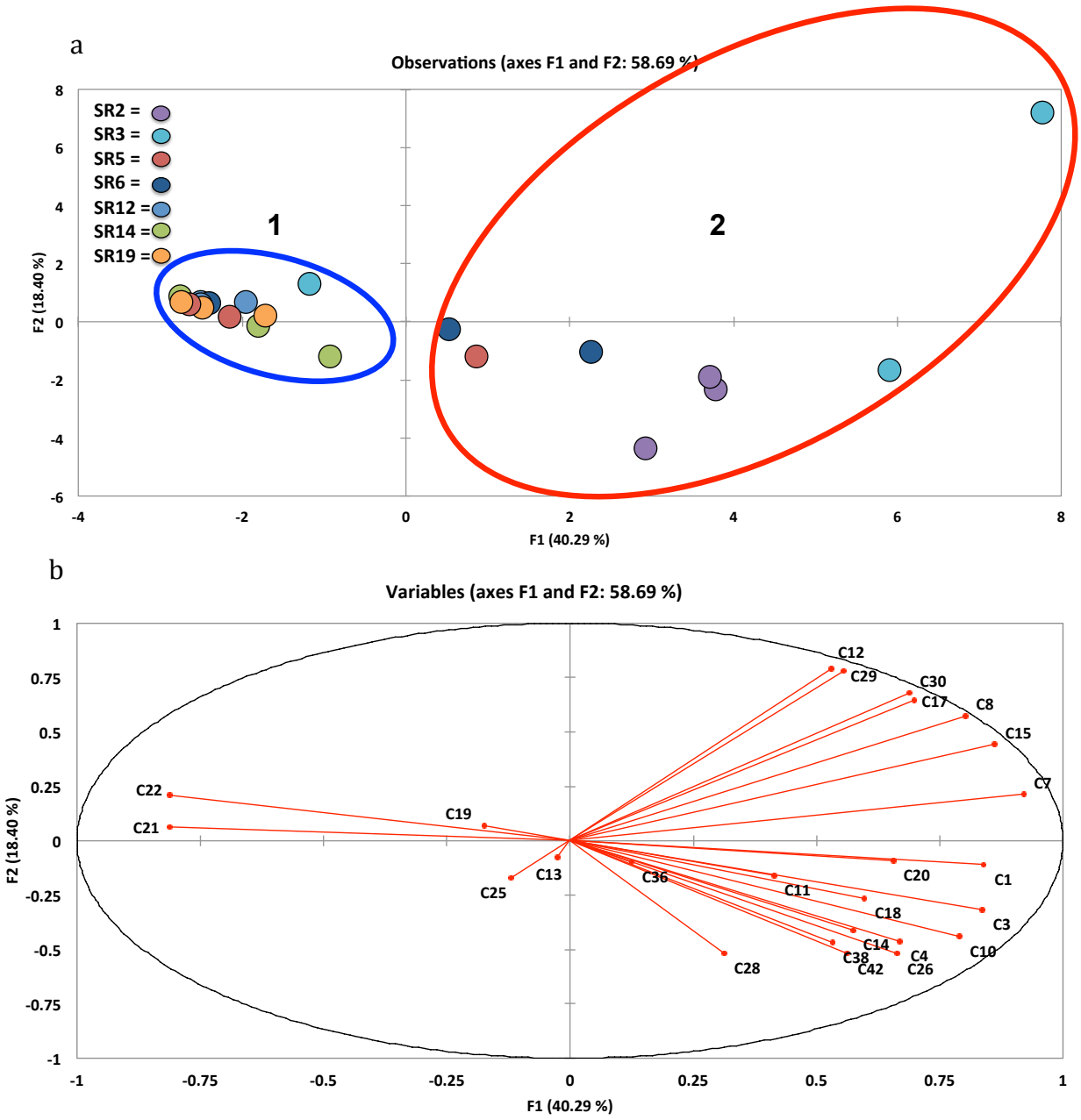

Figure 4.4. Scores plot (a) and loadings plot (b) from Principal Components Analysis of seven accessions of *E. sativa*, and the volatile organic compounds identified on 'Day 7'. Individual replicates were plotted to visualize variation within and between accessions. PC1 vs. PC2 (F1 & F2) accounted for 58.69% of the total variation within data. For compound identities refer to Table 4.1. SR codes refer to each of the seven accessions used in the experiment (SR2, SR3, SR5, SR6, SR12 SR14 & SR19). See inset for accession colour coding. Numbers and circles inset (a) refer to cluster discussed within the text.

4.4.4. Implications of detected VOCs on rocket quality and human nutrition

Between 1.0 ± 0.6% (SR3) and 6.8 ± 2.2% (SR5) of volatile compounds produced on 'Day 0' are isothiocyanates that may have potential benefits for human health (Verkerk et al., 2009). Although present at relatively low levels compared to other VOCs detected, isothiocyanates have been shown to be efficacious in eliciting

health benefits at very low concentrations in previous *in vitro* studies with other crops (10 μM; Hanlon, Webber & Barnes, 2007). Abundance of these compounds declined with storage to less than one percent on 'Day 7', indicating that by the time the rocket leaves reach the consumer, it is possible that there has been a substantial drop in nutritional value. Processes that precede packaging, such as harvest, transport and washing can be especially harsh on leaves, although some forms of stress can induce secondary metabolite production (Mewis et al., 2012). Thus, combined effects of storage and handling during the supply chain should be examined in relation to VOC loss.

4.5. Conclusions

Our results represent rocket plants grown under controlled environment conditions, and may differ from plants that are grown under variable field conditions. Many pre-harvest factors may affect the abundance and ratios of VOCs, such as disease status, soil type, light intensity and water status (Varming et al., 2004). Our method was established in order to minimize these stress effects and produce results based on genotypic and post-harvest storage factors, rather than pre-harvest environmental variation. Changes in phytochemical content between different growing environments are poorly documented in rocket species, and non-existent for VOCs. Future work will examine the effects that these factors may have on the crop and the end consumer by conducting experiments in a commercial, field trial setting.

Our study has shown that there are significant differences amongst cultivars in the relative abundance of volatiles they produce post-harvest and their retention during storage. Therefore, there is scope for plant breeders to consider basing selections, at least in part, on post-harvest VOC profiles in order to select for improved flavour and nutritional value traits. By assessing such data in the supply

chain alongside phytochemical screening at harvest, rocket lines can feasibly be bred to limit losses of important VOCs, such as ITCs. More research is needed to fully understand where exactly these losses occur within the supply chain, and what (if anything) can be done to mitigate such potential losses.

The sampling methodology established here might also have potential applications within the food processing industry as part of quality assurance methods for rocket leaves. Sampling itself is straightforward and requires little specialist knowledge. Samples could be taken at critical points during processing to assess effects on VOC profiles with the aim of keeping ITC losses to a minimum. Most, if not all, producers routinely take and store samples of different batches of rocket salad to assess visual traits. In the near future, it is likely that quality assurance will expand beyond this to both phytochemical and volatile traits of many crops, not just rocket salad.

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CHAPTER 5: Analysis of Seven Salad Rocket (*Eruca sativa*) Accessions: The Relationships Between Sensory Attributes and Volatile and Non-volatile Compounds

5.1. Introduction To Paper (accepted by *Food Chemistry*)

After determining the inherent variability of phytochemicals and headspace volatiles present between cultivars of rocket, a second phase of research was initiated. This was focused on the sensory characteristics of rocket leaves, and how phytochemical constituents might influence responses. Much of the previous research concerning rocket sensory attributes has been minimal, and often focusing on shelf life aspects rather than the actual variability in tastes, odours and flavours found between cultivars. Researchers and industrial processors have often assumed that these attributes are dependent on storage, rather than any genetic or chemical basis.

In this paper, we aimed to address these ideas and subject the same seven cultivars (as used in Chapter 4) to rigorous and detailed sensory analysis, combined with the phytochemical and volatile data presented in the previous chapters. Collaboration was formed as part of this experiment with Dr. Angelo Signore of the University of Bari, Italy, who performed the analysis of polyatomic ions.

5.2. Introduction

Rocket and other members of the *Brassicaceae* plant family have been consistently shown to contribute beneficial, health-promoting phytochemicals to the human diet (Holst & Williamson, 2004). Consumption of such vegetables, that contain glucosinolates (GSLs) and flavonols in particular, is associated with a reduced risk of numerous cancers (Higdon, Delage, Williams, & Dashwood, 2007)

and improved cardiovascular health (Podsedek, 2007). In this study we consider several phytochemical attributes that may also contribute to sensory traits of rocket, as well as influence nutritional 'quality'.

Glucosinolates react with myrosinase enzymes (thioglucoside glucohydrolase, EC 3.2.1.147) to form several classes of compound which have potential benefits to human health (Saha et al., 2012). These products (particularly isothiocyanates; ITCs, thiocyanates, nitriles and sulphates) are thought to be primarily responsible for the array of sensory perceptions that humans detect in *Brassicaceae* vegetables. ITCs can result in bitter taste perception due to thiourea moieties, such as those found in synthetic bitter compounds like 6-n-propylthiouracil (PROP; Lipchock & Mennella, 2013). ITCs are also known to contribute to the hot and burning perceptions on the tongue (Cartea, Velasco, Obregon, Padilla, & de Haro, 2008), as well as pungent aromas. Thiocyanates are thought to infer bitter taste (Drewnowski & Gomez-Carneros, 2000), and sulfates the sulfurous, 'rotten cabbage' aromas and flavours often experienced (Pasini, Verardo, Cerretani, Caboni, & D'Antuono, 2011). A previous study (Pasini et al. 2011) indicated that the individual glucosinolate and flavonol compounds in rocket contributed towards different sensory perceptions. The GSLs progoitrin/epiprogoitrin and dimeric-mercaptobutyl glucosinolate (DMB) were significantly associated with bitter taste, and total GSL content with perceived pungency. This study did not quantify the two forms of glucosativin separately however, (Cataldi, Rubino, Lelario, & Bufo, 2007), and it is unknown whether they infer differing sensory properties.

Flavonols are also thought to contribute towards the taste and aroma of *Brassicaceae* plants. Research is somewhat lacking in this area for the *Brassicaceae*, but studies conducted in other plants/foods (such as *Ribes rubrum,*

redcurrant juice) have found that flavonols are generally associated with astringent and bitter sensations (Schwarz & Hofmann, 2007).

The effect of polyatomic ion (PI) content and concentration on rocket sensory profiles has not been previously considered. PIs are covalently bonded atoms that act as single units or become dissociated from larger molecules, and can be created when small molecules become negatively charged. For example, hydrogen sulfate (HSO_4-) is the polyatomic anion of sulfuric acid (H_2SO_4). Rocket is known to accumulate high nitrate (NO_3-) concentrations (Jakše, Hacin, & Kacjan Maršić, 2013) but it is not known how this, and other PIs such as chlorides, phosphates and sulphates impact upon sensory attributes in the plant.

Free amino acids (AAs) are ubiquitous compounds found within foodstuffs and living organisms, and vary in relative concentration/abundance. They are known to contribute to sensory perceptions in foods, but to date no study has considered this in rocket. Some compounds such as glutamic acid infer savoury (umami) attributes in fruits such as tomato (Jinap & Hajeb, 2010) for example; whereas others may taste sweet (alanine), sour (asparagine), or bitter (leucine; Kirimura, Shimizu, Kimizuka, Ninomiya, & Katsuya, 1969). In this way, it is thought that they modify or enhance the flavours and tastes of food. The effects of sugars and organic acids (OAs) on taste/aroma/flavours has not been previously determined in rocket. It is widely known that sweetness reduces the perception of bitterness, but the degree to which this effect occurs in rocket leaves is poorly understood. OAs typically infer sour taste, and the relative abundances in crops such as tomato are known to infer changes to flavour (Jinap & Hajeb, 2010).

The rocket species *Eruca sativa* is commonly known as ‘salad’ or ‘cultivated’ rocket, and is notable for having hot, peppery and bitter attributes (Pasini et al. 2011). In this study a sensory profile of seven *E. sativa* accessions was developed, using a

trained sensory panel, to objectively quantify an agreed vocabulary of various sensory traits. The data were analysed in conjunction with chemical analyses of rocket, cultivated in controlled environment conditions, to determine which specific variables have an impact significantly on sensory properties. We hypothesised that the increased relative concentrations and abundances of the major GSL/ITC compounds alongside the concentration of PIs, free sugars, free AAs and OAs would be key influencing factors in the pungency and bitterness of the accessions.

5.3. Materials and Methods

5.3.1 Plant Material

For the source of each of the seven accessions used in this paper, and the exact controlled environment conditions under which plants were grown, see Bell, Oruna-Concha & Wagstaff (2015; Chapter 3). 20 accessions were analysed by this previous study, and the seven selected here represent a diverse range of GSL and flavonol profiles. Another factor for consideration was the availability of seed that could be provided by Elsoms Seeds Ltd. (Spalding, UK). SR2, SR5, SR6, SR12, SR14 and SR19 are accessions sourced from European germplasm collections, and SR3 is a commercially available cultivar sold by Elsoms Seeds Ltd.

Each accession was germinated in a Fitotron controlled environment room (Weiss-Technik UK, Loughborough, UK) after being sown in a random sequence (using random number allocation in Microsoft Excel; Microsoft Corp., Redmond, WA, USA). Growth of plants was staggered over seven days to ensure that all leaves were of the same age (30 days) on each of the sensory assessment days. Plants were harvested each morning of the study (~10.00 am). After transport, samples were washed with cold water to remove any soil detritus and prepared under food grade conditions. Leaves were stored in a fridge (~4°C) until ready to be served to

assessors (between 12.30 pm and 2.00 pm). Leaves were selected at random from zip-loc storage bags when preparing samples for presentation on plates.

For chemical analyses, the leaves of four plants were harvested together and collectively treated as one replicate. There were three 'blocks' of four plants for each accession, resulting in a total of three replicates per accession (n = 3); therefore a total of 12 plants were used as representative samples of each population. Leaves were harvested in an identical fashion as outlined above, but placed immediately into a -80°C freezer after transport. Samples were lyophilized in batches and ground into a fine powder using a miniature coffee grinder.

5.3.2. Sensory Analysis

Sets of sensory descriptors for rocket were established using an expert panel of eleven sensory assessors (see Table 5.1 for definitions of terms used). Panelists were selected and trained in accordance with ISO standards for sensory analysis (ISO 8586:2012) and are subject to performance monitoring (ISO 11132:2012). All panelists had a minimum of 6 months experience in sensory evaluation, and some up to eight years of experience.

Samples were presented in a random, coded fashion over the course of five, half-hour sessions on consecutive days. Assessors discussed, with the aid of a facilitator, the various sensory attributes associated with the appearance, odour, mouthfeel, taste, flavour and aftereffects of leaf samples. Reference standards were used where appropriate to ensure agreement of the descriptive terms chosen. For example, for mustard attributes, assessors used a jar of Colman's Mustard (Colman's, Norwich, UK) as a reference. Once a consensus set of descriptors was established, a formal sensory assessment was conducted.

Table 5.1. Definitions for sensory attributes associated with 7 *Eruca sativa* accessions

Attribute	Agreed definition
Appearance	
Leaf shape	Variability of leaf shape between the two presented; none – completely different
Depth of colour	Shade of green; light green – dark green
Leaf size	Small, medium or large in reference to a scale provided to assessors
Hairiness	Extent of visibility of hairs on leaf petiole and underside of lamina
'Purple' stem	Presence of pink, red or purple within the stem, petiole or midrib of leaves
Odour	
Sulfur	Aroma associated with eggs
Green	Aroma(s) associated with cut grass and freshness
Stalky	Dry aroma associated with dried leaves or grasses
Pepper	Pungent aroma associated with ground peppercorns
Earthy	Resembling or suggestive of earth or soil
Burnt rubber	An aroma reminiscent of burning rubber
Pungent	A sharp aroma; associated with perceived strength
Sweet	A pleasant, sugary aroma
Aromatic	A pleasant aroma associated with herbaceous oils
Mustard	Potent aroma associated with crushed mustard seeds or condiment mustard
Mouthfeel	
Initial heat	The initial burst of 'hotness' on the tongue momentarily after placing into the mouth and chewing
Spikiness	Sensation associated with the sharpness of any leaf hairs that may be present on samples
Crispiness	Brittle sensation on the teeth or tongue when chewing or biting leaves
Chewiness	Degree of ease with which leaves are chewed and swallowed
Toughness	Degree of ease with which leaf stems can be broken by the teeth
Moistness	Associated with the water content of the leaf samples ingested
Salivating	Degree to which samples induced the production of saliva in the mouth upon chewing
Astringent	Degree to which samples induced drying and/or the sensation of shrinkage of the tongue and soft palate
Tingliness	The sensation produced upon the tongue; associated with slight prickling or stinging
Warming	The sensation of increased temperature within the mouth while chewing; prolonged and separate from "initial heat"
Taste	
Sweet	Pleasant taste associated with sugary foods

Sour	Acidic sensation associated with vinegar
Bitter	Sharp, unpleasant or pungent taste upon the tongue
Savoury	Taste associated with slightly salty or spicy food
Flavor	
Green	Flavor associated with cut grass and freshness
Stalky	Flavor associated with dry, fibrous leaves
Peppery	Flavor associated with ground peppercorns
Mustard	Flavor associated with the potency of crushed mustard seeds or condiment mustard
Sulfur	A flavor associated with consumption of eggs
Earthy	A flavor resembling or suggestive of earth or soil
Aftereffects	
Bitter	A persistence of bitter taste after swallowing leaf samples
Sweet	A persistence of pleasant, sugary taste
Acid	Persistence of a sharp, unpleasant taste upon the tongue; reminiscent of vinegar
Savoury	Persistence of a salty or slightly spicy flavor upon the tongue
Peppery	Persistence of the flavor of peppercorns
Mustard	Persistence of the flavor of mustard seed/condiment mustard
Green	Persistence of a grassy, fresh flavor
Earthy	Persistence of flavours resembling or suggestive of earth or soil
Warming	A persistence of the sensation of heat/temperature within the mouth after swallowing

Sensory descriptors were entered into Compusense software (version 5.2; Guelph, ON, Canada) and assessors were asked to score each attribute on anchored unstructured line scales (15 cm, scaled 0-100), with each anchor corresponding to the agreed extremes of each attribute definition. Each accession was presented and assessed twice by each of the 11 panelists, and averaged. Odour, taste, flavour and aftereffects were assessed as an overall representation of the two leaves presented per accession (n = 22). Due to the variability of leaf morphology within gene bank accessions, the test was designed to ask assessors about the sensory characteristics of two leaves separately for appearance and mouthfeel descriptors (n = 44), which were then averaged.

Stem colour was the only attribute assessed using a multiple-choice question (categories: white/green or pink/red/purple). *E. sativa* accessions show gradations of colouring in the leaf stem and it is thought to be a desirable commercial trait. Colour can range from being absent, to pink, to red, to purple. If colour was present, assessors selected 'pink/red/purple' and were asked to score the degree of this coloration on a standard, anchored line scale. Assessors were presented with a size chart encompassing the extremes of rocket salad leaf sizes in order to standardise responses. This indicated into which range on the line scale they should enter their response based on the leaf area (three size examples were given).

Evaluation sessions were carried out under artificial daylight conditions in an air-conditioned room (~22°C), in isolated sensory booths within the Sensory Science Centre at the Department of Food & Nutritional Sciences, University of Reading, UK. Freshly harvested plant samples were presented twice to each assessor in a balanced order over five days (approximately two to three hours after harvest). Two random leaves from each accession were placed on a single plate with a randomly assigned, three-digit code. Panelists were provided with water (room temperature)

and frozen natural yoghurt (Yeo Valley Farms (Production) Ltd., Bristol, UK) for palate cleansing between samples. Warm water was also provided for assessors to wash their fingers between samples, to avoid carry-over of aromas to subsequent samples. No more than four samples were presented in any one session to avoid palate/trigeminal fatigue. There was a one-minute time delay between the finishing of one sample and the presenting of the next.

5.3.3. Reagents & Chemicals

All solvents and chemicals used were obtained from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. The EZ:faast Free (Physiological) Amino Acid Analysis by GC-MS kit was obtained from Phenomenex (Macclesfield, UK).

5.3.4. Glucosinolate & Flavonol Analysis

GSLs and flavonols were extracted and analysed by LC-MS and presented in Bell et al. (2015; Chapter 3). Briefly, lyophilized leaves were milled, and extracted using 70% methanol at 70°C. Crude extracts were filtered and diluted (n = 3) before being run on a HPLC and ion trap mass spectrometer (MS/MS) with an isocratic gradient of 95% water (0.1% ammonium formate) and 5% acetonitrile over a 40 minute run. GSLs and flavonols were quantified separately at two different wavelengths and quantified by two different external standards (GSLs: sinigrin hydrate; flavonols: isorhamnetin).

5.3.5. Polyatomic Ion Analysis By Ion Chromatograph

Lyophilized rocket powder for each accession (n = 3) was re-dried after transport (to the Dipartimento di Scienze Agro-Ambientali e Territoriali, University of Bari, Italy) at 65°C, and subsequently re-milled with a micrometric mill (IKA,

Germany). 0.5 g of the material was placed in a bottle of 100 ml, to which 50 ml of a solution composed of Na_2CO_3 (3.5 mM) + $NaHCO_3$ (1.0 mM) was added. The bottle was shaken for 20 minutes (145 rpm). Before inserting the solution into the ion chromatograph (IC), the supernatant was filtered using a 0.22 μm filter to remove any residual organic matter. A Dionex DX-120 Chromatograph (Dionex Corporation, Sunnyvale, CA, USA) was used to measure chloride, nitrate, phosphate and sulphate anions by comparison to a multi-anion standard (Dionex, Milan, Italy).

5.3.6. VOC Analysis

VOCs were extracted and analysed presented in Bell, Spadafora, Müller, Wagstaff & Rogers (2016; Chapter 4), using Thermal Desorption Gas Chromatography Time-Of-Flight Mass Spectrometer (TD-GC-TOF-MS); see this paper for detailed methodology.

Briefly, rocket leaves of each accession (70g) were placed into sealed bags and manually disrupted to release volatiles into the headspace (n = 3). Samples were collected using a hand-pump device attached to a portable thermal desorption tube, which was inserted through a port in the bag. Tubes were desorbed using a TD100 thermal desorption system (Markes International Ltd., Llantrisant, Wales, UK) and samples analysed using a BenchTOF-dx mass spectrometer (Almsco International, Cincinnati, OH, USA).

5.3.7. Free Amino Acid Analysis

Lyophilized rocket powder (50.0 mg; n = 3) was added to 0.5 ml of 25% acetonitrile in 0.01 M hydrochloric acid. Samples were vortexed for five minutes and left to settle for one hour at room temperature (~22°C), and then centrifuged. The supernatant of each sample was removed and filtered with 0.22 μm filter discs with a

low protein binding Durapore polyvinylidene fluoride (PVDF) membrane (Millex; EMD Millipore, Billerica, MA, USA).

A diluted aliquot of the filtrate (10 μl sample, 90 μl H_2O) was derivatized using the EZ:faast Free (Physiological) Amino Acid Analysis by GC-MS kit. GC-MS analysis of the derivatized samples was carried out using an Agilent 7890A/5795C GC-MS instrument as described by Elmore, Koutsidis, Dodson, Mottram, & Wedzicha (2005). Samples were quantified using an internal standard of norvaline.

5.3.8. Free Sugars & Organic Acid Analyses

Lyophilized rocket powder (0.4 g; *n* = 3) was suspended in 10 mL of 0.01 M hydrochloric acid (except SR19 where dried material of two replicates was depleted; *n* = 1). Each sample was stirred for 30 min at room temperature (~22°C), and the mixture was set aside to settle for 30 min. An aliquot of the supernatant (1.5 mL) was centrifuged for 30 min. The supernatant of the resulting extract was filtered with a Millex Millipore sterile syringe driven filter unit (0.22 μm) and analysed by capillary electrophoresis (CE). An external standard method for sugars (glucose, fructose, sucrose, and galactose) and OAs (malic acid and citric acid; ranging from 0.5 – 10 $mg.g^{-1}$) was used for the quantification of the analytes of interest.

The CE method used was adapted from Lignou, Parker, Oruna-Concha, & Mottram (2013) and Soga & Ross (1999). Briefly, a HP3D CE with DAD and Agilent ChemStation software (Santa Clara, CA, USA) was used to run sugars and OAs within the same chromatographic run. Electrophoretic separation was performed at a constant pressure of 50 mbar, with a six second injection of sample, followed by a four second injection of buffer. A G1600-61311 capillary (Agilent, Stockport, UK) was used which measured 75 μm id, 64.5 cm in length, with an effective length of 56 cm,

maintained at 15°C. An anion buffer was used for sample separation and the column was preconditioned for four minutes with buffer before each run.

5.3.9. Statistical analysis

5.3.9.1. ANOVA

To analyze the sensory profiling data, two-way analysis of variance (ANOVA; with accessions and assessors as treatment effects, and these main effects tested against their interaction) was carried out in Senpaq (Qi Statistics Ltd., Reading, UK). ANOVA was conducted using a 95% confidence interval and a tolerance of 0.0001%. A post-hoc Tukey's HSD test was used for multiple pairwise comparisons. This was chosen for the higher level of stringency than other pairwise comparison tests, such as Fisher's LSD Test.

The quantitative data for each compound identified in the CE, IC and GC analyses (sugars, OAs, PIs, AAs) were analysed independently by one-way ANOVA using XL Stat (Addinsoft, Paris, France). Significant differences between varieties were determined using Tukey's HSD test to generate pairwise comparisons.

5.3.9.2. Principal Component Analysis

The means for the sensory data were taken (as described in section *2.2.*) and used in Principal Component Analysis (PCA, Pearson *n*-1; XL Stat) to extract principal components (PCs). Sensory relationships were determined by coefficient analysis. Phytochemical data obtained from PIs, free sugars, organic acids, and free AAs were collated with data from Bell et al. (2015; Chapter 3) for GSLs and flavonols, and data from Bell et al. (2016; Chapter 4) for headspace VOCs. These were regressed onto the sensory PCA as supplementary data, and a correlation matrix was constructed to determine significant relationships. Sensory variables with

statistically significant correlations were identified at levels of $P<0.05$, <0.01 and <0.001.

5.4. Results and discussion

5.4.1. Sensory Attributes

5.4.1.1. Appearance

A summary table of sensory attribute scores can be found in Table 5.2, along with pairwise comparison statistical significances and the typical appearance of each cultivar can be seen in appendix II.

Leaf sizes varied greatly across each accession; SR14 and SR12 had very large leaves, whereas SR5 and SR19 were significantly smaller by comparison. The range of sizes could potentially give breeders traits to select within gene bank populations, where new, novel types can be identified. Significant differences were also found for, depth of colour, leaf shape, hairiness and the prevalence of 'purple stem' (Table 5.2; $P<0.05$). SR2 had a significantly higher degree of colouration in the stem than both SR5 and SR19, potentially making this a desirable accession to select this trait from.

5.4.1.2. Odour

There was a significant difference overall between samples for sulfur odour (Table 5.2; $P<0.05$). No other odour attributes were significantly different between accessions. The strength of sulfur traits may play a key role in this differentiation between rocket accessions and consumer preferences (Pasini et al. 2011), though consumer studies of rocket cultivars are lacking in the literature.

Table 5.2. Average values of sensory traits of *Eruca sativa* accessions rated by 11 trained panel assessors.

Sensory trait	Accession							Significance (*P* values)	
	SR2	SR3	SR5	SR6	SR12	SR14	SR19	Sample	Sample* assessor
Appearance									
Depth of leaf colour (A)	57.3^{a}	59.5^{a}	60.8^{ab}	61.0^{ab}	60.9^{ab}	63.5^{ab}	68.1^{b}	0.0111*	0.9228
Leaf shape (A)	40.5^{a}	41.8^{ab}	37.4^{a}	42.9^{ab}	57.7^{bc}	63.9^{c}	66.7^{c}	<0.0001*	0.3526
Leaf size (A)	51.3^{bc}	40.8^{abc}	31.4^{a}	38.9^{ab}	55.9^{c}	56.3^{c}	33.8^{a}	<0.0001*	0.9933
Hairiness (A)	10.6^{bc}	1.7^{ab}	1.2^{a}	0.2^{a}	4.1^{abc}	11.7^{c}	1.0^{a}	0.0002*	0.0001*
Purple stem (A)	36.0^{b}	31.0^{ab}	17.7^{a}	32.1^{ab}	25.3^{ab}	30.8^{ab}	13.7^{a}	0.2387	0.0009*
Odour									
Sulfur (O)	11.8^{ab}	15.4^{ab}	19.9^{b}	7.3^{a}	9.0^{a}	13.8^{ab}	11.3^{ab}	0.0198*	0.0088*
Green (O)	33.4	37.8	34.6	34.4	34.1	40.0	39.2	0.2663	0.7788
Stalky (O)	21.4	27.4	29.0	24.6	24.0	25.5	28.0	0.4152	0.2568
Pepper (O)	12.9	12.1	15.3	13.6	12.9	15.6	17.2	0.2489	0.4200
Earthy (O)	10.5	11.0	7.9	12.6	10.7	13.6	10.6	0.4603	0.1956
Burnt rubber (O)	6.6	5.5	11.3	4.3	4.3	5.9	7.2	0.4274	0.0193*
Pungent (O)	14.8	19.5	20.8	13.3	11.6	12.9	16.9	0.1334	0.1200
Sweet (O)	12.0	12.4	10.0	11.1	14.5	12.2	14.4	0.4247	0.0219*
Aromatic (O)	4.1	6.4	7.0	5.9	6.0	9.2	5.1	0.3226	0.9454
Mustard (O)	9.2	12.2	16.5	12.1	7.9	11.6	11.3	0.0958	0.2066
Mouthfeel									
Initial heat (MF)	21.3^{a}	20.8^{a}	37.8^{c}	21.8^{a}	21.5^{a}	24.6^{ab}	31.4^{bc}	<0.0001*	0.5755
Spikiness (MF)	2.9^{ab}	0.2^{a}	2.3^{ab}	1.4^{ab}	2.1^{ab}	4.3^{b}	1.1^{a}	0.1153	0.1393

Crispiness (MF)	14.2	15.4	16.3	17.4	15.8	17.4	18.7	0.5049	0.0114*
Chewiness (MF)	20.3^{ab}	17.9^{a}	23.1^{ab}	23.5^{ab}	21.2^{ab}	25.7^{b}	19.4^{ab}	0.0381*	0.0273*
Toughness (MF)	16.4	15.4	17.6	20.3	17.2	20.7	15.0	0.0418*	0.3210
Moistness (MF)	23.6	24.9	21.3	22.9	24.3	22.3	24.5	0.5499	0.3206
Salivating (MF)	19.5	18.0	17.2	19.0	17.2	19.9	21.9	0.3921	0.1493
Astringent (MF)	17.9	15.8	19.5	15.9	19.1	15.1	14.6	0.2518	0.0004*
Tingling (MF)	10.8^{abc}	8.5^{a}	18.8^{c}	9.7^{ab}	9.5^{ab}	13.0^{abc}	16.8^{bc}	0.0010*	0.1998
Warming (MF)	16.4^{a}	13.7^{a}	26.0^{b}	14.0^{a}	14.1^{a}	18.3^{ab}	22.4^{ab}	0.0008*	0.0847
Taste									
Sweet (T)	9.6	9.5	7.1	11.6	12.7	10.1	13.5	0.2740	0.4906
Sour (T)	6.5	6.1	8.5	5.1	5.0	5.4	6.7	0.4743	0.1067
Bitter (T)	20.1	23.2	29.2	23.4	25.3	21.7	24.6	0.1682	0.5437
Savoury (T)	11.6	15.7	17.9	14.0	12.3	16.7	15.1	0.0361*	0.4433
Flavour									
Green (F)	31.0	32.2	27.9	31.4	29.1	36.0	35.2	0.2599	0.5475
Stalky (F)	19.7	25.5	29.1	19.6	21.3	21.8	24.5	0.0613	0.6718
Peppery (F)	16.5^{abc}	12.8^{a}	21.2^{bc}	14.9^{ab}	14.2^{ab}	16.9^{abc}	22.6^{c}	0.0009*	0.7834
Mustard (F)	16.9^{abc}	15.4^{ab}	27.3^{c}	19.3^{abc}	11.6^{a}	16.7^{abc}	24.8^{bc}	0.0003*	0.8755
Sulfur (F)	8.2^{a}	10.5^{a}	20.7^{b}	10.8^{ab}	8.5^{a}	11.4^{ab}	11.6^{ab}	0.0076*	0.0697
Earthy (F)	8.6	6.8	10.2	8.2	10.7	10.0	7.8	0.5436	0.8714
Aftereffects									
Bitter (AE)	20.5	19.7	24.9	23.3	21.3	21.6	23.5	0.4921	0.7345
Sweet (AE)	6.8	5.9	3.6	7.8	8.4	6.7	6.2	0.4044	0.0375*

Acid (AE)	4.6^{ab}	4.2^{ab}	9.2^{b}	3.2^{a}	2.5^{a}	4.0^{ab}	4.7^{ab}	0.0175*	0.0544
Savoury (AE)	10.0	12.0	13.0	12.1	12.2	13.9	12.3	0.5462	0.8850
Peppery (AE)	15.9^{ab}	12.6^{a}	20.4^{b}	14.4^{ab}	14.5^{ab}	16.5^{ab}	19.3^{ab}	0.0116*	0.8395
Mustard (AE)	13.0^{a}	12.3^{a}	21.4^{b}	15.1^{ab}	12.0^{a}	14.2^{ab}	19.9^{ab}	0.0016*	0.8292
Green (AE)	21.5	24.9	20.9	21.8	21.1	23.6	26.6	0.2629	0.7435
Earthy (AE)	7.5	7.7	7.5	7.4	10.6	10.6	7.1	0.3296	0.9384
Warming (AE)	19.6^{abc}	13.8^{a}	26.2^{c}	16.4^{ab}	13.7^{a}	22.5^{bc}	23.9^{bc}	<0.0001*	0.9869

Abbreviations: A, appearance; O, odour; MF, mouthfeel; T, taste; F, flavor; AE, aftereffects. Significantly different values indicated by superscript letters within rows (ANOVA Tukey's HSD test, P ≤ 0.05). An absence of letters indicates no significant difference was observed. * Denotes significance (P = < 0.05) for the sample and sample*assessor interactions. For multi-leaf attributes (A & MF) n = 44, for all other attributes n = 22.

5.4.1.3. Mouthfeel

Significant differences between accessions for mouthfeel attributes were found for initial heat, spikiness, chewiness, tingliness and warming (Table 5.2; $P<0.05$). Accessions SR5 and SR19 were significantly different from SR2, SR3, SR6 and SR12 for initial heat, and also significantly higher in terms of tingling than SR3. SR5 was significantly different from SR2, SR3, SR6 and SR12 for warming mouthfeel. These data suggest a genetic component for inferring differing degrees of pungency between accessions, as SR5 in particular is scored highly in these traits.

SR14 was significantly chewier than SR3, and spikier than SR3 and SR19. The presence of hairs on leaves is not thought to be a desirable characteristic for consumers, and would need to be bred out of any potential future varieties (Bell & Wagstaff, 2014; Chapter 2).

5.4.1.4. Taste, flavour & aftereffects

There were significant differences in peppery, mustard flavour and sulfur between accessions (Table 5.2; $P<0.05$). Peppery flavour in SR19 and SR5 was significantly higher than in SR3; and mustard and sulfur flavour in SR5 was significantly higher than in SR12 and SR3, respectively. Acid, peppery, mustard and warming aftereffects were significantly different between some cultivars (P <0.05), though no statistically significant differences were found for taste attributes. These data suggest that pungency/warming effects are more important for discriminating between cultivars than bitterness as has been suggested in a previous study (Pasini et al. 2011).

5.4.2. Phytochemical Analyses

5.4.2.1. Previous phytochemical analyses

The analysis of GSLs, flavonols and headspace VOCs is presented in Bell et al. (2015 & 2016; Chapters 3 & 4). The data for the seven accessions used here are summarised in appendix III. The material used in these analyses was grown under identical conditions to those presented in this paper, and the data were combined with new analyses of PIs, AAs, OAs and sugars.

5.4.2.2. Polyatomic ions

Table 5.3 summarises the concentrations of four PI groups found in the rocket accessions: chlorides, nitrates, phosphates and sulphates. The PI content of the seven cultivars varied significantly. Nitrate concentrations are relatively low for all accessions compared to previous reports, but this is not unusual as large variations in cultivar accumulations are known to occur across growing methods, cultivars, and environments (Cavaiuolo & Ferrante, 2014).

Chloride concentration was lowest in accession SR12 (9.5 $g.kg^{-1}$ DW) and highest in SR5 (16.6 $g.kg^{-1}$ DW) and this was a significant difference ($P<0.05$). SR5 is also high in phosphate concentration (15.2 $g.kg^{-1}$ DW) and is significantly different from SR3, SR6 and SR14. SR19 accumulated significantly more phosphate than any of the other accessions tested. SR5 is conversely very low in nitrate concentration (10.0 $g.kg^{-1}$ DW) – almost five times less than SR19 (48.5 $g.kg^{-1}$ DW). SR19 was also relatively high in phosphate (20.7 $g.kg^{-1}$ DW) and sulphate (17.7 $g.kg^{-1}$ DW), making it distinct in terms of PI concentrations.

Table 5.3. Polyatomic ion (g.kg^{-1} DW), amino acid (μg.g^{-1} DW), sugar (mg.g^{-1} DW) and organic acid (mg.g^{-1} DW) concentration for seven accessions of *Eruca sativa* (*n* = 3) with standard errors (±).

	Accession						
	SR2	SR3	SR5	SR6	SR12	SR14	SR19*
Polyatomic ions (g.kg^{-1} DW)							
Chloride	12.6±3.0^{ab}	10.3±0.1^{ab}	16.6±0.9^{b}	13.1±0.7^{ab}	9.5±1.2^{a}	11.1±2.0^{ab}	14.0±1.1^{ab}
Nitrate	26.9±7.2^{ab}	24.2±1.0^{a}	10.0±0.8^{a}	21.6±7.1^{a}	15.4±6.7^{a}	13.0±1.3^{a}	48.5±5.1^{b}
Phosphate	14.6±0.4^{bc}	9.8±1.3^{ab}	15.2±0.7^{c}	9.4±0.4^{a}	13.4±1.7^{abc}	9.6±0.1^{ab}	20.7±0.6^{d}
Sulphate	12.8±2.8^{ab}	10.5±1.0^{a}	10.8±1.3^{a}	10.7±0.6^{a}	12.5±1.2^{ab}	12.3±1.4^{ab}	17.7±0.4^{b}
Amino acids (μg.g^{-1} DW)							
Alanine	65.1±8.5	59.6±4.5	39.1±5.3	61.9±5.2	63.8±13.4	46.8±6.8	52.5±8.1
Valine	11.1±6.4	7.2±5.9	nd	11.9±5.0	6.0±4.9	nd	12.0±4.9
Leucine	nd	3.5±2.9	nd	4.0±3.3	2.6±2.1	nd	nd
Threonine	21.8±2.2	24.1±1.3	12.9±1.1	25.0±1.1	19.7±3.9	13.7±1.1	22.3±3.6
Serine	63.0±31.5^{a}	167.2±20.5^{b}	70.7±14.4^{ab}	81.9±36.4^{ab}	113.6±43.5^{ab}	84.5±10.7^{ab}	117.3±33.2^{ab}
Proline	73.7±7.4	41.3±5.2	67.1±21.2	50.5±5.5	75.4±16.7	69.9±35.0	25.8±3.8
Asparagine	4.3±2.5	nd	nd	nd	nd	nd	3.2±2.6
Aspartic acid	155.8±16.4	155.8±17.5	93.9±4.0	162.8±5.9	117.3±22.2	80.5±16.4	138.2±22.5
Glutamic acid	143.1±69.5	158.2±8.8	120.2±6.7	222.9±34.0	177.1±61.6	146.9±15.9	148.0±26.1
Glutamine	90.8±12.3^{c}	83.0±5.6^{bc}	29.2±1.5^{a}	77.2±9.4^{abc}	49.3±13.0^{abc}	35.8±4.3^{ab}	65.1±8.5^{abc}
Lysine	nd	2.1±1.7	nd	nd	nd	nd	nd
Total AAs	628.9±107.1	701.9±31.9	433.0±45.0	698.2±44.6	624.8±128.8	478.2±81.5	584.4±97.8
Sugars (mg.g^{-1} DW)							

Fructose	3.7±0.6	3.6±0.1	2.8±0.3	2.0±0.2	3.2±0.6	4.9±0.9	1.9
Glucose	16.2±1.2	16.7±1.2	23.7±0.5	14.7±2.5	19.6±0.9	21.1±0.9	9.1
Galactose	3.9±1.0	3.5±1.5	4.6±1.0	2.6±0.8	4.1±0.3	6.6±0.5	0.7
Sucrose	2.1±0.6	3.8±1.8	2.7±0.7	1.4±0.3	1.7±0.3	5.3±1.9	3.1
Total sugars	25.9±2.4	27.6±3.4	33.8±0.7	20.8±4.9	28.6±0.3	37.9±3.2	14.8
Organic acids (mg.g^{-1} DW)							
Malic acid	83.5±24.1	67.7±0.2	62.9±4.3	54.4±9.1	46.8±0.7	47.2±0.1	59.0
Citric acid	19.1±1.2	30.9±5.0	14.0±0.8	10.4±7.3	20.0±2.0	28.3±0.4	22.8
Total OAs	102.7±25.4	98.5±4.8	77.0±3.5	64.8±16.4	66.8±2.7	75.5±0.5	81.9

Significant differences (ANOVA Tukey's HSD test, P <0.05). Different letters in each row indicates a significant difference; an absence of letters indicates no significant difference. * = *n* = 1 for sugar and organic acid analyses. nd = not detected.

5.4.2.3. Free amino acids

Table 5.3 shows the AA concentrations found in each of the seven accessions. In total 11 free AAs were detected and quantified, however only serine and glutamine showed significant differences between cultivars. The commercial cultivar SR3 had a high serine concentration of 167.2 $\mu g.g^{-1}$ DW, which was significantly greater than SR2 (63.0 $\mu g.g^{-1}$ DW). SR2 conversely had statistically higher glutamine concentration (90.8 $\mu g.g^{-1}$ DW) than SR5 (29.2 $\mu g.g^{-1}$ DW) and SR14 (35.8 $\mu g.g^{-1}$ DW). Aspartic acid and glutamic acid were the most abundant AAs detected overall, and were present in every accession. Valine, leucine, asparagine and lysine were not observed in several accessions, with concentrations very low where they were detected.

5.4.2.4. Free sugars & organic acids

Table 5.3 displays the free sugar content of each accession tested. No significant differences were found in the ANOVA, with the possible exception of SR19. Unfortunately leaf material of this accession was limited, and only one biological replicate could be analysed (not included in ANOVA).

Concentrations of free OAs are also presented in Table 5.3, and as with sugars, no significant differences between each accession were observed. This is perhaps due to the very large variation within some samples, particularly SR2, which had large variation in malic acid concentration.

5.4.3. Principal Component Analysis

5.4.3.1. Sensory attributes

PCA extracted six components, all of which had Eigenvalues >1.0; however the majority of information was contained in the first three PCs (78.6%; appendix IV).

On this basis PC1, PC2 and PC3 are presented. The majority of explained variation is found in PC1 (43.49%) and this component separates traits associated with pungency and bitterness, and coupled with the correlation matrix data (appendix V), many of these traits share significant relationships. PC2 identifies a dimension characterised by green and sweetness characters, as well as some appearance and mouthfeel traits. The information contained within PC3 is related to earthy attributes and aromatic odour, but also visual and morphological characteristics such as leaf size, toughness, chewiness and spikiness.

These separations are easily identifiable within the biplots presented in Figure 5.1. SR5 is distinctive in Figure 5.1a, characterised by a high degree of association with pungent attributes, acid aftereffects and bitterness. SR19 is also separate from the main cluster (lower left), but separates along PC2 in terms of the distinct difference in appearance from the other cultivars. SR14, SR6, SR12, SR3 and SR2 are broadly similar in these dimensions, and are characterised by a comparatively low bitterness, and lower scoring mustard, pepper, sulfur and initial heat mouthfeel attributes. This is coupled with an increase in relative perceptions of sweetness attributes, moistness mouthfeel and larger leaf shapes. In Figure 5.1b this pattern is broadly repeated, however SR19 separates along the negative axis of PC3 due to low scores for leaf hairiness, purple stem, spikiness, and earthy/aromatic attributes. The distinctiveness of SR5 and SR19 was also repeated in components PC4, PC5 and PC6 (plots not presented).

The purple stem attribute was correlated highest in PC5 (plot not presented), and in the Pearson's correlation analysis was inversely and significantly correlated to traits such as bitter taste, peppery flavour, mustard aftereffects, initial heat, tingly and warming aftereffects (r = -0.765, -0.791, -0.812, -0.823, -0.792, -0.758 respectively; all P<0.05). This may suggest that stem colouration could be used as a visual cue for

a

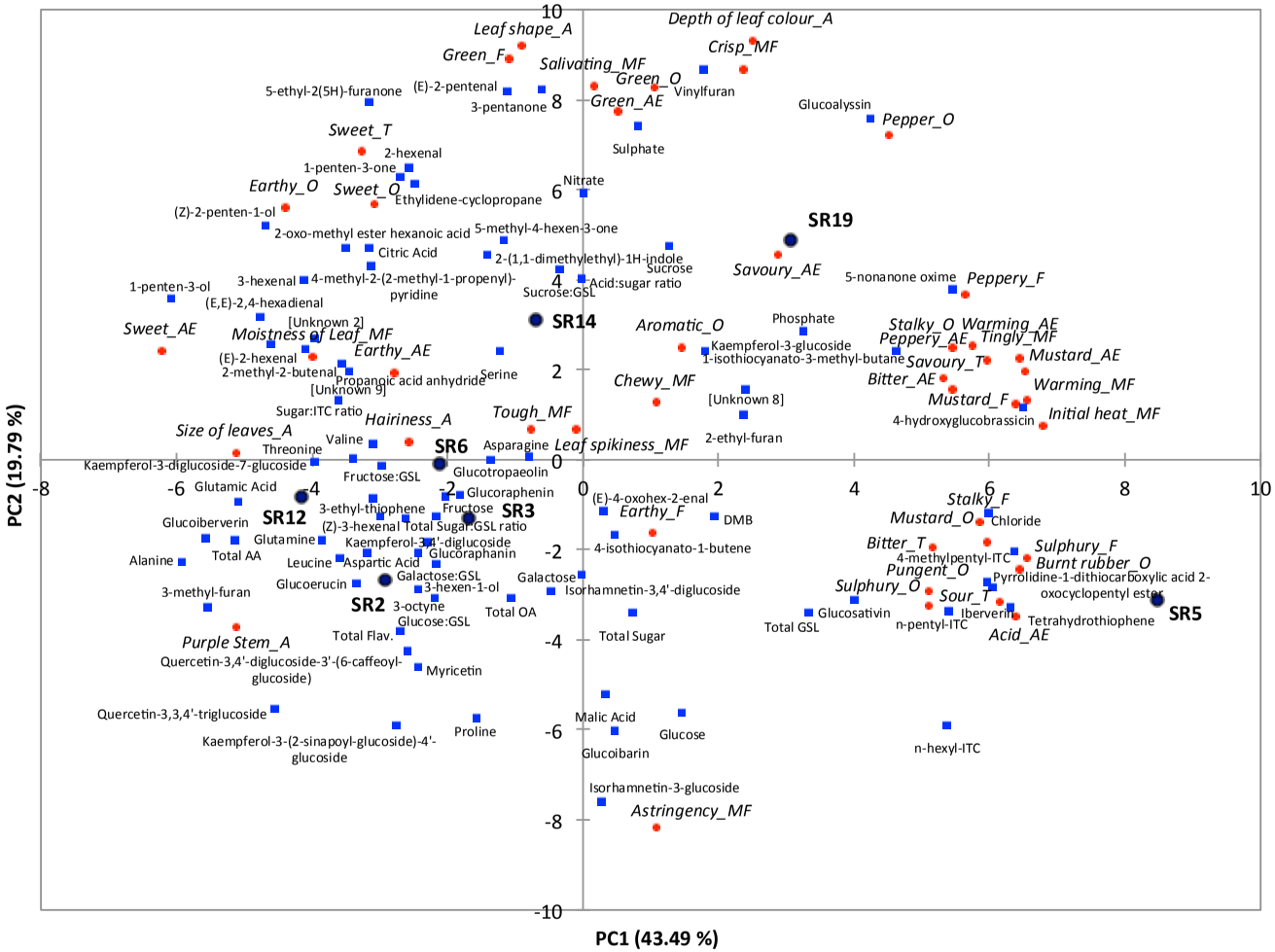

Figure 5.1. Principal component analysis biplots of sensory descriptive trait scores and regressed supplementary phytochemical data. PC1 vs. PC2 (a) accounts for 63.28% of the explained variation. PC1 vs. PC3 (b) accounts for 58.81% of the explained variation. Dark blue circles with bold labels indicate scores values for each respective accession. Red circles with italic labels indicate measured sensory attributes. Blue squares indicate supplementary data points of each phytochemical analysis: GSLs, flavonols, PIs, headspace VOCs, AAs, free sugars, organic acids, sugar-GSL ratios, sugar-ITC percentage abundance ratio and the sugar-OA ratio. Abbreviations: A, appearance; O, odour; MF, mouthfeel; T, taste; F, flavor; AE, aftereffects.

b

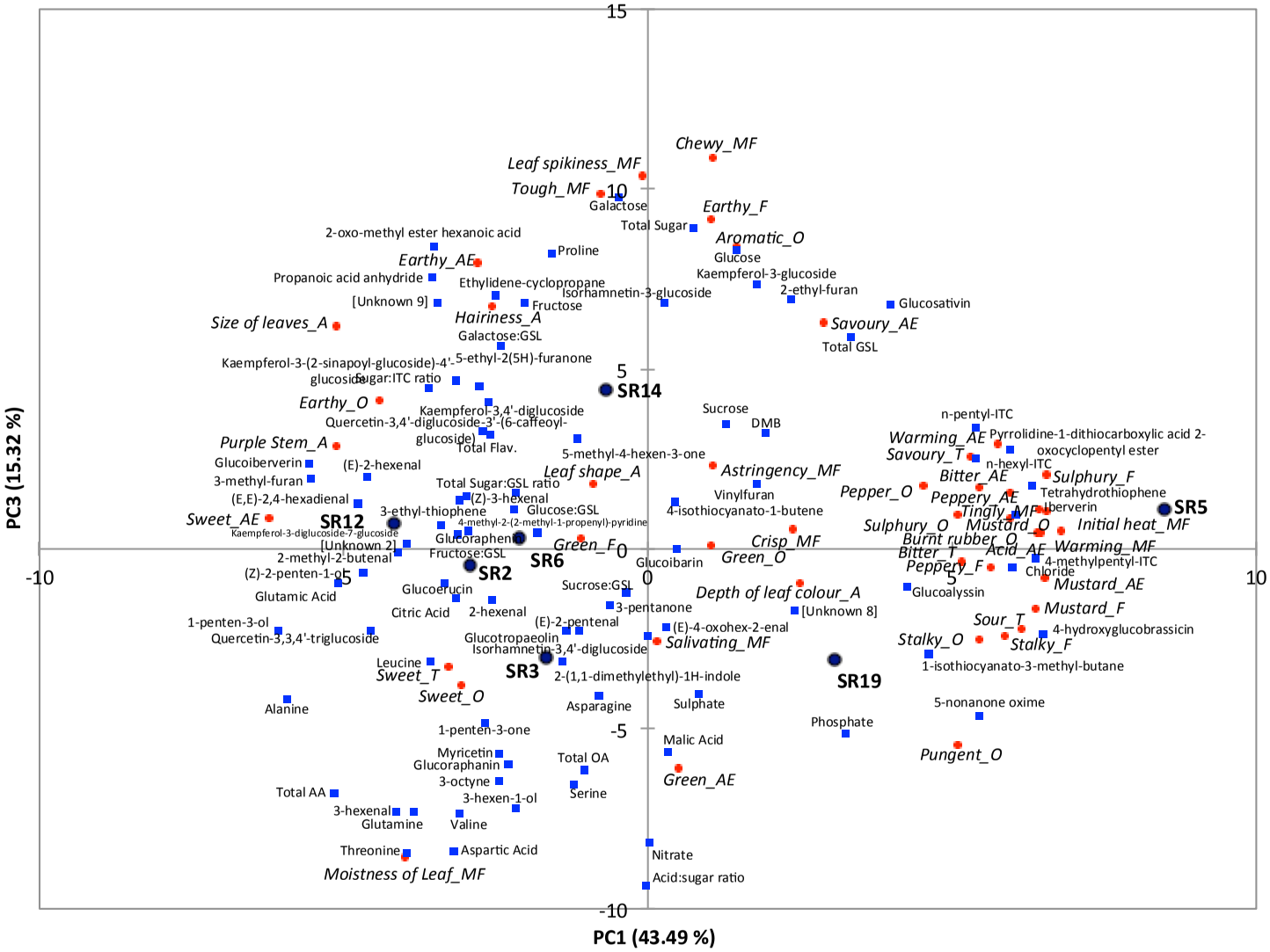

Figure 5.1. continued.

determining pungency/bitterness of leaves, although this would need to be assessed in more focused experiments.

Many of the sensory attributes assessed share highly significant correlations (appendix V). Briefly, the odour attribute of burnt rubber shares several significant relationships with traits such as sulfury flavour (r = 0.880; P<0.01), initial heat mouthfeel (r = 0.907; P<0.01) and warming mouthfeel (r = 0.921; P<0.01). Many of the perceptions associated with these types of pungent attributes are correlated and co-locate within the PCA in PC1. Peppery flavour is also significantly correlated with tingly mouthfeel (r = 0.956; P<0.001), and mustard flavour/aftereffects with initial heat (r =0.897, P<0.05; r = 0.956, P<0.001, respectively).

5.4.3.2. Phytochemical data

5.4.3.2.1. General

The regressed phytochemical data is presented in Figure 5.1, superimposed upon the sensory PCA, and illustrates the relationships found with these data across the three most informative principal components. Significant correlations (Pearson *n*-1) between phytochemicals and sensory attributes are also summarised in appendix V, and the regressed factor loadings of each variable are presented in appendix VI.

5.4.3.2.2. Glucosinolates

Eleven GSL compounds were detected in the seven rocket accessions by Bell et al. (2015; Chapter 3). These were 4-hydroxyglucobrassicin, glucotropaeolin, glucoraphanin, glucoiberverin, glucosativin, DMB, glucoalyssin, glucoerucin, glucoraphenin, diglucothiobeinin and glucoibarin. For the purposes of the analysis, data for diglucothiobeinin were not included, as it was only detected in one of the accessions analysed.

The major GSL of rocket, glucosativin (2.7 – 7.7 $mg.g^{-1}$ DW; Bell et al. 2015; Chapter 3), was significantly and positively correlated to earthy flavour (r = 0.863, P<0.05; appendix V) and was most positively correlated along PC3 (Figure 5.1b). Unlike other studies where glucosativin and its dimer (DMB) have been linked with bitterness, there was no significant relationship found here. DMB was most highly correlated along PC1 and positioned between earthy and pungent attributes but no significant correlations were observed.

Total GSL concentration separated along PC3 and PC1 (Figure 5.1b), and shared a significant correlation with bitter aftereffects (r = 0.766, P<0.05), and negatively with the perceived moistness mouthfeel of leaves (r = -0.803, P<0.05). These two correlations suggest an overall tendency for rocket GSLs to have a bitter component associated with them post-swallowing, and the intensity to be inverted to the levels of moisture.

SR6 had high concentrations of total GSLs (10.0 $mg.g^{-1}$ DW; Bell et al. 2015; Chapter 3), but the sensory profile of this accession is more similar to SR2, and is associated with sweet/green attributes (PC1 vs. PC3; Figure 5.1b). This indicates that individual GSLs may be more influential on sensory properties than the total concentration. SR6 was characterised by relatively high concentrations of glucoerucin (1.3 $mg.g^{-1}$ DW), for example. The absence of any significant correlations with this GSL and glucoraphanin are also of potential importance. We hypothesise that they do not directly contribute to the sensory profile of rocket, and could be increased through selective breeding to produce more nutritionally dense rocket varieties without affecting flavour.

Minor rocket GSLs such as 4-hydroxyglucobrassicin and glucoalyssin were reported in very low concentrations by Bell et al. (2015; Chapter 3) yet produced strong correlations with PC1 and PC2, respectively. This may be indicative of the role

minor GSLs play in sensory perceptions of rocket, and what creates distinctive flavours between cultivars. 4-hydroxyglucobrassicin for example was only detected in SR5 and SR19, and when these supplementary data were regressed onto the sensory principal components the presence of this compound is significantly correlated with bitter aftereffects (r = 0.794, P<0.05), pepper flavour and aftereffects (r = 0.901, 0.895; both P<0.01), mustard flavour (r = 0.908, P<0.01) and aftereffects (r = 0.959, P<0.001), the initial heat of leaves (r = 0.967, P<0.001), and tingly and warming mouthfeels (r = 0.936, 0.932; both P<0.01). It was observed that the presence of glucoalyssin had significant correlations with pepper odour (r = 0.950, P<0.01), pepper flavour (r = 0.838, P<0.05), and mustard aftereffects (r = 0.795, P<0.05).

Although concentrations/presence differs across accessions, it is not possible to know definitively if they are the cause of sensory differences without isolated standards. It is likely however that a higher diversity of 'minor' GSLs is associated with distinctive sensory attributes, such as pepperiness in SR19, and hotness/pungency in SR5, rather than total GSL concentration. D'Antuono, Elementi, & Neri (2009) similarly found that 4-hydroxyglucobrassicin was highly associated with "pleasant" taste and pungency. It may be that minor GSLs and their hydrolysis products contribute more to these effects than has been previously realized.

5.4.3.2.3. Flavonols

Eleven flavonol compounds were identified and quantified in the rocket accessions tested by Bell et al. (2015; Chapter 3). These were myricetin, kaempferol-3-glucoside, quercetin-3-glucoside, isorhamnetin-3-glucoside, kaempferol-3,4'-diglucoside, isorhamnetin-3,4'-diglucoside, kaempferol-3-diglucoside-7-glucoside, quercetin-3,3,4'-triglucoside, kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside,

quercetin-3,4'-diglucoside-3'-(6-caffeoyl-glucoside) and quercetin-3,4'-diglucoside-3'-(6-sinapoyl-glucoside). For the purposes of the analysis quercetin-3-glucoside and quercetin-3,4'-diglucoside-3'-(6-sinapoyl-glucoside) data were omitted as they were each only detected in one accession. Some of these compounds were highly correlated along PC3 (Figure 5.1b) and were generally associated with mouthfeel traits, and negatively with stalky and intense sensory attributes.

Flavonols and other polyphenols have been strongly linked with astringent sensory perceptions in studies of drink products, such as red current juice (Schwarz & Hofmann, 2007), red wine (Hufnagel & Hofmann, 2008), berry juice (Laaksonen, Ahola, & Sandell, 2013) and black tea (Scharbert, Holzmann, & Hofmann, 2004). Isorhamnetin-3-glucoside was the only compound where a significant correlation was observed with astringent mouthfeel (r = 0.762, P<0.05). This compound was significantly, negatively correlated with the perception of salivating mouthfeel (r = -0.856, P<0.05), implying a possible link with perceptions of moisture on the palate.

5.4.3.2.4. Polyatomic ions

Chloride and phosphate separated highly along PC1, and this was largely due to the relatively high concentrations present in both SR5 and SR19. Nitrate and sulphate however were highly correlated with PC2 (Figure 5.1a), opposite to SR5, which is characterised by low nitrate concentration.

In a previous study by Hufnagel & Hofmann (2008) on red wine fractions, both chloride and phosphate were linked with astringency and sourness. In this study, only chloride was positively correlated with sour taste (r = 0.821, P<0.05). One hypothesis for this association (which is usually caused by acids), might be that chloride ions react with thiol groups of some ITCs to produce hydrochloric acid (La Quèrè, Gierezynski, Langlois, & Sèmon, 2006), and thus create H+ ions which would be

perceived as sour on the tongue. Such reactions may also influence volatile formations (La Quèrè et al. 2006) and infer differing sensory properties according to relative abundances. Scores for sourness were low uniformly across accessions and non-significant in the ANOVA, but it is interesting to note SR5 was scored highest overall, as well as for acid aftereffects with which chloride ion concentration was also significantly correlated with (r = 0.837, P<0.05). Unfortunately studies of this kind are absent for rocket and other leafy vegetables.

Accession SR5 had the highest concentration of chloride, and SR19 the highest concentration of phosphate, and is again a distinguishing attribute in terms of sensory properties. Numerous significant correlations were observed between chloride ions and traditional rocket traits (appendix V); particularly of note is mustard flavour (r = 0.953, P<0.001) and aftereffects (r = 0.907, P<0.01). These correlations are of course not proof that they are the causative agents; however there does seem to be a relationship in these samples between sensory attributes and chloride concentrations.

The other three PIs also had significant correlations (P<0.05; appendix V). Phosphate was significantly correlated to peppery flavour (r = 0.802) and both sulphate and nitrate produced significances relating to salivating mouthfeel (r = 0.799, 0.818, respectively). Nitrate levels have been linked to differences in spinach flavour (Maga, Moore, & Oshima, 1976), however information regarding direct and specific effects of these ions in leafy vegetables is sparse within the literature.

5.4.3.2.5. VOCs

ITCs, sulfur volatiles and an oxime showed large separation along PC1, indicating that there is a strong relationship between their relative abundances and the hot, peppery, mustard and warming attributes present in accessions, such as

SR5 and SR19. Alcohols, aldehydes and ketone compounds separated along PC2 indicating a high degree of association with odour and some taste, flavour and mouthfeel attributes (appendix VI), such as stalky, sweet and green. A smaller number of compounds separated to a high degree on PC3, and included some furans, acids, a thiophene and a cyclopropane. This associates them with earthy and savoury attributes, as well as with accessions that were typically described as being chewy or tough. The relative distribution of these compounds with sensory attributes is presented in the PCA biplots (Figure 5.1). 3-ethyl-1,5-octadiene, *O*-methyloxime-butanal and oxalic acid diallyl ester were removed from the analysis as they were only detected in one accession, respectively (Bell et al. 2016; Chapter 4).

4-methylpentyl-ITC and iberverin showed significant correlations with bitter taste (r = 0.827, P<0.05; r = 0.940, P<0.01, respectively) and aftereffects (r = 0.802, 0.797; both P<0.05). *n*-pentyl-ITC and 1-isothiocyanato-3-methyl-butane also correlated strongly with bitter taste (r = 0.912, P<0.01; r = 0.781, P<0.05, respectively). Bitterness in ITC-containing compounds is well documented within the literature (Behrens, Gunn, Ramos, Meyerhof, & Wooding, 2013) and our data are in agreement with other studies in this regard.

Three other compounds that are not ITCs were also correlated with bitter taste: pyrrolidine-1-dithiocarboxylic acid 2-oxocyclopentyl ester (r = 0.852, P<0.05), tetrahydrothiophene (r = 0.783, P<0.05), and an unidentified compound (Unknown 8; r = 0.804, P<0.05). Only one significant negative correlation was found with bitter taste, which was 3-methyl-furan (r = -0.847, P<0.05). This latter compound was significantly positive in correlation with the purple stem attribute (r = 0.954, P<0.001). Purple stem was inversely related to 5-nonanone oxime (r = -0.775, P<0.05) and 1-isothiocyanato-3-methyl-butane (r = -0.957, P<0.001). This may again provide a possible visual cue for leaf pepperiness, bitterness and overall pungency.

n-hexyl-ITC and iberverin are correlated significantly with aroma perceptions such as mustard (r = 0.767, 0.815 respectively; both P<0.05), indicating that these compounds contribute heavily to rocket odour properties, despite their low relative abundance within the VOC bouquet. 4-methylpentyl-ITC (r = 0.912 P<0.01), *n*-hexyl-ITC and iberverin (r = 0.836, 0.786; both P<0.05) all correlated with burnt rubber aroma. These along with pyrrolidine-1-dithiocarboxylic acid 2-oxocyclopentyl ester (r = 0.784, P<0.05) were higher in relative abundance in SR5, which is separated along PC1 with sulfur and mustard odours/flavours, as well as with high GSL concentrations and relative ITC abundances.

5-nonanone oxime was significantly correlated with several attributes usually attributed to ITCs, and is correlated strongly with SR19 and PC1 (Figure 5.1). Significant correlations included pepperiness (odour, r = 0.760; P<0.05; flavour, r = 0.889, P<0.01; and after effects, r = 0.798, P<0.05), initial heat (r = 0.794, P<0.05), tingliness (r = 0.839, P<0.05), warming aftereffects (r = 0.829, P<0.05), and mustard flavour and aftereffects (r = 0.843, 0.835; both P<0.05, appendix V). These results infer that the sensations commonly associated with rocket are perhaps not wholly due to direct products of the GSL-myrosinase reaction, and that other VOCs may have a role.

Tetrahydrothiophene is a pungent chemical odourant (Swanston, 2000) and is likely an ITC derivative. It has significant correlations with burnt rubber odour (r = 0.911, P<0.01), initial heat (r = 0.898, P<0.01), warming (r = 0.844, P<0.05), tingliness (r = 0.817, P<0.05), sour taste (r = 0.851, P<0.05), bitter taste (r = 0.783, P<0.05), bitter aftereffects (r = 0.809, P<0.05), acid aftereffects (r = 0.927, P<0.01) and peppery aftereffects (r = 0.786, P<0.05; appendix V). In agreement with Jirovetz, Smith & Buchbauer (2002), we found this compound to be significantly correlated to mustard odour, flavour and after effects (r = 0.807, 0.822, 0.842; all P<0.05), as well

as sulfur flavour (r = 0.945, P<0.01). This compound has been linked with unpleasant odours, allium-like smells and 'cabbage' odour (Jirovetz et al. 2002), and our results suggest that it is an important component in the volatile mixture produced by rocket leaves.

At the low end of PC1, and opposite to the pungency/pepperiness of SR5 and SR19 are the 'green leaf volatiles', produced in higher relative abundances by accessions such as SR2. The initial heat of leaves (r = -0.854, P<0.05), and the aroma sensations of mustard (r = -0.796, P<0.05) and burnt rubber (r = -0.915, P<0.01) were negatively correlated with 1-penten-3-ol. This is an unexpected result as in previous studies 1-penten-3-ol has been linked with burnt and pungent attributes (Berger, Drawert, & Kollmannsberger, 1989; Buttery, Teranishi, Ling, & Turnbaugh, 1990), which is not consistent with our data.

Ketones are VOCs thought to play an active part in plant defense (Jimenez, Lanza, Antinolo, & Albaladejo, 2009) and as such it is unsurprising that as these compounds are released they contribute to the sensory profile of rocket. They are known to have pleasant odours, and 3-pentanone was significantly correlated with green odour, flavour (r = 0.881, 0.944; both P<0.01) and aftereffects (r = 0.862, P<0.05). 3-pentanone has been previously described as having an 'ether' odour (Berger et al. 1989).

Several alcohol, ketone, indole and aldehyde compounds were significantly correlated with sweet attributes, and separated highly along PC2. These include 2-(1,1-dimethylethyl)-1H-indole, 1-penten-3-ol, 1-penten-3-one, 2-hexenal, (Z)-2-penten-1-ol and (E)-2-pentenal (appendix VI). In previous studies 3-hexenal has been linked with green, stalky and aromatic attributes (Carrapiso, Jurado, Timón, & García, 2002), but no significant correlations with these was observed in our data. Green flavour was low in SR5, as was relative abundance of 3-hexenal. This may

suggest a tentative link between relative abundances of ‘green-leaf’ VOCs and the perception of pungency caused by sulfur-containing VOCs such as ITCs. From a plant defense point-of-view, this may be an evolutionary strategy to favour one biosynthetic pathway over another and *vice versa*. Differing genetic regulation of GSL synthesis/ITC formation and the octadecanoid pathway for ‘green-leaf’ VOCs in different cultivars may be responsible for the balance between ITC/sulfur volatile formation and ‘green-leaf’ volatiles (Ahuja, Rohloff, & Bones, 2010). The relative abundances of VOCs between these two pathways are likely to be a determining factor in rocket sensory properties.

5.4.3.2.6. Free amino acids

AA concentrations were primarily separated along PC4 (plot not presented), with the exception of proline on PC3. In Figure 5.1b AAs are co-located with sweetness attributes, and negatively associated with pungency. AA compounds are known to infer a variety of tastes, and sometimes flavours. Sweet tasting AAs include: alanine, threonine, serine, proline and glutamine; sour/umami tasting include: aspartic acid and glutamic acid; and bitter tasting include: valine and leucine (Nishimura & Kato, 1988; Solms, 1969).

No significant correlations with sweet attributes were observed for alanine, threonine, serine, proline or glutamine, however a general trend was observed for these AAs to correlate in the same spatial orientation of sweetness. Glutamic acid was significantly correlated with sweet aftereffects (r = 0.794, P<0.05), which is unexpected, as this AA has been previously described as having umami properties. As an observational trend, high AA concentrations are negatively correlated with strong and pungent rocket attributes in PC1, and concentrations are typically higher in the ‘milder’ accessions such as SR2 and SR3.

Glutamic acid and aspartic acid did not correlate with savoury (umami) or sour tastes (Kirimura et al. 1969). Aspartic acid was significantly inversely correlated with savoury aftereffects within the model (r = -0.825, P<0.05). The AAs known to be bitter (valine and leucine) showed no significant correlations with this attribute. This is unsurprising as these compounds were of very low concentration and were not detected at all in some samples.

5.4.3.2.7. Sugars

Few significant correlations were found for sugar concentration in the sensory PCA. The spatial positions of the free sugars can be seen in Figure 5.1b, in the lower half of the plot, and are associated generally with sweetness attributes along PC3. After this determination, the sugar-GSL ratio was calculated and added as supplementary data and regressed onto the sensory PCA. Previous studies have suggested that the role of sugar-GSL ratios may influence the perception of bitterness (Jones, Faragher, & Winkler, 2006). The correlation matrix revealed no significant correlations with bitter taste, however three significant negative correlations were observed for total sugar-GSL, glucose-GSL, and fructose-GSL ratios with bitter aftereffects (r = 0.773, 0.780, 0.851; all P<0.05). This indicates that a greater ratio infers a reduction in bitterness after the swallowing of leaves, but does not in turn correspond to a significant increase in sweetness attributes.

5.4.3.2.8. Organic acids

OAs have been linked with sourness (Tandon, Baldwin, & Shewfelt, 2000) and astringency (Hufnagel & Hofmann, 2008) in previous sensory analyses of other foods. Here we saw no such associations, which was unexpected considering the relatively high accumulations of OAs in rocket leaves compared with other

Brassicaceae. It is possible that an acid-sugar ratio should be considered, however only one significant negative correlation between this ratio and a sensory attribute (earthy flavour; r = -0.758, P<0.05) was observed (appendix V). Studies on apples have shown that the acid-sugar ratio affects sweetness and sourness (Kühn & Thybo, 2001) but in a crop such as rocket with so many bitter and pungent volatiles, it is difficult to separate and identify if such a ratio is truly affecting perceptions.

5.5. Conclusion

In this study six promising gene bank cultivars of rocket and one commercial comparator (SR3) were used to objectively elucidate the relationships between sensory characteristics and phytochemical content, as well as aspects of appearance. No study of rocket salad has previously encompassed such a wide range of analytical methods and chemical analyses in combination with sensory evaluation. It marks a significant step forward in understanding how compounds interact and influence perceptions. Whilst only a relatively few samples were tested here for practical reasons, it is recommended that in future other other cultivars/accessions of rocket to expand upon and elucidate the relationships identified.

There was a large amount of morphological variation between accessions, and this also seems to be the case for some sensory attributes, as these varied significantly for pungent traits such as sulfur, initial heat on the tongue, tingliness, warming sensations, pepperiness, mustard flavour and some of the associated aftereffects. It should be remembered that these accessions are not commercial products, but are effectively wild (with the exception of SR3). That being said, no truly domesticated rocket varieties currently exist because of the relatively short time in

which humans have actively bred the species compared to other crops (Bell & Wagstaff, 2014; Chapter 2).

It is important for breeders to have a wide range of traits to select for within germplasm collections, but this also makes producing a commercially viable end product much more difficult. Bell et al. (2015; Chapter 3) highlighted the diversity of GSL and flavonol accumulations in both commercial and germplasm accessions, which vary to a large degree regardless of the source or commercial availability.

Unlike previous sensory studies on rocket, bitterness was not a significantly variable attribute in these particular accessions, but the concentrations of bitter-causing sensations (such as ITCs) are highly variable. This indicates that the sugar-GSL ratio, and perhaps the relative abundances of 'green-leaf' VOCs and ITCs, plays an important role in rocket taste perceptions, and could be utilised and modified by plant breeders in creating new varieties. These relationships would benefit from more in-depth investigation in future studies.

Several ITC compounds were significantly correlated to the well-known hot and pungent rocket attributes, and some VOCs and AAs are negatively associated with these perceptions. ITCs typically constitute <9.0% of the overall VOC headspace bouquet (Bell et al. 2016; Chapter 4), suggesting that even in low abundances they have a very large impact upon sensory attributes. Selecting and breeding rocket plants with higher ITC headspace volatile abundance, by even a relatively small amount, may have large effects on the sensory properties of leaves.

The results presented indicate the possibility of elevating health beneficial compounds such as glucoraphanin and glucoerucin without any perceptible or negative changes in sensory attributes. In this study, no significant correlations were observed for these GSLs with any sensory attribute. High glucoraphanin content has been selectively bred for in *Beneforté* broccoli, for example, with no apparent

adverse effects on consumer acceptance (Traka et al., 2013). Low concentration GSLs such as 4-hydroxyglucobrassicin also seem to infer, or are related to, an increased perception of pungent attributes. Therefore selecting for 'minor' rocket GSL constituents and ITCs could feasibly lead to the creation of "hot rocket" varieties. This has been attempted commercially through conventional breeding methods, but varieties marketed as such are often unstable across growing environments and have problems with reliable seed production due to a lack of true domestication (Bell & Wagstaff, 2014; Chapter 2).

A consumer study of these same seven rocket salad accessions has been conducted; the results of which will be subsequently published. Future work will also consider the impact of the industrial supply chain on phytochemical constituents, and the implications this might have for sensory attributes.

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CHAPTER 6: Analysis of Seven Salad Rocket (*Eruca sativa*) Accessions: Consumer Preference Based On Perceived Sensory Attributes, TAS2R38 Genotype, and the Abundance of Volatile and Non-volatile Compounds

6.1. Introduction To Paper (submitted to *Food Chemistry*)

Immediately after the sensory analysis presented in Chapter 5, a consumer study of the same seven accessions of rocket was undertaken. As will be highlighted in this chapter, very little scientific evidence has been presented to determine on what basis consumers like/dislike/are indifferent to the leaves of the species. The aim of this study was to form a foundation for future research, by assessing both consumers' preferences and their perceptions of four key sensory attributes. Utilising the knowledge gained from Chapter 5, we identified key discerning attributes on which consumers base their preferences. We also determined the taste receptor genotype of individuals in the study, which has not been previously tested in respect to the sensory attributes found in rocket species.

The author wrote an ethics application to the University of Reading ethics committee, with the aid and guidance of Dr. Lisa Methven. Ethical approval was granted for the experiments, and volunteers were recruited from in and around the University of Reading. This was achieved by emailing consenting individuals, whose names were listed on a database held by the Food & Nutritional Sciences department. Others were recruited via electronic mailing lists, distribution of flyers and posters, and word of mouth.

This chapter is currently in preparation for submission to *Food Chemistry* for publication. Several subsequent studies conducted at the University of Reading have used this experiment as a model, in particular Project SOAR, which was funded by Bakkavör. Details of this collaboration are outlined in Chapter 8.

6.2. Introduction

Eruca sativa ("salad" rocket) and other species of rocket are popular leafy vegetables consumed all over the world as part of salads or as a garnish (Bennett, Carvalho, Mellon, Eagles, & Rosa, 2007). Previous research has largely focused on the diversity of phytochemical content and post-harvest quality. Studies have investigated the impacts of modified atmosphere and general sensory trends in rocket (Amodio, Derossi, Mastrandrea, & Colelli, 2015; D'Antuono, Elementi, & Neri, 2009; Lokke, Seefeldt, & Edelenbos, 2012; Martinez-Sanchez, Marin, Llorach, Ferreres, & Gil, 2006; Pasini, Verardo, Cerretani, Caboni & D'Antuono, 2011), however these made certain assumptions regarding what is the 'ideal' or 'preferred' rocket sensory profile of consumers. Few have taken into account the genetic and phytochemical variability of rocket varieties, and none have accounted for the genetic variability of consumers. Harvest, post-harvest and shelf life processes affect salad 'quality' (Amodio et al. 2015), but no study has tested consumers to determine the reasons for their liking/disliking of rocket. This is needed in addition to the quantification of sensory traits to plan and implement breeding and marketing strategies.

Studies by D'Antuono et al. (2009) and Pasini et al. (2011) have combined aspects from both sensory and consumer studies on *Eruca sativa* and *Diplotaxis tenuifolia*. While no scores for liking of traits were given, some subjective descriptive terms were used, such as "typical rocket salad flavour". Both studies used six untrained individuals but the minimum for profiling is eight trained assessors (Carpenter, Lyon, & Hasdell, 2012), and the minimum for a consumer study is 30 (Hough et al. 2006).

Based on these previous studies of preserving appearance and analysing sensory traits (Lokke et al. 2012; Pasini et al. 2011), it is difficult to propose modification of supply chains/breeding programs without knowing the effects of phytochemicals on consumer acceptance. It has yet to be determined which attributes consumers like, and if they are able to discriminate between varieties on the basis of quantifiable traits. Previous studies have been successful at identifying 'bad' sensory traits, such as leaf browning and off-odours (Lokke et al. 2012), as these are uniform across consumer groups. There has been less focus on identifying positive traits preferred by the consumer.

The reasons given why consumers like the taste and flavour of rocket salad are anecdotal. High levels of bitterness are quoted as being a negative aspect of consumer acceptance, but this is not universal (Hayes & Keast, 2011). Across *Brassicaceae* crops, it is has been demonstrated that bitter tastes contribute negatively to acceptance of products, and this could be part of a protective mechanism to prevent ingestion of harmful compounds, particularly at a young age (Tepper et al., 2009).

Bitterness is cited as the main taste attribute of rocket that consumers reject. It is an extremely complex taste sensation, with 25 putative G-protein-coupled TAS2R receptors existing in humans (Le Nevé, Foltz, Daniel, & Gouka, 2010). Glucosinolates (GSLs) and isothiocyanates (ITCs) have been linked with the gene *hTAS2R38* (Meyerhof et al. 2010) and the thiocyanate moiety (-N-C=S) confers the perception of bitterness and shows a bimodal distribution of two haplotypes: sensitive and insensitive (Tepper, 2008). Due to genetic recombination, three common diplotypes are present within the human population: PAV homozygotes ('supertasters'), heterozygotes ('medium-tasters'), and AVI homozygotes ('non-tasters'; Hayes, Bartoshuk, Kidd, & Duffy, 2008).

The *hTAS2R38* gene is known to confer varying bitter-tasting sensitivity for certain bitter compounds depending on the diplotype of the person (Wooding et al., 2004). Pasini et al. (2011) suggested that bitterness and pungency in rocket leaves has an association with the GSLs progoitrin/epiprogoitrin and dimeric-4-mercaptobutyl-GSL (DMB). Individuals who have the PAV/PAV 'supertaster' conformation theoretically perceive bitter compounds such as these and their myrosinase derivatives with greater intensity. Some consumers find these tastes overpowering or repulsive and avoid consuming *Brassicaceae* vegetables (Garcia-Bailo, Toguri, Eny, & El-Sohemy, 2009).

We hypothesised those individuals with PAV/PAV diplotype would score samples more intensely for bitter taste, and negatively for liking of rocket taste than those with PAV/AVI or AVI/AVI diplotypes. This study questioned which of seven *E. sativa* cultivars people preferred based on phytochemical composition and visual and textural characteristics. Data were combined with sensory analysis and phytochemical analyses presented in Bell, Oruna-Concha, & Wagstaff (2015; Chapter 3), Bell, Spadafora, Müller, Wagstaff, & Rogers (2016; Chapter 4), and Bell, Methven, Signore, Oruna-Concha, & Wagstaff (2017*,* Chapter 5) to determine which sensory attributes are most important for consumers in deciding if they like or dislike rocket. We also tested the hypothesis that sweetness, hotness and pepperiness are positive attributes in rocket consumer acceptance.

Perceptions of sweetness in other foods increase liking, and for some people, hotness is also a desirable characteristic; e.g. in hot peppers. Hotness is a trigeminal sensation, and consumers vary in their sensitivity according to the number of papillae they possess, and the abundance of associated trigeminal neurons (Reed & Knaapila, 2010).

The study aims were to (a) determine which sensory attributes contribute most to consumer liking of rocket, (b) determine if TAS2R38 diplotype status influences consumer liking, and (c) determine which specific phytochemical components influence liking and disliking of rocket.

6.3. Materials and methods

6.3.1. Plant Material

Plant material was grown and harvested under identical conditions to those presented in Bell et al. (2017*,* Chapter 5). SR2, SR5, SR6, SR12, SR14 and SR19 were sourced from European germplasm collections; see Bell et al. (2015; Chapter 3) for a list of institutes from which each was obtained. SR3 is a commercially available cultivar sold by Elsoms Seeds Ltd. (Spalding, UK).

6.3.2. Untrained Consumer Assessments

The untrained consumer study consisted of 91 consenting individuals, who were recruited from in and around the University of Reading (Reading, UK). Recruitment stipulated individuals must be over 18 years of age and be non-smokers. Anchored unstructured line scales were used to determine assessors' liking of overall appearance, leaf shape, mouthfeel and taste (extremely dislike – like extremely). Individual perception of selected sensory attributes (bitterness, hotness, sweetness and pepperiness) were rated using labeled magnitude scales (LMS). Scales ascended from 'not detectable', 'weak', 'moderate', 'strong', 'very strong' to 'strongest imaginable', where spacing between descriptors increased logarithmically. These values were then converted into antilog values and normalised for statistical analyses (Bartoshuk et al. 2003).

Consumers were asked what the likelihood would be of purchasing each of the samples if they were available in supermarkets (5 point category scale; 1 = low purchase intent, 5 = high purchase intent). The questionnaire was designed, and data acquired, using Compusense software (version 5.2; Guelph, ON, Canada). After the testing was complete, consumers were asked to complete a demographic questionnaire and answer questions regarding their usual rocket consumption (*n* = 90; 1 person declined to answer demographic questions).

Assessments were conducted in a similar manner to the trained sensory panel assessments presented in Bell et al. (2017*,* Chapter 5) over six weekdays. There were two main differences: consumers were presented with each accession only once, and were asked to assess the two leaves presented for each accession in combination rather than separately. Samples (random coded) were presented in a balanced design over two days (four samples at first visit, three samples at second) to avoid palate and trigeminal fatigue. On the second visit, volunteers were asked to provide a buccal swab sample (in duplicate) using C.E.P. ejectable buccal swabs (Fitzco International Ltd., Plymouth, UK).

6.3.3. DNA Extraction

Buccal DNA samples were extracted using an Omega Bio-Tek E.Z.N.A. Forensic DNA Kit (Norcross, GA, USA). 550µl of phosphate buffered saline (PBS) and 25µl of protease solution was added to each sample, a further 550µl of bacterial lysis buffer, then vortexed (30 s). Samples were incubated for 30 minutes at 60°C in a heat block with occasional mixing. Samples were subsequently centrifuged (14,000 x *g*), then 550 µl of 100% ethanol (Sigma, Poole, UK) was added, vortexed and centrifuged again. 700 µl of sample was passed through a Hi-Bind DNA mini column and centrifuged for 1 minute and repeated. 500 µl of isopropanol buffer was added to

columns and centrifuged for 1 minute. 700 μl of DNA wash buffer (diluted with 100% ethanol) was applied to columns and centrifuged, then repeated. Columns were dried by centrifugation for 2 minutes. DNA was eluted into sterile micro centrifuge tubes by adding 200 μl of preheated elution buffer (70°C) and left for 3 minutes at room temperature (~22°C). Samples were centrifuged for 1 minute and then the elution step was repeated. DNA was quantified using a NanoDrop ND 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and was subsequently stored at -20°C until analysis.

6.3.4. SNP Genotyping

SNP genotyping kits were obtained from Life Technologies Ltd. (Paisley, UK) according to the three most common alleles of the *hTAS2R38* gene: A49P (rs713598), A262V (rs1726866) and V296I (rs10246939). A reaction mixture of TaqMan Genotyping Mastermix (Life Technologies Ltd.) and primers was prepared as follows: 12.5 μl Mastermix, 1.25 μl primer, 6.25 μl d.H_2O and 5 μl of DNA template (25 μl total per reaction). 3 non-template controls were used on each genotyping plate. Analysis was performed on a 7300 Real Time PCR system (Applied Biosystems Inc., Foster City, CA, USA). PCR run parameters were as follows: 0 minutes at 55°C, 10 minutes at 95°C, 15 seconds at 92°C and 1 minute at 60°C. Alleles were automatically 'called' by RT-PCR software according to fluorescence probes. Genotype was determined by the presence/absence of the corresponding alleles; the diplotype of 69 individuals was successfully determined. The remaining 21 individuals either: 1) did not consent to having a sample taken (n = 1), 2) did not yield sufficient DNA for analysis (n = 2), or 3) failed to attend the second study visit (n = 19). The expected frequencies of diplotypes were determined by comparison to observations by Mennella, Pepino, Duke, & Reed (2010).

6.3.5. Phytochemical Analyses

Point-of-harvest GSL, flavonol, polyatomic ion (PI), headspace volatile organic compound (VOC), free amino acid (AA), free sugar and free organic acid (OA) data from previous studies were incorporated into a statistical analysis to determine significant correlations with consumer preferences and perceptions. These data can be found in Bell et al. (2015, 2016, 2017; Chapter 3, 4, & 5). All leaves were harvested 30 days after sowing (Hall, Jobling, & Rogers, 2012).

6.3.6. Statistical Analyses

To ensure an unbiased data set, only consumers who attended both tasting sessions were included in statistical analyses (n = 67). Preference and perception data underwent analysis of variance (ANOVA) with accessions as a treatment effect. Individual consumer TAS2R38 diplotypes were input as a nested effect in a separate ANOVA, testing genotype*sample interaction. All ANOVA were conducted using a 95% confidence interval and a tolerance of 0.0001%, and post-hoc Tukey's HSD test was used for multiple pairwise comparisons. Observed TAS2R38 diplotype frequencies were compared with expected frequencies (Mennella et al. 2010) by Pearson's chi-squared test. Any influence of bitter perception (normalised scores) on taste liking was tested by Pearson's correlation.

Agglomerative Hierarchical Cluster (AHC) analysis was used to identify liking and perception clusters; dissimilarity was determined by Euclidean distance, agglomeration using Ward's Method (automatic truncation). ANOVA was then carried out separately for each cluster. All clusters containing ≥20 people, plus clusters of ≤19 with significant discrimination between samples were included in subsequent Principal Component Analysis (PCA) analysis.

Taste liking data were used to extract principal components (PCs; Pearson *n*-1). Phytochemical data were fitted as supplementary variables, as well as the ratios between sugars and GSLs, sugars and ITCs, and organic acids and sugars (see Bell et al. 2017*,* Chapter 5), and cluster means. A correlation matrix was constructed as part of the analysis to determine significant correlations between variables ($P<0.05$, $P<0.01$ and $P<0.001$). Internal preference maps were produced using PCA of consumer data (firstly taste liking, secondly appearance liking), with sensory profiling data and AHC class centroids regressed as supplementary variables. The taste liking preference map also used AHC class centroids relating to mouthfeel liking as well as taste liking, and taste perception (normalised bitterness, sweetness, hotness and pepperiness) and purchase intent as supplementary variables. All analysis was carried out using XLStat (Version 12.0, Addinsoft, Paris, France).

6.4. Results and discussion

6.4.1. Consumer Demographics & Usual Rocket Consumption

Table 6.1 presents the summarised demographic data for this study. 77.7% of the participants were between the ages of 18 and 35. Recruitment around the University of Reading, led to high numbers of female participants (*n* = 69; 76.7%), and Asian and African (*n* = 24; 22.2% and 4.4% respectively) participants volunteering for the study. 72.2% of those who took part described themselves as having White ethnicity.

Participants were asked to answer one question about their usual rocket consumption: '*How often do you consume rocket when it is available?*' 36 people (40.0%) stated they sometimes eat rocket when available. 11 (12.2%) stated they never eat rocket, and only 4 (4.4%) said they always consume rocket when available. These responses indicate that the typical consumer makes conscious decisions

Table 6.1. Summary of study participant demographics (*n* = 90) and level of usual rocket consumption

Question	Number of individuals (%)
Age range	
18-25	40 (44.4%)
26-35	30 (33.3%)
36-45	15 (16.7%)
46-55	4 (4.4%)
56-65	1 (1.1%)
Ethnicity	
White European	26 (28.9%)
White British	37 (41.1%)
White Irish	2 (2.2%)
Asian Chinese	17 (18.9%)
White/Black Asian	1 (1.1%)
Black African	4 (4.4%)
Asian Bangladeshi	1 (1.1%)
Asian Indian	1 (1.1%)
Declined to answer	1 (1.1%)
Gender	
Male	21 (23.3%)
Female	69 (76.7%)
Rocket consumption	
Question: How often do you consume rocket when it is available?	
Never	11 (12.2%)
Rarely	19 (21.1%)
Sometimes	36 (40.0%)
Usually	20 (22.2%)
Always	4 (4.4%)

Table 6.2. Summary table of average consumer responses (*n* = 67), and class centroid values (determined by agglomerative hierarchical cluster analysis) for preference ('liking') and normalised antilog perception traits in seven accessions of rocket salad.

Trait	Mean score / AHC cluster means	No. in cluster (%)	SR2	SR3	SR5	SR6	SR12	SR14	SR19	*P*-value (sample effect)
Appearance liking	All		61.2^{ab}	57.5^{a}	62.8^{ab}	61.5^{ab}	62.5^{ab}	57.6^{a}	68.8^{b}	0.001
	Cluster 1	23 (34.3%)	64.5^{ns}	71.3^{ns}	64.2^{ns}	74.8^{ns}	73.3^{ns}	62.5^{ns}	70.5^{ns}	0.044
	Cluster 2	38 (56.7%)	55.1^{abc}	46.2^{a}	58.5^{bc}	51.2^{ab}	51.4^{ab}	48.7^{ab}	63.1^{c}	<0.0001
	Cluster 3	6 (9.0%)	87.2^{ab}	76.2^{a}	84.9^{ab}	76.0^{a}	91.3^{ab}	94.5^{ab}	98.3^{b}	0.011
Liking of colour	All		69.2^{ab}	63.8^{a}	68.5^{ab}	65.8^{ab}	64.6^{a}	65.2^{ab}	71.7^{b}	0.003
	Cluster 1	26 (38.8%)	71.8^{ns}	61.5^{ns}	68.7^{ns}	68.8^{ns}	64.5^{ns}	61.1^{ns}	68.7^{ns}	0.092
	Cluster 2	19 (28.4%)	81.8^{ns}	80.7^{ns}	83.7^{ns}	82.7^{ns}	81.1^{ns}	84.9^{ns}	84.9^{ns}	0.761
	Cluster 3	22 (32.8%)	55.5^{ab}	51.8^{a}	55.0^{ab}	47.5^{a}	50.4^{a}	53.1^{a}	63.9^{b}	0.001
Liking of shape	All		63.0^{ab}	58.3^{a}	59.6^{ab}	60.7^{ab}	63.3^{ab}	60.1^{ab}	68.6^{b}	0.026
	Cluster 1	20 (29.9%)	58.4^{ns}	51.2^{ns}	58.8^{ns}	53.5^{ns}	47.9^{ns}	44.4^{ns}	47.7^{ns}	0.096
	Cluster 2	24 (35.8%)	74.5^{ns}	75.7^{ns}	72.3^{ns}	66.4^{ns}	73.0^{ns}	74.3^{ns}	75.5^{ns}	0.511
	Cluster 3	23 (34.3%)	55.1^{abc}	46.3^{a}	46.9^{ab}	61.0^{bc}	66.7^{cd}	58.9^{abc}	79.4^{d}	<0.0001
Liking of mouthfeel	All		61.3^{ns}	62.7^{ns}	57.4^{ns}	61.6^{ns}	59.8^{ns}	60.3^{ns}	61.2^{ns}	0.586
	Cluster 1	28 (41.8%)	73.7^{ns}	75.1^{ns}	70.0^{ns}	74.6^{ns}	66.9^{ns}	72.5^{ns}	73.0^{ns}	0.453
	Cluster 2	7 (10.4%)	37.1^{a}	71.7^{b}	19.0^{a}	49.7^{ab}	43.6^{ab}	45.6^{ab}	39.2^{a}	0.001
	Cluster 3	32 (47.8%)	55.7^{ns}	49.8^{ns}	54.7^{ns}	52.9^{ns}	57.0^{ns}	52.9^{ns}	55.7^{ns}	0.429
Liking of taste	All		58.5^{ns}	62.2^{ns}	55.9^{ns}	59.2^{ns}	56.1^{ns}	58.1^{ns}	59.2^{ns}	0.420
	Cluster 1	25 (37.3%)	72.2^{ab}	80.1^{b}	69.4^{ab}	74.6^{ab}	63.5^{a}	70.7^{ab}	71.4^{ab}	0.079
	Cluster 2	36 (53.7%)	55.7^{ns}	51.8^{ns}	52.5^{ns}	53.4^{ns}	57.6^{ns}	53.1^{ns}	55.8^{ns}	0.685
	Cluster 3	6 (9.0%)	17.8^{ns}	49.9^{ns}	20.5^{ns}	30.0^{ns}	17.0^{ns}	35.3^{ns}	28.5^{ns}	0.074

Perception of bitterness	All		24.2^{ab}	22.7^{ab}	22.7^{ab}	21.8^{a}	27.1^{b}	25.8^{ab}	21.2^{a}	0.004
	Cluster 1	49 (73.1%)	19.9^{ab}	19.3^{ab}	18.6^{ab}	16.3^{a}	21.8^{ab}	22.5^{b}	17.8^{ab}	0.028
	Cluster 2	14 (20.9%)	30.4^{ab}	24.5^{a}	31.8^{ab}	33.1^{ab}	38.4^{b}	29.8^{ab}	26.0^{a}	0.002
	Cluster 3	4 (6.0%)	54.0^{ns}	57.0^{ns}	40.4^{ns}	50.0^{ns}	52.1^{ns}	53.0^{ns}	45.1^{ns}	0.371
Perception of hotness	All		16.0^{a}	16.3^{a}	18.9^{ab}	16.0^{a}	16.3^{a}	16.3^{a}	21.3^{b}	<0.0001
	Cluster 1	14 (20.9%)	9.4^{a}	12.9^{abc}	17.4^{bc}	11.8^{ab}	18.8^{c}	11.5^{ab}	12.1^{ab}	<0.0001
	Cluster 2	34 (50.7%)	17.5^{b}	14.8^{ab}	14.9^{ab}	13.8^{ab}	12.5^{a}	17.5^{b}	23.6^{c}	<0.0001
	Cluster 3	19 (28.4%)	18.3^{ab}	21.3^{abc}	27.1^{c}	23.0^{abc}	21.3^{abc}	17.6^{a}	24.0^{bc}	<0.0001
Perception of sweetness	All		12.5^{bc}	12.3^{bc}	8.6^{ab}	13.6^{c}	10.4^{abc}	11.5^{abc}	7.1^{a}	0.001
	Cluster 1	19 (28.4%)	23.3^{ns}	21.5^{ns}	19.6^{ns}	20.1^{ns}	19.8^{ns}	19.7^{ns}	12.2^{ns}	0.281
	Cluster 2	8 (11.9%)	3.9^{a}	17.6^{a}	7.2^{a}	35.8^{b}	10.1^{a}	14.3^{a}	7.9^{a}	<0.0001
	Cluster 3	40 (59.7%)	9.0^{b}	6.9^{ab}	3.7^{a}	6.1^{ab}	6.1^{ab}	7.0^{ab}	4.5^{a}	0.002
Perception of pepperiness	All		20.1^{ab}	21.5^{ab}	22.5^{ab}	21.4^{ab}	18.9^{a}	19.2^{ab}	23.2^{b}	0.011
	Cluster 1	44 (65.7%)	16.2^{a}	19.2^{ab}	19.9^{ab}	19.3^{ab}	18.4^{a}	19.4^{ab}	23.5^{b}	0.001
	Cluster 2	5 (7.5%)	5.8^{ns}	8.2^{ns}	9.4^{ns}	5.9^{ns}	6.3^{ns}	6.1^{ns}	7.7^{ns}	0.934
	Cluster 3	18 (26.9%)	33.6^{c}	30.8^{abc}	32.6^{bc}	23.7^{ab}	23.7^{ab}	22.2^{a}	26.7^{abc}	0.001
Purchase intent	All		3.1^{ns}	3.3^{ns}	3.0^{ns}	3.1^{ns}	3.0^{ns}	3.1^{ns}	3.3^{ns}	0.449
	Cluster 1	31 (46.3%)	3.6^{ns}	4.0^{ns}	3.5^{ns}	3.9^{ns}	3.4^{ns}	3.5^{ns}	3.8^{ns}	0.070
	Cluster 2	15 (22.4%)	2.2^{a}	2.6^{abc}	3.3^{abc}	2.5^{ab}	3.4^{bc}	2.4^{ab}	3.7^{c}	<0.0001
	Cluster 3	21 (31.3%)	2.8^{ns}	2.7^{ns}	2.0^{ns}	2.4^{ns}	2.1^{ns}	2.9^{ns}	2.1^{ns}	0.009

Differences in superscript letters within rows indicate significances according to ANOVA with Tukey's HSD test (P<0.05). ns = not significant.

about the rocket they consume, and there are sensory attributes on which they base these decisions. Rocket from diverse growing regions are currently all used the same way for each salad product sold on the market. Due to this blanket approach to the species, and the inherent sensory diversity present between varieties/growing regions, consistency within products is not guaranteed. For the consumer this could affect the likelihood of re-purchase, and affect how often they choose to consume rocket.

6.4.2. Consumer Preference, Perceptions & Purchase Intent

6.4.2.1. General

The response of consumers for each perception and preference modality tested is presented in Table 6.2. Each of the attributes assessed by consumers were consistently divided into three clusters in each respective AHC analysis. The average scores of all consumers are summarised, as well as the results of ANOVA Tukey HSD test pairwise comparisons. Within the text, clusters where a significant difference was observed (Tukey HSD test, $P<0.05$) are denoted by *. Clusters with <20 individuals, but contained significant differences between consumer scores, are denoted by ^.

6.4.2.2. Appearance liking

Appearance liking scores differed significantly between some accessions (Appendix II). The appearance of SR19 was liked significantly more than SR3 (commercial cultivar) and SR14. SR19 closely resembles the leaf morphology of *Diplotaxis tenuifolia* (“wild” rocket), even though it is *E. sativa*. This demonstrates consumers have generally come to like and accept this leaf appearance, as it is the

type they are most familiar with. SR3 and SR14 typically have much broader, less serrated leaf profiles.

From AHC analysis, appearance liking Cluster 2* (C2; *n* = 38, 56.7%) was the largest, and consumers differentiated their liking of appearance; generally these scores were lower than the total average. SR19 was again the most liked, and was significantly different from the commercial cultivar SR3. Appearance liking C3*^ was composed of only six individuals (9.0%), but showed a propensity for higher than average scores, and discriminated significantly between SR19, SR3 and SR6.

In terms of colour liking consumers discriminated significantly, again favouring SR19 over SR3 and SR12. Cluster analysis identified some consumers (C3*; *n* = 22, 32.8%) liked the dark green leaf colour of SR19 significantly more than the lighter coloured SR3, SR6, SR12 and SR14.

The liking of leaf shape was also significantly different between accessions. SR19 scored significantly higher than SR3 across all consumers. C3* individuals (*n* = 23, 34.3%) showed a high degree of preference for SR19 over SR2, SR3, SR5, SR6 and SR14, but C1 (*n* = 20, 29.9%) and C2 (*n* = 24, 35.8%) did not show any significant preference. C1 uniformly scored lower than average for all accessions, whereas C2 scored much higher for their leaf shape. These data indicate some people discriminate based on leaf shape, favouring a “wild” rocket-type leaf, but over two thirds show no significant preference.

6.4.2.3. Mouthfeel liking

The smallest cluster (C2*^; *n* = 7, 10.4%) showed a significant preference for SR3 over SR2, SR5 and SR19. Generally this attribute can be described as being comparatively unimportant with regards to most consumers’ preferences, with only a minority discriminating in their liking of these accessions.

6.4.2.4. Taste liking

Considering the whole consumer group there was no significant difference in the liking of taste between samples, and this was reflected in the largest cluster (C2, *n* = 36; 53.7%). The minority cluster (C3^, *n* = 6; 9.0%) disliked the taste of most rocket samples (scoring <50). For C1* (*n* = 25; 37.3%) there was a significant difference between accessions where the taste of the commercial sample (SR3) was liked significantly higher than for SR12. These people were generally very accepting of all seven samples (scoring >63.4), yet still differentiated significantly between them.

These data suggest over half of the people tested are indifferent to the taste of the tested cultivars, whereas a proportion of people like all rocket, but especially the milder cultivar (SR3). A small percentage of people conversely reject rocket taste to a large degree, and they do not discriminate for this modality.

6.4.2.5. Bitterness perception

The perception of bitterness has long been held as a defining criterion of whether individuals accept or reject *Brassicaceae* vegetables. The role diplotype of the TAS2R38 taste receptor plays in this response will be explored in following sections, but irrespective of genetics, consumers could differentiate bitterness significantly between some cultivars.

SR12 was perceived as more bitter than SR6 and SR19. Bitter perception C1* was the largest cluster (*n* = 49, 73.1%) and scores were low compared to the average. These people found SR14 to be significantly more bitter than SR6, whereas C2*^ (*n* = 14; 20.9%) conformed to the significance observed in the total average

scores (Table 6.2). These individuals scored higher by comparison to the average and to C1*, but not as high as the minority cluster C3^ (*n* = 4, 6.0%).

Neither SR12 nor SR14 contain especially high concentrations of GSLs (Bell et al. 2015; Chapter 3) or volatile ITCs (Bell et al. 2016; Chapter 4). Following the assumption these compounds are generally responsible for bitterness in rocket, one would expect SR5 to be perceived as the most bitter as it has been found to contain 11.5 $mg.g^{-1}$ dw in total GSL concentration, and observed to have a high percentage of volatile ITCs within the headspace. This suggests other compounds present within leaves contribute to bitterness to a greater degree than has been previously realised. The counter-hypothesis is the bitterness caused by GSL-related compounds are masked to some degree, either by sugars, amino acids, or green-leaf VOCs (Bell et al. 2017*,* Chapter 5).

6.4.2.6. Hotness perception

The perception and level of hotness has been used anecdotally to characterise the 'ideal' rocket leaf. As a whole cohort, consumers perceived SR19 to be the hottest and significantly different from SR2, SR3, SR6, SR12 and SR14. SR19 was shown to contain lower concentrations of GSLs than all of these accessions (with the exception of SR3, Bell et al. 2015; Chapter 3), and as with bitterness, indicates other compounds influence the perception of hotness, such as the sugar-ITC ratio (see *3.5.2.7.*).

Hotness was the only attribute measured in which all clusters discriminated significantly between accessions. C2* was the largest cluster (*n* = 34, 50.7%) and mirrored the consumer average, perceiving SR19 to be hotter than all of the other accessions. The smaller clusters did not follow this trend – in particular C3*^ (*n* = 19; 28.4%) perceived SR5 to be hotter than SR2 and SR14, and C1*^ (*n* = 14, 20.9%)

found SR12 to be the hottest and significantly different from SR2, SR6, SR14 and SR19. The apparent differences in perceptions between each of the clusters infers a genetic component is responsible, but further study of papillae numbers and specific genes involved would be required to draw any meaningful conclusions. As observed for attributes associated with heat in Bell et al. (2017*,* Chapter 5; initial heat, tingliness, warming) the hotness attribute measured here has a significant degree of variability. This suggests heat is a key characteristic in determining the liking of rocket, rather than bitterness, as has been observed in other crops (Schonhof, Krumbein, & Brückner, 2004).

6.4.2.7. Sweetness perception

Several significant differences were observed for sweetness perception on average and in the AHC analyses. Overall, the consumers found SR6 to be sweeter tasting than SR5 and SR19, which have been previously noted for high levels of hotness (Bell et al. 2017*,* Chapter 5).

C3* was the largest cluster for this attribute (*n* = 40; 59.7%) and scores were generally much lower than the average, and those of C1^ (*n* = 19; 28.4%) and C2*^ (*n* = 8, 11.9%). C3* found SR2 to be significantly sweeter than SR5 and SR19, and C2*^ found SR6 to be significantly sweeter than all the other accessions. C1^ individuals displayed no discrimination between samples, despite their scores being higher than the average. These data suggest the pungent compounds found in accessions such as SR5 and SR19 mask sweetness perception, which in turn mask bitterness. To develop new varieties of rocket that are more acceptable to the consumer, hotness, sweetness and bitterness must be considered together, not in isolation.

6.4.2.8. Pepperiness perception

SR19 was again scored significantly higher than SR12 for pepperiness overall, and higher than SR2 and SR12 in C1* (*n* = 44; 65.7%). C3*^ (*n* = 18; 26.9%) scores were by comparison higher than the average, but SR2 was perceived as being more peppery than SR14. The differences between the two main clusters (C1* and C3*^) suggest a subset of people perceive this attribute more intensely. Further study is needed in this area, as no previous data have been published in relation to rocket and consumer perceptions/liking of this trait.

6.4.2.9. Purchase intent

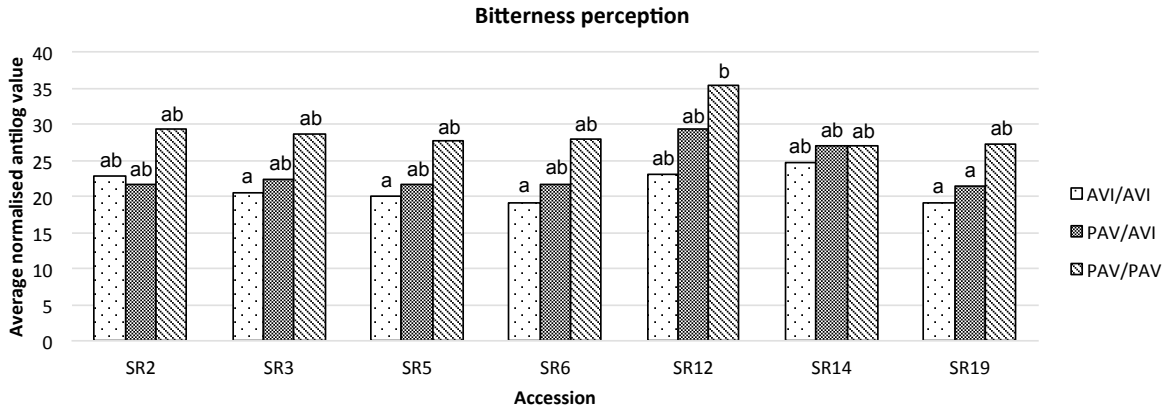
Overall there were no significant differences found for purchase intent, or for C1 (*n* = 31, 46.3%) and C3 (*n* = 21, 31.3%). C1 scores were generally higher than average, indicating the largest proportion of the cohort would consider buying most of the accessions were they all commercially available. C3 by comparison had lower than average scores, and would likely not buy any of the rocket accessions. Significant differences were observed for the smallest cluster, C2*^ (*n* = 15, 22.4%). These individuals would be significantly more likely to purchase SR19 than SR2, SR6 or SR14. These varieties are typically milder and sweeter, according to the cohort averages. The basis of preference is likely to be a combination of appearance and perception traits, with SR19 consistently being scored favorably for liking of appearance, hotness and pepperiness.

6.4.3. Effects of TAS2R38 diplotype

6.4.3.1. Taste liking and bitterness perception

Table 6.3 presents the numbers of each observed diplotype within the study. There was no significant difference between the observed and expected frequencies (Mennella et al. 2010; chi squared, *P* = 0.95). Figure 6.1 shows their respective average responses for perceived intensities of bitterness (a) and liking of taste (b).

a



b

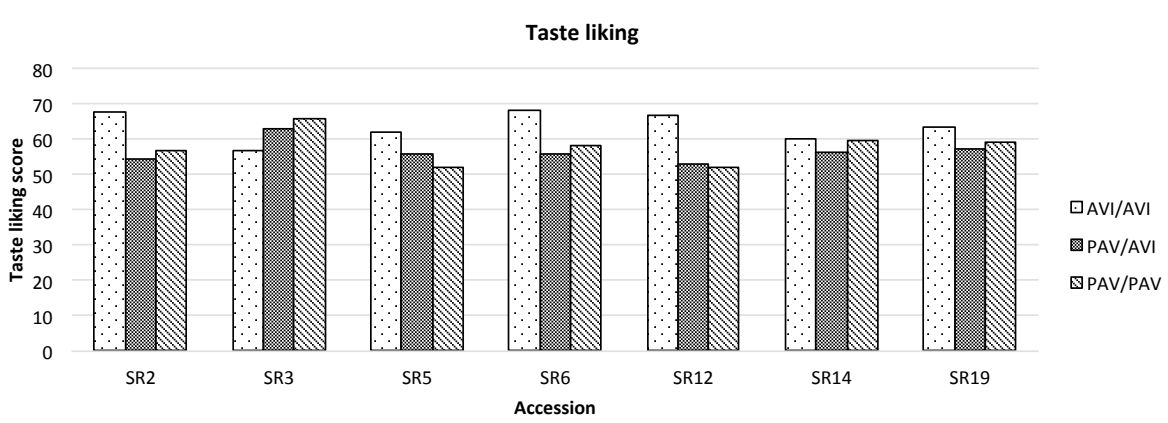


Figure 6.1. Consumer scores for bitterness perception (a) and taste liking (b) for seven accessions of Eruca sativa according to TAS2R38 taste receptor diplotype. Perception scores are given as normalised antilog values (a); differences in letters at the top of each bar indicate significant differences of ANOVA pairwise comparisons within and between accessions (P<0.05). An absence of letters indicates no significant differences were observed. See inset for diplotype colour coding.

TAS2R38 genotype had a significant effect on bitterness perception (P<0.02) (Figure 6.1a), and the effect of consumer genotype on bitterness scores was P<0.02 (ANOVA sum of squares analysis). This suggests a significant effect on bitter perceptions, but in the ANOVA there were no significant differences between genotypes within a specific rocket accession. The effect of diplotype is not as

Table 6.3. Summary of consumer TAS2R38 diplotype numbers (*n* = 69). Observed vs. expected numbers and percentages for the whole cohort and AHC taste liking clusters C1* (*n* = 25) and C2 (*n* = 36).

Diplotype	Observed number (%)	Expected %
Total cohort		
PAV/AVI	35 (52.2%)	51.1%
PAV/PAV	16 (23.9%)	24.3%
AVI/AVI	18 (26.9%)	24.6%
*Taste liking C1**		
PAV/AVI	12 (48.0%)	51.1%
PAV/PAV	6 (24.0%)	24.3%
AVI/AVI	7 (28.0%)	24.6%
Taste liking C2		
PAV/AVI	16 (47.1%)	51.1%
PAV/PAV	7 (20.6%)	24.3%
AVI/AVI	11 (32.4%)	24.6%
Undetermined[$]	2	-

Expected numbers determined by comparison to observations in Mennella et al. (2010), but not including the frequency of rare diplotypes. Chi-squared tests found no significant differences with expected frequencies (Total cohort, $P = 0.95$; C1*, $P = 0.918$; C2, $P = 0.564$). Chi-squared found no statistically significant differences between the observed frequencies in cluster C1* and C2 ($P = 0.919$).

* = Significant differences observed between scores (ANOVA, $P<0.05$; refer to Table 2).

[$] = Individuals present in taste liking cluster C2 but declined to provide a DNA sample; not included in % determination

pronounced as was originally hypothesised, but a general trend for ‘non-tasters’ to score bitterness of rocket lower than ‘medium’ or ‘supertasters’ is apparent.

The effect of consumer genotype was significant for liking of taste (P<0.004; ANOVA sum of squares analysis) however pairwise comparison scores (Figure 6.1b) were not significant when the interaction with the sample was taken into account. AVI/AVI individuals generally scored higher for liking in some accessions of rocket, however this pattern was reversed in accessions where bitter scores were low (SR3). In this instance, SR3 has been noted for high concentrations of AAs (Bell et al. 2017*,* Chapter 5), and for PAV/PAV ‘supertasters’ the relatively low concentration of GSLs and volatile VOCs infer higher liking.

The disparity between bitter perceptions and taste liking suggests TAS2R38 diplotype is only one of (potentially) many factors influencing an individual’s preference. A correlation test was performed independently of diplotype status on the total cohort data, comparing taste liking with bitterness perception. This test showed a significant negative relationship between the two attributes (r = -0.227, P<0.0001) and infers as bitter perception increases taste-liking decreases.

A similar observation was made by Shen, Kennedy, & Methven (2016) for perceptions of bitterness and liking in raw broccoli and white cabbage. Influences on liking according to TAS2R38 diplotype were observed, but this determination alone was not an accurate predictor of whether an individual would like or dislike *Brassica*-type vegetables. Other factors, such as consumer demographics, fungiform papillae density, familiarity with the food, and the conformation of other TAS2R taste receptors may also influence liking and preference in rocket.

6.4.3.2. TAS2R38 diplotype frequencies between agglomerative hierarchical clusters

The individuals in the two largest clusters for taste liking (C1* and C2) were scrutinised to see if the respective TAS2R38 diplotype frequencies therein conformed to the expected population frequency. As previously stated, C1* individuals tended be more discriminating of accessions (preferring SR3 overall) and C2 were indifferent. We hypothesised the frequency of PAV/PAV individuals would be higher in C1*, which would account for their preference of a non-bitter accession of rocket. The frequencies of each diplotype in each cluster were compared to total expected population frequencies (Mennella et al. 2010; Table 6.3) by chi-squared tests. No significant differences were found between the observed and expected frequencies in either cluster (C1*: $P = 0.918$; C2: $P = 0.564$). There was no significant difference in diplotype frequencies between the two clusters either ($P = 0.919$), further suggesting TAS2R38 status is not a singularly determining factor in consumer preference of rocket. The basis for preference is likely due to learned responses and/or other sensory factors as mentioned in the previous section (Shen et al. 2016).

6.4.4. Principal Component Analysis

6.4.4.1. Correlations between consumer preference & perceptions

Two biplots from the PCA are presented in Figure 6.2 and PCs were extracted on the basis of consumer taste liking scores. A total of six components were generated, all with Eigenvalues >1.0, but only the first five contained >10% of the explained variation. PC1 explained the largest amount of variance (24.9%) and predominantly separated SR12 from all other products. The other dimensions (PCs 2 to 5) all gave differing separations of the remaining accessions. PCs 1 vs. 4, and 1 vs. 5 have been selected for discussion as they represented the highest correlations with the supplementary AHC centroid scores and phytochemical variables according

a

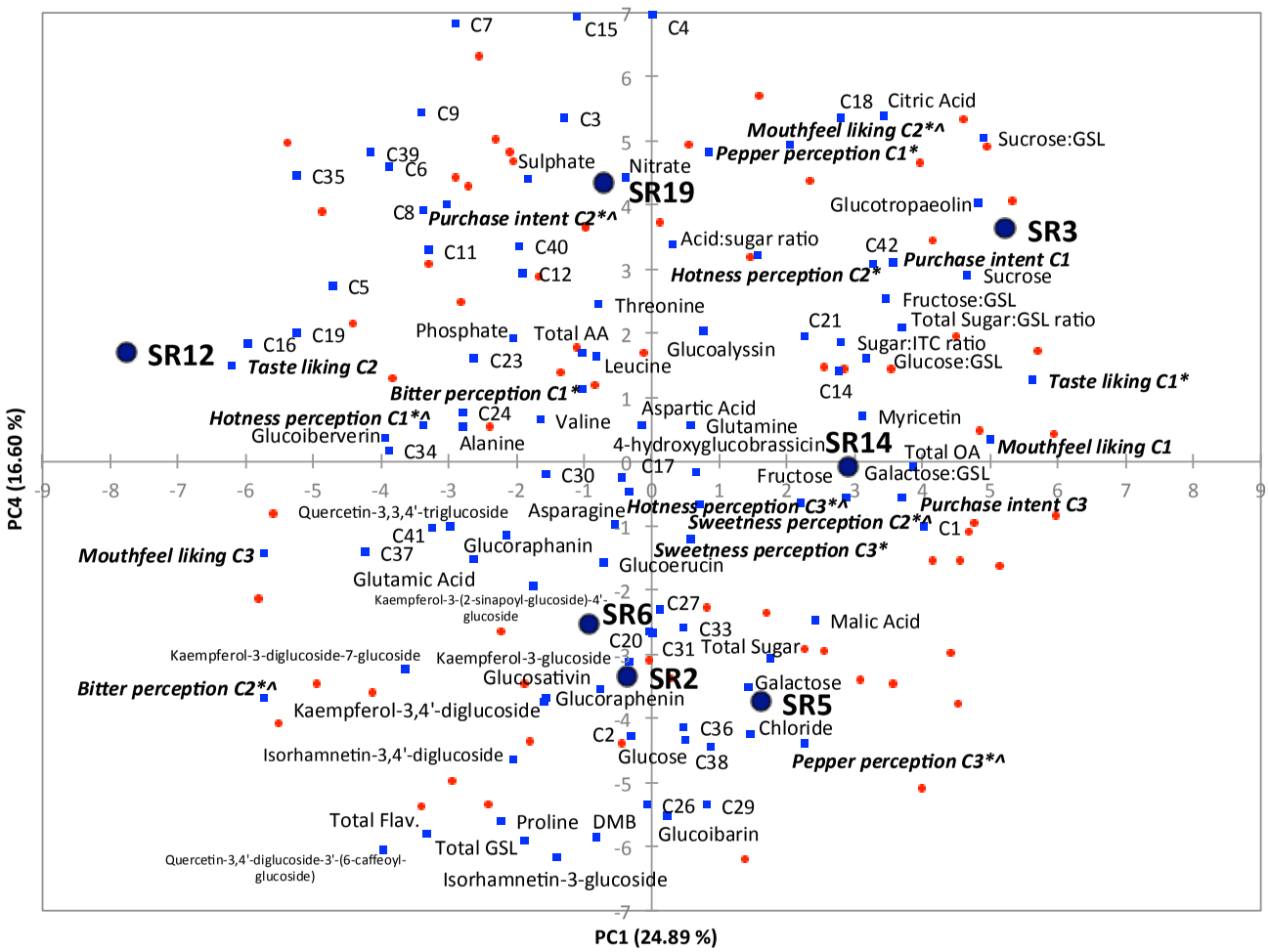


Figure 6.2. PCA biplot of consumer taste liking with phytochemical and AHC analysis (in bold italic; refer to Table 2) data regressed as supplementary variables. * = Significant differences observed with ANOVA (P<0.05). ^ = AHC cluster with <20 individuals. PC1 vs. PC4 (a) represents 41.5% of variation within the data, and PC1 vs. PC5 (b) represents 37.1% of variation within the data. Red circles = individual consumer responses; blue squares = supplementary variables; dark blue circles = rocket accession factor scores. VOC compound abbreviations (C#) are summarised in supplementary appendix VII, but can also be found in Bell et al. (2016).

b

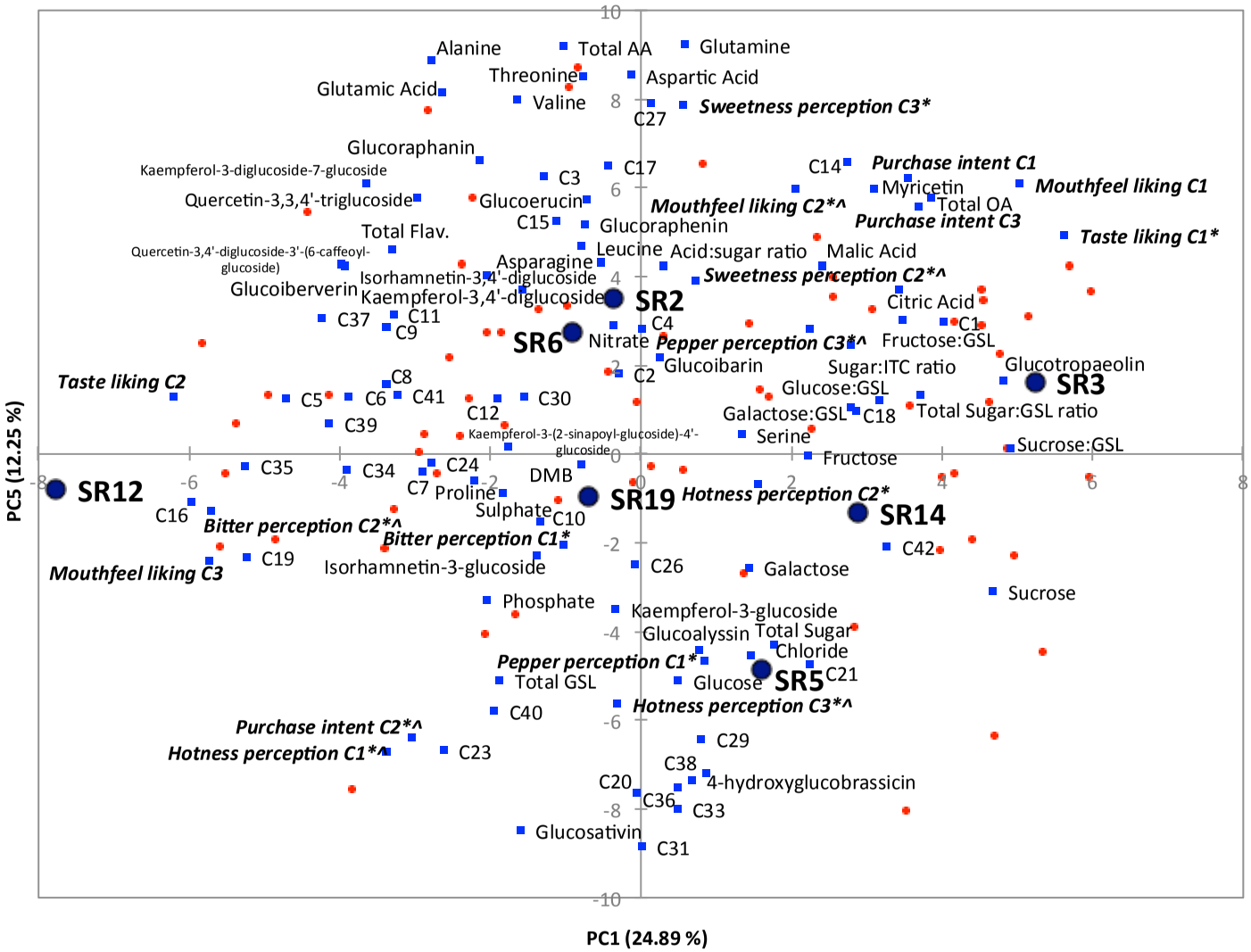


Figure 6.2. continued.

to their respective loadings scores; they are most informative for the purposes of this discussion. Cumulatively, these PCs illustrate 53.7% of the total variation within the data. For respective cluster scores for each accession refer to Table 6.2.

Mouthfeel liking C1 and taste liking C1* correlated highest along PC1 (Figure 6.2). These clusters locate closely with SR3 and purchase intent C1, indicating a preference of the commercial cultivar for some consumers. The bitterness of accessions such as SR12, to the extreme left of PC1 and away from SR3, indicates this preference is in part due to bitterness being perceived more intensely between accessions.

Sweetness perception C3* correlated most strongly with PC5, as did purchase intent C1. These attributes again co-locate near SR3 and SR2, further indicating bitterness and hotness are not desirable traits for a subset of the cohort. Similarly pepper perception C1* correlates most strongly along PC4. In the top right corner of Figure 6.2a, this attribute is associated with SR3 and SR19, and this suggests some individuals favor mild, peppery cultivars most. The individuals correlating highest along PC4 generally co-locate with SR19 and purchase intent C2*^ (Figure 6.2a). Combined with the relatively low perceptions of bitterness, these data indicate SR19 would be well suited to develop into a commercial product. Individuals showing a high degree of preference for SR19 would therefore be more likely to purchase rocket if it had more heat and pepperiness, and a low level of bitterness.

6.4.4.2. Correlations with phytochemical content

6.4.4.2.1. General

A summary table of all phytochemical-AHC correlation coefficients and significances is presented in supplementary appendix VII.

6.4.4.2.2. Glucosinolates

In the PCA biplot presented in Figure 6.2, concentrations of GSLs yielded significant correlations with consumer preference and perception AHC centroids. Glucosativin was significantly inversely correlated with scores for purchase intent C1 and mouthfeel liking C1 (r = -0.841, -0.766; both P<0.05). Individuals in these clusters were non-discriminatory but gave higher than average scores for each accession. Glucosativin is the most abundant GSL in these samples, and a high abundance infers reduced liking.

Glucoraphanin concentration has no significant positive or negative effects on consumer preferences or perceptions, indicating it and its hydrolysis products do not have an inherent taste. The compound separates strongly on PC5 (Figure 6.2b), and towards the upper left, away from the positions of perception clusters. The broccoli variety *Beneforté* has been bred for very high concentrations of glucoraphanin/sulforaphane, and no significant impacts on taste or flavour have been reported (Traka et al. 2013).

Another health beneficial GSL is erucin, which separates along PC5, and significantly with sweetness perception C2*^ (r = 0.894, P<0.01). Glucoraphenin is also significantly correlated with this attribute (PC5; r = 0.851, P<0.05), but is only found in small concentrations in SR2 and SR6 (Bell et al. 2015; Chapter 3). These compounds are unlikely to be causing sweetness themselves, but are more abundant in sweet-tasting accessions (Bell et al. 2017*,* Chapter 5*;* Bell et al. 2015, Chapter 3). Future rocket breeding should perhaps be selective for individual health beneficial GSLs such as glucoraphanin and glucoerucin, as suggested by Ishida et al. (2014).

Glucoalyssin was significantly correlated with pepper perception C1* and hotness perception C2* scores (r = 0.896, 0.764; P<0.01 and P<0.05, respectively). 4-hydroxyglucobrassicin was positively correlated with scores from hotness

perception cluster C3*^ and negatively with sweetness perception C3* (r = 0.805, -0.826; both P<0.05). These observations were also made by Bell et al. (2017, Chapter 5) and indicate 'minor' GSLs of rocket contribute significantly to taste and flavour perceptions. Just as glucoraphanin is selected to produce health beneficial properties in plants, minor GSLs could also be selected to produce enhanced sensory properties.

6.4.4.2.3. Flavonols

Negative correlations were observed for isorhamnetin-3-glucoside with hotness perception C2* (r = -0.859), and quercetin-3,3,4'-triglucoside and kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside with pepper perception C1* (r = -0.767, -0.793; all P<0.05). The reduction in perceptions implies an increased abundance of these flavonols is associated with reduced pungency.

Another significant positive correlation observed was for bitter perception C1*, the largest bitter perception cluster, and kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside (r = 0.782, P<0.05). It is unusual for a flavonol to have bitter taste, though in the complex matrix of the rocket leaf, consumers could have interpreted astringency as bitterness. It is likely field-grown rocket would have produced higher concentrations of flavonols due to higher light intensities than controlled environment (Bell et al. 2015, Chapter 3; Jin et al., 2009), and therefore might have produced stronger effects within the data. Further study is needed to properly determine the extent that flavonol glycosides influence taste attributes in rocket.

6.4.4.2.4. Polyatomic ions

Nitrate and sulfate were both correlated with the largest hotness perception cluster (Figure 6.2, C2*; r = 0.817, 0.871, both P<0.05). In Figure 6.2a, these compounds are closely associated with SR19, which is likely responsible for the significant correlations.

Nitrate and sulfate assimilation pathways are known to be integral to GSL and amino acid metabolism within leaves (Hirai et al. 2004). By comparison to the other cultivars, GSL concentration was not high in SR19 (Bell et al. 2015; Chapter 3), which suggests total GSL content alone is not a good indicator of hotness of rocket. The diversity of GSLs and VOCs, and the relative concentrations of accumulated PIs and free sugars likely to interact to determine the heat perceived. Future studies should therefore explore and take these aspects into consideration when conducting sensory and phytochemical analyses of rocket.

6.4.4.2.5. VOCs

C numbers in bold within the text refer to VOCs labeled in Figure 6.2; see appendix VII for a list of compounds and their corresponding abbreviations.

An unexpected association with sweetness perception C3* was observed with 3-methyl-furan (**C27**; r = 0.979, P<0.01), and a corresponding negative correlations with hotness perception C3*^ and pepper perception C1* (r = -0.840, -0.841, both P<0.05). Bell et al. (2017*,* Chapter 5) observed that this compound was significantly inversely correlated with bitter perception, but no corresponding association with sweetness. C3* was the largest cluster for sweetness perception, and the high degree of separation along PC5 (Figure 6.2b) means the compound could be utilised as a chemical marker for non-pungent, sweeter varieties of *E. sativa*. The compound was also significantly correlated with increased purchase intent C3 (who generally

would not buy rocket), and inversely correlated for purchase intent C2*^ (who discriminated for the hot accession SR19). This suggests hotness is preferable for one group of consumers, but is rejected by another.

Sweetness perception C3* also shared corresponding significant negative correlations with 4-methylpentyl-ITC (**C20**; *r* = -0.764), 1-isothiocyanato-3-methylbutane (**C23**; *r* = -0.869), iberverin (**C33**; -0.844), pyrrolidine-1-dithiocarboxylic acid 2-oxocyclopentyl ester (**C36**; -0.778) and an unknown compound (**C40,** *r* = -0.832; all *P*<0.05). Individually, very little is known about the aroma characteristics of these compounds, but ITCs and their derivatives are generally known for sulfurous, pungent and unpleasant attributes (Engel, Baty, Le Corre, Souchon, & Martin, 2002). These data suggest higher abundance has a powerful masking effect on sweetness. This is particularly evident in Figure 6.2b where these compounds are clustered near to SR5 and SR19, which are both noted for their hotness (Table 6.2).

The same compounds were positively correlated with hotness perception C2* and C3*^ (**C20** *r* = 0.814**, C23** *r* = 0.794**, C36** *r* = -0.778; all *P*<0.05; **C33**, *r* = 0.881, *P*<0.01). Additionally, 5-nonanone oxime (**C21**, *r* = 0.790) and tetrahydrothiophene (**C38** *r* = 0.765; both *P*<0.05) were also associated with these clusters. The later compound in particular has been previously associated with hotness and pungency in rocket (Bell et al. 2017*,* Chapter 5).

Pepper perception C1* (discriminated for SR19) was negatively correlated with 3-methyl-furan (**C27**, *r* = -0.841), as with hotness perception C3*^ (Figure 6.2b). Pepperiness perception C3*^ shared negative correlations with several volatiles, such as 2-hexenal (**C7**, *r* = 0.783), (E)-2-pentenal (**C10**, *r* = -0.772), 5-ethyl-2(5H)-furanone (**C12**, *r* = -0.840) and ethylidene-cyclopropane (**C24**, *r* = -0.798; all *P*<0.05). The green-leaf VOCs **C7** and **C10** were noted by Bell et al. (2017*,* Chapter 5) for being linked with sweeter-tasting cultivars, and detracting from the sensations of

bitterness and pungency. **C12** has previously been observed in tomato as a degradation product of (Z)-3-hexenal (**C16**; Buttery & Takeoka, 2004). The presence of these compounds within the headspace of rocket has important implications for consumer perceptions of pungent traits.

The dichotomy between those individuals who prefer hotter accessions and those who prefer milder can be seen in highly significant correlations with the ITC **C23**. Purchase intent cluster C2*^ (who discriminated for SR19) are positively correlated with this compound (r = 0.937, P<0.01) and purchase intent cluster C3 (who had uniformly low scores for purchase intent) is the inverse of this (r = -0.913, P<0.01). This implies part of the reason why the latter individuals (31.3%) scored the accessions so low is because of the abundance of ITCs. Taking into account the fact that glucoraphanin shared no significant correlations with sensory perceptions, it is desirable to breed rocket with reduced pungency and maintain health beneficial components. This would cater to the previously undefined demographic of consumers who reject rocket because of the hotness of leaves.

6.4.4.2.6. Free amino acids

High free AA concentrations detracted from the perception of pungent compounds such as ITCs in Bell et al. (2017*,* Chapter 5). In this study only one significant negative correlation was observed between pepper perception C1* and proline concentration. Proline is spatially distant at the bottom of the plot (Figure 6.2a), separating negatively along PC4 from the peppery accession SR19.

Threonine correlated significantly with purchase intent C1 (r = 0.755, P<0.05) and is known to have sweet taste (Nelson et al. 2002). AAs correlated along PC5 (Figure 6.2b) and are more highly associated with the milder accessions SR2 and SR6. This indicates amino acid content is generally in opposition to hotness, but

further study is needed to determine the full extent of the effects. Repeat experiments with other cultivars of rocket would help to confirm or reject this hypothesis.

6.4.4.2.7. Free sugars, organic acids and compound ratios

Fructose concentration was positively correlated with purchase intent C3 (r = 0.755, P<0.05), further suggesting these individuals would prefer rocket sweeter and less hot. Correlations with sugar-GSL and sugar-ITC ratios were more numerous. Purchase intent C3 (where scores were uniformly low) was correlated with high fructose-GSL, galactose-GSL and sugar-ITC ratios (r = 0.838, 0.791, 0.820; all P<0.05). This suggests the ratios between sugars and GSLs/ITCs are more important in determining consumer acceptance than the concentrations of each compound individually. The sugar-ITC ratio had a negative correlation with hotness perception C3*^ (r = -0.777, P<0.05), inferring higher sugar content masks hotness for a proportion of consumers, but not all, as no corresponding correlations were observed for C1*^ or C2*.

The sucrose-GSL ratio negatively correlated with bitterness perception C2*^. This ratio is almost directly opposite to SR12 (Figure 6.2b), separating strongly along PC1. SR12 was noted for high perceptions of bitterness (Table 6.2), and these data infer, for a proportion of the cohort (20.9%), the effect was an important determining factor in their responses. As this was not seen in the other clusters, other factors such as TAS2R receptor status and fungiform papillae density could impact the effect sugar-GSL ratios have upon perceived bitterness.

6.4.5. Internal preference map PCA

6.4.5.1. Sensory perceptions

Figure 6.3a presents a preference map of consumer taste liking scores, where sensory panel data for all attributes (taken from Bell et al. 2017*,* Chapter 5; except appearance traits; see following section) and AHC centroids for mouthfeel liking, taste liking, perceptions and purchase intent have been regressed as supplementary variables. A summary table of relevant correlations is presented in appendix VIII.

Six PCs were extracted from the consumer liking data, with all having Eigenvalues >1.0. PCs 1 – 5 contained >10% of explained variation, respectively, but PC1 and PC2 discriminated most strongly for consumer responses, AHC centroid scores and sensory attribute scores. As such these two components were selected for presentation and 44.4% of the total variation is explained.

Of note are several correlations between sweet perception C3* and sensory analysis scores. Centroid scores for this cluster (which were discriminatory, but generally low) were inversely correlated with attributes such as stalky odour (r = -0.820, P<0.05), bitter taste (r = -0.906, P<0.01), bitter aftereffects (r = -0.836, P<0.05) mustard aftereffects (r = -0.822, P<0.05) and initial heat mouthfeel (r = -0.815, P<0.05). These correlations suggest perceptions of sweetness for these individuals are low predominantly because of the pungency, heat and bitterness of leaves (such as in SR5 and SR19) masking the taste.

Taste liking C1* was negatively correlated with earthy flavour attributes identified by the trained assessors (r = -0.872, P<0.05). This was also seen for purchase intent C1 (r = -0.950, P<0.01), where scores were generally high for all accessions, but lower where earthy flavour was more prominent (SR12; Figure 6.3a). Taste liking C2 by comparison was negatively correlated with mustard odour (r = -0.782, P<0.05). Purchase intent C3 was negatively correlated with bitter taste (r = -

a

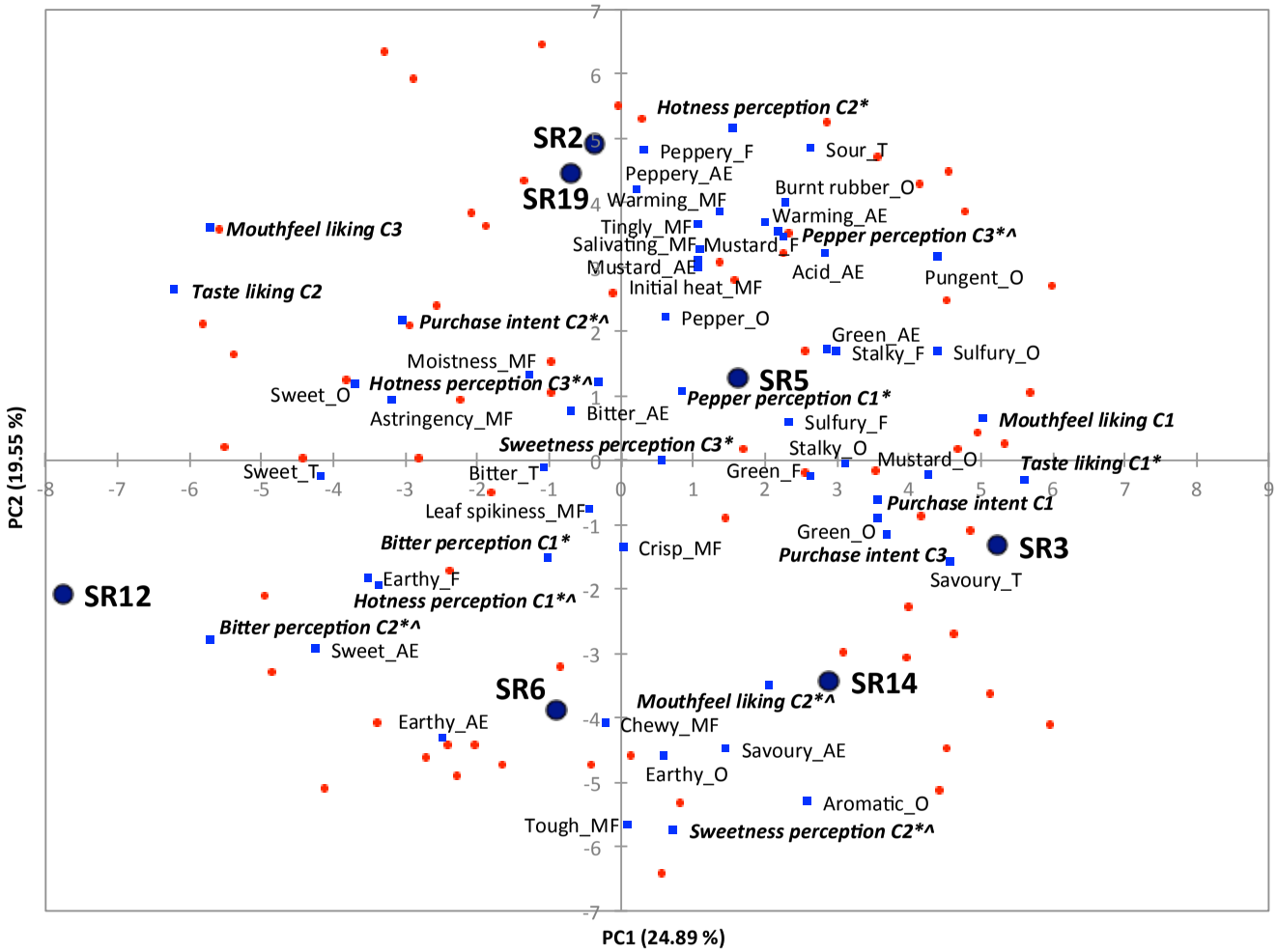


Figure 6.3. Internal preference map PCA biplot of consumer taste liking (a) and consumer appearance liking (b) with AHC analysis (in bold italic; refer to Table 6.2) and sensory data regressed as supplementary variables (obtained from Bell et al. *in press*) PC1 vs. PC2 (a) represents 44.4% of variation within the data, and PC1 vs. PC3 (b) represents 44.3% of variation within the data. Red circles = individual consumer responses; blue squares = supplementary variables; dark blue circles = rocket accession factor scores. Sensory variable suffix abbreviations: A = appearance; O = odour; T = taste; F = flavour; MF = mouthfeel; AE = aftereffects.

b

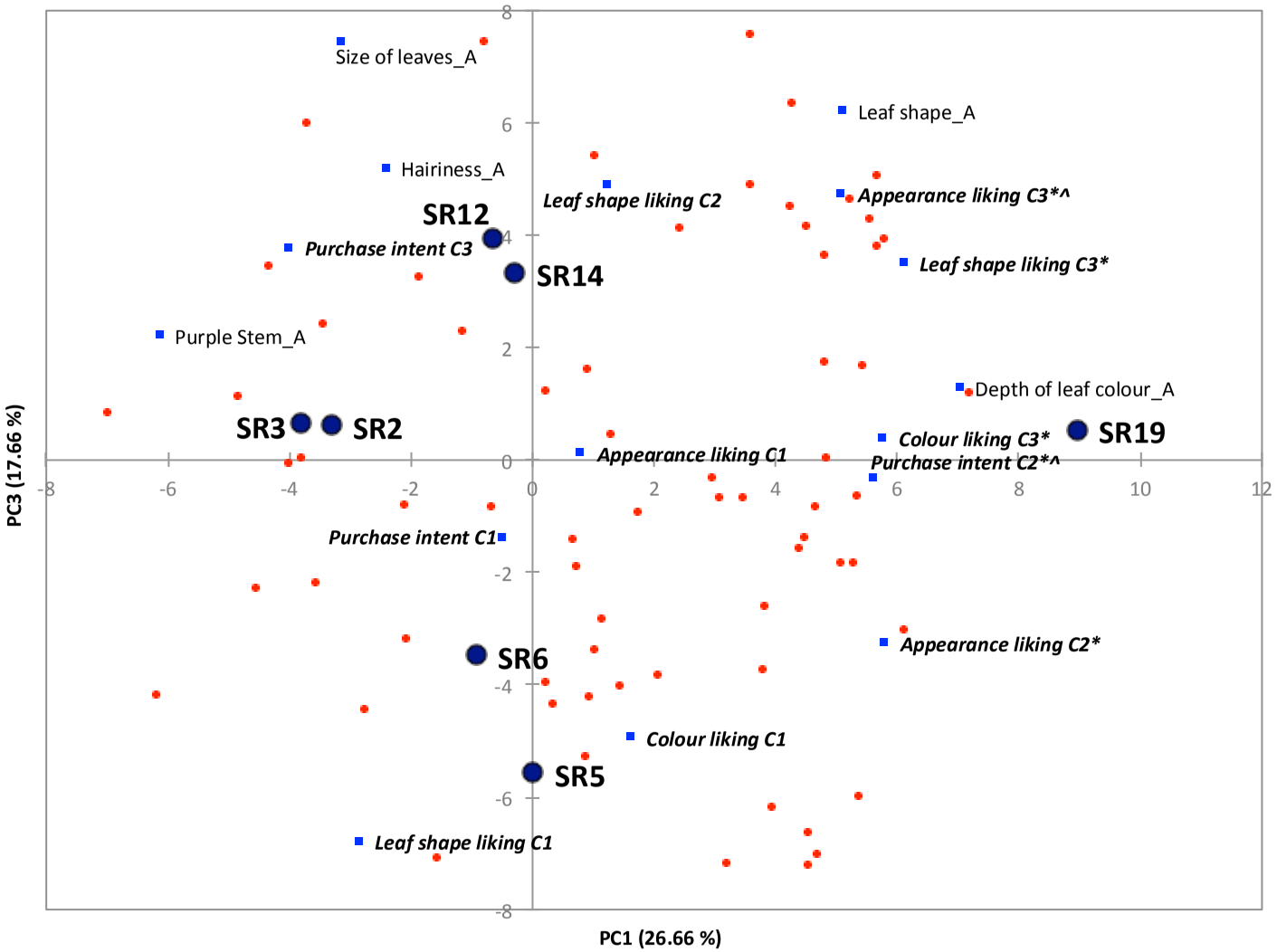


Figure 6.3. continued.

0.855, *P*<0.05) and further implies a uniform dislike of rocket because of their perceptions of bitterness and hotness.

6.4.5.2. Appearance liking

Figure 6.3b illustrates a preference map of consumer appearance liking scores, where sensory data for appearance traits (Bell et al. 2017*,* Chapter 5), and AHC centroids for appearance liking traits and purchase intent have been regressed onto the PCA. A summary table of relevant correlations is presented in appendix IX. Six PCs were extracted from the data, with all scoring >1.0 Eigenvalues and >10% explained variability, respectively. PCs 1 and 3 discriminated the supplementary variables to the highest degree, and were selected for presentation (44.3% of data variation is explained).

A disparity between leaf shape clusters was observed. Leaf shape liking C1 was negatively correlated with leaf shape uniformity scores from the sensory analysis (*r* = -0.887, *P*<0.01), whereas leaf shape liking C3* was positively correlated (*r* = 0.798, *P*<0.05). C3* individuals, who discriminated for SR19 and the traditional rocket shape, prefer this type of leaf and the relative uniformity of the accession. C1 individuals did not discriminate significantly, but tended towards liking the shape of the broad-leaved accessions. A proportion of people therefore find the novel leaf types unobjectionable, but another proportion prefers the more familiar “wild” type. This dichotomy in preference can be observed in Figure 6.3b where these clusters are in opposing quadrants of the biplot, and associated with SR19 in the upper right of the plot, and SR5 and SR6 in the lower left.

Correlations along PC1 indicate many consumers overall preferred the appearance of SR19. The high concentration of data points to the right is indicative of this, and the shape, colour, serrated and dark green leaf type of this accession has

likely driven this trend in the consumers. There is an indication of a general and substantial preference of this accession over the less familiar, round-shaped leaves overall. SR2, SR3, SR12 and SR14 are associated with attributes such as leaf hairiness and purple stem. It is perhaps unsurprising that hairiness is an undesirable attribute, but the purple stem has previously been thought of as a unique selling point for varieties, such as in the variety *Dragon's Tongue* (Tozer Seeds). This trait was significantly and inversely correlated to purchase intent C2*^ (r = -0.932, P<0.01), indicating a proportion of individuals found this trait to be undesirable.

6.5. Conclusions

This study has for the first time conducted a consumer analysis of *E. sativa* accessions in conjunction with sensory, phytochemical and human genotype analyses. The hypothesis all consumers reject bitter tasting cultivars is not fully supported by the data presented, even when human TAS2R38 diplotype of consumers is considered. Genotype effects are significant in determining the degree to which a person will rate the bitterness of rocket and their liking of taste; but when considered with sample effects, pairwise comparisons did not reveal significant differences with any specific cultivar tested. 'Supertaster' (PAV/PAV) individuals generally scored higher for bitterness and lower for taste liking, whereas AVI/AVI individual were the opposite of this (with the exception of the commercial cultivar, SR3). When these data are viewed in combination with AHCs and phytochemical correlations, it seems the predominant basis of acceptance/rejection is actually more related to the perceived hotness of leaves, rather than bitterness.

Distinct clusters of consumer have been identified that show preferences for different accessions on the basis of phytochemical content and sensory properties, such as for and against ITCs and potent sulfur-containing VOCs. Our second

hypothesis that hotness, pepperiness and sweetness were positive traits was therefore not wholly accurate. Consumers preferred peppery cultivars like SR19, but a substantial proportion of people within the study preferred the ‘milder’ cultivar SR3. Many of the consumers were indifferent to any of the accessions, and roughly a third would generally not purchase these cultivars.

The results run in opposition to the general dogma that a) rocket varieties should all be hot, but not bitter, and b) consumers either like or dislike varieties on this basis. The present study has shown this is an oversimplification of reality, and reduced hotness is a desirable sensory trait for a subset of consumers. Some of the consumers analysed preferred the hotness, pepperiness and appearance of SR19, perhaps making it the most accepted “all-round” accession tested in this study. By comparison, SR12 was perceived negatively due to its high levels of bitterness, and SR5 was not favored because of its high levels of hotness and low levels of sweetness.

High concentrations of specific phytochemicals that typically contribute towards hot and bitter sensations are not acceptable to some consumers. Breeding varieties for high total GSL/ITC content is an unsophisticated approach that does not account for these differences in consumer preference. Some preferred the hot ITC and sulfur compounds that are produced from and associated with the GSL-myrosinase reaction (as in SR19), but a substantial proportion rejected accessions because of low sugar-ITC ratios.

It is also important to note the health beneficial GSL glucoraphanin had no significant effect on consumer perceptions and preferences. This adds weight to our hypothesis that specific GSLs can be increased through breeding without having a negative impact on sensory attributes (Bell et al. 2017*,* Chapter 5). With regular consumption of rocket and sulforaphane (the ITC of glucoraphanin) consumers could

potentially improve their long-term health and reduce the risk of developing chronic diseases, such as cardiovascular disease and some forms of cancer (Traka et al. 2013).

The results of this study illustrate consumers of rocket leaves are able to differentiate between accessions, and are much more sophisticated in their evaluation of leaves than has been previously realised. Not all consumers of rocket are alike, and as such desire products that match their tastes. Plant breeders and processors must attempt to amalgamate positive visual, sensory and phytochemical traits in rocket to expand the market to individuals who at present are not specifically catered for. This can be achieved in the short term by selection of varieties that can produce a known and consistent standard of expected 'quality', and are well suited to specific growing regions or climates. In the long term, new varieties must be produced that account for the diverse preferences of consumers, such as those who prefer sweet and 'milder' leaves, and those who prefer hot and peppery leaves. These products must also be marketed appropriately; just as different types of apples are known for their differing sweet and sour tastes, rocket types could also be subdivided according to sensory properties and their intended consumer demographic.

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CHAPTER 7: Changes In Rocket Salad Phytochemicals Within The Commercial Supply Chain: Glucosinolates, Isothiocyanates, Amino Acids, & Bacterial Load Increase Significantly Over Time

7.1. Introduction To Paper (submitted to *Food Chemistry*)

After performing comprehensive phytochemical, sensory and consumer analyses on rocket cultivars under controlled conditions, a second phase of research was initiated. Cultivars from the previous analyses were selected to move forward into a 'real-world' research environment. This involved extensive collaboration with the industrial sponsors Elsoms Seeds and Bakkavor, and utilisation of their resources and infrastructure to grow, transport and process leaf material. The presented work was also in collaboration with a fellow PhD student, H. Nadia Yahya, who conducted the bacterial experiments. These data are presented here to provide a holistic view of the experiment, and highlight the previously unknown links between glucosinolates, isothiocyanates and bacterial leaf populations.

With a sizeable body of work already conducted, the aim of this study was to determine if the conclusions drawn previously would hold true for industrial produce. The experiment was large in scope and scale, and to the author's knowledge, is the first whole supply chain phytochemical analysis experiment conducted for any *Brassicaceae* crop that extends from field, to processing, to post-harvest shelf life.

7.2. Introduction

The majority of rocket (*Eruca sativa* & *Diplotaxis tenuifolia*) consumed in the UK is imported from Italy (Bell, Spadafora, Müller, Wagstaff, & Rogers, 2016; Chapter 4). In 2015 sales of bagged rocket salad in the UK increased 3.9% on the

previous year (Dr. Lorraine Shaw, Bakkavor, Spalding, UK; personal communication, 2016) and this trend is expected to continue in future.

Leaves are typically harvested by machine from long, linear beds in open fields, polytunnels or glasshouses. Time from sowing to harvest can be between 20 to 40 days depending on the growing region and species (Bell, Oruna-Concha, & Wagstaff, 2015; Chapter 3), and has been reported to extend up to 99 days in winter months (Hall, Jobling, & Rogers, 2012). Growing methods vary according to region and grower preference. Produce for the bagged salad market is generally processed in the same way; after harvesting, leaves are vacuum chilled and stored under cool-chain conditions (<5°C) until processing. This may be at the site of harvest, a nearby facility, or after transport to the country where it will be sold and consumed. Leaves enter a 'low care' environment, and are typically washed in chlorinated water (Rico, Martín-Diana, Barat, & Barry-Ryan, 2007) with mechanically induced water turbulence to remove detritus. Leaves are spin-dried in a high care environment to remove excess water, and then passed into a 'high care' environment, where it is weighed and bagged. Products use micro or laser perforated bags that contain modified or unmodified atmosphere to preserve and prolong self life (Hall, Jobling, & Rogers, 2013). Bags are shipped through a cold-chain to supermarkets and other vendors who store them in open-fronted chiller cabinets (Hall et al. 2013). Shelf life of rocket has been reported to range from seven to 14 days depending on environmental conditions (Martínez-Sánchez, Allende, Cortes-Galera, & Gil, 2008).

The stressful nature of the supply chain on leafy produce has led to questions regarding how nutritional value is affected (Verkerk et al., 2009). It is known that adverse storage conditions post harvest have a negative impact upon the appearance and odour of leaves (Lokke, Seefeldt, & Edelenbos, 2012). Cutting and processing material also makes it more perishable during storage (Watada, Ko, &

Minott, 1996), and temperature is the predominant means by which degradation is controlled (Lokke et al. 2012). There has been little research into how nutritional traits are affected by the industrial supply chain in leafy salads. Studies have covered parts of the supply chain for different *Brassicaceae*, such as effects of cutting and washing (Martínez-Sánchez et al. 2008), post harvest storage (Bell et al. 2016; Chapter 4), and packaging treatments (Rangkadilok et al., 2002).

In this study, a commercial supply chain was utilised to assess phytochemical profiles of rocket salads across multiple time points – immature leaves, harvest, processing, and throughout shelf life. Building upon previous phytochemical, sensory and consumer analyses (Bell et al. 2015, Chapter 3; Bell et al. 2016, Chapter 4; Bell, Methven, Signore, Oruna-Concha, & Wagstaff 2017*,* Chapter 5), six underutilised germplasm accessions and one commercial variety were tested for glucosinolate (GSL), isothiocyanate (ITC), free amino acid (AA), and free sugar concentrations. The aim of our work is to inform the breeding selections and practices of industrial collaborators to create new, sensorially and nutritively enhanced varieties of rocket. The accessions used throughout have been shown to vary significantly in phytochemical composition under controlled environmental conditions, but it is unknown how these might change under industrial circumstances.

Rocket is well known for accumulating GSL compounds, which are hydrolysed by myrosinase enzymes into ITCs, nitriles, and other degradation products (Bell & Wagstaff, 2014; Chapter 2). ITCs such as erucin (4-(methylthio)-butyl-ITC) and sulforaphane (4-(methylsulfinyl)-butyl-ITC) are both present in rocket species, and their potential anticarcinogenic properties are well studied in the literature (Traka et al. 2013). Other ITCs present in rocket are not well understood. The GSLs DMB (dimeric 4-mercaptobutyl-GSL), glucosativin (4-mercaptobutyl-GSL), diglucothiobeinin (4-(β-D-glucopyranosyldisulfanyl)-butyl-GSL), and their respective

myrosinase degradation products are poorly understood in terms of abundance and anti-cancer properties. As demonstrated in Bell et al. (2017*,* Chapter 5) some of the volatile derivatives of the GSL-myrosinase reaction, infer significant associations with sensory attributes such as bitterness and pungency. Some GSLs such as glucoerucin and glucoraphanin have no significant sensory properties associated with them.

In Bell et al. (2017*,* Chapter 5), total AA concentration was negatively correlated with the perceptions of bitterness and pungency, leading to the hypothesis that certain AAs contribute to sensory qualities of the crop (Solms, 1969). The way AAs respond to commercial processing may therefore impact upon sensory traits, and are an important indicator of senescence and tissue breakdown (Buchanan-Wollaston et al. 2003). Free sugars may also impact sensory attributes by masking bitter and pungent sensations, though it is unknown how they are affected by processing in rocket.

Another important aspect of rocket in the supply chain is the presence of bacteria (which are naturally present on leaves). Usually these are non-pathogenic strains and do not pose a health concern for humans, but can contribute to spoilage and shorten shelf life (Lokke et al. 2012). It has been known for over 20 years that chlorinated or chemically treated water does not erradicate bacterial populations from leaves, but does have a role to play in ensuring santitation of recirculated water in processing facilities. Strict field technical control protocols are followed to prevent contamination with pathogenic strains (Dr. Lorraine Shaw, Bakkavor, Spalding, UK; personal communication, 2016), however native leaf bacteria reside within cells and crevaces on the leaf surface, making it impossible to fully remove them from fresh-cut produce (Watada, Ko, & Minott, 1996). ITCs are known to have antibacterial effects (Vig, Rampal, Thind, & Arora, 2009) but this relationship has not been studied

in the context of the commercial supply chain. Free sugars may also provide a food source for bacteria, and we question how natural populations respond to concentrations within leaves during commercial processing and shelf life.

With the aforementioned aspects in mind (Verkerk et al., 2009), we hypothesised that GSL and ITC content would decline significantly over time due to a combination of GSL hydrolysis and leaching into wash water. We theorised that this would lead to a reduction in the nutritive and health beneficial properties of leaves. We also hypothesised that with a decrease in potentially anti-microbial compounds (ITCs) bacterial populations would increase and peak during shelf-life. The results presented in this paper show however that these hypotheses could be rejected, and that processing of rocket leaves may add nutritional value to the crop.

7.3. Materials & Methods

7.3.1. Plant Material

The five non-commercial accessions used in this paper (*E. sativa*) were originally sourced from European germplasm collections. See Bell et al. (2015; Chapter 3) for information regarding the supplying institutes. Due to the small amounts of seed given, each cultivar was individually bulked by open pollination in separate glasshouse compartments at Elsoms Seeds Ltd. (Spalding, UK) in the spring/summer of 2014. The amount of seed produced for each cultivar weighed >500 g. The commercial variety *Torino* (*Diplotaxis tenuifolia*) used as a comparator to gene bank-sourced cultivars.

7.3.2. Growing & Industrial Supply Chain Conditions

Plants were grown in an open field at a Bakkavor supplier, (Dorchester, England) from the 3rd to the 25th of July 2014. Cultivars were sown using a tractor

mounted air drill in parallel beds measuring approximately 50 meters in length. *Torino* was sown as a guard crop surrounding the trial beds, and crop protection and irrigation of the trial was as per standard commercial practice.

Plants were harvested on the morning of 25th of July 2014 (22 days old) by machine. Due to the slower growth of *Torino*, plants drilled on the same date as the *E. sativa* cultivars were not harvested. Leaves were loaded into crates, which were placed into a waiting trailer. From harvest (H) onwards, five temperature data loggers (Tinytag Transit 2, -40 to +70 °C sensitivity range; Gemini Data Loggers Ltd., Chichester, UK) were added to crates and set to record one data point every five minutes for the remainder of the trial. See appendix X for a temperature-time plot of averaged data. The temperature on the day of harvest was unusually hot for UK summer time, and the recorded average was 34.8 °C.

A tractor-trailer loaded with samples was driven approximately one mile to a storage facility. Crates were unloaded into a vacuum cooler, which removed field heat from the produce. Samples were stored in a 4 °C cold store, in the dark, for two days; the average temperature for this period was 4.9 °C. Samples were transported on the third day after harvest to a Bakkavor processing site via temperature-controlled lorry. Produce was stored in a 4 °C environment for the remainder of that day, but temperatures ranged between 2.2 °C and 8.6 °C during this time.

The following day, samples were processed using a commercial wash line with mild water chlorination. Each cultivar was entered into the line separately with a five-minute gap between to prevent mixing. Leaves were spin-dried, before being transferred by conveyor belt to be bagged in unmodified atmosphere, micro-perforated bags. Produce was stored overnight under controlled conditions; temperatures averaged 5.1 °C in the processing environment. The day after, samples were transported via courier in a temperature-controlled vehicle to the University of

Reading (UoR), but temperatures as high as 14.3 °C were recorded during this time, representing a potential breach in the cold-chain (appendix X). The temperature upon arrival at UoR was 21.7 °C.

7.3.3. Shelf-life Storage Conditions

Samples were then stored in the dark continuously, for nine days in a controlled temperature storage room set to 4 °C. Temperatures varied, reaching an average low of 3.9 °C, and an average high of 6.4 °C. Storage conditions represent typical refrigeration temperatures used for storing rocket salad, although a range between 0 °C and 4 °C is considered optimal within the literature (Dekker, Verkerk, & Jongen, 2000).

7.3.4. Sample Collection

Leaf samples were taken at ten time points (n = 3), spanning the previously described supply chain, with each bagged sample treated as one replicate. Bacterial count samples were taken separately, with each replicate weighing ~30 g (n = 3).

The first samples were taken 12 days after sowing and included all *E. sativa* samples but not *Torino* because of the disparity in growth stages. These samples were designated 'preharvest' (PH) and represent produce at an immature growth stage. Both leaf and cotyledon were sampled and taken from random points along the complete length of each bed to avoid any potential bias from localised field effects. At harvest (H) *Torino* was again not sampled, as it was not of marketable leaf size. Samples were taken from multiple crates of harvested material spanning the length of the trial to again avoid bias. For both PH and H, samples were placed immediately into Ziploc freezer bags and frozen on dry ice in polystyrene containers to prevent phytochemical changes during transit. Samples were transported by car to

UoR, taking approximately two hours. Upon arrival samples were placed into a -80 °C freezer.

Sampling at delivery to the Bakkavor processing site was designated ‘post transport’ (PT). It was at this time that the commercial variety *Torino* was first sampled. Samples were taken from a crop from the same producer and harvested on the same day, but had been sown approximately seven days before the *E. sativa* cultivars.

The following day, two time point samples were taken and designated ‘pre-wash’ (PR) and ‘post-wash’ (PW), and were again taken from random crates to avoid bias, and frozen on dry ice. Transit time from the Bakkavor processing site to UoR was approximately one hour.

Upon arrival at UoR the following day, transported bagged samples were taken and placed directly into a -80°C freezer. This time point was designated ‘day 0’ of shelf life (D0). Subsequent samples were taken at ‘day 2’ (D2), ‘day 5’ (D5), ‘day 7’ (D7; commercial display-until date, DUD), and ‘day 9’ (D9; DUD +2) in an identical fashion.

All samples were lyophilized in batches for three days. Dried tissue was milled using a Mini Cutting Mill (Thomas Scientific, Swedesboro, NJ, USA) into fine powder. Samples were stored in a cool, dry, dark place until analyses began. All time points were examined by analytical methods (see following sections), with the exception of PH and H time points for *Torino* (as explained previously), and D9 for bacterial counts. Due to the time consuming nature of extraction, only time points PT and D7 were analysed for GSL hydrolysis products in each cultivar.

7.3.5. Bacterial Counting

7.3.5.1. General

Total plate count (TPC) of the plant materials was determined at nine different processing points (PH – D7) for the cultivars of *E. sativa*. Samples of *Torino* began at time point PT.

7.3.5.2. Preparation Of Nutrient Agar For TPC

11.75 g of standard plate count agar (APHA; Oxoid Ltd., Basingstoke, UK) was diluted in 500 ml of distilled water and stirred until boiling, giving a final concentration of 2.4% (w/v). Agar was sterilised (15 minutes at 121 °C) and subsequently kept in a 50 °C water bath to maintain molten state.

7.3.5.3. Preparation Of Maximum Recovery Diluent For Sample Preparation & Enumeration

For sample preparation, 9.5 g of maximum recovery diluent (MRD; Sigma, Gillingham, UK) was diluted in 1 L of distilled water (0.95% w/v) and stirred until completely dissolved. 90 ml of MRD was poured into 100 ml bottles and then sterilised (15 minutes at 121 °C). The mixture was left to cool, or was kept in a 4 °C cold room for longer-term storage. For enumeration, MRD was prepared in an identical fashion. 9 ml of MRD was then transferred to a bottle, sterilised and cooled, as above.

7.3.5.4. Total Plate Count

10 g of rocket leaves was added to the 90 ml preparation of MRD and placed in a stomacher (400 Circulator; Seward, Worthing, UK) and shaken for 120 seconds (230 rpm) to create a 10^{-1} dilution (w/v). 1 ml of the homogenised inoculum was

sampled and serially diluted into the 9 ml MRD preparation to obtain 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. 1 ml of each respective solution was added to 15 ml nutrient agar (45 – 50 °C) in petri dishes, using the pour plate technique. Plates were swirled to mix evenly. Inoculated plates were allowed to cool at room temperature (~22 °C) until the liquid solidified, and incubated at 30 °C in inverted condition. After 72 ± 3 hours, the number of colonies per plate were counted using a colony counter. Bacterial numbers for each sample were estimated in colony forming units ($cfu.g^{-1}$).

7.3.6. Glucosinolate Extraction & Analysis By LC-MS/MS

Glucosinolates were extracted and analysed according to the protocol in Bell et al. (2015; Chapter 3) with the following alterations: Extracts were filtered with 0.22 μm Arcrodisc syringe filters with Supor membrane (hydrophilic polyethersulfone; VWR, Lutterworth, UK) after extraction. Analysis was performed using an Agilent 1200 Series LC system (Agilent, Stockport, UK) equipped with a variable wavelength detector (GSLs quantified at 229 nm), and coupled with a Bruker HCT ion trap (Bruker, Coventry, UK). A Gemini 3 μm C_{18} 110 Å (150 x 4.6 mm) column was utilised (with Security Guard column, C_{18}; 4mm x 3mm; Phenomenex, Macclesfield, UK), and separation was optimised for use with the Bell et al. (2015; Chapter 3) isocratic gradient, at a flow rate of 0.4 ml/min. A six point sinigrin hydrate calibration curve was prepared (r^2 = 0.977, y = 7.763; Jin et al. 2009). Compounds were identified using literature ion data and characteristic ion fragments (Table 7.1). Quantification was performed using Bruker Daltonics HyStar software (Bruker) with relative response factors.

Table 7.1. Identification of intact glucosinolates by LC-MS/MS, and myrosinase hydrolysis products by GC-MS in *Eruca sativa* and *Diplotaxis tenuifolia* by comparison to literature ion data.

Common name	Chemical name	$[M-H]^-$ *m/z*	MS^2 (Base ion in bold)	References
Glucosinolates				
Glucoerucin	4-(methylthio)-butyl-GSL	420	340, 275, **259**, 242, 195	Rochfort, Trenerry, Imsic, Panozzo, & Jones (2008)
Glucoraphanin	4-(methylsulfinyl)-butyl-GSL	436	**372**, 259	
Glucoiberverin	3-(methylthio)-propyl-GSL	406	275, **259**, 227	
4-hydroxyglucobrassicin	4-hydroxy-3-indolylmethyl-GSL	463	383, **285**	
Epi/progoitrin	(*R,S*)-2-hydroxy-3-butenyl-GSL	388	332, **259,** 136	
Diglucothiobeinin	4-(b-D-Glucopyranosyldisulfanyl)-butyl-GSL	600	**521**	Lelario, Bianco, Bufo, & Cataldi (2012)
Glucosativin	4-mercaptobutyl-GSL	406	326, 275, **259**, 228, 145	
DMB	Dimeric 4-mercaptobutyl-GSL	811	**731**, 469, 405	

Common name	Chemical name	MS spectra *m/z* (Base ion in bold)		References
Myrosinase hydrolysis products				
-	4-isothiocyanato-1-butene	113	85, **72**	Arora et al. 2014; Guo, Yang, Wang, Guo, & Gu (2014)
Sativin	4-mercaptobutyl-ITC	147	**114**, 87, 72, 60	Cerny, Taube, & Battaglia (1996)
-	Bis (4-isothiocyanatobutyl) disulfide	292	146, **114**, 87, 72, 55	
Erucin	4-(methylthio)-butyl-ITC	161	**115**, 72, 61	Arora et al. 2014
Erucin nitrile	1-cyano-4-(methylthio)-butane	129	114, **82**, 61	Vaughn & Berhow (2005)
Sulforaphane nitrile	5-(methylsulfinyl)-pentane-nitrile	145	82, 64, **55**, 41	Chiang, Pusateri, & Leitz (1998)
Sulforaphane	4-(methylsulfinyl)-butyl-ITC	177	**160**, 114, 72, 55	Chiang, Pusateri, & Leitz (1998); Arora et al. (2014)

7.3.7. Free Amino Acid Analysis By GC-MS & Free Sugar Analysis By Capillary Electrophoresis

Free sugars and free AAs were extracted and analysed using the protocol and instrumentation presented in Chapter 5.

7.3.8. Glucosinolate Hydrolysis Product Extraction & Analysis By GC-MS

Samples were extracted and run in a random sequence to avoid bias (as for all other analyses). 0.5 g of lyophilized rocket powder was mixed with 10 ml of deionized water. Tubes were incubated for three hours at 30 °C in a temperature-controlled room. The mixture was subsequently centrifuged for ten minutes (4,600 rpm) and supernatant collected. This last step was repeated twice more and supernatants were combined and filtered (0.45 μm syringe filters, Sartorius Minisart cellulose acetate, surfactant free membrane; Sartorius, Epsom, UK) into glass centrifuge tubes. An equal volume of dichloromethane (DCM) was added, vortexed and centrifuged (3,500 rpm) for ten minutes. The organic phase was collected using glass Pasteur pipettes and transferred into a new glass centrifuge tube. Sample was salted with sodium sulphate (2 g; Sigma) and filtered using Whatman Grade 1 filter paper into a round-bottomed flask. Filtrate was dried using a rotary evaporator (37 °C) and re-dissolved in 1 ml of DCM. This volume was filtered again with a 0.22 μm filter (VWR) into glass GC-MS vials for analysis.

GC-MS was performed on an Agilent 7693/5975 GC-MS with autosampler (Agilent, Manchester, UK). Sample was injected onto a HP-5MS 15 mm wax plus column (0.25 μm film thickness, 0.25 mm I.D.; Agilent). Injection temperature was 250 °C in split mode (1:20); oven temperature was programmed from 40 – 320 °C at a rate of 5 °C/min until 250 °C. Carrier gas was helium, with a flow rate of 1.1 ml/min and a pressure of 7.1 psi. Mass spectra were obtained by electron ionization at 70

eV, and mass scan from 35 – 500 amu. 1 μl of sample was injected, and separation occurred within a 42 minute run. Compounds were identified using literature ion data (Table 7.1) and quantified based on integrated peak areas of an external standard calibration curve of sulforaphane (Sigma). Standards for the other ITCs and nitrile compounds detected were unobtainable. Five concentrations of sulforaphane were prepared from a stock of 5 $mg.ml^{-1}$ in DCM: 0, 0.175, 0.25, 0.375, and 0.5 $mg.ml^{-1}$ (r^2 = 0.947; y = 4E+08). Data analysis was performed using ChemStation for GC-MS (Agilent).

7.3.9. Statistical Analysis

Results from three biological replicates of each sample (n = 3) at each time point, for all compounds analysed were averaged. All statistical analyses were performed using XL Stat (Addinsoft, Paris, France).

ANOVA followed by Tukey's HSD test was used to conduct multiple pairwise comparisons and determine significant differences (P<0.05) between cultivars at each respective time point (i.e. SR2 vs. SR5 at H), and between time points for each cultivar (i.e. H vs. D7 for SR6).

Averaged data were entered in to Principal Component Analysis (PCA) with correlation matrix (Pearson, n-1) in two separate tests. The first test extracted principal components (PCs) using GSL, AA, sugar and bacterial count data, with time point regressed as a supplementary, qualitative variable. The second test extracted PCs using data from time points PT and D7 (for each of the aforementioned analyses) with the addition of GSL hydrolysis product data. Significance thresholds of P<0.05, 0.01, and 0.001 were applied to each respective analysis.

7.4. Results & Discussion

7.4.1. Bacterial Counts & Phytochemical Composition Of Rocket Extracts Within The Commercial Supply Chain

7.4.1.1. Bacterial Counts

Bacterial count data for each time point and cultivar are presented in Figure 7.1. The general trend in the data matched our hypothesis that bacterial populations would increase during shelf life, which is in agreement with Martinez-Sanchez, Marin, Llorach, Ferreres, & Gil (2006). With the exception of SR12 and *Torino*, all other cultivar TPC numbers peaked on D7 (DUD); and with the exception of SR12

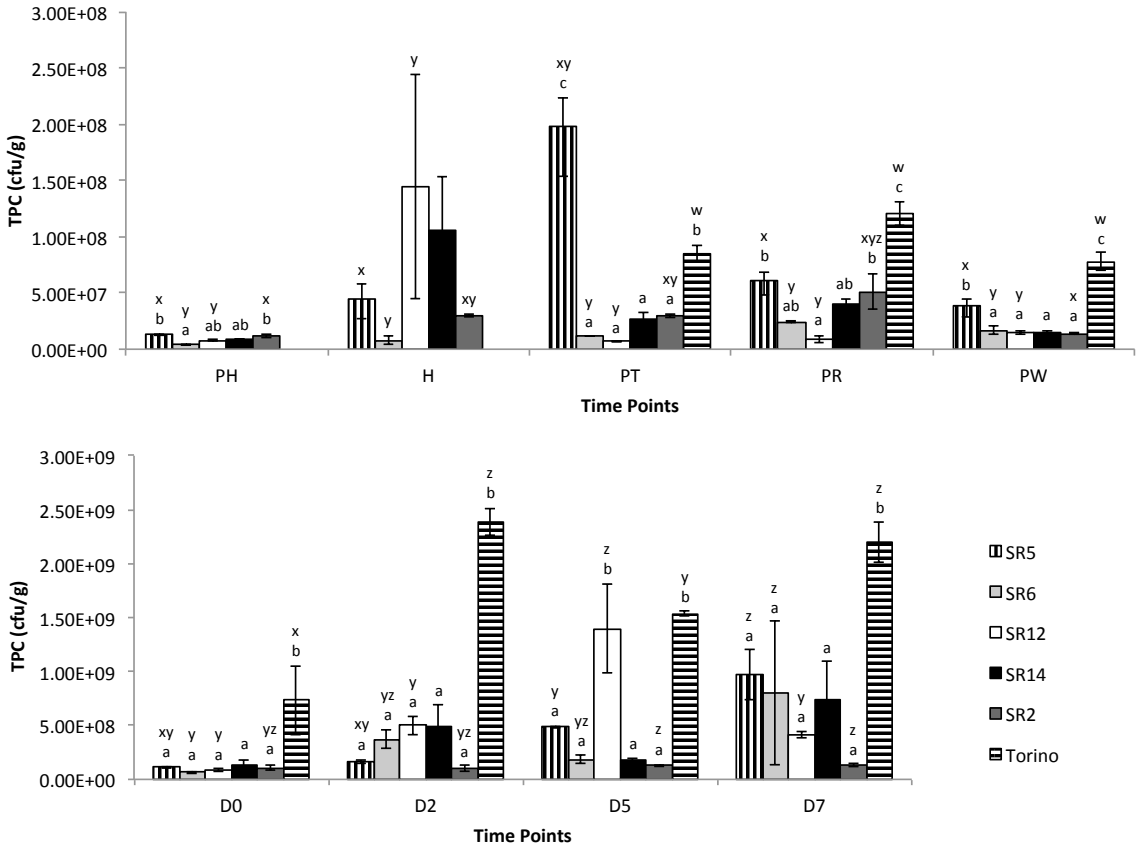


Figure 7.1. Total plate count (TPC) numbers of bacteria from rocket salad leaves (cfu.g^{-1}) at each respective time point during the commercial supply chain (top) and shelf life (bottom) periods which are both part of the same ANOVA with Tukey's HSD pairwise comparison tests. Error bars represent standard errors of the mean TPC. Letters a, b, c: bars not sharing a common letter differ significantly (P<0.05) between accessions for each individual time point. Letters w, x, y, z: bars not sharing a common letter differ significantly (P <0.05) across time points for each individual accession. Abbreviations: PH, preharvest (12 days old); H, harvest (22 days old); PT, post transport; PR, pre-wash; PW, post wash; D0, day 0 shelf life; D2, day 2 shelf life; D5, day 5 shelf life; D7, day 7 shelf life (DUD; display until date).

and SR14, these values were significantly higher than PR levels. Torino had significantly greater numbers of bacteria present from PW through to D7; possibly due to the difference in leaf morphology of *D. tenuifolia*.

The breaches in the cool-chain combined with high summer field temperatures, likely contributed to the high bacterial counts. Previous data presented under pseudo-commercial conditions for rocket (*D. tenuifolia*; Spadafora et al. 2016) showed that produce stored above 10 °C for 14 days (~4.0 cfu.g^{-1} fw) has significantly more bacteria on the leaves than those stored at 5 °C and 0 °C. The samples in this experiment were stored for only nine days, and bacterial counts were highest on D2 of shelf life, and several log units greater in abundance (Torino; Figure 7.1). Conversely, the cultivar SR14 saw no significant changes in bacterial load throughout the entire supply chain. This indicates that there may be a genotypic component imparted by each cultivar on the endemic leaf bacteria that determines their proliferation. Appendix X demonstrates that temperatures breached the 10 °C threshold twice after harvest, but without independent data it is difficult to determine if this is the absolute cause for the high bacterial numbers seen for the other cultivars in the subsequent days.

Bacteria continued to propagate during shelf life on all accessions, possibly due to the unmodified air and high relative humidity within bags (Watada et al. 1996). The aforementioned factors likely allowed the natural bacterial populations present within/on leaves to proliferate. Non-pathogenic field bacteria are likely to be resistant to extremes of temperature due to the variable climate of the UK, and so are likely to grow even under cold-chain conditions. Further experimentation is needed on commercial produce in order to properly elucidate these effects.

7.4.1.2. Glucosinolates

GSL concentrations for each cultivar are presented in Figure 7.2, and LC-MS/MS ion data used for identification are presented in Table 7.1. At each respective time point, total GSL concentrations between cultivars did not differ significantly.

The highest total GSL concentration was in *Torino* on D7 (11.5 $mg.g^{-1}$ dw) and the lowest in SR12 at PR (1.0 $mg.g^{-1}$ dw). The trend for GSL concentrations was to increase over time from H, before lowering at D9 (Figure 7.2), which was contrary to our hypothesis. SR5 had significantly higher GSL accumulation at D7 (9.7 $mg.g^{-1}$ dw) compared with PH (1.9 $mg.g^{-1}$ dw) and H (2.0 $mg.g^{-1}$ dw) and this was also seen in SR6: H = 1.3 $mg.g^{-1}$ dw, PW = 7.7 $mg.g^{-1}$ dw, D0 = 9.3 $mg.g^{-1}$ dw, D5 = 8.4 $mg.g^{-1}$ dw'; and in SR12: H = 1.8 $mg.g^{-1}$ dw, D2 = 8.1 $mg.g^{-1}$ dw. These GSL concentrations at PH (12 days old) and H (22 days old) are low compared to controlled environment (Bell et al. 2015; Chapter 3), as no cultivar contained >3.6 $mg.g^{-1}$ dw (SR14).

The relatively low concentrations at PH are likely due to the low dry matter content at this immature stage of growth. Work conducted in *Arabidopsis thaliana* (Brown, Tokuhisa, Reichelt, & Gershenzon, 2003) has indicated that dry matter and leaf number are related to total GSL concentration. One would therefore have expected GSLs in rocket to increase over this 10 day gap in growth and sampling (PH to H) but no significant differences in the concentrations were observed between the two time points. This implies that concentrations measured in the H samples were possibly reduced due to the damage induced by harvesting and the high ambient field temperature (appendix X). Further study is needed to ascertain the true effects of harvesting on GSL concentrations of commercial rocket.

When the data at H are compared to controlled environment conditions (Bell et al. 2015; Chapter 3), concentrations are 48.5% lower for SR2, 82.6% lower for SR5, 87.0% lower for SR6, 78.6% lower for SR12, and 52.0% lower for SR14. Despite this

difference in observed GSL abundances, only SR6 and SR12 failed to recover during shelf life and exceed concentrations previously reported (Bell et al. 2015; Chapter 3).

When looking at the respective GSLs over time for each cultivar, it is apparent that concentrations are highly dynamic. Concentrations of glucosativin varied significantly between time points and most of the changes occurring in total GSL concentration are because of the relative increases/decreases of this GSL.

DMB was also observed at each respective time point, though no significant differences were seen until D9 (SR5; 2.4 $mg.g^{-1}$ dw). The propensity for certain rocket accessions and varieties to accumulate DMB and glucosativin in differing ratios has been documented by Bell et al. (2015; Chapter 3), though relatively few studies have acknowledged that it is an independent GSL and is naturally present in rocket leaves. This was originally proposed by Cataldi, Rubino, Lelario, & Bufo (2007), and our study lends further support to the hypothesis that both monomeric glucosativin and DMB should be identified and quantified separately.

Glucoerucin and glucoraphanin did not show any significant difference across either time points or cultivars. The lack of any significant changes in glucoraphanin concentration is in agreement with a previous study in broccoli florets (Winkler, Faragher, Franz, Imsic, & Jones, 2007). This GSL seems to be far more stable than others in rocket, such as glucosativin and glucoerucin (Figure 7.2).

Several other GSLs were also observed. These were: diglucothiobeinin, glucoiberverin, 4-hydroxyglucobrassicin, and epi/progoitrin. Diglucothiobeinin had significant changes over the course of the trial in both SR5 (Figure 7.2b) and *Torino* (Figure 7.2f). No significances were observed for glucoiberverin, which was transient between time points. 4-hydroxyglucobrassicin was only detected in SR5 and SR6 in small amounts (<0.3 $mg.g^{-1}$ dw), though this GSL may infer important sensory attributes as suggested in Bell et al. (*in press,* Chapter 5), where it was correlated

with pungent sensations. Epi/progoitrin was only observed in SR5 at D0 (0.3 $mg.g^{-1}$ dw; Figure 7.2b) but has been previously linked with bitter taste in rocket (Pasini, Verardo, Cerretani, Caboni, & D'Antuono, 2011).

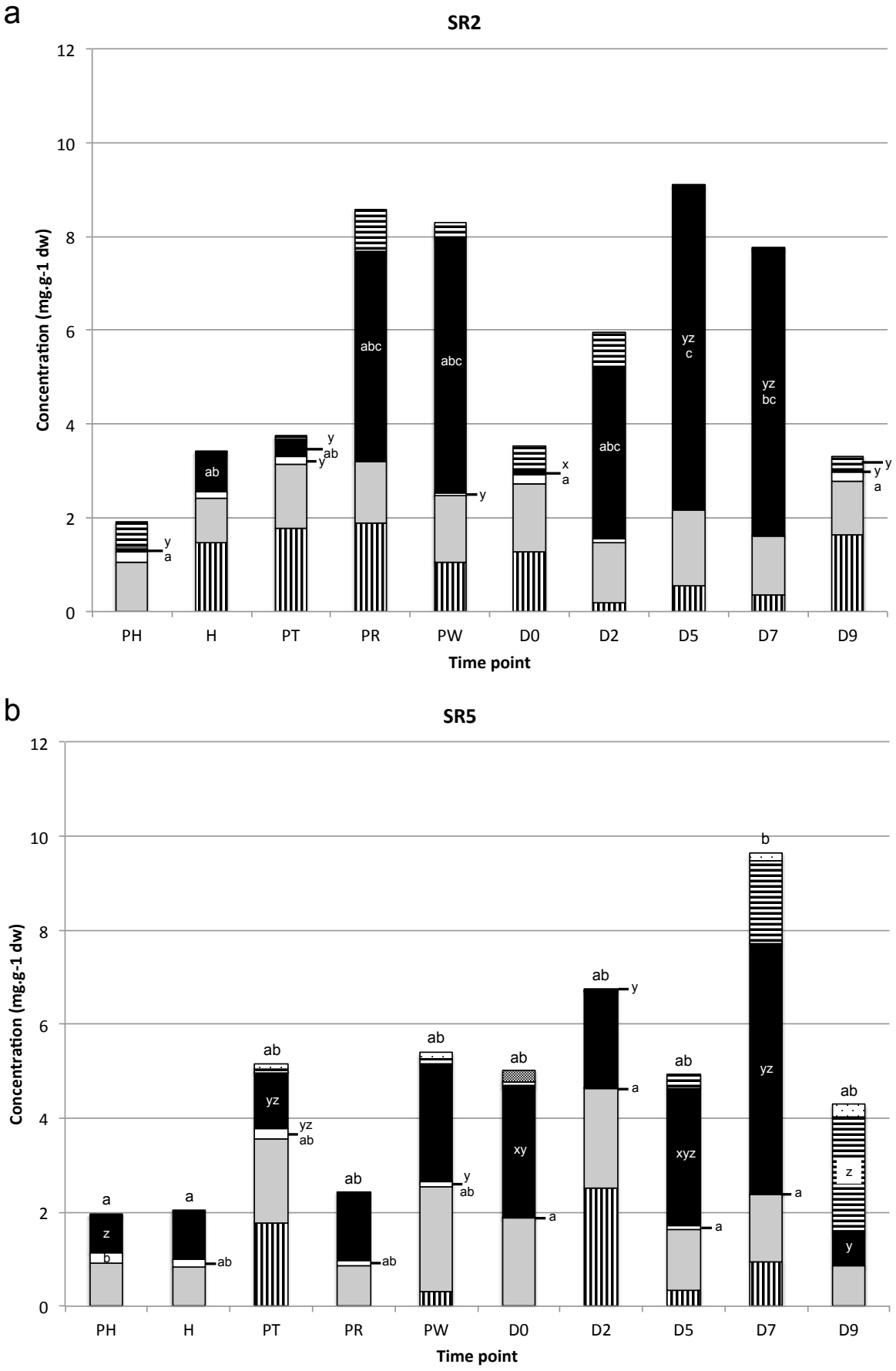
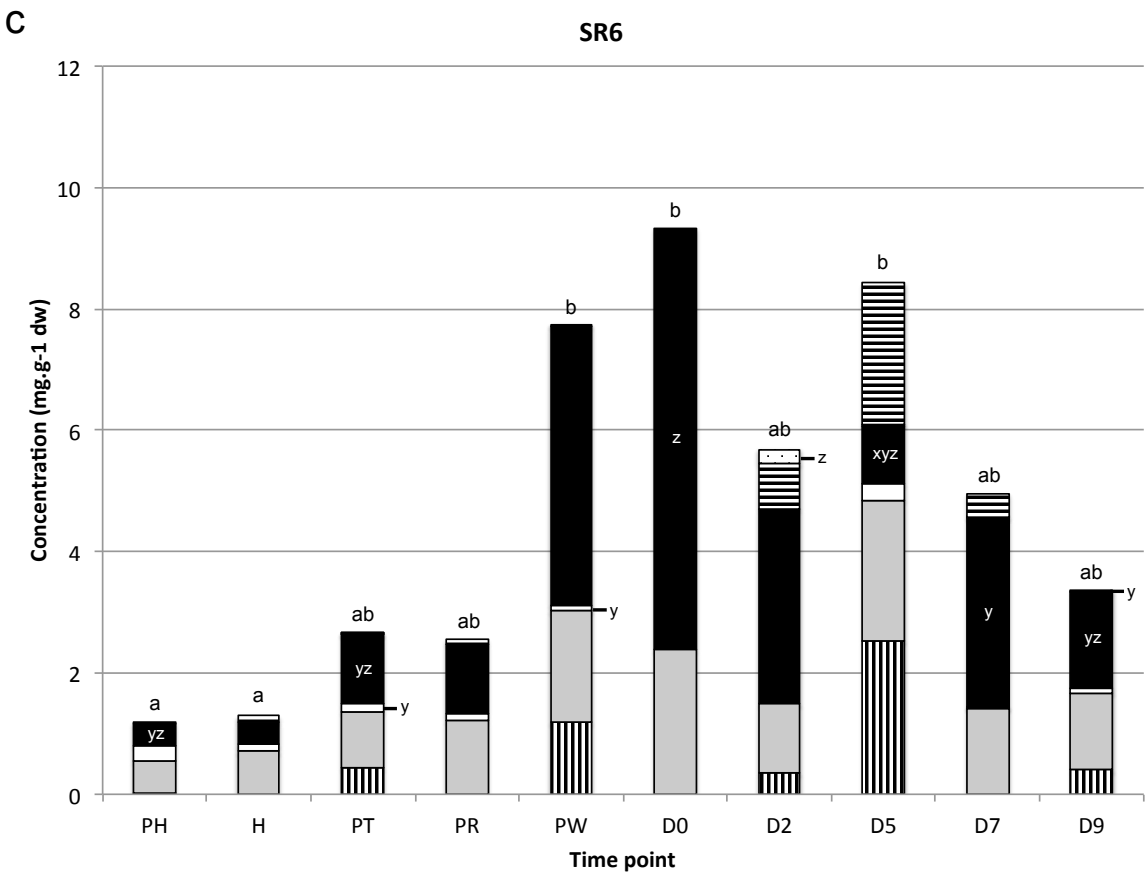


Figure 7.2. Glucosinolate (GSL) concentrations within each cultivar at each time point (mg.g^{-1} dw). Letters a, b, c: bars not sharing a common letter differ significantly (P<0.05) between time points for each individual accession. Letters x, y, z: bars not sharing a common letter differ significantly (P<0.05) across accessions for each time point. Letters above bars refer to differences in total GSL concentration; letters within/beside bars refer to differences in individual compounds. An absence of letters above/within bars indicates no significant differences were observed for the total GSL concentration/individual compounds. Abbreviations: PH, preharvest (12 days old); H, harvest (22 days old); PT, post transport; PR, pre-wash; PW, post wash; D0, day zero shelf life; D2, day two shelf life; D5, day five shelf life; D7, day seven shelf life (DUD; display until date); D9, day nine shelf life.



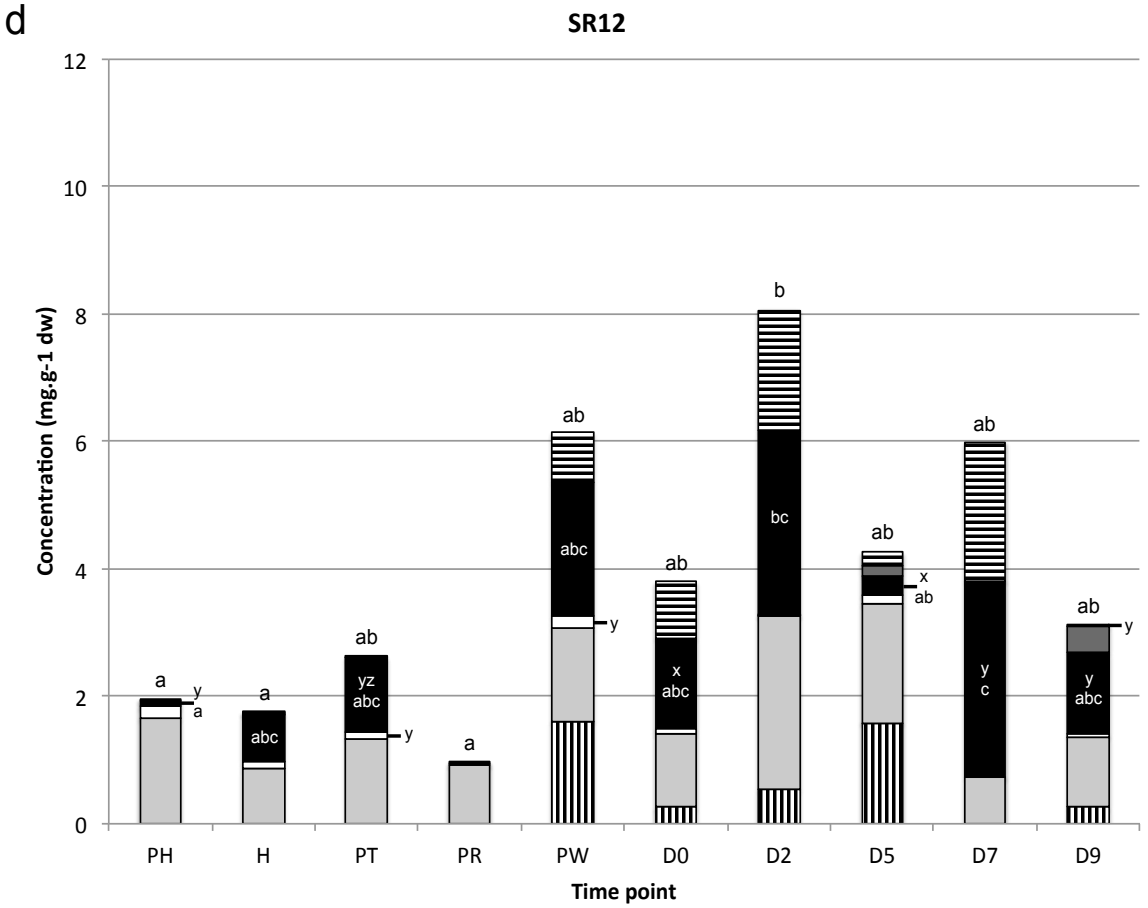


Figure 7.2 – continued.

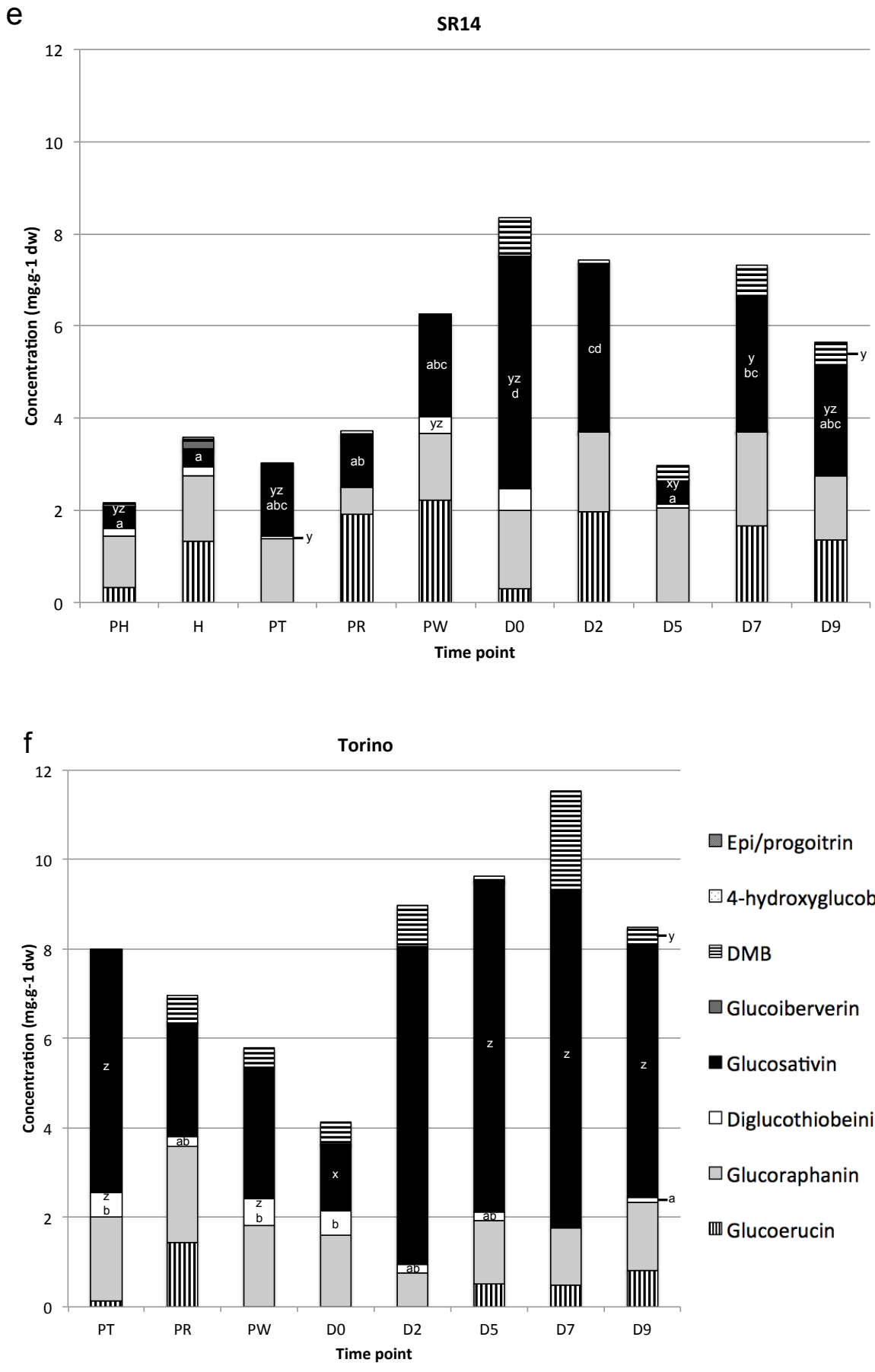


Figure 7.2 – continued.

7.4.1.3. Glucosinolate Hydrolysis Products

ITC and nitrile concentrations are presented in Figure 7.3, and GC-MS ion data used for identifications are presented in Table 7.1. All concentrations are expressed as equivalents of sulforaphane.

Total GSL hydrolysis products were predominantly composed of the ITCs sativin and sulforaphane (derived from glucosativin/DMB and glucoraphanin, respectively). The nitriles of erucin and sulforaphane were also observed, as well as a sativin degradation product, bis(4-isothiocyanatobutyl)-disulfide. Total concentrations varied significantly for SR2, SR12, and SR14 between the two time points analysed. Total hydrolysis products were significantly higher in *Torino* at PT (0.4 $mg.g^{-1}$ dw) than the *E. sativa* cultivars, but by D7 there were no significant

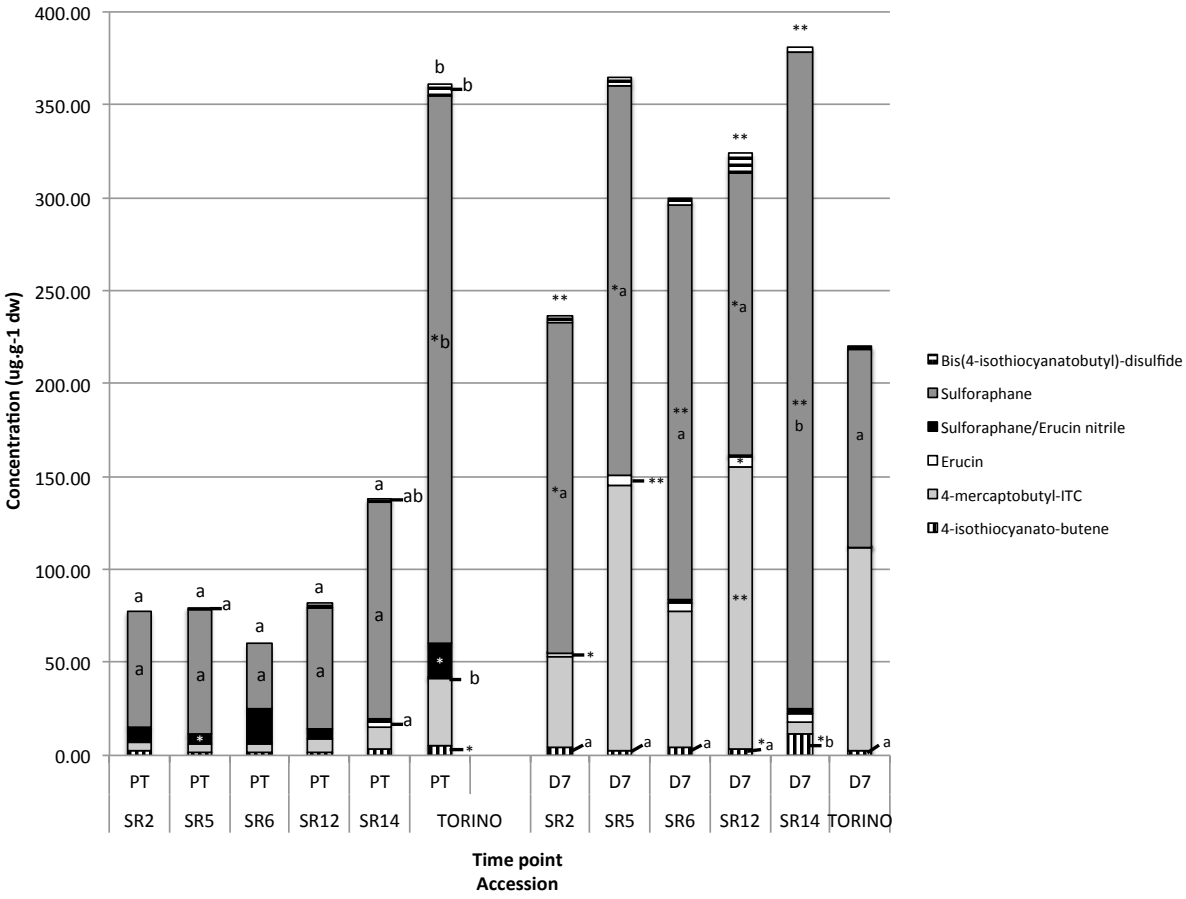


Figure 7.3. Glucosinolate (GSL) hydrolysis product concentrations at time points PT and D7 ($\mu g.g^{-1}$ dw; sulforaphane equivalents). Letters a, b: bars not sharing a common letter differ significantly (P<0.05) within each respective time; point PT (post transport) and D7 (day seven shelf life, DUD; display until date). Asterisks indicate significantly higher concentrations between each time point for each respective compound (within/to the side of bars) and total amounts (above bars). * = P<0.05; ** = P<0.01.

differences between the cultivars. The decline in sulforaphane concentration between PT and D7 was significant in *Torino*, measuring 0.3 $mg.g^{-1}$ dw (PT) and falling to 0.1 $mg.g^{-1}$ dw (D7). This suggests that although glucoraphanin concentration remains stable over time, this may not translate into consistent ITC formation. All of the *E. sativa* cultivars saw significant increases in sulforaphane between PT and D7, and SR14 saw significantly higher concentrations than any of the other cultivars (0.4 $mg.g^{-1}$ dw). This is contrary to the reductions seen in headspace ITC concentrations (Bell et al. 2016; Chapter 4), indicating this method of analysis may not be reflective of abundance within leaves, or of GSL concentration as has been sugested by Spadafora et al. (2016).

Sulforaphane (derived from glucoraphanin) was the most abundant ITC detected, which does not mirror the GSL composition of rocket salad. Logically, one would expect that sativin would be the predominant ITC, due to the large concentrations of glucosativin/DMB on D7. The observations were variable, and did not generally exceed those for sulforaphane. Significant differences were only observed for SR12 between each time point, despite the obvious large differences seen in the other cultivars (Figure 7.3). We hypothesise that the high variability of sativin concentration may be due to its unstable nature, when compared to sulforaphane, for example.

Another anomaly observed in our data are the low concentrations of erucin. Erucin increased significantly from PT to D7 in SR2, SR5 and SR12, and declined significantly in *Torino*. The highest concentration was only 5.7 $\mu g.g^{-1}$ dw however (SR12; D7). A study comparing ITC extraction methods in *E. sativa* seeds (Arora et al., 2014) showed that erucin recovery was dependent on the homogenisation time and GC-MS injection temperature, which may account for the low concentrations

observed here. It may be that the extraction method has a significant impact on determining the relative abundances of ITCs, as well as their inherent volatility.

4-isothiocyanato-1-butene was observed, and has been quoted in the literature as a breakdown product of gluconapin (Guo et al. 2014). No gluconapin was observed in the samples, and we hypothesise that this compound may be a breakdown product of either sulforaphane or erucin (Arora et al., 2014). Concentrations were significantly higher in SR14 (11.6 $\mu g.g^{-1}$ dw) on D7 than any other cultivar, and SR14 also has high concentrations of both erucin and sulforaphane.

Another point of note is the low amounts of nitrile compounds detected in leaves. This may depend greatly upon the acidity of hydrolysis conditions (Bell & Wagstaff, 2014; Chapter 2), however nitrile formation over ITC in broccoli has been shown to account for a substantial reduction in potential health promoting properties (Matusheski & Jeffery, 2001). Our data infer that the prevalence of ITC formation in rocket may have important implications for health benefits to the consumer. ITCs do survive commercial processing, and increase significantly post harvest. This suggests that consumers are able to ingest ITCs (particularly sulforaphane) from rocket salad bags, and that processing actually enhances this property of leaves.

In a hypothetical scenario where SR14 contains 1.97 $\mu mol.g^{-1}$ dw (0.35 $mg.g^{-1}$ dw) of sulforaphane, a commercial 50 g bag would therefore contain approximately 9.85 μmol, assuming 0.2 $\mu mol.g^{-1}$ fresh weight with 10% dry matter. Cooked broccoli contains ~5.8 $\mu mol.g^{-1}$ dw after boiling for four minutes, ~2.0 $\mu mol.g^{-1}$ dw after eight minutes, and ~1.2 $\mu mol.g^{-1}$ dw after 12 minutes (Ghawi, Methven, & Niranjan, 2013). This means that weight for weight, SR14 contains almost as much sulforaphane as a typical broccoli cultivar after cooking for eight minutes. Rocket requires no cooking in order to be eaten, and the present study data suggest that consuming rocket after

commercial processing could be an effective way for consumers to enhance their intake of sulforaphane. Clinical studies testing the direct and indirect effects of sulforaphane consumption are few, and the concentrations needed to elicit health beneficial effects in humans are ambiguous within the literature. Nevertheless, the weight of consensus suggests that increased consumption of sulforaphane in the diet has important long-term health benefits (Traka et al. 2013).

7.4.1.4. Amino Acids

Free AA concentrations are presented in Figure 7.4, and 18 compounds were detected and quantified. Significant differences between cultivars and time points are presented in appendix XI.

There were numerous significant differences between the abundances of respective AAs of *Torino* (Figure 7.4f) and the *E. sativa* cultivars (Figure 7.4a-e). *Torino* had significatly higher concentrations of valine, threonine, asparagine, aspartic acid, and phenylalanine, and significantly lower concentrations of proline. The increases seen in proline in *E. sativa,* and asparagine in *D. tenuifolia,* is possible evidence of stress signalling and response within tissues (Okumoto, Funck, Trovato, & Forlani, 2016). This change may also impact upon sensory attributes, though in what manner is difficult to predict, as no sensory study has previously compared *D. tenuifolia* with *E. sativa* by examining AAs. Relatively little is known about the specific influence of AAs in other *Brassicaceae* species, but it is thought that glycine and alanine influence sweetness, valine and leucine create bitterness, and aspartic acid creates sourness (Park et al. 2014).

There is a significant trend for AA concentrations to increase throughout shelf life, and a substantial proportion of this is due to elevations of glutamine, which peaked in accessions between D0 and D9. Increases in free glutamine are

associated with leaf senescence and are a result of protein breakdown, enzymatic conversion, and nitrogen transport (Buchanan-Wollaston et al. 2003). This has the possibility to impact bitter and pungent notes as glutamine is known to infer sweetness (Nelson et al. 2002). Concentrations observed in Bell et al. (2017*,* Chapter 5) did not exceed 90.8 $\mu g.g^{-1}$ dw (SR2; Figure 7.4a) in freshly harvested leaves. In this study they reached as high as 1.4 $mg.g^{-1}$ dw in *Torino* (D5; Figure 7.4f) – over 15 times higher. For the consumer, this may have a significant impact in the pleasurability and acceptance of leaves, especially if it masks other attributes such as pungency. Further study is needed on consumer preference and sensory properties during shelf-life for rocket.

Total AA concentrations were highest in SR3 (0.7 $mg.g^{-1}$ dw; characterised as a 'mild' accession) and lowest in SR5 (0.4 $mg.g^{-1}$ dw; characterised as having hot, pungent and bitter attributes, Bell et al. 2017*,* Chapter 5). In this study, total AA concentrations were substantially higher overall, being highest in *Torino* (Figure 7.4f) on D5 (2.7 $mg.g^{-1}$ dw) and lowest in SR5 (Figure 7.4b) at H (0.2 $mg.g^{-1}$ dw). Thus, depending on the time point at which the produce is hypothetically consumed, AA concentration may have a greater or lesser effect on perceived pungency and bitterness.

In Bell et al. (2017*,* Chapter 5), alanine was determined to have a strong influence on rocket sensory properties. In this study, alanine varied significantly across time points for SR5 (Figure 7.4b), SR14 (Figure 7.4e) and *Torino* (Figure 7.4f). SR14 displayed a trend for alanine concentrations to decline post D0, and this is even more pronounced in *Torino.* As free alanine can confer sweetness, this may indicate a loss in some sweet taste attributes during shelf-life (Solms, 1969). Previously, the highest concentration observed was 65.1 $\mu g.g^{-1}$ dw in SR2 (Bell et al. 2017*,* Chapter 5), whereas in this study, alanine was highest in SR14 (61.6 $\mu g.g^{-1}$ dw;

D0) and was not detected at all in *Torino* on D2, D5, D7 and D9. This may infer a stronger perception of pungent and bitter tastes in *Torino*.

Leucine however showed the opposite trend, and is known to have bitter taste properties (Solms, 1969). The change in relative abundance of this AA may have implications for bitter perception also. The compound increased in the *E. sativa* cultivars potentially making them more bitter; but declined in *Torino* from PT. In Bell et al. (2017*,* Chapter 5), leucine concentrations were low, only reaching 4.0 $\mu g.g^{-1}$ dw (SR6). Here, leucine was also highest in SR6 (D2; Figure 7.4c), but measured 24.7 $\mu g.g^{-1}$ dw. This coupled with the losses of alanine may increase bitterness during shelf life for *E. sativa* cultivars, although it is unknown if this would significantly enhance the bitterness caused by ITCs, or be mitigated by the large increases in glutamine concentrations.

Methionine is conspicuous by its absence in or results. No concentrations were detected in any of the samples tested, and the analysis by Bell et al. (2017*,* Chapter 5) similarly found no concentrations. This is puzzling, as methionine is the predominant precursor AA to aliphatic GSLs. Graser, Schneider, Oldham, & Gershenzon (2000) observed that methionine is involved in the synthesis of glucoerucin in rocket. The lack of any detectable free methionine in this study suggests that it is perhaps stored in another form, possibly as one of the precursor molecules postulated by Graser et al. (2000). This may explain some of the dynamic fluctuations seen in GSL concentrations between time points, facilitating rapid synthesis. The disparity between aliphatic GSL and free methionine concentration has yet to be addressed within the literature.

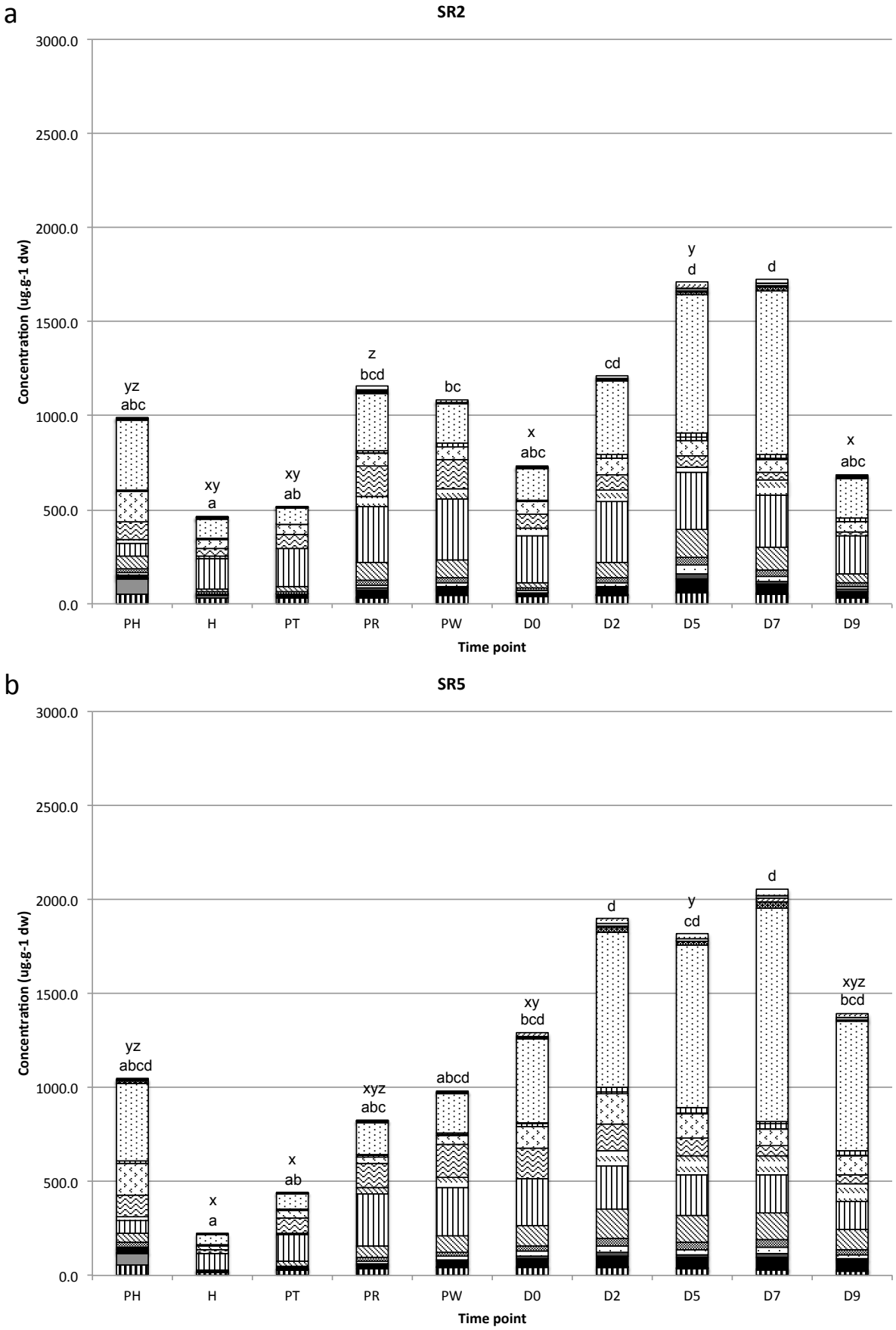
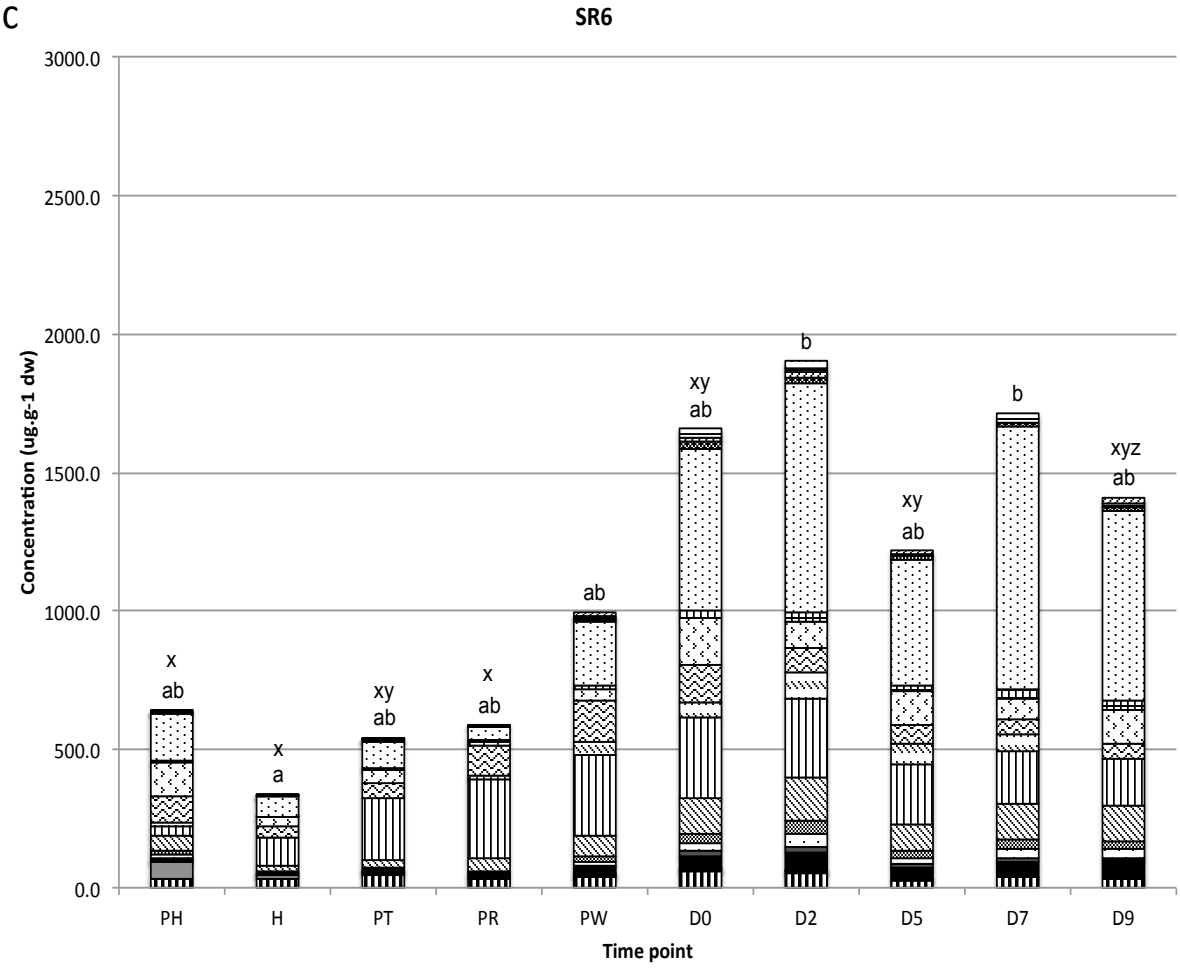
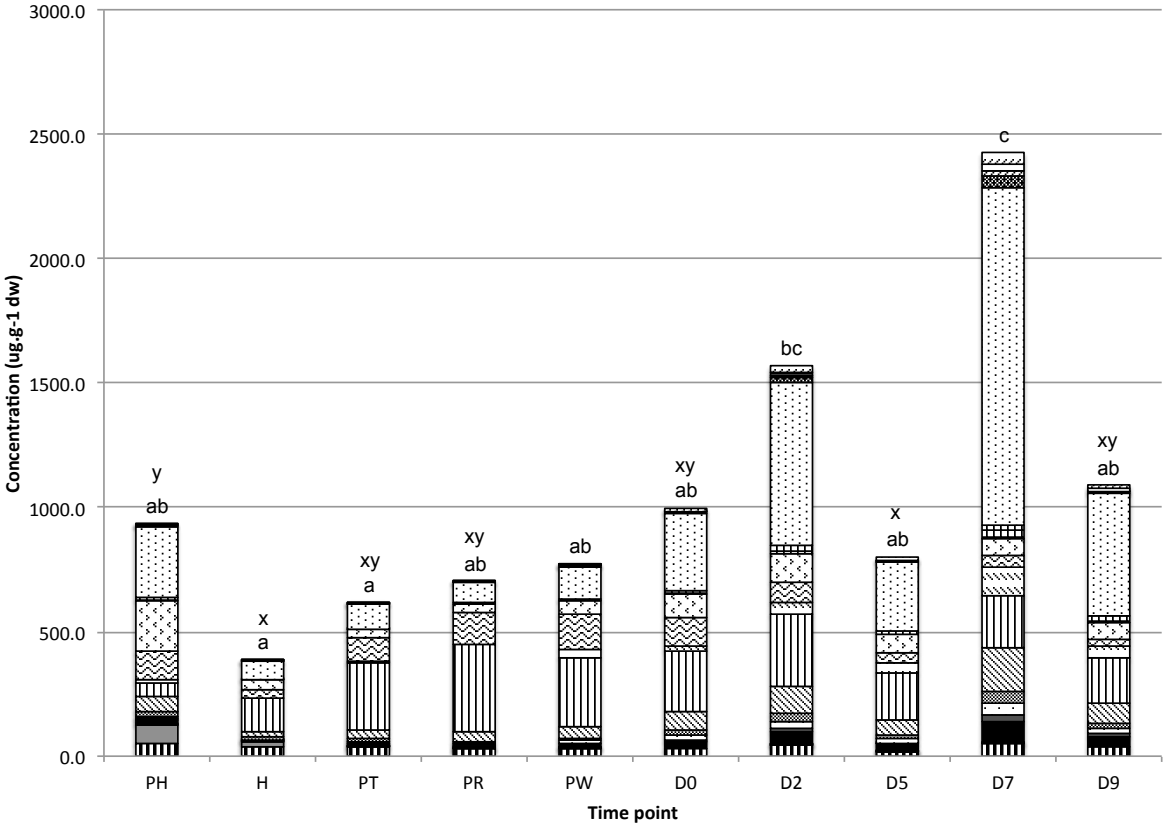


Figure 7.4. Amino acid concentrations for each cultivar at each time point (µg.g-1 dw). Letters above bars refer to total concentration. Letters a, b, c, d: bars not sharing a common letter differ significantly ($P<0.05$) between time points for each individual accession. Letters x, y, z: bars not sharing a common letter differ significantly ($P<0.05$) across accessions for each time point. Significant differences for each individual AA are presented in appendix XII. Abbreviations: PH, preharvest (12 days old); H, harvest (22 days old); PT, post transport; PR, pre-wash; PW, post wash; D0, day zero shelf life; D2, day two shelf life; D5, day five shelf life; D7, day seven shelf life (DUD; display until date); D9, day nine shelf life.

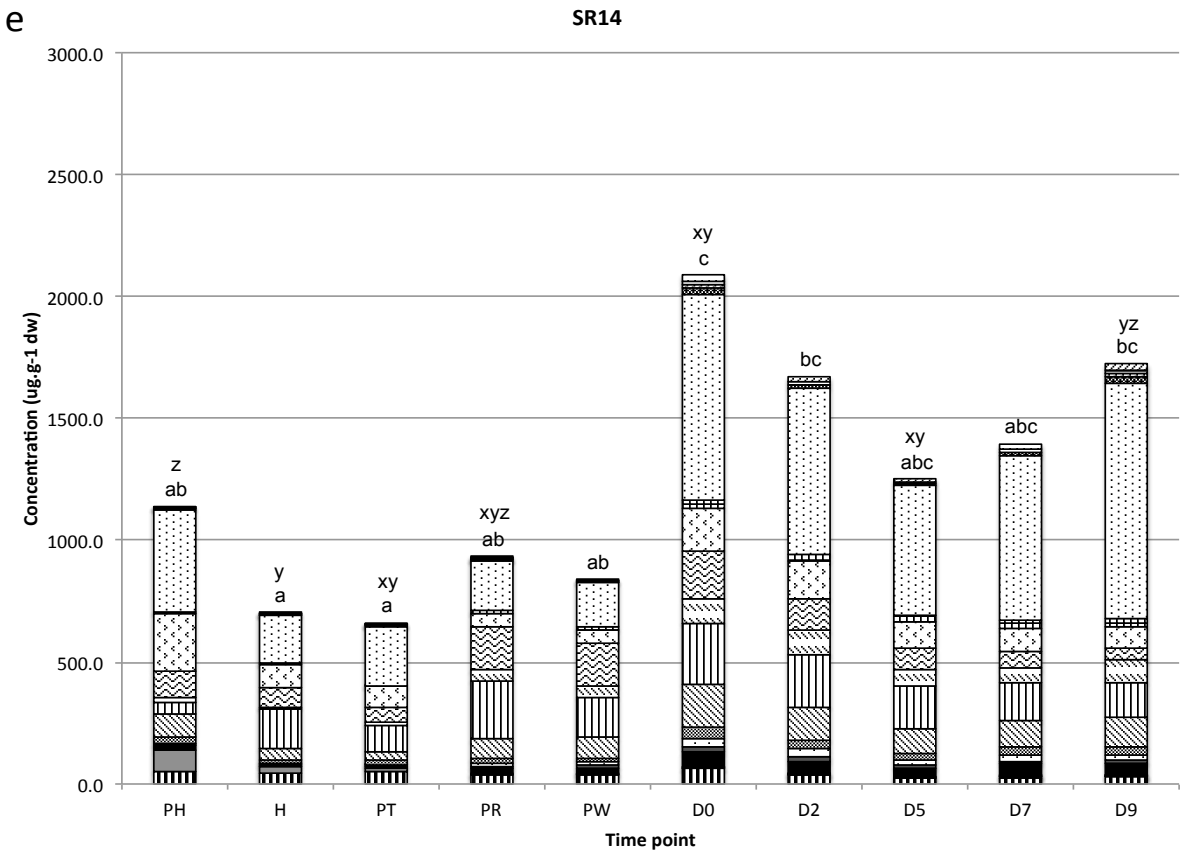
c


SR6
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
Concentration (ug.g-1 dw)
x ab
x a
xy ab
x ab
ab
xy ab
b
xy ab
b
xyz ab
PH
H
PT
PR
PW
D0
D2
D5
D7
D9
Time point

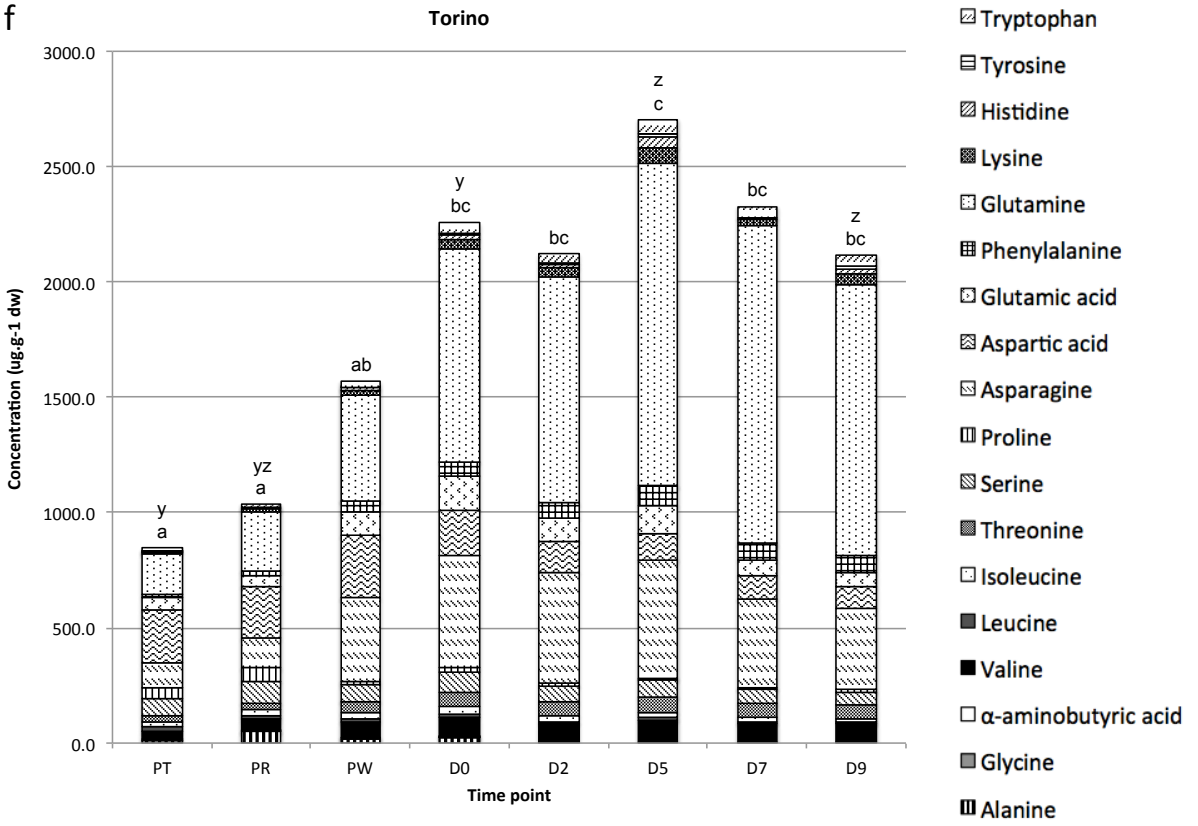
d


SR12
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
Concentration (ug.g-1 dw)
y ab
x a
xy a
xy ab
ab
xy ab
bc
x ab
c
xy ab
PH
H
PT
PR
PW
D0
D2
D5
D7
D9
Time point

e


SR14
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
Concentration (ug.g-1 dw)
z
ab
y
a
xy
a
xyz
ab
ab
xy
c
bc
xy
abc
abc
yz
bc
PH
H
PT
PR
PW
D0
D2
D5
D7
D9
Time point

f


Torino
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
Concentration (ug.g-1 dw)
y
a
yz
a
ab
y
bc
bc
z
c
bc
z
bc
PT
PR
PW
D0
D2
D5
D7
D9
Time point
Tryptophan
Tyrosine
Histidine
Lysine
Glutamine
Phenylalanine
Glutamic acid
Aspartic acid
Asparagine
Proline
Serine
Threonine
Isoleucine
Leucine
Valine
α-aminobutyric acid
Glycine
Alanine

7.4.1.5. Sugars

Relative concentrations of free sugars are presented in appendix XII. Few significant differences were observed overall, though *Torino* showed a trend to accumulate lower amounts than the *E. sativa* cultivars. SR2 contained significantly more fructose (46.1 $mg.g^{-1}$ dw) and total sugar (141.9 $mg.g^{-1}$ dw) at H than SR5 (7.2 $mg.g^{-1}$ dw and 52.7 $mg.g^{-1}$ dw, respectively). A significant difference in total sugar was also observed at PW between SR2 (161.1 $mg.g^{-1}$ dw) and *Torino* (32.4 $mg.g^{-1}$ dw).

During shelf life significant differences became more numerous between samples at each time point. On D0, SR14 had significantly higher glucose (118.8 $mg.g^{-1}$ dw) and total free sugars (143.1 $mg.g^{-1}$ dw) than *Torino* (14.9 $mg.g^{-1}$ dw; 28.5 $mg.g^{-1}$ dw). By D7 *Torino* contained significantly less fructose (2.6 $mg.g^{-1}$ dw), glucose (16.6 $mg.g^{-1}$ dw) and total free sugar (32.4 $mg.g^{-1}$ dw) than both SR12 (20.0 $mg.g^{-1}$ dw, 131.7 $mg.g^{-1}$ dw, and 164.7 $mg.g^{-1}$ dw, respectively) and SR2 (32.6 $mg.g^{-1}$ dw, 111.0 $mg.g^{-1}$ dw, and 156.6 $mg.g^{-1}$ dw, respectively).

Bell et al. (2017*,* Chapter 5) observed that high free sugar concentrations in and of themselves do not correspond to milder taste, and that the ratio between sugars and GSLs/ITCs is the more significant attribute in determining pungency and bitterness. As GSLs and ITCs increase over time, and sugars are relatively stable, this is likely to have a large impact on how leaves taste.

7.4.2. Principal Component Analyses

7.4.2.1. General

Figure 7.5 displays the PCA for all phytochemical and time point data of each cultivar. Figure 7.5a (loadings) and b (scores) are plotted with GSL, sugar, and AA

data for all time points. Figure 7.5c and d are plotted with these same data for time points PT and D7, with the addition of GSL hydrolysis product data.

From the data used to generate Figure 7.5a and b, 31 principal components (PCs) were extracted, with the first eight having Eigenvalues >1.0. Of these only PC1 and PC2 contained >10.0% of the explained variability (51.5% cumulatively) and as such were selected for presentation. PC1 separates for bacterial counts, the major GSL compounds of rocket (total, glucosativin, DMB), and amino acid concentrations. PC2 separates for sugar and proline concentration.

In the analysis presented in Figure 7.5c and 7.5d, 11 PCs were extracted. PCs 1 – 8 had Eigenvalues >1.0, but only PCs 1 – 3 explained >10.0% of the variation (72.4% cumulatively). PC1 vs. PC2 and PC1 vs. PC3 were selected for presentation as biplots. PC1 separates for glucosativin/DMB and the associated ITC hydrolysis products, as well as bacterial counts and amino acid concentrations. PC2 separates for sugars, alanine, glycine and proline concentrations, and PC3 separates for glucoraphanin, sulforaphane and 4-isothiocyanato-1-butene. Loadings values can be found in appendix XIII and correlation matrices in appendix XIV for each of the PCA analyses.

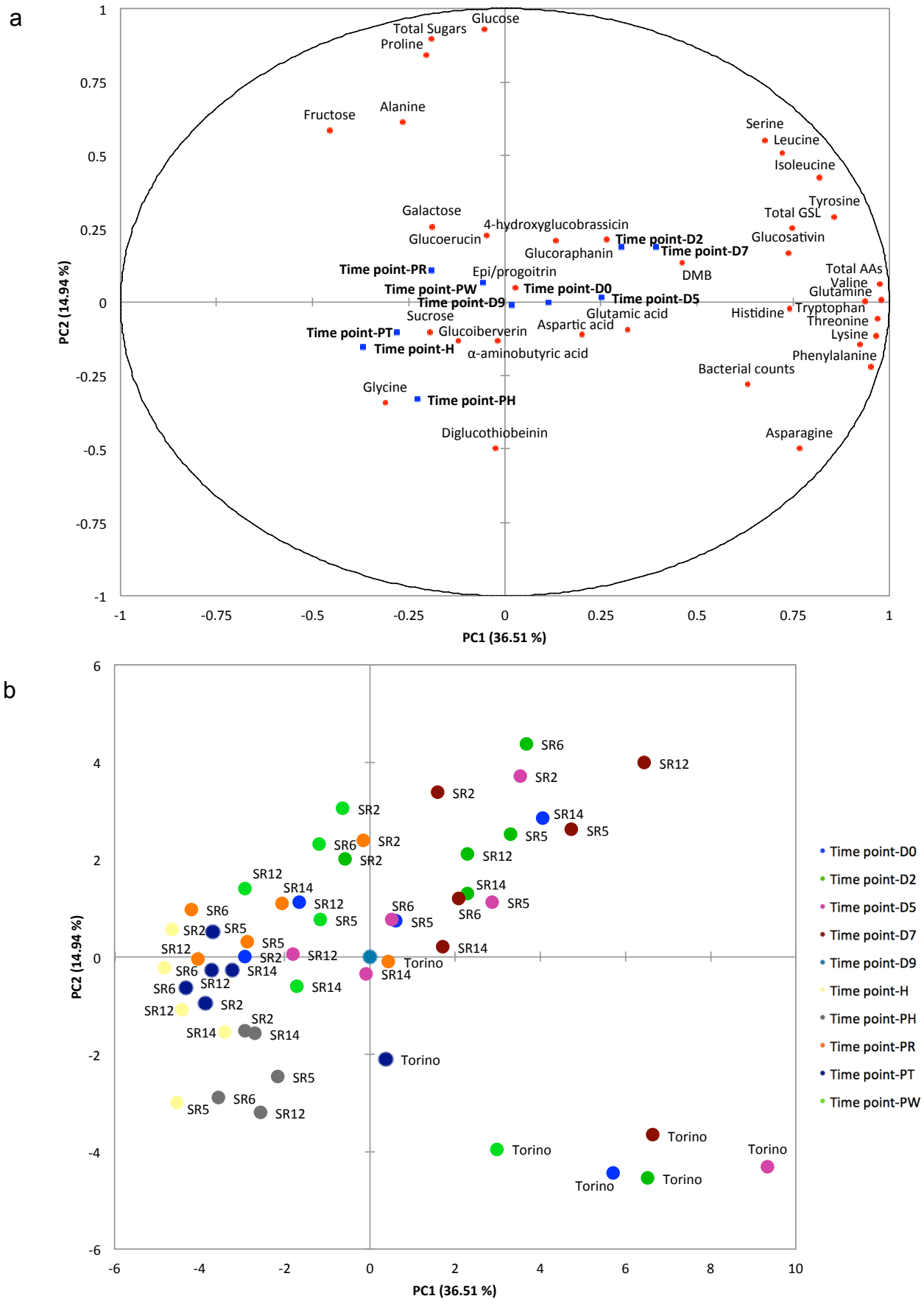
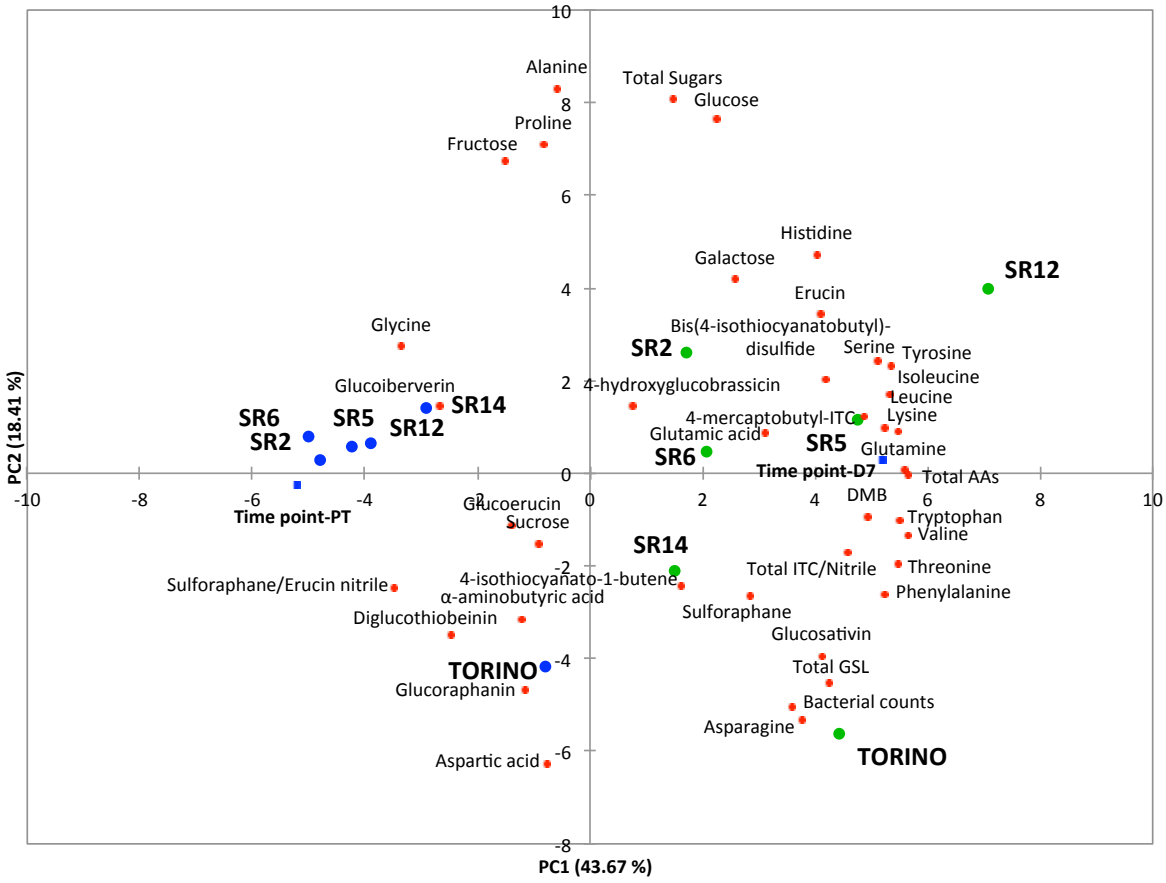
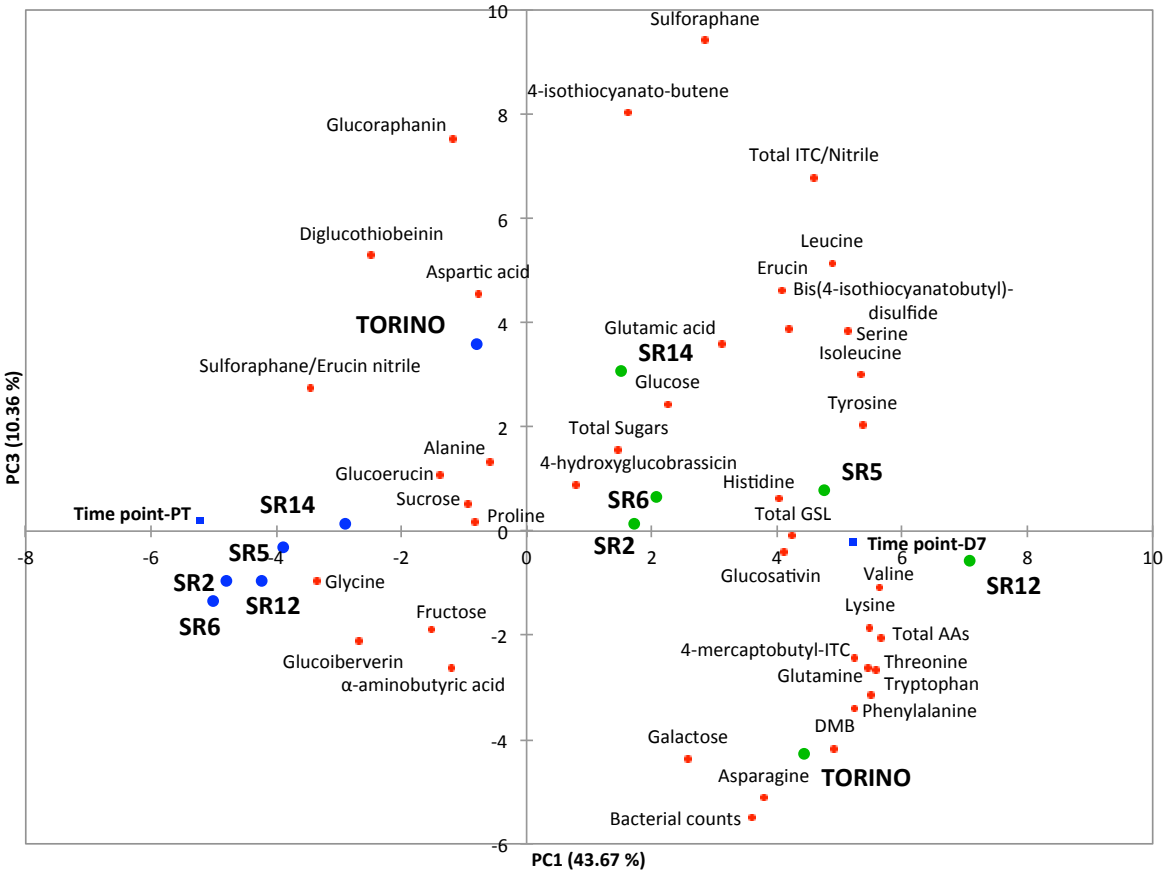


Figure 7.5. PCA loadings (a) and scores (b) plot for glucosinolate, sugar, amino acid and time point data for the five cultivars tested (PC1 vs. PC2; 51.5% variation explained). PCA biplots (c: PC1 vs. PC2, 62.1% variation explained; d: PC1 vs. PC3, 54.0% variation explained) for all phytochemical data, including ITCs and nitriles, at time points PT and D7. Plots a, c, d: red = active variables, blue = supplementary variables. Plot b: see inset for score plot key. Plots c, d: blue circles = time point PT, green circles = time point D7. Abbreviations: PH, preharvest (12 days old); H, harvest (22 days old); PT, post transport; PR, pre-wash; PW, post wash; D0, day zero shelf life; D2, day two shelf life; D5, day five shelf life; D7, day seven shelf life (DUD; display until date); D9, day nine shelf life.

c


PC2 (18.41 %)
PC1 (43.67 %)
Alanine
Total Sugars
Glucose
Proline
Fructose
Histidine
Galactose
SR12
Erucin
Glycine
Bis(4-isothiocyanatobutyl)-
disulfide
SR2
Serine
Tyrosine
Isoleucine
Glucoiberverin
4-hydroxyglucobrassicin
SR14
SR6
SR5
SR12
SR2
Leucine
4-mercaptobutyl-ITC
Lysine
Glutamic acid
SR5
SR6
Glutamine
Time point-D7
Total AAs
DMB
Time point-PT
Glucoerucin
Tryptophan
Sucrose
Valine
SR14
Threonine
Total ITC/Nitrile
4-isothiocyanato-1-butene
Phenylalanine
Sulforaphane/Erucin nitrile
α-aminobutyric acid
Sulforaphane
Diglucothiobeinin
Glucosativin
TORINO
Total GSL
Glucoraphanin
Bacterial counts
Asparagine
Aspartic acid
TORINO

d


PC3 (10.36 %)
PC1 (43.67 %)
Sulforaphane
4-isothiocyanato-butene
Glucoraphanin
Total ITC/Nitrile
Diglucothiobeinin
Leucine
Erucin
Aspartic acid
Bis(4-isothiocyanatobutyl)-
disulfide
TORINO
Glutamic acid
Serine
SR14
Isoleucine
Sulforaphane/Erucin nitrile
Glucose
Tyrosine
Total Sugars
Alanine
4-hydroxyglucobrassicin
SR5
Glucoerucin
Histidine
SR6
Time point-PT
SR14
Sucrose
Total GSL
Proline
Time point-D7
SR5
SR2
Glucosativin
Valine
SR12
Glycine
Lysine
SR2
SR12
Fructose
Total AAs
SR6
4-mercaptobutyl-ITC
Threonine
Glucoiberverin
Glutamine
Tryptophan
α-aminobutyric acid
Phenylalanine
DMB
Galactose
Asparagine
TORINO
Bacterial counts

7.4.2.2. Bacterial Counts

The most unexpected result from this study was the significant correlation between bacterial counts present on leaves and phytochemical composition. Our hypothesis was that higher GSLs/ITCs would reduce bacterial load, but the exact opposite was observed (Figure 7.5). Significant correlations were recorded with glucosativin (r = 0.442, P<0.001), DMB (r = 0.391, P<0.01) and total GSL concentration (r = 0.428, P<0.01, Figure 7.5a; appendix XIV). It has been previously hypothesised (Schreiner, Krumbein, & Ruppel, 2009) that under nutrient limited conditions some bacterial strains may use GSLs as a source of carbon. In our study nutrients were not limited, and were abundant in leaves due to the high free sugar concentrations. Bacterial counts were in fact inversely correlated with total sugars (r = -0.318, P<0.05) and fructose (r = -0.325, P<0.05). No significant correlations were observed between bacterial counts and GSL hydrolysis products, indicating that any ITCs formed throughout the supply chain and shelf life have no discernable anti-microbial effect on endemic bacteria.

We hypothesise microbes on rocket leaves are highly adapted to that environment, and have evolved a tolerance or for high ITC concentrations, or a way to circumvent the GSL-myrosinase system. It has been documented that soil bacteria (*Citrobacter* spp.) possess a glucoside hydrolase family 3 (GH3) β-glucosidase enzyme, which may potentially aid them in the scavenging of glucose from GSLs (Albaser et al., 2016). The same may be true of bacteria that live on leaves, but very little research has been conducted in this area. Adaptation by insects to the GSL-myrosinase system is well documented (Alan & Renwick, 2002), but how bacteria have adapted is poorly understood.

Positive correlations were observed for some AAs and bacterial numbers, whereas others displayed a negative association. Positive correlations (appendix XIV) were seen for valine (r = 0.603, P<0.001), isoleucine (r = 0.337, P<0.01), threonine (r = 0.611, P<0.001), asparagine (r = 0.659, P<0.001), phenylalanine (r = 0.685, P<0.001), glutamine (r = 0.651, P<0.001), lysine (r = 0.558, P<0.001), histidine (r = 0.308, P<0.001), tyrosine (r = 0.439, P<0.001), tryptophan (r = 0.632, P<0.001), and total AA concentration (r = 0.584, P<0.001). Negative correlations were with alanine (r = -0.566, P<0.001) and proline (r = -0.323, P<0.05). Coupled with the trends seen for GSLs as a potential carbon source, we hypothesise that bacteria may similarly utilise free amino acids as a nutrient source.

7.4.2.3. Glucosinolates & Hydrolysis Products

Aside from the aforementioned correlations with bacterial counts (Figure 7.5; appendix XIV), several other significant correlations are present in the results. Total GSLs were significantly correlated with numerous AAs and total AA concentration. This association may be reflective of the underlying degradation of proteins due to senescence (Buchanan-Wollaston et al. 2003) and up-regulation of secondary defense compounds (Jin et al., 2009).

GSLs and respective hydrolysis products correlated significantly (Figure 7.5c & 5d) as was expected. Total GSL concentration correlated with both sativin (r = 0.621, P<0.05) and total ITC/nitrile concentration (r = 0.590, P<0.05). Glucoraphanin correlated significantly with both sulforaphane (r = 0.578, P<0.05) and 4-isothiocyanato-1-butene (r = 0.585, P<0.05), further supporting the hypothesis that the latter ITC is a degradation product of the former.

7.4.2.4. Sugars & Amino Acids

The free sugars fructose (r = 0.308, P<0.05), glucose (r = 0.536, P<0.001) and galactose (r = 0.318, P<0.05) shared significant correlations with alanine (Figure 7.5a; appendix XIV), and fructose (r = 0.560, P<0.001) and glucose (r = 0.746, P<0.001) with proline. It is interesting to note that these AAs are known to have sweet tastes (Solms, 1969), which could potentially influence the sensory properties of leaves. They were also negatively correlated with bacterial growth (alanine: r = -0.566, P<0.001; proline: r = -0.323, P<0.05), perhaps indicating a relationship between sugar/AA content and bacterial load of leaves, though in what respect is presently unclear.

7.4.2.5. Time Points

Several of the studied phytochemical components correlated significantly with specific time points (Figure 7.5; appendix XIV). Many of these have important implications for rocket breeding and commercial supply chain management.

In the PCA (Figure 7.5b) the profile of D7 samples separate along PC1, and are indicative of significant phytochemical changes by this point of the trial. Time point PH and H form a distinct and tight cluster to the lower left of the plot on the PC1 axis, blending with PT and PW before separating towards the top right, loosely according to shelf life time point. *Torino* is very distinct, separating away to the bottom right. This is due to the high bacterial load of these samples (PC2), as well as low sugar, and high GSL/AA concentration during shelf life (Figure 7.5a). This pattern is similar in Figure 7.5c and 5d where *E. sativa* PT samples are tightly clustered, with D7 separating along the PC1 axis in a more dispersed fashion to the right. The two *Torino* time points are isolated however, associated again with high bacterial counts,

asparagine, aspartic acid, and high total GSL concentration. The *E. sativa* cultivars trend towards higher total sugars and sweet AAs (PC2).

PH was distinct in several aspects from the subsequent time points (Figure 7.5a). Glucosativin (r = -0.306, P<0.05) and total GSL concentrations (r = -0.394, P<0.01) were typically low, and were negatively correlated. There were also several AAs significantly correlated with PH, and not with any subsequent time point. Glycine (r = 0.961, P<0.001), alanine (r = 0.289, P<0.05), α-aminobutyric acid (r = 0.358, P<0.05) and glutamic acid (r = 0.580, P<0.001) are all relatively higher in abundance, whereas others, such as proline (r = -0.392, P<0.01), leucine (r = -0.308, P<0.05) and tryptophan (r = -0.286, P<0.05) were negatively correlated with PH.

At H, significant negative correlations can be seen with glucoraphanin (r = -0.295, P<0.05), glucosativin (r = -0.262, P<0.05) and total GSL concentration (r = -0.327, P<0.05); this is perhaps indicative of GSL depletion during the harvest procedure. Numerous AAs and total AA concentrations were also low and negatively correlated (Figure 7.5a).

D7 (DUD) is perhaps the key time point within the trial, as total GSL (r = 0.339, P<0.01), glucosativin (r = 0.341, P<0.01) and DMB (r = 0.404, P<0.01) concentrations were all significantly correlated (Figure 7.5c & 5d; appendix XIV). Total ITC/nitriles (r = 0.707, P<0.05), sativin (r = 0.720, P<0.01) and erucin (r = 0.683, P<0.05) were also significantly correlated, demonstrating that all cultivars displayed the ability to re-synthesize GSLs/ITCs to a high level during shelf life. Total AAs (r = 0.410, P<0.01) and glutamine (r = 0.528, P<0.001) shared a strong correlation with this time point.

There seems to be juxtaposition between the point of highest nutritional content (GSLs/ITCs) and the high degree of tissue and protein breakdown evidenced by the increases in free AAs. This may lead to visual and aroma changes that

consumers will reject, and may dissuade them from consuming leaves that contain the highest ITC concentrations.

7.5. Conclusions

This study is the first to demonstrate the phytochemical and bacteriological effects of an entire commercial supply chain on rocket leaves. It is clear from our results that total GSL concentration increases post processing. Importantly, glucoerucin and glucoraphanin are not significantly reduced by processing, suggesting that GSLs are not lost due to leaching or myrosinase action in wash water as we originally hypothesised.

ITCs (particularly sulforaphane) increase significantly during shelf life in *E. sativa*, and this could have positive health benefits for the end consumer. We have elucidated significant changes in AA composition of leaves, and that free sugars remain relatively stable throughout processing and shelf life. The fluctuations in relative abundance of GSLs, ITCs and AAs may have important implications for consumer acceptance and sensory properties of leaves.

We have demonstrated a possible link between GSL and AA concentrations with the bacterial load of leaves. At present it is unknown by what mechanism this is achieved, but further study and identification of bacterial strains on rocket leaves may provide insight. We hypothesise that bacterial populations have evolved to survive on GSL-producing plants, perhaps utilising GSLs as a carbon source and free AAs as a nitrogen source. We speculate that such native bacterial loads are non-pathogenic, however their presence and metabolism of sulphur-containing compounds (such as GSLs) within sealed bags may produce off-odors that consumers might reject (Spadafora et al. 2016).

Finally, we have demonstrated that GSL/ITC profiles observed in controlled studies are not fully representative of commercially processed material. The data presented here illustrate how dynamic GSL profiles are over time. Future studies may wish to consider the impact of the whole supply chain when attempting to analyse crops for phytochemicals, and not just the point of harvest.

7.6. References

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CHAPTER 8: Overall Discussion, Related Projects & Future Work

8.1. Overall Discussion

The final summary chapter of this thesis will highlight the various outputs and collaborations of this PhD project, and summarise the key findings. The results presented in the preceding chapters highlight the potential for rocket, and in particular the species *E. sativa,* to be further developed into high quality varieties for human consumption. The inherent natural variation within the species means breeders have a broad palette of traits with which to diversify breeding lines and create novel varieties. This may range from unusual visual traits such as leaf shape, size and colour, to phytochemical attributes that improve sensory properties, consumer acceptance, and health benefits.

The results presented have significantly advanced research into the sensory and health promoting compounds of rocket, and their interactions with the consumer. Key outcomes have been the diversity of GSL profiles present in different cultivars of rocket (Chapter 3), particularly the propensity for some to accumulate more glucosativin in monomeric or dimeric form. The exact reasons for this are unknown, though it has been elucidated in Chapter 5 that the two forms may infer differing sensory properties. Similarly in Chapter 7 it was noted that glucoraphanin was consistently maintained across processing and shelf life. The differing abundances in sulforaphane at the two time points tested would suggest that it is myrosinase which is most adversely affected by the supply chain, not GSL content. The results in Chapter 5 suggest that glucoraphanin and glucoerucin impart very little effect on the overall taste and flavour of rocket leaves, and combined with results from Chapter 4, it is apparent that relative VOC abundances affect sensory perceptions. This may be beneficial for breeding new varieties of rocket, as high accumulators of glucoraphanin

can be selected for without imparting significant negative effects on sensory properties or consumer acceptance (Chapter 6).

The increases in phytochemicals within the supply chain after processing (Chapter 7) are contrary to some previous literature findings. It was previously shown that GSL concentrations generally decrease under varying environmental conditions (Jones, Faragher, & Winkler, 2006), but analysis of how ITC formation is affected during shelf life is poorly understood, with no papers directly measuring ITC concentrations over time for any crop (to the authors' knowledge). The results in Chapter 4 would seem to support the assumption that ITCs decrease over time in terms of VOC production, but due to the nature of the analysis, this is a representation of the relative abundances of ITCs within headspace, not tissues. It would have been interesting to study all of the time points in Chapter 7 for their ITC content in order to elucidate any other fluctuations during the entirety of the processing trial, rather than just PT and D7. Due to budget and time constraints this was not possible however.

There is a danger that GSLs are used as a proxy measurement of ITC abundance over shelf life. The results of Chapter 7 demonstrate that GSL abundance does not necessarily reflect specific ITC concentrations, particularly with regards to glucoraphanin and sulforaphane. Concentrations of glucoraphanin did not vary significantly over the course of the experiment, however sulforaphane concentration increased significantly by D7. This suggests that myrosinase activity is reduced after harvest and recovers over time and after processing.

Work relating to the bacterial load of commercial salad leaves also an unexpected but intriguing result. The current literature thinking espouses the view that GSLs and ITCs are highly bactericidal and the differing profiles of cultivars should in theory produce differing levels of colonisation. By this logic, one would have

expected *Torino* to contain low levels of bacteria, but this was not the case. Publications have previously suggested the bacteria on rocket leaves play a role in producing off odours (Nielsen, Bergstrom, & Borch, 2008), however no study has tied the endogenous bacterial populations to the glucosinolate concentrations of the plant. We hypothesise that endemic bacterial populations within/on rocket leaves are highly specialised to that environment, and may have evolved to evade the GSL myrosinase system, or alternatively, evolved to tolerate ITCs and other volatiles much as some insects have (Nielsen, Nagao, Okabe, & Shinoda, 2010). There is clearly some form of relationship between the two, however it is not absolutely certain if bacterial load increases because of high GSLs or *vice versa*. The hypothesis put forward by Schreiner, Krumbein, & Ruppel (2009) that some bacteria utilise GSLs as a carbon source is interesting, but needs much more study in order to be verified. Our data would seem to suggest that nitrogen (from amino acids) and sulphur (from GSLs/ITCs) are more likely candidates for bacterial assimilation, as carbon is abundant in the form of free sugars.

The specialisation of bacteria to GSL containing plants is only just being elucidated, and is more related to root systems than leaves at the present time (Albaser et al., 2016). The assumption that all ITCs have a general anti-microbial or anti-fungal effect may be misguided. The results presented in Chapter 7 suggest that there may be resistance to such compounds, and indeed an ability to thrive under high GSL conditions, as high ITC concentrations do not have any effect on bacterial numbers during shelf life. There is also the possibility that some leaf bacteria have formed a symbiotic relationship with rocket plants, but until specific strains can be identified there is little evidence for this at the present time. This work will continue to be investigated by the Wagstaff Group at the University of Reading.

8.2. Industrial Relevance, Collaboration & Related Projects

8.2.1. Elsoms Seeds Ltd. Commercial Breeding Program

As stated in Chapter 1 the Elsoms Seeds rocket-breeding program ran in parallel to this PhD project. The results of sensory and consumer analyses (Chapters 5 & 6) in particular have influenced plant selections and breeding goals. Due to commercially sensitive information, specific details about current breeding lines and their characteristics cannot be given. Several elite lines are however undergoing commercial trials.

8.2.2. Bakkavor Group Ltd. Project SOAR

Following the results presented in Chapters 3, 4, 5 & 6, Bakkavör initiated Project SOAR with the Wagstaff Group at the University of Reading. Specific details about the project cannot be given for commercial sensitivity reasons, but multiple rocket salad varieties from several geographical regions were compared in terms of GSL, free sugar, sensory and consumer attributes over the course of one year. The project confirmed several aspects of the research presented in this thesis, and also highlighted numerous other areas for investigation.

8.2.3. E. sativa Genome Sequencing Project

An ambitious project was initiated as part of this PhD to fully sequence the genomes of three *E. sativa* elite inbred lines (referred to as A, B & C) from the Elsoms Seeds breeding program. The aim is to produce two mapping populations, created by crossing A with B, and A with C. The Genome Analysis Centre (TGAC; The John Innes Centre, Norwich, UK) is conducting the project, and at the time of writing, sequencing and assembly has been completed, with single nucleotide polymorphism (SNP) calling currently underway.

8.2.4. BBSRC LINK Project: 'Smart Breeding For Salad Rocket'

The data generated by the aforementioned genome-sequencing project will feed into a BBSRC LINK funded project developed in collaboration with Elsoms Seeds and Bakkavör. The author is a co-applicant researcher named on the grant along with Dr. Carol Wagstaff and Dr. Lisa Methven, and it is the first BBSRC LINK award ever to be given to the University of Reading, and is worth over £900k across three years.

The TGAC project will yield substantial SNP data for each of the assessed parent lines, enabling the eventual construction of genetic linkage and quantitative trait loci (QTL) maps of their progeny. Genotyping of each of the mapping population lines for GSL, ITC, amino acid and sugar concentrations across environments will be conducted. These will be in controlled environment, a field environment (UK), and a polytunnel environment (Italy). This will investigate the stability of phytochemical traits in unprecedented detail for rocket, and determine if any QTL co-locate across environments. Extreme lines will be selected for further sensory analysis, utilising two trained panels – one that is composed of the 'supertaster' PAV/PAV TAS2R38 diplotype, and one that is composed of the 'non-taster' AVI/AVI diplotype. The research presented in Chapter 6 revealed that each group perceived bitterness differently, and their differing responses will (in theory) allow us to detect QTL relating to the intensity of these perceptions within the plant genome. Each panel will be presented with leaves from commercial environments in the UK and Italy. Results from this study, in combination with phytochemical and transcriptomic data, will allow us to establish if sensory trait QTL co-locate with any related to phytochemicals or pathways, giving a better understanding of how plant genetics influences sensory attributes.

With genomic and transcriptomic data, other experiments will also be conducted to examine orthologous sequences with *Arabidopsis* and *Brassica oleracea*. It is hoped that similar genes involved in GSL biosynthesis and ITC formation pathways can be elucidated in rocket, and ultimately be fast-tracked into generating SNP markers for breeders at Elsoms. The mechanisms responsible for high glucoraphanin accumulation in broccoli are now well understood (Traka et al., 2013) and it is hoped that similar MYB gene sequences will be identified in cultivars of *E. sativa*.

In terms of future rocket breeding strategy, much has changed in terms of which phytochemical and sensory attributes should be selected for in order to produce a variety of improved quality. Firstly, the definition of quality has itself changed due to the results presented in this thesis. It was initially assumed that all consumers liked the same attributes of rocket, but it has been shown that this is not the case and that people like/dislike rocket for different reasons. This has influenced the breeding strategy in that certain compounds such as ITCs and sulfur volatiles can be selected for and against in order to produce hot and mild varieties. We have also elucidated that the ratio with sugars and amino acids should also be considered in order to mitigate the intensity of hotness in leaves. This was the defining attribute on which consumers based their liking of the cultivars tested, not bitterness as had been originally assumed.

The screening of breeding lines for all of these aspects will likely become more common practice in breeding programs, and will eventually tie-in with genomic information generated from the upcoming LINK project. High-throughput in-house analysis of these compounds is not possible in the breeding company at the present time, and it can be expensive to outsource these types of analysis. This is perhaps the largest ‘gap’ in terms of full implementation of our results into a breeding

program. With the development of SNP markers linked to specific genes in rocket, this process will become significantly improved.

8.3. Conclusion

Rocket species have been cultivated for millennia (Hall, Jobling, & Rogers, 2012), and are well known and documented in folk medicine and cuisine. Many of the therapeutic properties associated with the eating of leaves have only started to be properly understood in the last 15-20 years. In many respects, we are rediscovering the importance of vegetables in our daily lives, which particularly in Western societies, has been lost. People in urban communities have become disconnected from their food supply, and general knowledge and interest in food production is low. Increasingly, supermarkets dictate the foods that people have access to and can afford, and so in poor urban communities this has a significant impact on the amount and variety of vegetables consumed (McCaffrey, 2012). It is often the poorest in such places who are unable to access healthier food items, and as such have a higher risk of acquiring chronic health problems such as type-2 diabetes, cardiovascular disease, and cancer (World Health Organization, 2003).

On a more positive side, it is likely that due to renewed interest and research in the health-beneficial properties of minor crops like rocket (and vegetables in general) that these will become much more prominent in future decades within personalised nutrition regimes. More people are becoming interested in the nutritional properties of foods, and this is encouraging; but such interest often comes with improved socioeconomic status, and in people least likely to benefit from dietary changes.

The *in vitro* and *in vivo* studies analysing the effects of ITCs and other GSL-hydrolysis products are very promising, but should be interpreted cautiously. Effects

in cell and animal models are not necessarily occurring in the human body, and it should not be assumed that the same mechanisms are occurring therein. Much more work is needed to properly understand how GSL-containing plants interact with gut microbiota, and how different individuals assimilate, metabolise and excrete ITCs. The systemic effects of regular consumption are only now being properly investigated, but are poorly understood in terms of what mechanisms are responsible for the anti-carcinogenic effects observed in clinical and epidemiological studies.

Food chemists and qualified nutritionists should perhaps be more vocal and precise about the effects certain types of food could have on their health. As an example, rocket, kale, and other *Brassicaceae* are often mentioned for having anti-cancer properties, and are portrayed by the mainstream media and on social media as "super foods" that will ensure people live longer and don't get cancer. As every food scientist and cancer biologist knows, things aren't quite so simplistic. Evidence is abundant within the literature that diets rich in *Brassicaceae* lower the risk of certain types of cancer (van Poppel, Verhoeven, Verhagen, & Goldbohm, 1999), and that the more general consumption of fruits and vegetables also lowers the risk (Block, Patterson, & Subar, 1992). It is in explaining the concept of these risks and statistics that problems lie, which often leads to consumers having unrealistic expectations of their food, or distorted views of what constitutes "healthy" or "natural" produce (Grunert, 2005).

In a time where anyone with a computer can start a blog on "nutrition", more should perhaps be done to improve scientific literacy and understanding of how food is produced, rather than offering inaccurate and inarticulate generalisations. During the course of this PhD, several social media "nutritionists" have risen to prominence advocating the consumption of "natural" foods, and promoting pseudoscientific theories with disturbing success. If experts do not take this role, charlatans will step

in and fill the void and mislead those who are uninformed, which will ultimately be to the detriment of efforts to improve food for the consumer.

8.4. References

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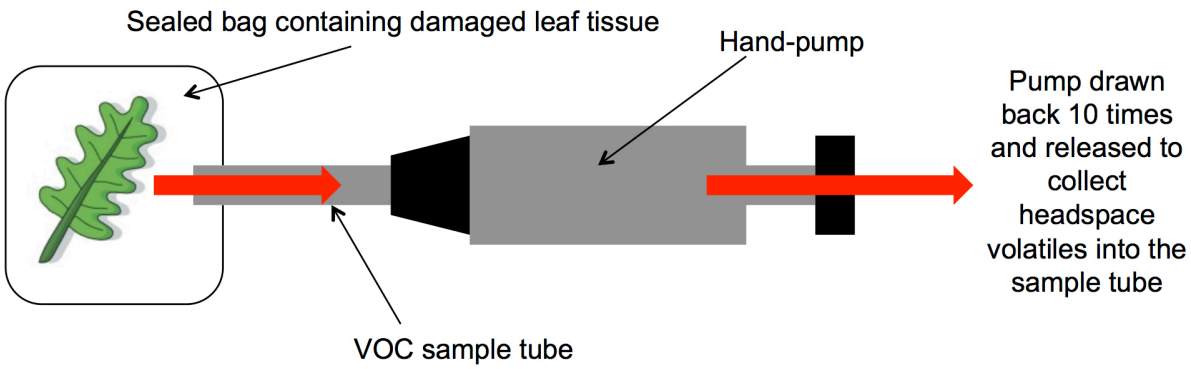
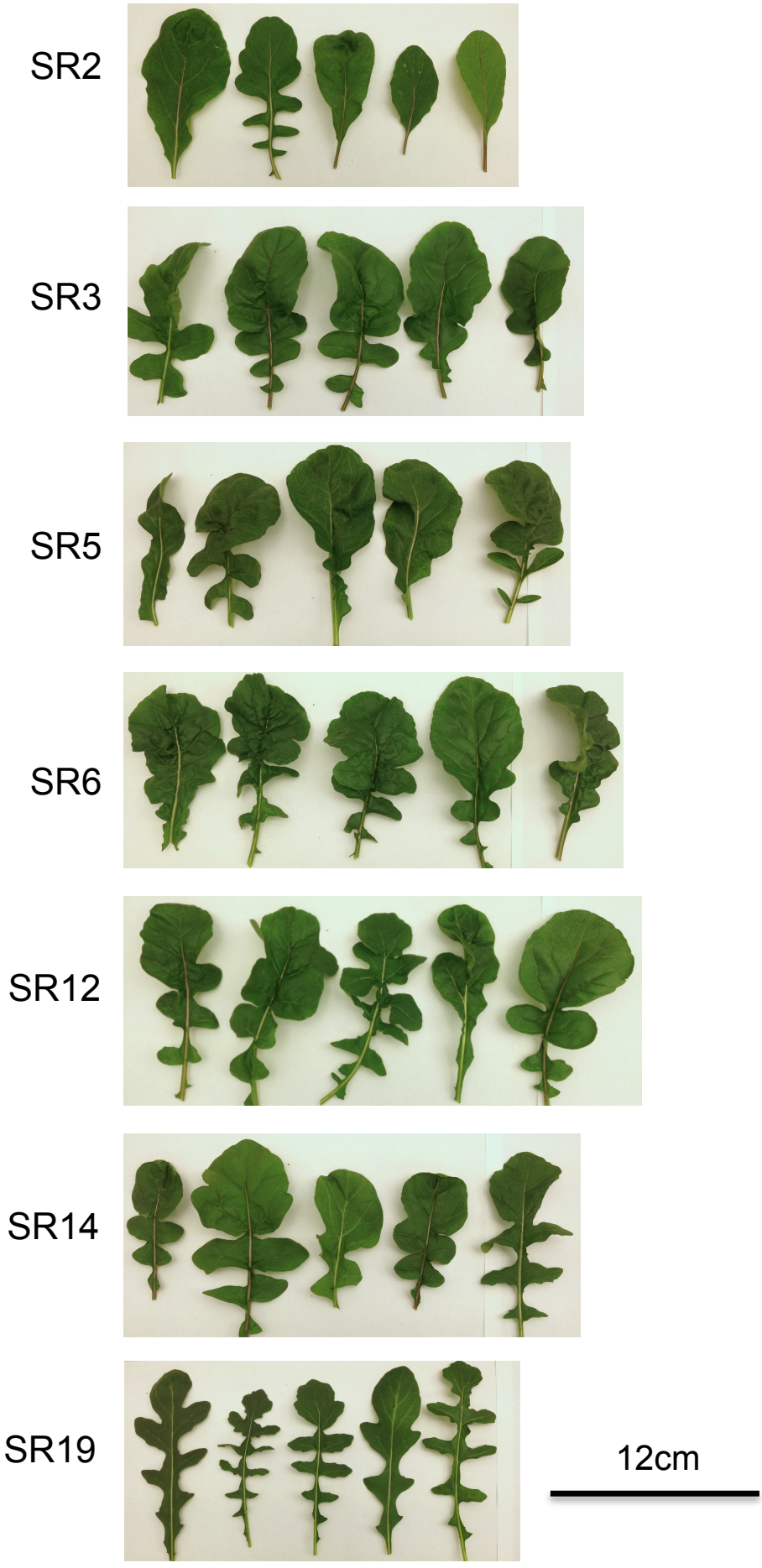
Appendix I


Sealed bag containing damaged leaf tissue
Hand-pump
Pump drawn back 10 times and released to collect headspace volatiles into the sample tube
VOC sample tube

Appendix II



Appendix III Summary of glucosinolate and flavonol average concentrations, and headspace VOC relative abundances (at point of harvest) for the seven accessions analysed (± standard error). Data previously presented by Bell et al. (2015; 2016).

Accession	SR2	SR3	SR5	SR6	SR12	SR14	SR19
Glucosinolates (mg.g-1 dw)							
4-hydroxyglucobrassicin	nd	nd	0.1±0.1	nd	nd	nd	0.1±0.1
Glucotropaeolin	nd	0.1±0.1	nd	nd	nd	<0.1±<01	nd
Glucoraphanin	0.4±0.1	0.3±0.1	0.2±0.1	0.6±0.4	0.3±0.2	nd	0.3±0.1
Glucoiberverin	<0.1±<0.1	<0.1±<0.1	nd	0.1±<0.1	0.1±<0.1	<0.1±<0.1	nd
Glucosativin	3.5±0.5	2.7±0.2	7.7±0.8	3.5±0.2	5.7±0.7	5.1±0.4	3.4±0.5
DMB	2.3±0.4	1.2±0.3	3.3±0.3	4.4±0.4	1.9±0.4	2.2±0.5	2.2±0.6
Glucoalyssin	nd	nd	<0.1±<0.1	<0.1±<0.1	nd	<0.1±<0.1	0.1±0.1
Glucoerucin	0.3±0.1	0.6±0.2	0.1±0.1	1.3±0.3	0.5±0.3	0.1±0.1	nd
Glucoraphenin	<0.1±<0.1	nd	nd	0.2±0.2	nd	nd	nd
Glucoibarin	0.1±0.1	nd	<0.1±<0.1	nd	nd	nd	nd
Average total GSL	6.6±0.6	4.9±0.6	11.5±0.9	10.0±1.1	8.4±0.8	7.5±0.7	6.3±0.8
Flavonols (g.kg-1 dw)							
Myricetin	<0.1±<0.1	<0.1±<0.1	nd	nd	nd	nd	nd
Kaempferol-3-glucoside	0.1±<0.1	0.1±<0.1	0.3±<0.1	0.4±0.1	0.2±0.1	0.3±0.1	0.2±0.1
Isorhamnetin-3-glucoside	0.1±<0.1	0.1±<0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.1±<0.1	nd
Kaempferol-3,4'-diglucoside	0.5±0.1	0.4±0.1	0.5±0.1	1.1±0.1	0.6±0.1	0.6±0.1	0.3±0.1
Isorhamnetin-3,4'-diglucoside	0.5±0.1	0.2±0.1	0.4±0.1	0.6±0.1	0.3±0.1	0.1±0.1	0.4±0.1
Kaempferol-3-diglucoside-7-glucoside	0.2±0.1	<0.1±<0.1	<0.1±<0.1	0.2±0.1	0.1±0.1	0.1±0.1	0.1±0.1
Quercetin-3,3,4'-triglucoside	0.2±0.1	0.1±<0.1	<0.1±<0.1	<0.1±<0.1	0.1±0.1	nd	<0.1±<0.1
Kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside	0.2±0.1	0.1±0.1	0.1±0.1	0.1±<0.1	0.2±0.1	0.1±0.1	<0.1±<0.1
Quercetin-3,4'-diglucoside-3'-(6-caffeoyl-glucoside)	0.4±0.1	0.2±0.1	0.3±0.1	0.4±0.1	0.3±0.1	0.3±0.1	0.2±0.1
Average total flavonols	2.5±0.5	1.3±0.2	1.9±0.3	3.2±0.4	2.3±0.5	1.7±0.3	1.4±0.3
Headspace VOCs (relative % abundance)							
(E)-4-oxohex-2-enal (C1)	22.4±0.4	14.8±2.9	10.5±8.6	4.7±3.8	2.5±2.0	13.4±8.6	12.3±5.1
4-isothiocyanato-1-butene (C2)	nd	nd	<0.1±<0.1	0.1±<0.1	nd	nd	nd
1-penten-3-ol (C3)	1.2±0.1	1.5±0.3	0.5±0.1	1.2±0.2	1.5±0.2	1.3±0.1	1.2±0.2
1-penten-3-one (C4)	1.9±0.1	2.0±0.2	1.3±0.5	1.4±0.3	1.8±0.2	1.9±0.2	2.2±0.3
2-(1,1-dimethylethyl)-1H-indole (C5)	nd	nd	nd	<0.1±<0.1	<0.1±<0.1	nd	<0.1±<0.1
2-methyl-2-butenal (C6)	nd	<0.1±<0.1	nd	<0.1±<0.1	0.1±<0.1	<0.1±<0.1	<0.1±<0.1
2-hexenal (C7)	4.3±0.5	7.7±2.1	4.7±1.6	7.3±1.8	9.9±1.7	7.7±2.4	8.9±0.9
(E)-2-hexenal (C8)	0.7±0.2	3.1±1.6	0.4±0.1	3.5±1.5	5.5±1.7	3.4±1.9	1.9±1.0
(Z)-2-penten-1-ol (C9)	0.1±<0.1	0.2±0.1	nd	0.2±0.1	0.3±0.1	0.2±0.1	0.2±<0.1
(E)-2-pentenal (C10)	1.0±<0.1	1.2±0.2	1.0±0.3	1.0±0.1	1.3±0.2	1.3±0.1	1.5±0.1
(E,E)—2,4-hexadienal (C11)	0.4±0.1	1.0±0.4	0.2±0.1	1.4±0.6	1.6±0.5	1.1±0.5	0.8±0.2
5-ethyl-2(5H)-furanone (C12)	0.2±<0.1	0.2±0.1	<0.1±<0.1	0.4±<0.1	0.4±<0.1	0.5±0.1	0.4±0.1
3-hexen-1-ol (C14)	39.3±5.4	36.9±14.1	14.1±1.9	17.0±1.4	14.3±1.1	15.7±1.9	24.2±5.2
3-hexenal (C15)	12.2±2.6	18.7±5.6	6.9±1.6	15.3±1.8	16.5±1.2	11.7±3.3	17.4±3.2
(Z)-3-hexenal (C16)	nd	nd	nd	0.1±<0.1	1.4±1.1	nd	nd
3-octyne (C17)	0.1±<0.1	<0.1±<0.1	<0.1±<0.1	<0.1±<0.1	<0.1±<0.1	nd	<0.1±<0.1
3-pentanone (C18)	0.2±<0.1	0.1±0.1	0.1±<0.1	0.1±<0.1	0.1±<0.1	0.2±0.1	0.3±0.1
5-methyl-4-hexen-3-one (C19)	nd	nd	0.9±0.4	2.3±1.4	3.3±1.4	1.8±1.4	2.2±1.8
4-methylpentyl isothiocyanate (C20)	1.2±0.2	0.7±0.3	4.3±1.0	1.1±0.3	1.3±0.2	0.8±0.1	2.4±0.5
5-nonanone oxime (C21)	0.1±<0.1	0.1±<0.1	0.2±<0.1	0.1±<0.1	<0.1±<0.1	0.1±<0.1	0.3±<0.1
1-isothiocyanato-3-methylbutane (C23)	nd	nd	<0.1±<0.1	<0.1±<0.1	<0.1±<0.1	nd	<0.1±<0.1
Ethylidene-cyclopropane (C24)	<0.1±<0.1	<0.1±<0.1	<0.1±<0.1	0.1±<0.1	0.1±<0.1	0.1±<0.1	<0.1±<0.1
2-ethyl-furan (C26)	1.9±0.3	1.3±0.3	2.8±0.4	3.2±0.3	1.9±0.3	2.7±0.4	2.1±0.1
3-methyl-furan (C27)	<0.1±<0.1	<0.1±<0.1	nd	<0.1±<0.1	<0.1±<0.1	<0.1±<0.1	nd
Hexyl isothiocyanate (C29)	0.4±0.1	0.2±0.1	1.8±0.7	0.4±<0.1	0.3±<0.1	0.2±0.1	0.2±0.1
2-oxo-methyl ester hexanoic acid (C30)	10.4±1.4	7.0±2.9	6.2±1.1	10.8±1.3	12.1±1.6	16.7±3.5	8.9±1.8
Pentyl isothiocyanate (C31)	nd	0.1±<0.1	0.4±0.2	0.1±<0.1	0.1±<0.1	0.1±<0.1	0.1±<0.1
Iberverin (C33)	nd	<0.1±<0.1	0.1±0.1	<0.1±<0.1	<0.1±<0.1	<0.1±<0.1	<0.1±<0.1
Propanoic acid anhydride (C34)	nd	nd	nd	2.2±0.9	3.9±0.5	3.1±1.5	nd
4-methyl-2-(2-methyl-propenyl)-pyridine (C35)	nd	0.1±<0.1	nd	0.2±<0.1	0.5±0.1	0.2±0.1	0.2±0.1
Pyrrolidine-1-dithiocarboxylic acid 2-oxocyclopentyl ester (C36)	1.5±0.9	2.4±1.1	41.7±16.8	15.8±7.0	7.4±3.2	8.2±2.5	11.2±1.6
3-ethyl-thiophene (C37)	nd	nd	nd	<0.1±<0.1	<0.1±<0.1	nd	nd
Tetrahydrothiophene (C38)	0.3±<0.1	0.2±0.1	1.2±0.1	0.4±0.1	0.2±0.1	0.3±<0.1	0.5±<0.1
[Unknown 2] (C39)	nd	0.1±<0.1	nd	0.1±<0.1	0.1±<0.1	<0.1±<0.1	<0.1±<0.1
[Unknown 8] (C40)	0.1±<0.1	0.3±0.1	0.3±0.1	0.3±<0.1	0.3±0.1	0.2±0.1	0.3±0.1
[Unknown 9] (C41)	nd	nd	nd	9.0±0.2	7.7±0.4	6.9±3.0	nd
Vinylfuran (C42)	<0.1±<0.1	<0.1±<0.1	<0.1±<0.1	<0.1±<0.1	<0.1±<0.1	0.1±<0.1	0.1±<0.1

nd = not detected

Appendix IV Loadings of the first three principal components for sensory trait analysis, and the Eigenvalues, variability, and cumulative variability explained by each component.

Sensory attribute	PC1	PC2	PC3
Odour			
Sulfury	**0.733**	-0.316	0.081
Green	0.150	**0.802**	0.010
Stalky	**0.784**	0.240	-0.216
Pepper	0.650	**0.702**	0.149
Earthy	-0.634	**0.543**	0.351
Burnt rubber	**0.929**	-0.235	0.041
Pungent	**0.735**	-0.285	-0.463
Sweet	-0.441	**0.551**	-0.324
Aromatic	0.209	0.243	**0.717**
Mustard	**0.858**	-0.176	0.074
Taste			
Sweet	-0.471	**0.663**	-0.278
Sour	**0.884**	-0.305	-0.190
Bitter	**0.741**	-0.188	-0.030
Savoury	**0.764**	0.176	0.220
Flavour			
Green	-0.157	**0.865**	0.027
Stalky	**0.843**	-0.132	-0.206
Peppery	**0.812**	0.356	-0.045
Mustard	**0.919**	0.122	-0.141
Sulfury	**0.941**	-0.213	0.175
Earthy	0.149	-0.158	**0.783**
Aftereffects			
Bitter	**0.783**	0.150	0.148
Sweet	-0.895	**0.235**	0.074
Acid	**0.921**	-0.338	0.037
Savoury	0.415	0.441	**0.539**
Peppery	**0.858**	0.214	0.135
Mustard	**0.938**	0.191	-0.071
Green	0.073	**0.750**	-0.519
Earthy	-0.402	0.189	**0.681**
Warming	**0.828**	0.246	0.247
Appearance			
Depth of leaf colour	0.359	**0.904**	-0.082
Leaf shape	-0.129	**0.893**	0.153
Size of leaves	-0.738	0.016	**0.528**
hairiness	-0.368	0.039	**0.575**
Purple Stem	-0.737	-0.363	0.244
Mouthfeel			
Initial heat	**0.976**	0.075	0.041
Leaf spikiness	-0.013	0.067	**0.885**
Crisp	0.342	**0.843**	0.048
Chewy	0.155	0.123	**0.928**
Tough	-0.112	0.067	**0.843**
Moistness of Leaf	-0.573	0.223	-0.729

Salivating	0.023	**0.806**	-0.218
Astringency	0.155	-0.793	0.199
Tingly	**0.927**	0.219	0.094
Warming	**0.942**	0.130	0.092
Eigenvalues	19.135	8.709	6.743
Variability (%)	43.488	19.793	15.324
Cumulative variability (%)	43.488	63.281	78.605

Bold = highest correlation

Appendix VI Loadings of the first three principal components for suplementary phytochemical variables regressed onto the sensory PCA data.

Phytochemical variable	PC1	PC2	PC3
Glucosinolates			
4-hydroxyglucobrassicin	**0.936**	0.114	-0.201
Glucotropaeolin	-0.194	-0.001	-0.195
Glucoraphanin	-0.332	-0.177	-0.510
Glucoiberverin	-0.802	-0.169	**0.203**
Glucosativin	0.576	-0.301	**0.582**
DMB	**0.279**	-0.124	0.276
Glucoalyssin	0.612	**0.737**	-0.091
Glucoerucin	-0.480	-0.267	-0.080
Glucoraphenin	-0.263	-0.077	**0.037**
Glucoibarin	0.068	-0.584	0.001
Total GSL	0.479	-0.328	**0.504**
Flavonols			
Myricetin	-0.350	-0.446	-0.485
Kaempferol-3-glucoside	0.259	0.232	**0.626**
Isorhamnetin-3-glucoside	0.039	-0.737	**0.585**
Kaempferol-3,4'-diglucoside	-0.375	-0.125	**0.348**
Isorhamnetin-3,4'-diglucoside	-0.002	-0.248	-0.209
Kaempferol-3-diglucoside-7-glucoside	-0.491	0.002	0.057
Quercetin-3,3,4'-triglucoside	-0.654	-0.539	-0.192
Kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside	-0.397	-0.574	0.385
Quercetin-3,4'-diglucoside-3'-(6-caffeoyl-glucoside)	-0.374	-0.414	**0.270**
Total Flav.	-0.389	-0.370	**0.277**
Free amino acids			
Alanine	-0.854	-0.220	-0.356
Valine	-0.445	0.034	-0.630
Leucine	-0.515	-0.213	-0.267
Threonine	-0.571	-0.003	-0.722
Serine	-0.178	0.233	-0.561
Proline	-0.228	-0.557	**0.699**
Asparagine	-0.116	0.006	-0.349
Aspartic Acid	-0.458	-0.200	-0.716
Glutamic Acid	-0.734	-0.092	-0.080
Glutamine	-0.554	-0.174	-0.625
Total AA	-0.741	-0.174	-0.579
Free sugars			
Fructose	-0.292	-0.079	**0.585**
Glucose	0.210	-0.544	**0.708**
Galactose	-0.070	-0.283	**0.835**
Sucrose	0.184	0.462	0.298

Total Sugar	0.106	-0.331	**0.763**
Organic acids			
Malic Acid	0.047	-0.506	-0.480
Citric Acid	-0.456	0.456	-0.118
Total OA	-0.152	-0.299	-0.523
Headspace VOCs			
(E)-4-oxohex-2-enal	0.042	-0.109	-0.183
4-isothiocyanato-butene	0.066	-0.162	**0.111**
1-penten-3-ol	-0.876	**0.348**	-0.193
1-penten-3-one	-0.388	**0.609**	-0.414
2-(1,1-dimethylethyl)-1H-indole	-0.203	**0.443**	-0.265
2-methyl-2-butenal	-0.591	**0.238**	-0.009
2-hexenal	-0.369	**0.628**	-0.121
(E)-2-hexenal	-0.663	**0.248**	0.172
(Z)-2-penten-1-ol	-0.673	**0.505**	-0.055
(E)-2-pentenal	-0.162	**0.795**	-0.196
(E,E)-2,4-hexadienal	-0.688	**0.309**	0.109
5-ethyl-2(5H)-furanone	-0.453	**0.773**	0.400
3-hexen-1-ol	-0.312	-0.226	-0.615
3-hexenal	-0.595	**0.387**	-0.623
(Z)-3-hexenal	-0.430	-0.122	0.123
3-octyne	-0.352	-0.279	-0.549
3-pentanone	-0.089	**0.798**	-0.131
5-methyl-4-hexen-3-one	-0.167	**0.474**	0.263
4-methylpentyl-ITC	**0.918**	-0.198	-0.023
5-nonanone oxime	**0.786**	0.369	-0.396
1-isothiocyanato-3-methyl-butane	**0.663**	0.236	-0.249
Ethylidene-cyclopropane	-0.358	0.594	**0.600**
2-ethyl-furan	0.341	0.096	**0.592**
3-methyl-furan	-0.797	-0.319	0.169
n-hexyl-ITC	**0.774**	-0.573	0.214
2-oxo-methyl ester hexanoic acid	-0.506	0.459	**0.720**
n-pentyl-ITC	**0.778**	-0.326	0.289
Iberverin	**0.871**	-0.275	0.081
Propanoic acid anhydride	-0.511	0.208	**0.647**
4-methyl-2-(2-methyl-1-propenyl)-pyridine	-0.451	**0.417**	0.035
Pyrrolidine-1-dithiocarboxylic acid 2-oxocyclopentyl ester	**0.859**	-0.263	0.238
3-ethyl-thiophene	-0.447	-0.084	**0.118**
Tetrahydrothiophene	**0.909**	-0.318	0.150
[Unknown 2]	-0.569	**0.263**	0.011
[Unknown 8]	0.346	**0.152**	-0.145
[Unknown 9]	-0.498	0.192	**0.586**
Vinylfuran	0.258	**0.843**	0.153
Polyatomic ions			

Chloride	**0.862**	-0.114	-0.041
Nitrate	0.002	**0.577**	-0.698
Phosphate	**0.468**	0.279	-0.438
Sulphate	0.118	**0.720**	-0.343
Compound ratios			
Total Sugar:GSL ratio	-0.312	-0.122	0.133
Glucose:GSL	-0.316	-0.300	0.096
Fructose:GSL	-0.426	-0.011	0.045
Galactose:GSL	-0.348	-0.201	0.483
Sucrose:GSL	-0.050	0.410	-0.104
Sugar:ITC ratio	-0.518	0.130	0.383
Acid:sugar ratio	-0.004	**0.391**	-0.799

Bold = highest correlation

Appendix VII Summary table of correlation coefficients between agglomerative hierarchical clusters and phytochemical variables within a PCA of consumer liking scores.

Variables	Mouthfeel liking C1	Mouthfeel liking C2*^	Mouthfeel liking C3	Taste liking C1*	Taste liking C2	Purchase intent C1	Purchase intent C2*^	Purchase intent C3	Bitter perception C1*	Bitter perception C2*^	Hotness perception C1*^	Hotness perception C2*	Hotness perception C3*^	Sweetness perception C2*^	Sweetness perception C3*	Pepper perception C1*	Pepper perception C3*^
Glucosinolates																	
4-hydroxyglucobrassicin	-0.206	-0.670	0.289	-0.190	-0.098	-0.249	0.702	-0.683	-0.375	-0.206	0.317	0.455	**0.805**	-0.400	**-0.826**	0.663	0.203
Glucotropaeolin	0.441	**0.789**	**-0.854**	0.686	-0.603	0.551	-0.339	0.555	0.223	-0.600	-0.166	-0.127	-0.295	0.159	0.272	-0.043	-0.053
Glucoraphanin	0.344	0.190	0.042	0.261	0.199	0.521	-0.102	-0.210	-0.742	0.135	-0.232	-0.133	0.195	0.535	0.115	-0.095	0.490
Glucoiberverin	-0.273	0.454	0.019	-0.214	0.331	-0.001	-0.228	0.082	0.256	0.648	0.246	-0.705	-0.365	0.489	0.364	-0.481	-0.332
Glucosativin	**-0.766**	-0.737	0.407	-0.678	-0.006	**-0.841**	0.365	-0.484	0.300	0.540	0.730	-0.315	0.420	-0.344	-0.532	-0.020	-0.125
DMB	0.095	-0.432	0.093	-0.055	-0.184	-0.021	-0.105	-0.330	-0.630	0.358	-0.038	-0.186	0.431	0.557	-0.318	0.042	0.328
Glucoalyssin	0.107	-0.370	0.167	-0.067	-0.028	0.015	0.544	-0.403	-0.387	-0.344	-0.125	**0.764**	0.444	-0.041	-0.656	**0.896**	-0.217
Glucoerucin	0.306	0.491	-0.394	0.358	-0.198	0.521	-0.402	0.045	-0.455	0.269	-0.071	-0.614	0.016	**0.894**	0.186	-0.291	0.236
Glucoraphenin	0.397	0.140	-0.180	0.265	-0.140	0.416	-0.385	-0.012	-0.640	0.244	-0.300	-0.292	0.084	**0.851**	0.089	-0.134	0.295
Glucoibarin	0.070	-0.468	0.330	-0.044	0.144	-0.252	-0.364	0.230	0.017	0.036	-0.292	0.055	-0.165	-0.514	0.441	-0.590	0.648
Total GSL	-0.447	-0.671	0.288	-0.452	-0.115	-0.503	0.170	-0.557	-0.241	0.628	0.504	-0.427	0.570	0.216	-0.541	-0.021	0.144
Flavonols																	
Myricetin	0.517	0.518	-0.440	0.638	-0.209	0.504	-0.521	0.591	0.040	-0.491	-0.422	-0.062	-0.387	-0.163	0.667	-0.495	0.528
Kaempferol-3-glucoside	-0.115	-0.261	-0.064	-0.202	-0.290	-0.185	0.003	-0.239	-0.197	0.373	0.166	-0.220	0.271	0.598	-0.450	0.245	-0.278
Isorhamnetin-3-glucoside	-0.463	-0.312	0.066	-0.334	-0.162	-0.487	-0.250	-0.119	0.181	0.708	0.516	**-0.859**	0.126	0.155	-0.014	-0.611	0.187
Kaempferol-3,4'-diglucoside	0.121	0.167	-0.169	0.049	-0.118	0.176	-0.410	0.023	-0.311	0.504	-0.056	-0.566	-0.042	**0.869**	0.110	-0.279	0.021
Isorhamnetin-3,4'-diglucoside	0.229	-0.304	0.275	0.044	0.170	0.181	-0.111	-0.287	-0.748	0.254	-0.240	-0.035	0.306	0.355	-0.011	-0.119	0.597
Kaempferol-3-diglucoside-7-glucoside	0.078	-0.199	0.534	-0.242	0.620	-0.085	-0.293	0.138	-0.118	0.372	-0.446	0.117	-0.384	0.049	0.501	-0.384	0.122
Quercetin-3,3,4'-triglucoside	-0.152	0.148	0.314	-0.142	0.544	-0.095	-0.296	0.252	0.302	0.320	-0.041	-0.321	-0.469	-0.298	0.705	**-0.767**	0.241
Kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside	-0.504	-0.010	0.196	-0.380	0.250	-0.529	-0.288	0.306	**0.782**	0.470	0.322	-0.565	-0.485	-0.413	0.511	**-0.793**	-0.104
Quercetin-3,4'-diglucoside-3'-(6-caffeoyl-glucoside)	-0.156	-0.355	0.517	-0.362	0.465	-0.292	-0.346	0.000	-0.092	0.674	-0.114	-0.329	-0.198	0.118	0.395	-0.629	0.280
Total Flav.	-0.026	-0.164	0.255	-0.185	0.236	-0.074	-0.398	-0.034	-0.278	0.664	-0.092	-0.485	-0.086	0.493	0.281	-0.531	0.266
Amino acids																	
Alanine	0.191	0.453	0.115	0.121	0.499	0.323	-0.370	0.303	-0.017	0.182	-0.322	-0.197	-0.503	0.150	0.725	-0.552	0.174
Valine	0.456	0.217	0.170	0.276	0.418	0.506	-0.143	0.074	-0.495	-0.145	-0.545	0.289	-0.151	0.129	0.405	-0.098	0.396
Leucine	0.177	0.699	-0.465	0.360	-0.160	0.564	-0.180	-0.031	-0.300	0.174	0.147	-0.632	0.051	**0.773**	0.099	-0.167	0.071
Threonine	0.533	0.591	-0.179	0.498	0.195	**0.755**	-0.185	0.115	-0.507	-0.212	-0.427	0.043	-0.127	0.398	0.383	-0.081	0.336
Serine	0.129	0.750	-0.495	0.395	-0.162	0.533	0.288	-0.035	0.017	-0.422	0.194	-0.015	0.075	0.147	-0.134	0.366	-0.243
Proline	-0.510	-0.378	0.369	-0.537	0.232	-0.716	-0.381	0.230	0.617	0.674	0.225	-0.539	-0.398	-0.282	0.416	**-0.803**	-0.034
Asparagine	0.268	-0.239	0.423	0.026	0.452	0.026	-0.129	0.222	-0.091	-0.245	-0.578	0.579	-0.287	-0.513	0.448	-0.170	0.366
Aspartic Acid	0.585	0.449	-0.167	0.544	0.114	0.719	-0.286	0.146	-0.573	-0.235	-0.480	0.024	-0.080	0.312	0.426	-0.208	0.573
Glutamic Acid	0.281	0.380	-0.020	0.148	0.256	0.388	-0.440	0.172	-0.316	0.348	-0.327	-0.336	-0.303	0.667	0.476	-0.394	0.124
Glutamine	0.646	0.494	-0.196	0.573	0.117	0.680	-0.451	0.398	-0.367	-0.323	-0.636	0.100	-0.333	0.178	0.644	-0.333	0.515
Total AA	0.449	0.657	-0.202	0.450	0.208	0.664	-0.354	0.264	-0.301	-0.076	-0.379	-0.174	-0.307	0.383	0.576	-0.347	0.309
Free sugars																	
Fructose	-0.047	0.191	-0.240	-0.016	-0.177	-0.283	-0.547	**0.755**	**0.850**	-0.038	-0.153	-0.170	-0.749	-0.270	0.578	-0.511	-0.343
Glucose	-0.506	-0.309	-0.036	-0.336	-0.297	-0.619	-0.187	0.057	0.530	0.444	0.518	-0.637	-0.006	-0.159	-0.045	-0.467	-0.057
Galactose	-0.267	-0.091	-0.157	-0.208	-0.282	-0.505	-0.483	0.485	0.733	0.280	0.131	-0.452	-0.458	-0.120	0.309	-0.545	-0.263
Sucrose	0.221	0.227	-0.473	0.253	-0.470	0.029	-0.150	0.519	0.486	-0.570	-0.241	0.359	-0.350	-0.188	0.047	0.263	-0.447
Total Sugar	-0.342	-0.146	-0.175	-0.214	-0.355	-0.533	-0.329	0.342	0.699	0.234	0.287	-0.459	-0.274	-0.196	0.131	-0.439	-0.222
Organic acids																	
Malic Acid	0.463	-0.053	-0.064	0.446	-0.095	0.280	-0.356	0.299	-0.259	-0.419	-0.464	0.187	-0.073	-0.360	0.434	-0.382	**0.823**
Citric Acid	0.528	**0.879**	-0.718	0.593	-0.338	0.612	-0.348	0.641	0.228	-0.557	-0.424	0.125	-0.538	0.331	0.377	0.115	-0.388
Total OA	0.683	0.329	-0.374	0.695	-0.240	0.540	-0.501	0.571	-0.156	-0.653	-0.640	0.238	-0.305	-0.210	0.589	-0.325	0.640
Headspace VOCs																	
(E)-4-oxohex-2-enal (C1)	0.527	0.006	-0.167	0.434	-0.178	0.180	-0.459	0.637	0.115	-0.631	-0.676	0.472	-0.419	-0.494	0.544	-0.255	0.417
4-isothiocyanato-1-butene (C2)	0.246	-0.057	-0.190	0.189	-0.309	0.279	-0.202	-0.246	-0.701	0.283	-0.045	-0.367	0.389	**0.818**	-0.230	0.005	0.340
1-penten-3-ol (C3)	0.176	**0.809**	-0.196	0.170	0.321	0.393	-0.218	0.446	0.326	-0.062	-0.260	-0.034	-0.669	0.244	0.567	-0.142	-0.480
1-penten-3-one (C4)	0.183	0.460	0.024	0.116	0.386	0.260	0.153	0.308	0.315	-0.476	-0.350	0.607	-0.481	-0.332	0.311	0.273	-0.440
2-(1,1-dimethylethyl)-1H-indole (C5)	-0.179	0.031	0.387	-0.295	0.538	0.116	0.510	-0.584	-0.412	0.341	0.172	0.103	0.292	0.391	-0.353	0.447	-0.296
2-methyl-2-butenal (C6)	-0.362	0.515	0.023	-0.254	0.369	0.054	0.241	-0.186	0.233	0.430	0.418	-0.401	-0.134	0.394	-0.023	0.053	-0.627
2-hexenal (C7)	-0.269	0.455	0.049	-0.223	0.371	0.097	0.469	-0.231	0.199	0.123	0.290	0.050	-0.071	0.230	-0.210	0.455	**-0.783**
(E)-2-hexenal (C8)	-0.333	0.539	-0.035	-0.250	0.313	0.004	0.072	-0.004	0.374	0.446	0.331	-0.437	-0.309	0.412	0.111	-0.060	-0.701
(Z)-2-penten-1-ol (C9)	-0.154	0.577	0.022	-0.161	0.432	0.177	0.168	-0.019	0.208	0.245	0.111	-0.107	-0.312	0.391	0.105	0.158	-0.706
(E)-2-pentenal (C10)	-0.174	0.289	0.143	-0.198	0.401	0.011	0.523	-0.074	0.348	-0.228	0.063	0.515	-0.185	-0.231	-0.151	0.582	**-0.772**
(E,E)—2,4-hexadienal (C11)	-0.148	0.561	-0.076	-0.132	0.280	0.172	-0.001	-0.010	0.132	0.412	0.163	-0.381	-0.267	0.614	0.115	-0.009	-0.596
5-ethyl-2(5H)-furanone (C12)	-0.037	0.260	0.068	-0.235	0.290	-0.035	-0.027	0.206	0.306	0.170	-0.199	0.190	-0.478	0.341	0.119	0.236	**-0.840**
3-hexen-1-ol (C14)	0.611	0.435	-0.295	0.627	-0.059	0.555	-0.416	0.541	-0.089	-0.603	-0.596	0.245	-0.374	-0.231	0.636	-0.313	0.519
3-hexenal (C15)	0.258	**0.792**	-0.226	0.317	0.256	0.631	0.136	0.057	-0.115	-0.247	-0.140	0.109	-0.196	0.306	0.182	0.232	-0.263
(Z)-3-hexenal (C16)	**-0.810**	0.004	0.530	-0.711	0.691	-0.488	0.390	-0.384	0.457	**0.760**	0.696	-0.479	-0.060	-0.121	-0.030	-0.217	-0.483
3-octyne (C17)	0.316	0.086	0.208	0.219	0.379	0.239	-0.252	0.292	-0.097	-0.232	-0.481	0.258	-0.323	-0.384	0.611	-0.400	0.515
3-pentanone (C18)	0.430	0.347	-0.227	0.294	-0.027	0.300	-0.019	0.468	0.226	-0.694	-0.575	**0.773**	-0.451	-0.179	0.186	0.452	-0.453
5-methyl-4-hexen-3-one (C19)	-0.563	-0.128	0.481	-0.653	0.541	-0.340	0.514	-0.526	0.077	0.617	0.443	-0.080	0.137	0.260	-0.396	0.346	-0.694
4-methylpentyl isothiocyanate (C20)	-0.370	**-0.809**	0.370	-0.323	-0.094	-0.440	0.588	-0.703	-0.302	0.057	0.467	0.164	**0.814**	-0.408	**-0.764**	0.380	0.324

5-nonanone oxime (C21)	0.144	-0.416	0.124	0.092	-0.119	0.038	0.557	-0.369	-0.365	-0.589	-0.084	**0.790**	0.536	-0.445	-0.582	0.746	0.172
1-isothiocyanato-3-methylbutane (C23)	-0.485	-0.595	0.554	-0.480	0.304	-0.337	**0.937**	**-0.913**	-0.315	0.117	0.540	0.346	**0.794**	-0.317	**-0.869**	0.701	-0.071
Ethylidene-cyclopropane (C24)	-0.231	0.061	0.168	-0.413	0.272	-0.233	-0.009	0.046	0.285	0.444	0.008	-0.042	-0.332	0.413	-0.006	0.139	**-0.798**
2-ethyl-furan (C26)	0.002	-0.462	0.045	-0.166	-0.270	-0.202	-0.117	-0.198	-0.339	0.341	-0.019	-0.116	0.282	0.498	-0.354	0.129	0.000
3-methyl-furan (C27)	0.310	0.410	-0.161	0.224	0.105	0.162	**-0.846**	**0.793**	0.332	0.104	-0.520	-0.303	**-0.840**	0.120	**0.979**	**-0.841**	0.128
Hexyl isothiocyanate (C29)	-0.327	-0.699	0.132	-0.205	-0.346	-0.433	0.201	-0.476	-0.214	0.217	0.489	-0.277	0.681	-0.203	-0.541	-0.031	0.483
2-oxo-methyl ester hexanoic acid (C30)	-0.136	0.088	0.135	-0.349	0.267	-0.318	-0.339	0.471	0.610	0.325	-0.235	0.006	-0.732	0.133	0.411	-0.200	-0.739
Pentyl isothiocyanate (C31)	-0.565	-0.613	0.151	-0.383	-0.273	-0.543	0.471	-0.618	-0.016	0.281	0.749	-0.280	0.717	-0.208	-0.750	0.202	0.087
Iberverin (C33)	-0.372	-0.608	0.101	-0.219	-0.334	-0.335	0.514	-0.710	-0.321	0.138	0.626	-0.147	**0.881**	-0.096	**-0.844**	0.356	0.263
Propanoic acid anhydride (C34)	-0.504	0.116	0.201	-0.546	0.340	-0.396	-0.045	0.021	0.511	0.709	0.323	-0.456	-0.368	0.328	0.103	-0.199	-0.751
4-methyl-2-(2-methyl-propenyl)-pyridine (C35)	-0.529	0.214	0.364	-0.527	0.624	-0.177	0.471	-0.370	0.242	0.513	0.443	-0.151	-0.061	0.187	-0.165	0.219	-0.734
Pyrrolidine-1-dithiocarboxylic acid 2-oxocyclopentyl ester (C36)	-0.323	-0.702	0.136	-0.246	-0.346	-0.386	0.381	-0.633	-0.340	0.204	0.508	-0.140	**0.809**	-0.029	**-0.778**	0.285	0.278
3-ethyl-thiophene (C37)	-0.180	0.149	0.125	-0.210	0.256	0.073	-0.025	-0.308	-0.288	0.676	0.237	-0.563	0.103	0.720	-0.042	-0.157	-0.108
Tetrahydrothiophene (C38)	-0.276	**-0.790**	0.212	-0.225	-0.295	-0.412	0.361	-0.571	-0.319	0.095	0.404	0.002	**0.765**	-0.243	-0.689	0.235	0.405
[Unknown 2] (C39)	-0.437	0.461	0.099	-0.334	0.434	-0.030	0.299	-0.206	0.308	0.446	0.454	-0.358	-0.148	0.293	-0.036	0.067	-0.681
[Unknown 8] (C40)	-0.469	-0.018	0.032	-0.255	-0.021	-0.070	**0.757**	**-0.787**	-0.204	0.224	**0.775**	-0.216	0.714	0.218	**-0.832**	0.587	-0.295
[Unknown 9] (C41)	-0.253	0.165	0.054	-0.336	0.178	-0.160	-0.182	0.021	0.184	0.659	0.153	-0.481	-0.266	0.633	0.085	-0.170	-0.554
Vinylfuran (C42)	0.358	0.036	-0.205	0.183	-0.225	0.126	0.048	0.290	0.096	-0.592	-0.476	**0.764**	-0.198	-0.063	-0.129	0.615	-0.460
Polyatomic ions																	
Chloride	0.068	-0.739	0.189	-0.014	-0.270	-0.144	0.267	-0.469	-0.592	-0.133	0.014	0.313	0.707	-0.140	-0.588	0.359	0.531
Nitrate	0.382	0.090	0.194	0.202	0.356	0.428	0.350	-0.137	-0.433	-0.506	-0.454	**0.817**	0.077	-0.142	-0.066	0.564	0.041
Phosphate	-0.237	-0.556	0.659	-0.354	0.508	-0.248	0.725	-0.560	-0.210	-0.148	0.096	0.698	0.421	-0.642	-0.435	0.529	0.065
Sulphate	-0.041	-0.213	0.515	-0.256	0.572	-0.064	0.563	-0.241	-0.044	-0.268	-0.205	**0.871**	0.020	-0.441	-0.199	0.618	-0.331
Compound ratios																	
Total Sugar:GSL ratio	0.194	0.550	-0.555	0.362	-0.356	0.131	-0.488	0.751	0.650	-0.371	-0.174	-0.155	-0.623	-0.190	0.551	-0.384	-0.165
Glucose:GSL	0.096	0.528	-0.521	0.319	-0.334	0.091	-0.458	0.659	0.635	-0.263	-0.034	-0.312	-0.540	-0.201	0.529	-0.479	-0.073
Fructose:GSL	0.307	0.588	-0.492	0.398	-0.230	0.210	-0.533	**0.838**	0.619	-0.444	-0.366	0.009	-0.748	-0.219	0.683	-0.379	-0.164
Galactose:GSL	0.063	0.358	-0.427	0.162	-0.317	-0.107	-0.636	**0.791**	0.753	-0.089	-0.152	-0.313	-0.715	-0.124	0.614	-0.573	-0.221
Sucrose:GSL	0.431	0.569	-0.623	0.521	-0.428	0.377	-0.191	0.600	0.362	**-0.761**	-0.360	0.367	-0.413	-0.133	0.224	0.222	-0.299
Sugar:ITC ratio	0.227	0.637	-0.567	0.292	-0.306	0.153	-0.603	**0.820**	0.649	-0.194	-0.279	-0.202	**-0.777**	0.151	0.595	-0.349	-0.436
Acid:sugar ratio	0.534	0.155	0.056	0.386	0.215	0.591	0.217	-0.108	-0.614	-0.547	-0.514	0.699	0.144	-0.009	-0.015	0.459	0.286

Numbers in bold indicate a significant correlation (Pearson *n*-1); green = significance at *P*<0.05; orange = significance at P<0.01. * = significant differences observed between rocket accessions; ^ = agglomerative hierarchical cluster with <20 individuals

Appendix VIII Summary table of correlation coefficients between mouthfeel liking, taste liking and perception agglomerative hierarchical clusters, and sensory data variables within a PCA of consumer liking scores.

Sensory attribute	Mouthfeel liking C1	Mouthfeel liking C2*^	Mouthfeel liking C3	Taste liking C1*	Taste liking C2	Purchase intent C1	Purchase intent C2*^	Purchase intent C3	Bitter perception C1*	Bitter perception C2*^	Hotness perception C1*^	Hotness perception C2*	Hotness perception C3*^	Sweetness perception C2*^	Sweetness perception C3*	Pepper perception C1*	Pepper perception C3*^
Sulfury_O	-0.041	-0.299	-0.274	0.158	-0.599	-0.198	0.079	-0.006	0.130	-0.382	0.226	0.038	0.334	-0.456	-0.319	0.087	0.290
Green_O	0.305	0.339	-0.408	0.284	-0.302	0.255	0.126	0.285	0.205	-0.652	-0.282	0.592	-0.189	-0.016	-0.151	0.634	-0.562
Stalky_O	0.000	-0.087	-0.320	0.192	-0.526	0.123	0.566	-0.455	-0.281	-0.449	0.352	0.241	0.707	-0.029	**-0.820**	**0.765**	-0.023
Pepper_O	-0.059	-0.524	0.277	-0.249	0.014	-0.247	0.508	-0.328	-0.133	-0.242	-0.064	0.742	0.327	-0.225	-0.606	**0.785**	-0.328
Earthy_O	0.373	0.560	-0.344	0.209	-0.045	0.307	-0.508	0.610	0.219	-0.043	-0.519	0.033	-0.691	0.559	0.471	-0.062	-0.545
Burnt rubber_O	-0.157	-0.730	0.162	-0.111	-0.308	-0.373	0.325	-0.360	-0.159	-0.199	0.228	0.256	0.593	-0.527	-0.543	0.255	0.401
Pungent_O	0.246	-0.118	-0.364	0.458	-0.593	0.251	0.224	-0.214	-0.388	-0.600	0.107	0.174	0.611	-0.253	-0.436	0.313	0.588
Sweet_O	-0.287	0.281	0.390	-0.331	0.732	-0.034	0.480	-0.128	0.330	0.032	0.101	0.345	-0.265	-0.273	0.065	0.290	-0.623
Aromatic_O	-0.140	0.049	-0.395	-0.058	-0.519	-0.247	-0.126	0.223	0.455	0.014	0.192	-0.191	-0.121	0.186	-0.202	0.129	-0.545
Mustard_O	0.169	-0.330	-0.388	0.295	**-0.782**	0.056	0.101	-0.257	-0.424	-0.311	0.154	0.001	0.669	0.108	-0.617	0.349	0.403
Sweet_T	-0.083	0.266	0.346	-0.239	0.667	0.160	0.380	-0.222	-0.072	0.138	-0.068	0.319	-0.141	0.191	-0.021	0.387	-0.539
Sour_T	-0.027	-0.613	0.088	0.048	-0.323	-0.192	0.309	-0.348	-0.283	-0.322	0.160	0.278	0.623	-0.519	-0.486	0.242	0.561
Bitter_T	-0.549	-0.497	0.202	-0.351	-0.127	-0.356	0.724	**-0.855**	-0.241	0.239	**0.811**	-0.187	**0.898**	-0.115	**-0.906**	0.437	0.075
Savoury_T	0.082	-0.151	-0.460	0.219	-0.753	-0.009	0.183	-0.113	-0.073	-0.405	0.180	0.132	0.444	0.035	-0.610	0.518	-0.062
Green_F	0.503	0.371	-0.289	0.313	-0.094	0.351	-0.141	0.494	0.124	-0.584	-0.647	0.685	-0.472	0.103	0.185	0.445	-0.503
Stalky_F	-0.151	-0.270	-0.185	0.094	-0.475	-0.089	0.508	-0.430	-0.131	-0.365	0.463	0.112	0.694	-0.346	-0.697	0.470	0.207
Peppery_F	-0.125	-0.746	0.438	-0.275	0.072	-0.323	0.576	-0.487	-0.268	-0.216	0.029	0.703	0.529	-0.461	-0.634	0.649	0.049
Mustard_F	0.131	-0.618	0.086	0.067	-0.323	-0.034	0.400	-0.481	-0.589	-0.327	-0.004	0.487	0.735	-0.143	-0.700	0.606	0.380
Sulfury_F	-0.191	-0.600	-0.049	-0.071	-0.520	-0.291	0.339	-0.479	-0.262	-0.064	0.416	-0.015	0.751	-0.140	-0.745	0.347	0.285
Earthy_F	**-0.849**	-0.632	0.579	**-0.872**	0.316	**-0.950**	0.199	-0.273	0.569	**0.773**	0.603	-0.352	0.008	-0.301	-0.204	-0.230	-0.417
Bitter_AE	-0.221	-0.711	0.303	-0.302	-0.111	-0.285	0.519	-0.717	-0.511	0.166	0.302	0.192	**0.785**	0.081	**-0.836**	0.569	0.099
Sweet_AE	-0.066	0.430	0.198	-0.202	0.586	0.097	-0.203	0.195	0.191	0.445	-0.149	-0.208	-0.559	0.384	0.494	-0.285	-0.441
Acid_AE	-0.086	-0.655	0.025	0.000	-0.443	-0.282	0.233	-0.330	-0.228	-0.206	0.232	0.130	0.627	-0.400	-0.531	0.195	0.494
Savoury_AE	-0.263	-0.084	-0.223	-0.187	-0.389	-0.261	0.274	-0.159	0.246	0.020	0.365	-0.017	0.206	0.174	-0.569	0.497	-0.616
Peppery_AE	-0.257	**-0.852**	0.466	-0.381	0.029	-0.492	0.527	-0.484	-0.151	-0.079	0.148	0.549	0.527	-0.502	-0.637	0.519	0.049
Mustard_AE	-0.081	-0.684	0.218	-0.130	-0.200	-0.191	0.574	-0.622	-0.483	-0.190	0.183	0.459	**0.785**	-0.199	**-0.822**	0.681	0.203
Green_AE	0.479	0.448	-0.337	0.457	-0.123	0.555	0.259	0.134	-0.137	**-0.805**	-0.390	0.742	-0.042	-0.038	-0.127	0.710	-0.270
Earthy_AE	-0.587	0.087	0.182	-0.568	0.297	-0.562	-0.027	0.204	**0.875**	0.502	0.346	-0.316	-0.522	-0.104	0.191	-0.240	**-0.830**
Warming_AE	-0.034	**-0.758**	0.257	-0.188	-0.177	-0.387	0.266	-0.203	-0.109	-0.242	-0.088	0.592	0.348	-0.432	-0.472	0.443	0.076
Initial heat_MF	-0.264	-0.740	0.252	-0.249	-0.200	-0.380	0.599	-0.604	-0.250	-0.125	0.349	0.357	0.746	-0.381	**-0.815**	0.580	0.138
Leaf spikiness_MF	-0.295	-0.466	0.318	-0.493	0.120	-0.697	-0.331	0.374	0.638	0.347	-0.113	0.015	-0.502	-0.292	0.279	-0.351	-0.370
Crisp_MF	0.065	-0.083	0.009	-0.070	-0.050	0.103	0.475	-0.357	-0.304	-0.174	-0.030	0.510	0.328	0.307	-0.623	**0.874**	-0.492
Chewy_MF	-0.221	-0.410	0.080	-0.370	-0.184	-0.492	-0.242	0.079	0.218	0.451	0.051	-0.209	-0.103	0.274	-0.118	-0.091	-0.345
Tough_MF	-0.017	-0.099	-0.147	-0.142	-0.270	-0.214	-0.461	0.235	0.121	0.432	-0.076	-0.379	-0.226	0.569	0.071	-0.223	-0.284
Moistness of Leaf_MF	0.183	0.679	-0.072	0.253	0.391	0.511	0.157	0.091	0.007	-0.287	-0.136	0.181	-0.251	-0.033	0.302	0.092	-0.128
Salivating_MF	0.485	0.019	0.081	0.188	0.168	0.297	0.068	0.189	-0.215	-0.533	-0.695	**0.912**	-0.207	-0.057	0.051	0.539	-0.203
Astringency_MF	-0.699	-0.543	0.497	-0.563	0.260	-0.657	0.157	-0.405	0.199	0.671	0.668	-0.588	0.283	-0.384	-0.102	-0.516	0.302
Tingly_MF	-0.210	**-0.774**	0.324	-0.279	-0.113	-0.410	0.550	-0.493	-0.184	-0.174	0.188	0.526	0.599	-0.451	-0.715	0.594	0.057
Warming_MF	-0.213	**-0.778**	0.300	-0.257	-0.151	-0.425	0.512	-0.459	-0.150	-0.189	0.201	0.486	0.590	-0.502	-0.681	0.527	0.115

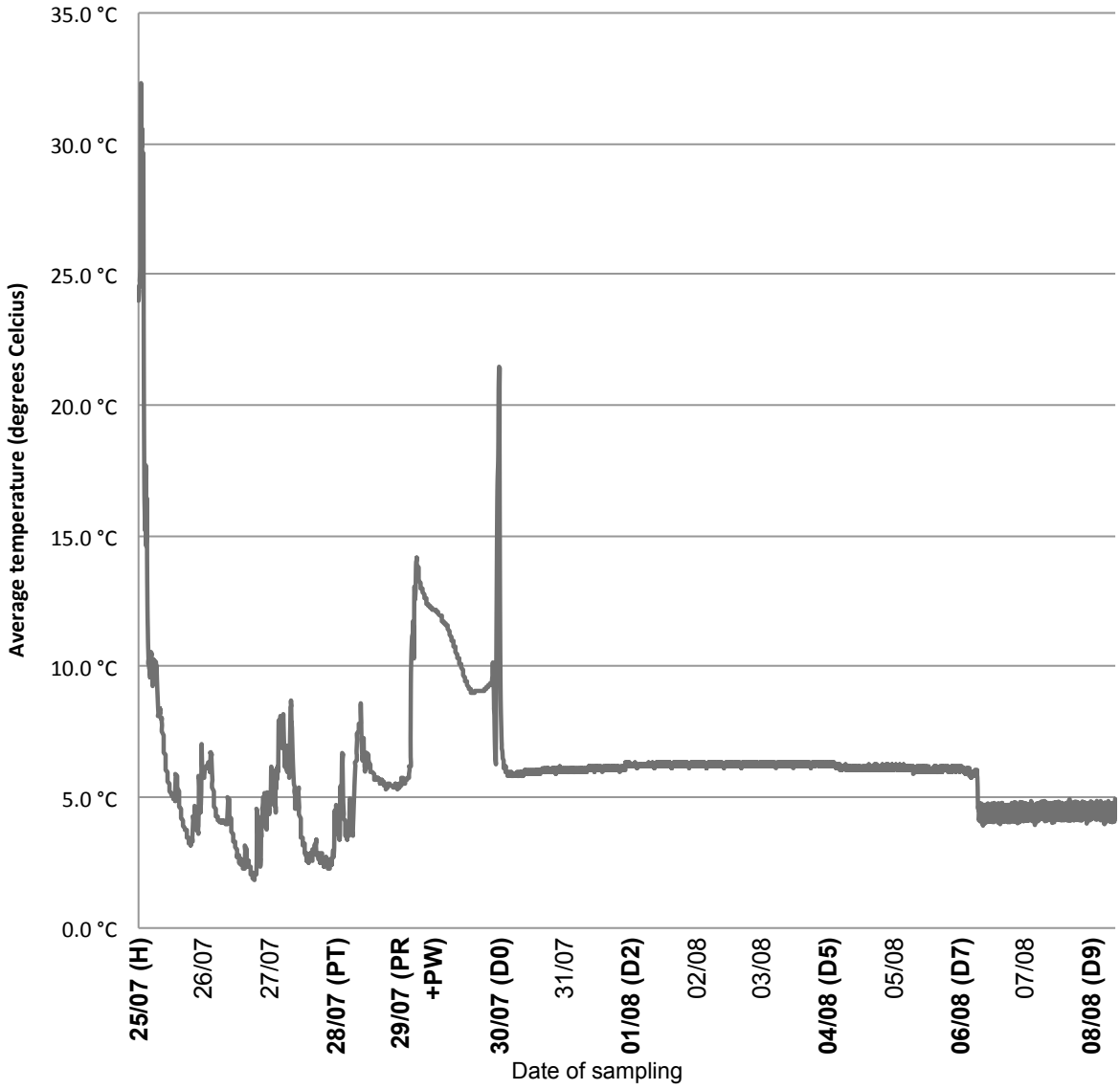
Numbers in bold indicate a significant correlation (Pearson *n*-1); green = significance at *P*<0.05; orange = significance at P<0.01. Abbreviations: A = appearance; O = odour; T = taste; F = flavour; MF = mouthfeel; AE = aftereffects. * = significant differences observed between rocket accessions; ^ = agglomerative hierarchical cluster with <20 individuals

Appendix IX Summary table of correlation coefficients between agglomerative hierarchical clusters for appearance traits and sensory data variables within a PCA of consumer appearance liking scores

Appearance attribute	Appearance liking C1	Appearance liking C2*	Appearance liking C3*^	Colour liking C1	Colour liking C3*	Leaf shape liking C1	Leaf shape liking C2	Leaf shape liking C3*	Purchase intent C1	Purchase intent C2*^	Purchase intent C3
Depth of leaf colour_A	0.084	0.507	0.656	-0.117	0.626	-0.635	0.180	0.753	-0.005	0.636	-0.360
Leaf shape_A	0.032	0.178	**0.819**	-0.355	0.426	**-0.887**	0.339	**0.798**	-0.207	0.425	-0.045
Size of leaves_A	-0.176	-0.543	0.274	-0.386	-0.360	-0.412	0.173	0.049	-0.372	-0.415	0.554
Hairiness_A	-0.684	-0.264	0.391	-0.149	-0.003	-0.160	0.356	-0.099	-0.383	-0.563	0.738
Purple Stem_A	-0.039	-0.749	-0.492	-0.138	-0.655	0.128	-0.203	-0.438	0.267	**-0.932**	**0.800**

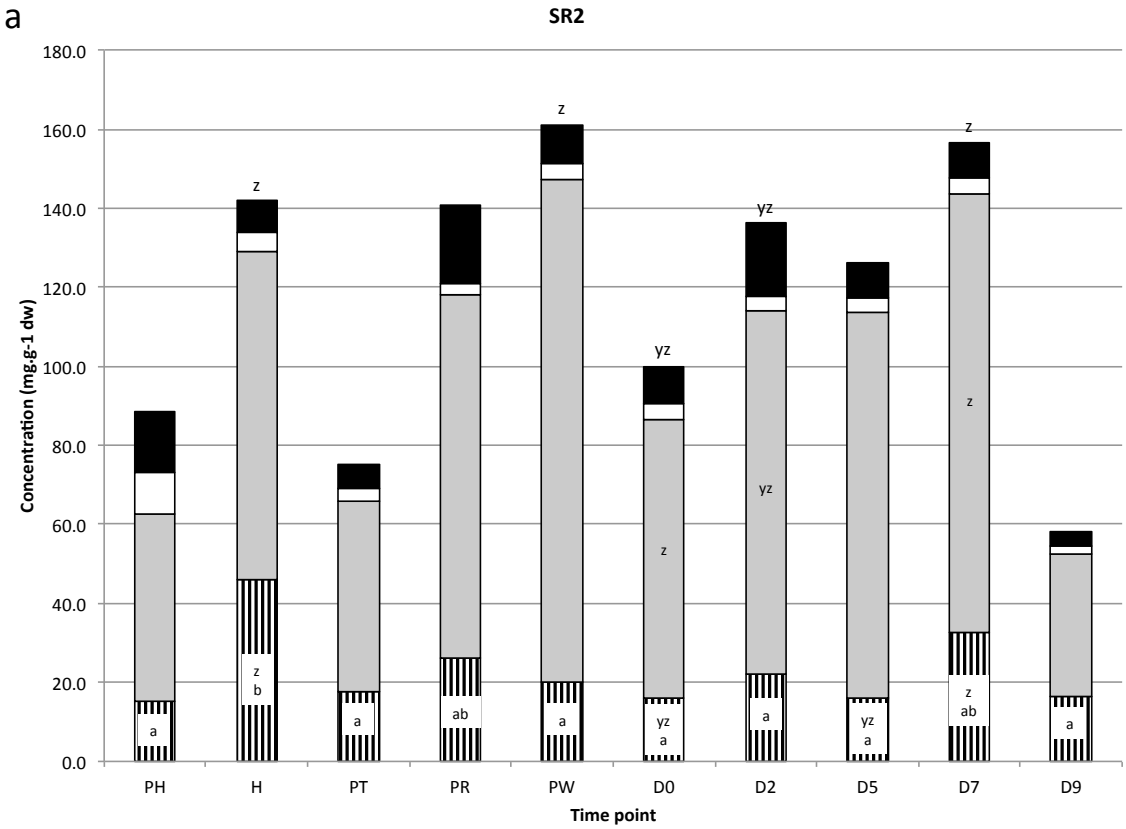
Numbers in bold indicate a significant correlation (Pearson n-1); green = significance at P<0.05, orange = significance at P<0.01. Abbreviations: A = appearance; O = odour; T = taste; F = flavour; MF = mouthfeel; AE = aftereffects. * = significant differences observed between rocket accessions; ^ = agglomerative hierarchical cluster with <20 individuals

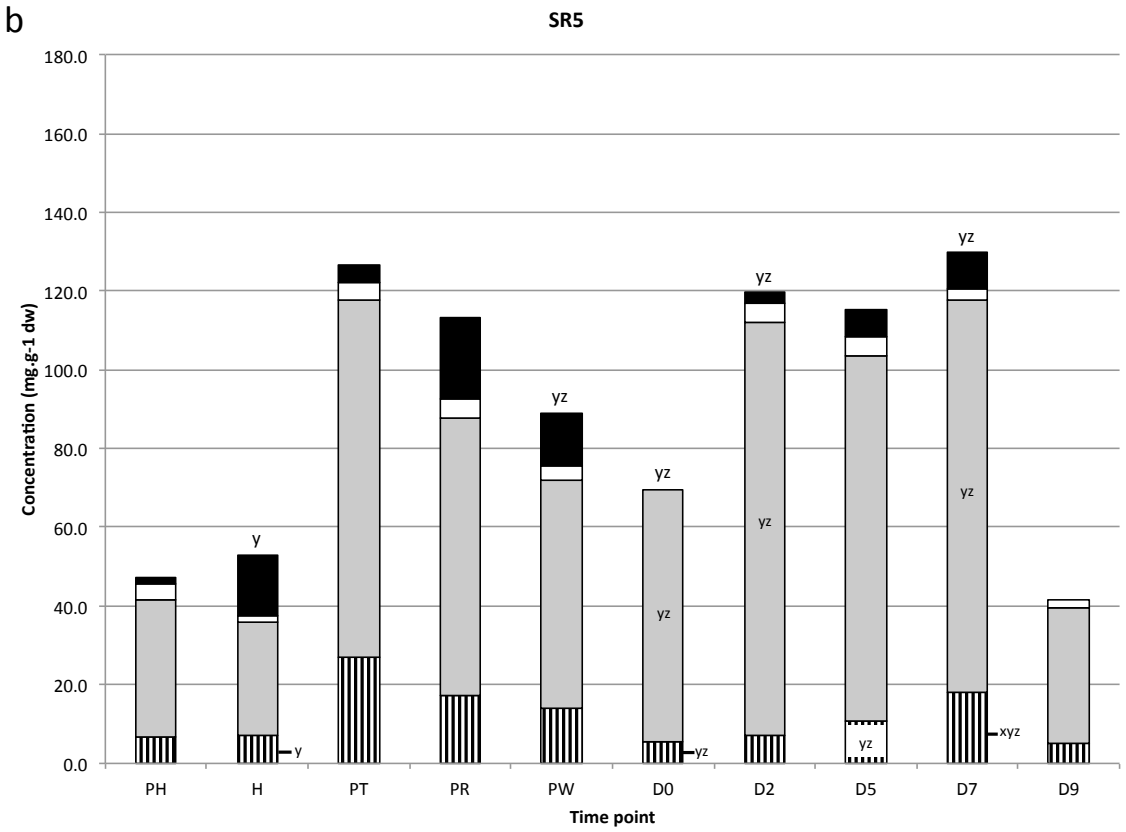
Appendix X



Appendix X. Average temperature data recorded during the rocket field, processing and shelf life experiment. Dates refer to 2014, with sample points highlighted in bold text.

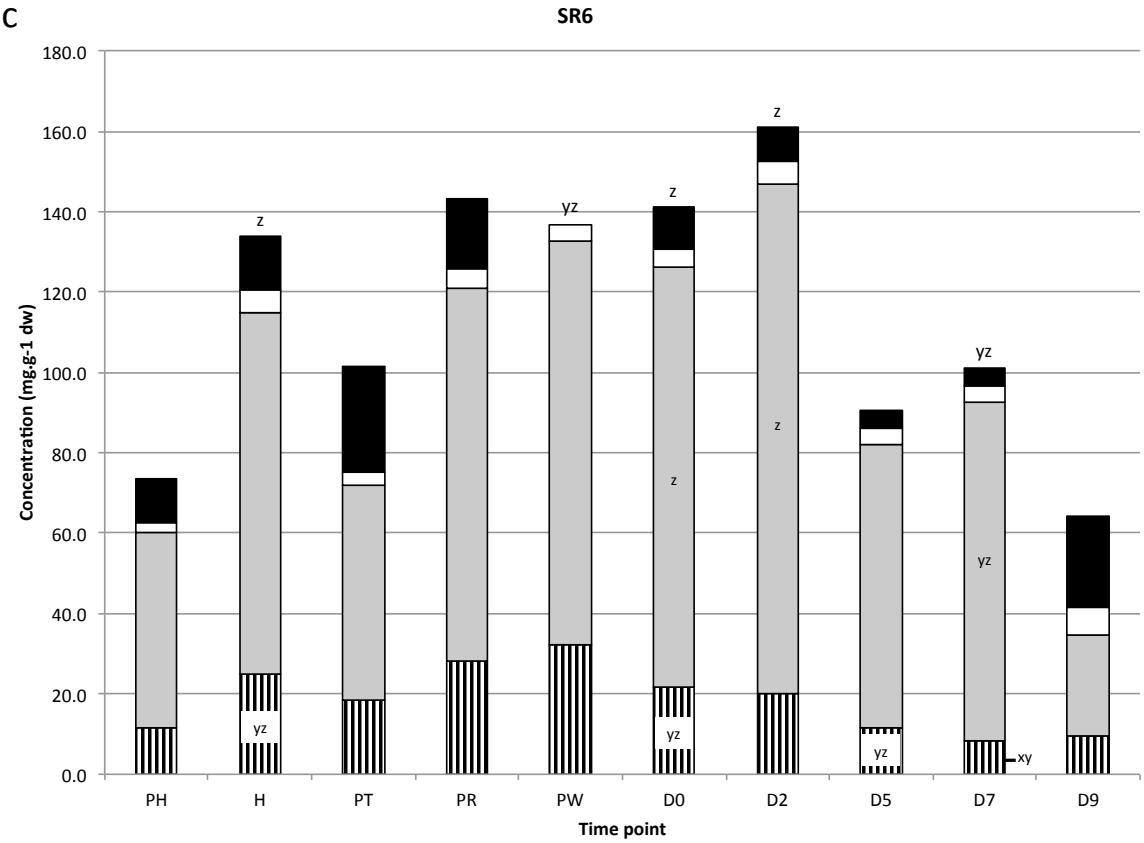
Appendix XII



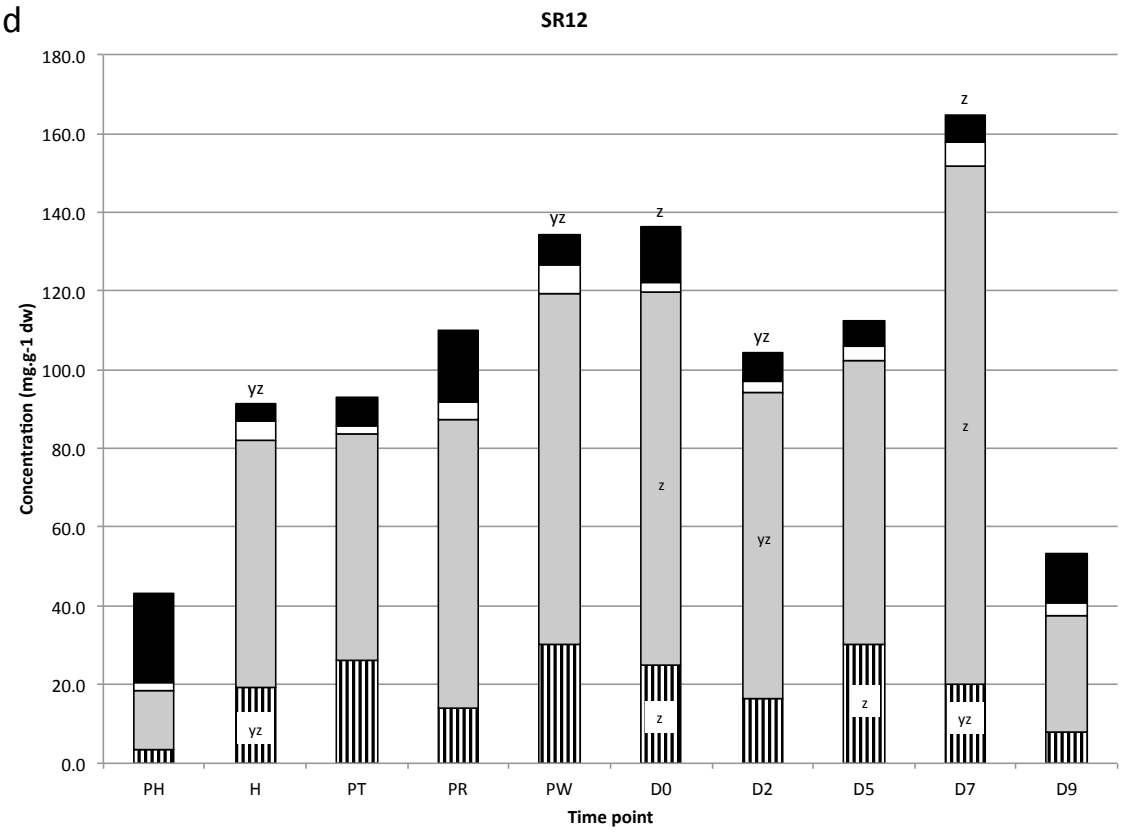


Appendix XII. Free sugar concentrations within each cultivar at each time point (mg.g^{-1} dw). Letters a – b indicate significant differences within each cultivar over time. Letters y – z indicate significant differences between each cultivar across time points. An absence of letters indicates no significant differences were observed (ANOVA + Tukey's HSD test; P = <0.05). Letters above bars refer to total concentration; letters within/beside bars refer to individual compounds. See inset for colour coding.

c

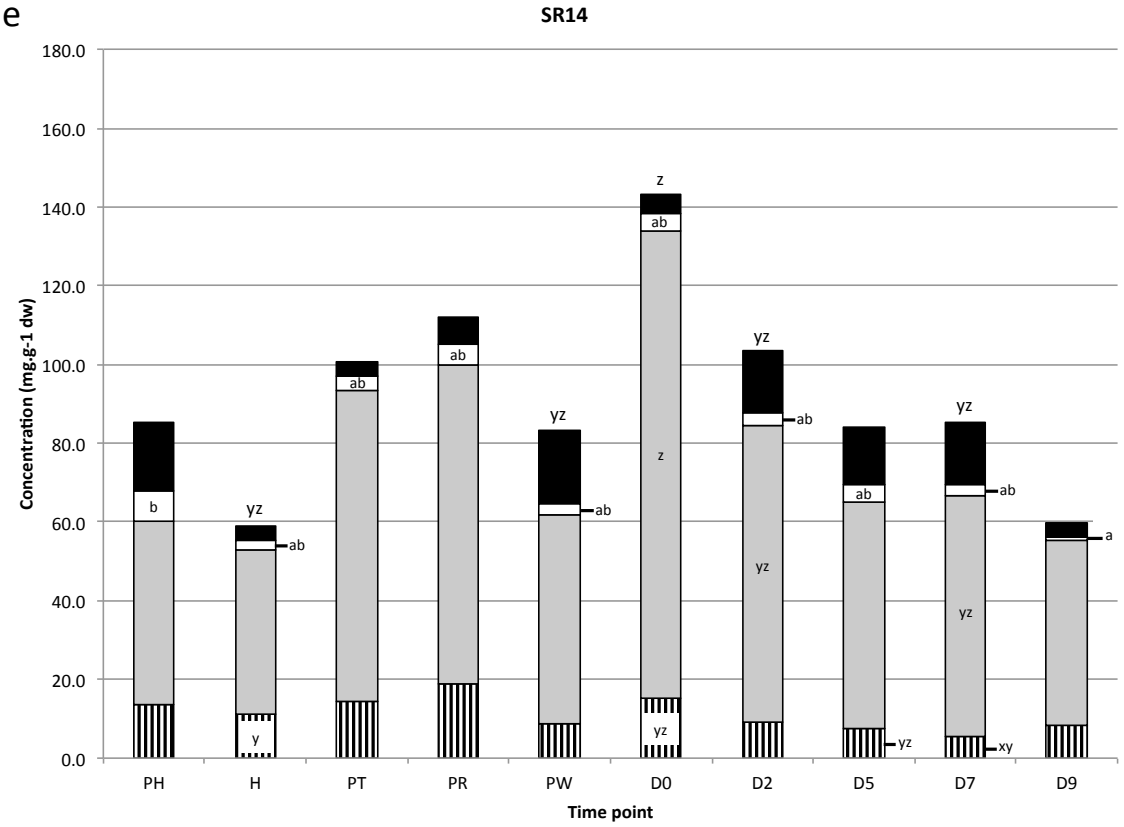


d

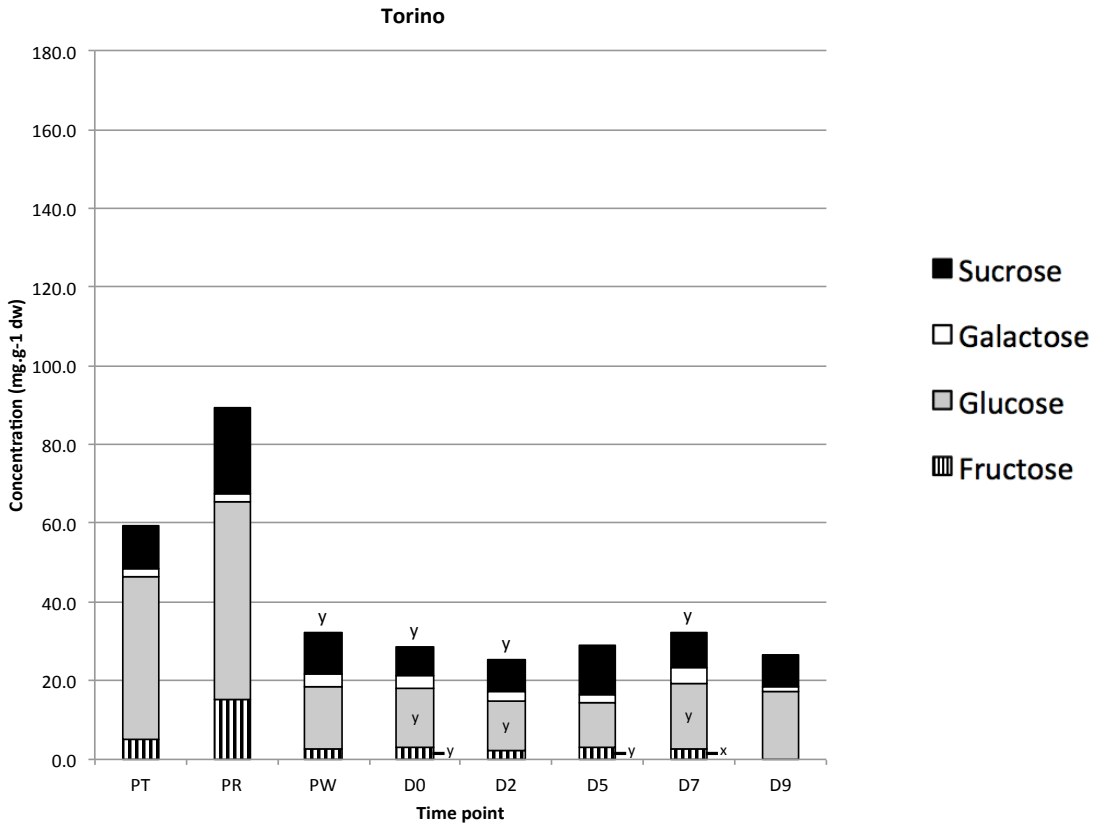


Appendix XII. continued.

e



f



Appendix XII. continued.

Appendix XIII Loadings of the first two principal components of analyses presented in Figure 7.5, and the Eigenvalues, variability, and cumulative variability explained by each component for each respective analysis.

Figure 7.5 a & b	PC1	PC2
4-hydroxyglucobrassicin	0.134	0.209
Glucoerucin	-0.046	0.227
Glucoraphanin	0.263	0.212
Epi/progoitrin	0.025	0.048
Diglucothiobeinin	-0.026	-0.500
Glucosativin	**0.737**	0.167
Glucoiberverin	-0.121	-0.134
DMB	**0.461**	0.134
Total GSL	**0.747**	0.250
Bacterial counts	**0.630**	-0.281
Fructose	-0.456	**0.582**
Glucose	-0.053	**0.927**
Galactose	-0.190	0.257
Sucrose	-0.194	-0.102
Total Sugars	-0.193	**0.894**
Alanine	-0.265	0.611
Glycine	-0.312	-0.345
α-aminobutyric acid	-0.017	-0.133
Valine	**0.979**	0.008
Leucine	**0.720**	0.509
Isoleucine	**0.816**	0.425
Threonine	**0.966**	-0.116
Serine	**0.677**	0.552
Proline	-0.206	**0.841**
Asparagine	**0.766**	-0.500
Aspartic acid	0.200	-0.112
Glutamic acid	0.318	-0.095
Phenylalanine	**0.951**	-0.219
Glutamine	**0.935**	0.001
Lysine	**0.922**	-0.146
Histidine	**0.742**	-0.021
Tyrosine	**0.857**	0.289
Tryptophan	**0.969**	-0.057
Total AAs	**0.974**	0.060
Time point-D0	0.112	-0.003
Time point-D2	0.302	0.186
Time point-D5	0.251	0.016
Time point-D7	**0.395**	0.186
Time point-D9	0.018	-0.010
Time point-H	-0.367	-0.153

Time point-PH	-0.228	-0.329
Time point-PR	-0.191	0.108
Time point-PT	-0.281	-0.104
Time point-PW	-0.057	0.066
Eigenvalue	12.413	5.080
Variability (%)	36.507	14.940
Cumulative %	36.507	51.448

Figure 7.5 c & d	PC1	PC2	PC3
4-isothiocyanato-butene	0.280	-0.272	**0.674**
4-mercaptobutyl-ITC	**0.904**	0.110	-0.205
Erucin	**0.706**	0.383	0.389
Sulforaphane/Erucin nitri	-0.597	-0.277	**0.231**
Sulforaphane	0.493	-0.296	**0.791**
Bis(4-isothiocyanatobutyl	**0.723**	0.226	0.327
Total ITC/Nitrile	**0.792**	-0.191	0.571
4-hydroxyglucobrassicin	0.133	**0.161**	0.074
Glucoerucin	-0.238	-0.127	**0.090**
Glucoraphanin	-0.201	-0.525	**0.631**
Diglucothiobeinin	-0.427	-0.391	**0.444**
Glucosativin	**0.710**	-0.442	-0.034
Glucoiberverin	-0.463	**0.164**	-0.179
DMB	**0.849**	-0.106	-0.350
Total GSL	**0.733**	-0.509	-0.008
Bacterial counts	**0.620**	-0.565	-0.461
Fructose	-0.263	**0.755**	-0.157
Glucose	0.389	**0.858**	0.205
Galactose	0.445	**0.469**	-0.367
Sucrose	-0.160	-0.170	**0.043**
Total Sugars	0.253	**0.903**	0.132
Alanine	-0.100	**0.930**	0.113
Glycine	-0.579	**0.306**	-0.081
α-aminobutyric acid	**-0.206**	-0.352	-0.222
Valine	**0.975**	-0.150	-0.090
Leucine	**0.842**	0.137	0.432
Isoleucine	**0.920**	0.189	0.252
Threonine	**0.943**	-0.219	-0.220
Serine	**0.884**	0.272	0.324
Proline	-0.143	**0.794**	0.014
Asparagine	**0.653**	-0.596	-0.430
Aspartic acid	-0.133	-0.704	**0.382**
Glutamic acid	**0.540**	0.098	0.301
Phenylalanine	**0.904**	-0.292	-0.285
Glutamine	**0.963**	0.006	-0.225
Lysine	**0.947**	0.100	-0.155

Histidine	**0.696**	0.527	0.051
Tyrosine	**0.925**	0.258	0.171
Tryptophan	**0.950**	-0.113	-0.263
Total AAs	**0.977**	-0.004	-0.171
Time point-PT	-0.900	-0.031	**0.018**
Time point-D7	**0.900**	0.031	-0.018
Eigenvalue	17.467	7.362	4.145
Variability (%)	43.667	18.405	10.362
Cumulative %	43.667	62.073	72.434

Bold = highest correlation