Defining, predicting and delivering ‘fresh’ in the context of leafy salads

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Abstract

Fresh is a general term used to encapsulate the qualities of a leafy-salad product, but the term does not have any well-defined meaning that can be empirically measured. Currently, the information provided to the consumer about the condition of a leafy salad product is provided by either a "best-before" or "use-by" date, dependent on if the product is sold as ready-to-eat or not. The current dating system evolved from a stock management system developed in the 1980's when supermarkets and their increasing volume of products necessitated the inclusion of sell-by dates on packaging to aid store workers. From sell-by dates, "best-before" and "use-by" were developed to inform the consumer about the condition of the product. However, using set chronological dates for indicating biological change is inherently inaccurate and requires a wide margin of

error to ensure that consumer safety is not compromised. One issue with having a wide margin of error is that there is a larger potential for waste from the consumer where a product is discarded that may have still been acceptable to consume. Due to climate change, a growing global population and a general under-consumption of fruits and vegetables, there has been renewed interest in the value of on-pack dates. This thesis presented data that focused on the variation of shelf life caused by crop growth environment and storage conditions. To understand better how the visual and physiological properties of leafy salads change over time and how the changes can be predicted, three areas of the agri-food system were investigated. Firstly, the preharvest factors of temperature and cultivar were varied for rocket leaves, and then the impact of the different growth factors on the key nutritional phytochemicals were analysed over a typical shelf life period. Secondly, a more in-depth analysis of the postharvest changes that occur in commercial rocket and lettuce were evaluated as well as the development of non-destructive methods for the postharvest monitoring of leafy salad crops. Variables measured for potential postharvest indicators included aerobic colony count, ammonia, chlorophyll, nitrates, visual quality (by imaging and visual

assessment), glucosinolates and glucosinolate hydrolysis products. Finally, the consumer's attitude towards leafy salads was evaluated in order to be able to relate potential biological markers with consumer rejection. The results show that, as with all biological organisms, growth, development and degredation of leafy salads crops is highly variable as there are many different factors by which they are influenced. In order to provide more accurate, up-to-date information to the consumer about the status of any given product, a dynamic non-destructive method of analysing a product is required. Of all the variables analysed in this thesis, tissue ammonia concentration is the most consistent potential indicator of postharvest status for iceberg lettuce. Furthermore, detectors capable of dynamically monitoring ammonia concentration are able to be incorporated into flexible packaging have been used for high-value products such as red meat but are yet to be tried for leafy salads. For rocket salads, there was no single variable that was consistent in its variation of the postharvest period as to be a useful indicator on its own. However, when considering the initial concentrations of soluble sugars, ammonia and nitrate, the combination may be useful in assessing the postharvest potential of the crop. Implementing this analysis

at the point of intake to the packhouse would enable on-pack dates to be flexed in accordance with crop quality and thereby to reduce food waste. It was found that consumers were quite variable in how they view leafy salads, but it was found that age and experience were important when trying to understand the reasons as to why consumers discard products. Older adults tended to rely less on on-pack dates and more on their visual perception of a product when deciding whether to consume the product. Ultimately, to reduce food waste from the consumer, large-scale data acquisition would be required along with dynamic monitoring to develop real time post-retail indicators on pack that would inform the consumer of the quality and safety of the product within.

Acknowledgements

First and foremost, I would like to thank Carol Wagstaff, as without out her, none of this would exist. Carol contributed and inspired much of this work but, more importantly, for me, provided an environment to pursue my interests and develop in all aspects of life. I would also like to thank all the staff I have encountered at Reading University who have been very helpful throughout my time as a researcher. Special thanks go to Stephen Elmore, Luke Bell and Martin Chadwick for thier time, support and guidance. I'd like to thank my sponsors BBSRC, and Waitrose, for all their help and resources. And thanks to Eloise, without whom I would have never considered going to university (as she often likes to remind me) and for her continued support.

The right place at the right time.

Declaration

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

Jake Louis Jasper

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List of Abbreviations

4MOB 4-methoxy-3-indolylmethyl

ABA Abscisic Acid

ACC Aerobic Colony Count

ANOVA Analysis of Variance

BBSRC Biotechnology & Biological Sciences Research Council

BITC Benzyl Isothiocyanate

CNN Convolutional Neural Network

D0 Day 0 of shelf life

D4 Day 4 of shelf life

D7 Day 7 of shelf life

DAD Diode Array Detector

DCM Dichloromethane

DEFRA Department for Environment Food and Rural Affairs

DGBT 4-(β-D-glucopyranosyldisulfanyl)

DGTB Diglucothiobeinin

DMB Dimeric 4-mercaptobutyl

EC European Commission

EEC European Economic Community

EM Electromagnetic Spectrum

EU European Union

EUFIC European Union Food Information Council

FAO Food and Agriculture Organization

FDA Food and Drug Administration

FSA Food Standards Agency

GER Glucoerucin

GHP Glucosinolate Hydrolysis Product

GNAS Gluconasturtiin

GRA Glucoraphanin

GRAS Generally Regarded as Safe

GRM Glucorucolamine

GRP Glucoraphanin

GSL Glucosionlate

GSV Glucosativin

HI Hyperspectral Imaging

HPLC High Performance Liquid Chromatography

HSD Honest Significant Difference

HSV Hue Saturation Value

IA Image Analysis

IL1 Iceberg Lettuce Trial 1

IL2 Iceberg Lettuce Trial 2

IL3 Iceberg Lettuce Trial 3

ILA Iceberg Lettuce video A

ILB Iceberg Lettuce video B

IPCC Intergovernmental Panel on Climate Change

ISO International Organization for Standardization

ITC Isothiocyanate

LAB CIELAB color space Lightness, a-axis, b-axis

LC Liquid Chromotography

LM Listeria Monocytogenes

LOD Limits of Detection

LOQ Limits of Quantification

MAP Modified Atmosphere Packaging

MNLR Multiple Non-Linear Regression

NDVI Normalized Difference Vegetation Index

NGB Neoglucobrassicin

NIR Near-Infrared

PC Principal Components

PCA Principal Component Analysis

PRO Progoitrin

QC Quality Control

QDA Quantitative Descriptive Analysis

R1 Rocket trial 1

R2 Rocket trial 2

R3 Rocket trial 3

RA Rocket Video A

RB Rocket Video B

RFID Radio-Frequency Identification

RGB Red Green Blue color space

RTE Ready To Eat

SF Sulforaphane

SI Statutory Instrument

SL Shelf Life

SPME Solid Phase Micro Extraction

TD-GC-TOF-MS Thermal Desorption with Gas Chromatography-Time-of-Flight Mass Spectrometry

TTI Time Temperature Indicator

UV Ultraviolet

VOC Volatile Organic Compound

Chapter 1

Introduction

In the context of leafy salads, the word “fresh” is generally used as a quality attribute by the consumer, almost certainly because of how products are marketed (Loades, 2017). With “fresh” being a synonym for “recent”, freshness is exclusively controlled by time. Although freshness does degrade, in the mind of the consumer, it is an abstract binary concept whereby something is either fresh or it isn’t. Regarding leafy salads, there is not a set definition of what qualifies of “fresh”. Becker, T. (2000) said the following when referring to meat: “Freshness is the only salient intrinsic credence quality attribute cue used by consumers”. This definition is applicable to leafy salads where “fresh” is a general catch-all term that encap-

sulates many different quality attributes such as visual appearance, aroma, and texture (Freidberg, 2010). From the perspective of the retailer and the consumer, the idea of "fresh", quality and safety all change over time, and a product at time t_0 would be considered optimal, and at t_{+1}, the product would be lower value, and at t_{+2} lower value still. Currently, for many leafy salads, the condition of the product is communicated to the consumer in the form of on-pack dates such as the "use-by" and "best-before". The best-before date can be considered a guarantee whereby before the date on the pack, the product is as advertised. After this point, the seller does not assume liability, although it is never acceptable to sell a product that is unsafe.

Fresh-cut, which is also referred to as minimally processed, in the context of leafy salads, refers to products that have some form of added value over the raw product – typically washing and cutting. With the desire by supermarkets to add add value and increase the profit margin of products, and with increased demand from consumers for convenience, ready-to-eat minimally processed leafy salads have risen in popularity over the last decade. One of the impli-

cations of this is that lettuce, depending on the processing or lack thereof, could be sold without a date label, with a best-before date or a use-by date. For spinach, which is often used in cooking, and therefore processed by the consumer, but also consumed raw with other salad leaves, it is not uncommon to see identical products in both the chilled and ambient section of supermarkets with use-by and best-before dates respectively. Ultimately when considering leafy salads, if there is an indication on the packaging that it is ready to eat, it has to have a use-by date and be stored in refrigerated cond-tions.

1.1 Online grocery shopping

Online grocery shopping has become more prominent over the last couple of decades as access to the internet has become a utility. As the food supply chain has evolved, so too has the way in which information is provided to the consumer. Grocery shopping moved from many small shops, where employees would serve one customer and personally relate information about the attributes of a product to the construction of large quasi-warehouses with complex stock arrange-

ments requiring product information to be included with the product. Online grocery shopping has provided further change in the way information about a product is given to the consumer as the quantity of information that is able to be provided is theoretically limitless. Currently (July 2021) several supermarkets give an indication of shelf life in the form of a "guarantee", typically it is stated as "Product life guaranteed for 2 days excluding delivery day". Given that consumers are often confused about the differences between "use-by" and "best-before" (Wilson et al., 2017; Alongi et al., 2019) it remains to be seen if a guarantee will improve this confusion or add to it. The meaning of "guarantee" has legal implications which can vary significantly and are generally explained within the terms and conditions for each website. The guarantee is typically related to the given on-pack-date, either "use-by" or "best-before". When purchasing products online, currently the consumer cannot see the product, and often in reference to the guarantee, the terms and conditions will state that if the product does not meet the "guaranteed" standards the consumer can reject the product upon delivery. This is not a functionality that is necessary for fresh produce when purchased in-store as presumably if a consumer does not accept the quality of

the product they will not purchase it. On-pack dating for indication of the product condition and its change over time, is relatively controversial (Neff et al., 2019) and remains to be seen how it will evolve going forward. As there is the possibility for increased information relating to a leafy salad product to accompany basic descriptions online, it may be the case that environmental information may be added in future to give consumers and indication as to the environmental impact of the product.

1.2 The environmental emissions associated with leafy salad waste

As well as the economic issues and the potential health implications of food waste, whereby consumers may be failing to consume their recommended five fruit and vegetables per day, the carbon emissions associated with waste are also worth considering. As with quality, carbon emissions are hard to state precisely as much depends on the season and production method, but approximate CO_2 equivalents (CO_2e) for 1 kg of lettuce account for 1.4 kg CO_2e / kg (Theurl et

al., 2017; Martinez-Mate et al., 2018). This can be highly variable as the largest proportion of this is from production and transport, which changes depending on the time of year. In the winter months, when lettuces are imported from Spain, the CO_2e can double due to the cost of refrigerated transport or the cost of heating in the UK (Hospido et al., 2009). From the value of 1.4 kg CO_2e / kg: production to the processor has a value of 0.45; processing 0.13; transport to retail 0.02; in-store 0.02; retail to home 0.1; domestic storage 0.13; landfill 0.5 and composting 0.045 (Poore and Nemecek, 2018).

After production, landfill is the biggest contributor to carbon emissions (Poore and Nemecek, 2018). Approximately 80 % of all food waste in the UK ends up in landfill, of which about half is derived from households (Facchini et al., 2018). To reduce the environmental impacts from food waste, reducing the amount of food that the consumer does not eat would have a relatively large impact in reducing carbon emissions from the food supply chain and increased the use of valorisation initiatives such as composting (Moult et al., 2018). Of the 10 - 20 % of leafy salads that end up as waste, processed products generally have less waste than fresh salads as some

of the leaves that the consumer would remove had been removed by the processor (Quested and Murphy, 2014). It is clear that, after production, the largest gains to be made in reducing carbon emissions associated with leafy salads are from reducing the food that is wasted and better handling the food that is wasted. There are many reasons why environmental information is often not displayed on the packaging, space and design being just one factor. However, with increased awareness by the consumer of environmental concerns, showing the environmental impact represents an opportunity for suppliers to differentiate their products and allow them to make ethical decisions. Foundation-Earth (https://www.foundation-earth.org/) are currently trialling an eco-label for food packaging in the form of a red-amber-green scale to indicate impact. The eco-label considers four parts of the food production chain of farming, processing, packaging and transport. Although it is not at the forefront of the consumers decision making when purchasing a leafy salad at the moment, as very few packs have this information, the environmental impact may soon be an important factor when purchasing.

Consumer safety must be the priority of the entire food supply chain, but this leads to a conservative approach to dating produce, particularly for anything subject to a "use-by" date. Without an ability to record quality and safety attributes in real time, the risk to the consumer will remain that they may be exposed to a pathogen if the supply chain conditions have been sub-optimal. Equally, many items or packs of fresh produce end up becoming food loss and waste because they are discarded when they would in actual fact be both safe and pleasant to consume. A date based system is inevitably based on chronology, but biology of leaves is determined by environmental and developmental cues. The same biological processes associated with senescence, degradation and death of leaves happen to each leaf that is harvested, but they happen over variable lengths of time, depending on what conditions the leaf is exposed to both pre and post harvest. This results in management of a food supply chain that is at odds with the processes happening to the food within it, and which is unable to be resilient or agile enough to deal with the seasonal and supply chain variability that fresh produce routinely experiences.

1.3 Structure of the thesis

To achieve the aims and objectives, various experiments primarily involving commercial ready to eat iceberg lettuce (*Lactuca sativa*) and wild rocket (*Diplotaxis tenufolia*) were carried out. In this thesis, the reports of the study are divided into seven chapters.

Chapter 1: This is a concise introductory chapter. The chapter outlined the background information for leafy salads and discussed how freshness and quality are defined and communicated. The aims and objectives of this thesis are also outlined in this chapter.

Chapter 2: This chapter is a published literature review that discusses shelf life with respect to leafy salads. The leafy salad industry is mapped out and put in context with land use and economics in the review. The historical development of the legislation for shelf life is investigated and discussed to understand the strengths and limitations of the current system. The three primary factors influencing product quality are reviewed, which are microbiology, visual appearance and aroma. Finally, the technological advances allowing for the continuous monitoring of products using imaging is dis-

cussed. Imaging enables up-to-date information about the condition of a given product to be conveyed to the consumer. I researched, wrote and managed the publication process for this review paper.

Chapter 3: This chapter is a published research paper. This chapter investigates how preharvest factors affect the biochemical properties of rocket leaves over shelf life. Two different cultivars for both *Eruca sativa* and *Diplotaxis tenufolia* were grown in growth chambers at three different temperatures 20, 30 and 40 °C and harvested twice. The characteristic compounds of rocket, glucosinolates and their subsequent breakdown products were assessed over a seven day shelf life period under optimal storage conditions. I conducted the experiments, collected and analysed the data, and co-wrote the paper with Luke Bell.

Chapter 4: Is a research article assessing the physiological, biological and microbiological quality of ready to eat iceberg lettuce leaves over a three week storage period at 4 °C. The purpose of this is to evaluate several established and potential markers of quality in order to predict shelf life more accurately. Three different trials were carried out across three different seasons and suppliers. I conducted

the experiments, performed the analysis, led the writing of the paper and it is now submitted to Postharvest Biology & Technology.

Chapter 5: Similar to Chapter 5, a research article assessing the physiological, biological and microbiological quality of ready to eat wild rocket leaves over a three week storage period at 4 °C. The purpose of this is to evaluate several established and potential markers of quality with in order to more accurately predict shelf life. Three different trials were carried out across three different seasons and suppliers. I conducted the experiments, performed the analysis, led the writing of the paper and it is now submitted to Postharvest Biology & Technology.

Chapter 6: This chapter is a research article that amalgamates consumer data from primary and secondary sources to assess how the consumer views leafy salads. The primary data consisted of time-lapse video captured of both rocket and lettuce stored in a domestic refrigerator and then shown to consumers as part of an online survey where they were asked to indicate when they would no longer consumer the product. From the responses to the time lapse video, the colour profile of rocket and lettuce that the majority of consumers

would not consume was then calculated. Furthermore, a novel data source of consumer comments left on supermarket websites was analysed using sentiment analysis to uncover how consumers view leafy salads. I conducted the experiments, performed the analysis, led the writing of the paper and it is now submitted to Food Quality and Preference.

Chapter 7: This is the General discussion and concluding chapter. This chapter discusses the conclusions derived from this thesis and also outlines suggestions for future work.

1.4 Aims of the thesis

(A) To understand how shelf life is defined, both in terms of legislation and by quality attributes, and therefore uncover the limitations and potential improvements that can be made to reduce food waste from the consumer. In addition, to ascertain methods that could be used to monitor a product dynamically and therefore give real-time feedback as the condition of the produce, reducing the reliance on estimation (Chapter 2).

(B) To understand how the modulation of the preharvest factors of temperature and cultivar affect the post-harvest life of rocket leaves by measuring the principal quality component for taste and aroma – glucosinolates and their subsequent breakdown products. (Chapter 3).

(C) To monitor and evaluate potential physiological, biological and microbiological markers of shelf life, from leafy salads that are stored under optimal conditions, that could be used to more accurately represent the condition of a leafy salad product (Chapters 4-5).

(D) To understand from a consumer perspective how the current shelf life dating system aligns with consumer expectation and to what extent the system is valued by the consumer. Furthermore, we aim to evaluate how the consumers define quality from a visual perspective and relate the findings to the previous shelf life testing (Chapters 4-6).

1.5 Objectives of the thesis

(A_1) Map out the legal progression of current laws for shelf life and establish how legislation is applied to different leafy salad products.

(A_2) To assess the primary factors (microbiology, visual appearance and aroma) that are used by suppliers and retailers to determine the quality and safety of a product.

(A_3) Suggest how the current system can be improved and quantify the improvement in terms of economic savings.

(B_1) To determine the effects of varying growth temperature, cut and species on the abundance of characteristic compounds of rocket leaves.

(B_2) To determine the effects of varying growth temperature, cut and species on the abundance of characteristic compounds of rocket leaves over shelf life.

(C_1) To evaluate how potential markers of shelf life change over

time for both ready-to-eat lettuce and rocket stored under optimal domestic conditions.

(C_2) To determine the feasibility of image analysis techniques to reliably measure changes in quality that are not apparent by visual perception.

(D_1) To evaluate the motivations of UK consumers for purchasing and discarding leafy salad products.

(D_2) To evaluate the rate of rejection of both iceberg lettuce and rocket leaves over shelf life by consumers and quantify the colour profile of the leaves at the point of rejection.

(D_3) Model the rate of rejection for the colour profile of iceberg lettuce and rocket leaves.

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Chapter 2

Determining the quality of leafy salads: Past, present and future

The following section was originally published as follows:

Jasper, J., Elmore, J.S., Wagstaff, C., 2021. Determining the quality of leafy salads: Past, present and future. *Postharvest Biology and Technology* **180**, 111630. https://doi.org/10.1016/j.postharvbio.2021.111630

2.1 Contribution to the project

The majority of the research and writing was done by myself with review and editing by C. Wagstaff and S Elmore. The final manuscript was the result of a team effort between C. Wagstaff, S. Elmore and myself. Contribution breakdown percentage by each author respectively [20, 10, 70].

2.2 Abstract

The relatively high proportion of avoidable waste from leafy salads and the under-consumption of fruits and vegetables generally is contributing toward renewed interest in the value of on-pack dates, particularly those that indicate quality. Current methods of predicting shelf-life in fresh vegetables and salad are relatively conservative due to the high variability of the product and few reliable markers that can be used to predict shelf-life. This is evidenced by the proportion of wastage in this category where fresh vegetables and salad account for almost a quarter of all avoidable food waste by weight. We have looked at the historical context in which date markings have

been derived, how they function currently and look at how the current system could be improved. We review the three primary factors that influence the quality of a product – microbiology, visual quality, aroma – and suggest that if more accurate predictions of shelf-life are to be obtained non-destructive methods of testing need to be developed in order to provide the consumer with accurate information about the current condition of the product.

2.3 Introduction

2.3.1 The fresh produce industry

Fresh produce is a category that encompasses farmed horticultural products, most commonly fruits and vegetables. Globally the yield and value of this sector has been increasing steadily over the last decade, and this trend is set to continue. From 2008 to 2018 global vegetable production increased from 4.4×10^8 to 6.4×10^8 tonnes and was forecast to maintain this growth (Euromonitor International, 2019). In Europe, 2.2 million hectares of land were used to produce fresh vegetables with nearly half coming from just three countries:

Italy, Spain and Poland. Within that, approximately 17.8 % of the land is used for leafy and stalked vegetable production (De Cicco, 2016). The United Kingdom (UK) dedicates 78,000 hectares to vegetable and salad production (DEFRA, 2018a).

In the UK, which historically has one of the highest consumptions of fruit and vegetables in Europe (Eurostat, 2018), 46 grams of leafy salads were purchased per person per week (DEFRA, 2018b). In the last decade, the number of prepared leafy salad items purchased has doubled in the UK from a spend of 519 to 1100 million pounds showing an increase in the desire to consume more conveniently prepared leafy vegetables as part of a balanced diet (Kantar World Panel, 2018). The desire for more leafy vegetables, along with increases in population, has resulted in a significant increase in importing leafy vegetables to the UK over the last couple of decades (Figure 2.1).

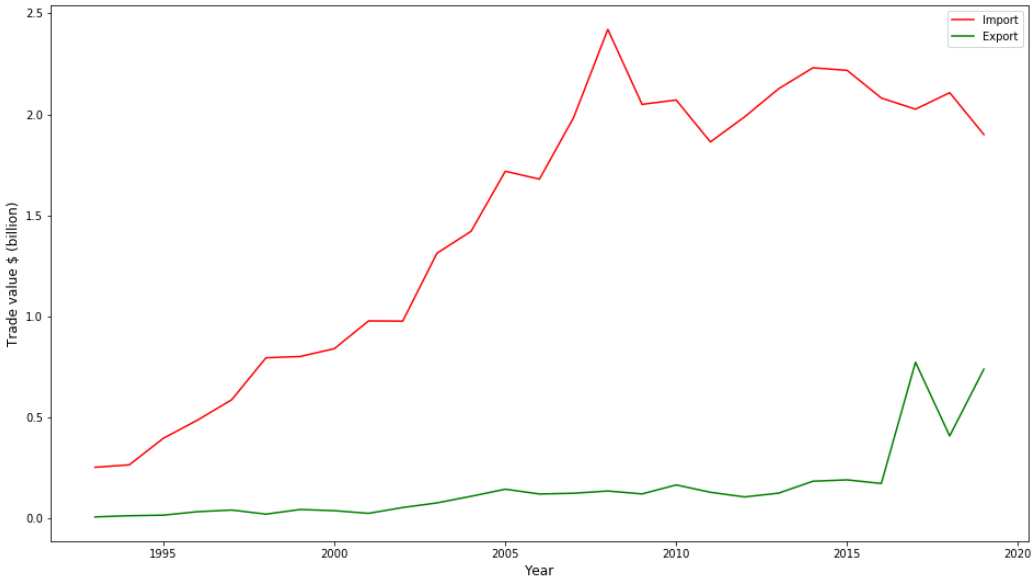

Figure 2.1: The UK trade balance of leafy vegetables from the Comtrade database comprised of lettuce, spinach and chicory (https://comtrade.un.org/).

2.3.2 Challenges facing the fresh produce industry

There is a mounting pressure on the entire global food system to increase sustainable food production, to cope with the growth in population numbers and the dietary changes that occur as populations become more affluent (Gerbens-Leenes et al., 2010). It is estimated that food production will have to increase by 70 % by the year 2050; not only will it have to increase in volume, but also in safety and nutrition (SEC(2010)379).

Alongside pressure from an increasing population, there are guide-

lines from governments and health organisations to increase consumption of fruits and vegetables. The World Health Organisation (WHO) recommends that people consume 400 g of fruits and vegetables per day to improve overall health. However, this goal is not commonly achieved (EUFIC, 2012). Increased production of fruits and vegetables is one part of the solution, another is increasing the consumption of those which have been grown, harvested and purchased. The majority of food waste in countries with highly developed food chains, occurs with consumers, and the longer the consumer keeps the food after purchase the less likely they are to consume it (Porat et al., 2018). As the produce ages the consumer views it as less valuable, due to its perceived decline in quality and safety. Often food that is acceptable to eat is wasted; depending on the classification method, waste estimates for leafy salads tend to be around 20 % (Quested and Murphy, 2014).

Food waste is a multifactorial problem and losses are not always avoidable. However, there are many aspects to improve on and these are covered by Sustainable Development Goal 12.3 (FAO, 2019). Food waste starts with losses in the field due to abiot and biotic fac-

tors as well as systematically overproducing crops in order to meet certain quality levels set by retailers. Then there are losses due to improper transportation and processing, follwed by waste arising from overstocking in supermarkets. One particularly important area is on-pack dates. In the majority of cases, where a date is present on the pack (best-before or use-by) it is indicating either safety or quality of the pack contents. With respect to safety there are robust scientific methods (Table 2.1) that are used to define the date, although a margin of error is usually applied, which itself may increase waste. With quality the consequences of errors are less serious for consumer health and, as such, the ways in which the dates are derived are often quite rudimentary. This leaves larger margins for error and can potentially mislead the consumer, causing them to discard the salad when it is still safe to consume. Approximately 70 % of the time consumers use on-pack dates to decide whether or not a salad is 'okay' to consume. Similarly, the appearance is also cited as a deciding factor 70 % of the time; in contrast, less than 10 % of respondents said that smell was used (Lyndhurst, 2008). This highlights the importance of providing accurate information to the consumer and that consumers often rely on visual cues when evaluating a product.

The situation is further complicated by the fact that consumers often open the bag and consume some of the product immediately afterwards, but then often keep the remainder for another day (Blanke, 2015). The combination of changing the gaseous atmosphere inside the bag and manual handling of the leaves often renders the 'use-by' date aspirational, to the extent that some suppliers advise that bags are guaranteed until the 'use-by' date or 24 hours after opening the bag, whichever is soonest. Educating sustainability-minded consumers, which is estimated to be 40 % of consumers (Sustainable, 2021), about what constitutes real deterioration may help to alleviate some of the waste that occurs when consumers throw away product prior to the end date on the pack. Equally, encouraging disposal of waste salad into compost rather than landfill may have benefits for sustainability in the home. Retail waste can be on a much larger scale, for example when shelves are stacked with salad products in anticipation of good weather, only to find that unseasonable rain and cold weather (a common feature of a UK summer) drives consumers away from salad purchase. In these cases developing better systems for collection and valorization of wasted leaves and packaging are needed to improve sustainability goals.

One of the biggest barriers the industry has to being able to provide accurate information to the consumer is the lack of reliable tests for markers of quality (Spadafora et al., 2016; Tsironi et al., 2017), and those that do exist measure the current status of the product rather than providing any predictive information relating to shelf-life (SL). As a consequence, the quality indication given by use-by is often tenuous; furthermore, when it is suspected that quality will be diminished and a shorter SL is required, there is little evidence to back this up and the date on pack often stays the same regardless of what quality assessments were made at harvest or at factory intake.

This review will explore the options available to suppliers and retailers that would help reduce the volume of food loss and waste that occurs in the ready-to-eat salad industry. This will include an evaluation of the technologies available for predicting shelf life of the leaves before they are packed, ways of dynamically assessing quality loss during shelf life, and advice that may be given to consumers that would help prevent food waste from bagged salads in the home.

2.4 Shelf-life: brief history and definitions

2.4.1 A history of shelf-life legislation

As long as there has been trade there have been rules and customs. Early food law was primarily concerned with food adulteration (Sophia, 2014). With the rise of centralised distribution in the food supply network starting in the 1970's more advanced methods of stock control were required (Moore, 1991). Marks and Spencer introduced sell-by dates in the UK in 1973 to keep track of stock (Marks and Spencer, 2020), but that was not intended to convey information to the consumer. It was not until 1980 that there was a statute requiring dates to be included on packaging informing consumers of quality. A date of 'minimum durability', now commonly known as 'best-before', was introduced in the UK (SI1980/1849) soon after similar legislation (79/112/EEC, 1978) was introduced to the European Economic Community (EEC). Use-before dates were introduced in the same document, and later revised to the wording 'use-by' (89/395/EEC, 1989). A year later the UK introduced use-by dates into its own legislation in an amendment to the Food Labelling

Regulations (SI 1984/1305, 1984). The introduction of a date of minimum durability was first discussed by Codex Alimentarius in 1965, where the committee agreed with a statement from the UK delegates (ALINORM 65/22, 1965):

"Much depends on the quality and freshness of ingredients and on distribution and storage conditions."

The next mention of a date of minimum durability was in 1972 when a standard list of date markings was discussed, to consolidate the markings being used (ALINORM 72/22, 1972). The first appearance of the definition in a similar form as it is today was presented by the Federal Republic of Germany:

"If the minimum durability date was applied in such a manner so that foods exceeding the date and which are still in good condition were not removed from the market, then both the producer and the consumer would benefit, the latter in terms of possibly lower priced foods."(ALINORM 74/22, 1974).

They also stated that: *"without such an application of this type of date marking provision, the risk existed of restricting distribution to the larger, higher volume retailers."*

However, in the UK the attitude was still of the view that the date of minimum durability was unnecessary other than for stock control purposes, and that minimum durability was 'open to interpretation', and argued that the SL would be variable depending on the storage conditions used by the consumer (Sawyer et al., 1980). The best-before date remains controversial (Neff et al., 2019) and the definition is still being discussed (REP18/FL, 2018).

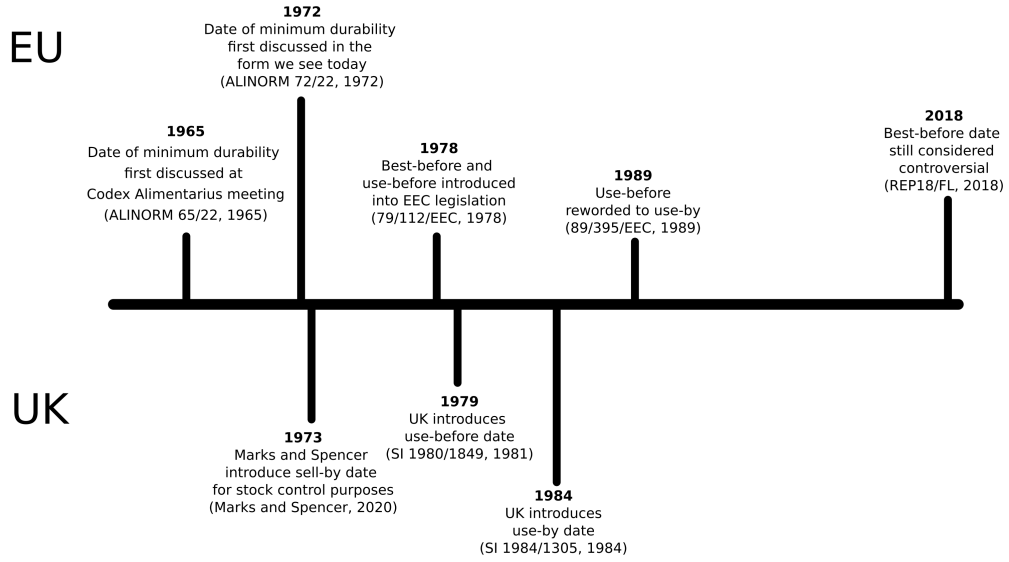

Figure 2.2: A timeline showing key milestones in the formation of on-pack date labeling that we see today in the UK and EU.

2.4.2 Legal definitions relating to shelf-life

The European Union set out two different formats for on-pack date labels; the first, 'use-by', is for products that are likely to be injurious to health at a certain point in time. The second date label is the 'date of minimum durability', or 'best-before' which is the 'date until which the food retains its specific properties when properly stored' (1169/2011). These static dates on the packs of fresh produce can be considered the SL of the produce within. However, 'shelf-life' is not specifically used in EU labelling legislation, but it does appear in (2073/2005) related to the microbiological criteria of food (Article 2,f):

> *"Shelf-life means either the period corresponding to the period preceding the 'use-by' or the minimum durability date, as defined respectively in Articles 9 and 10 of Directive 2000/13/EC."*

The words 'Shelf-life' do appear in a statuary instrument in the UK, but the definition refers to 'use-by' and 'best-before' definitions in EU legislation (SI 2014/1855).

One of the problems with on-pack dates is the conservative nature of the dates when applied in practice. Suppliers of fresh produce will choose a date that is a set number of days after the produce is packed. This date has a margin of error, perhaps two days, and this interval will stay static throughout the year, with the occasional adjustment downwards if the crop is known to have a significant reduction in quality. Therefore, the date on the pack is set to cover the worst-case scenario, which is good for ensuring public health, but sub-optimal for minimising waste (Lee et al., 2015).

Unlike other categories in the food industry, the fresh produce industry has limited options when it comes to food processing and preservation. Because of this, the life of fresh produce is particularly short postharvest. This is certainly true of leafy salads, where products are not expected to last longer than two-weeks postharvest (Araneda et al., 2008; Bell et al., 2017). The most significant reason for accurate communication of SL by a use-by date is ensuring microbiological safety. Any product designated as ready-to-eat (RTE) must carry a use-by date (EC 2073/2005) and, since bagged salad leaves are usually in this category, the suppliers do not have a choice

but to impose a use-by date rather than a best-before date. It is a criminal offence to sell food that has passed its use-by, but this is not true for food that is past its best-before date (178/200, 2002) although retailers often do not sell food past its best-before date.

Although leafy salads tend to have use-by dates, some products carry a best-before date where it is assumed further processing, e.g., cooking will occur in the home – for example with products such as sliced kale or spinach. This leads to some anomalies in the current retail system: spinach sold as a single line bagged salad is classed as RTE and is subject to a 'use-by' date. The same type of leaf is sold as a different line with other leafy green vegetables that are marketed for cooking and therefore has a 'best-before' date on pack. Since there is nothing to stop a consumer using a vegetable spinach in a salad, or blending sliced kale into a smoothie, it is clear that the distinction between use-by and best-before is a somewhat artificial construction that doesn't necessarily protect consumers who are consuming them raw from microbiological safety breaches. Best before dates are set to give the consumer an indication of the decline in the quality of the product. As the decline in quality is a result of decay and

senescence, which are biological processes, there are many different inputs and pressures that influence the decline (Wagstaff et al., 2007). Variance in salad crops are attributed to differences in growing conditions such as light intensity (Fu et al., 2012), and irrigation strategy (Luna et al., 2012; Allende and Monaghan, 2015). As well as the agronomic inputs, the genetic factors such as species and cultivar, influence the variability in the postharvest longevity of the product (Ntsoane et al., 2016; Bell et al., 2017; Jasper et al., 2020). Furthermore, as the leaves of the plant mature at different rates, there will be significant differences in the quality of leaves from the same plant. Because of this, the quality of the leaves within the individual bags may be highly variable. All of the pre and postharvest factors make accurately assessing a product's SL difficult, and interplant variability is one of the biggest challenges.

Commercially, all produce within a particular plot of land is planted and harvested at the same time. Within the plot there will inevitably be some variation in rates of growth and development, plus there will be leaves of different developmental stages within the same plant. Therefore, leaves of different maturities are harvested to-

gether, meaning that the physiology and chemical composition will be different between leaves in the same bag. Whenever a RTE salad bag is assessed the SL will be based on the average for all the leaves within the pack. This variation increases the difficulty in defining SL, and as to why the date on packs is set conservatively.

Food waste associated with leafy salad products could be mitigated by retail and domestic purchasers with better planning and logistical tools (for retailers) to improve the relationship between supply and demand. However, with current supply chains requiring several days between harvest and point of sale, and with rapidly fluctuating weather conditions driving very short-term fluctuations in what consumers choose to eat with a crop that takes several weeks to develop from sowing to harvest, managing supply to consumption patterns is challenging. Alternative supply chains are discussed in the section below that may enable shortened crop cycles and more localised supply chains that may both improve quality and reduce waste.

There is a vast amount of literature, assessing different facets of fresh-produce physiology and biochemistry over SL (e.g. Wagstaff et al., 2007; De Corato, 2020). However, there is a disconnect be-

tween the information gathered in academia, and the dates that are placed on the packs of consumer goods. This is often because the advanced methods used in academia, do not translate to industry, due to practical, economic and technological constraints.

There are many ways of quantitatively assessing quality attributes that are linked to SL. The challenge for those wishing to implement such measures is that the underpinning biology that regulates leaf degradation and quality loss is highly variable depending on factors linked to plant development, agronomy and postharvest handling. The following sections explore quality attributes linked to SL, providing information on the biological factors underpinning the measurable symptoms, methods for quantitatively analysing each factor or its symptom, and a review of available technologies that can currently predict the development of a quality marker.

2.4.3 The supply chain of RTE leafy salads

The food supply chain for ready to eat or ready to cook cut fresh vegetables can be rather long, given the delicate nature and cellular vulnerability of these plant products. For example, if a product is

grown in southern Spain for consumption in the UK it can be 24h between harvest and starting its journey, during which time it is imperative to remove the field heat from the crop as rapidly as possible (Bell et al., 2017) and to thereafter keep it at optimal storage temperature so that metabolic processes are arrested without causing chill damage. It can take three days to transport the crop by road to the UK, with temperatures often highly variable between different parts of the lorry (Jedermann et al., 2009). On arrival in the UK the crop may spend another 24-48 hours being washed, processed and packed before it is distributed to a retail outlet (Table 5.1). Typically a best-before date on pack can be five to seven days after packing, meaning that the product has to meet quality threshold criteria relating to appearance, safety and organoleptic characteristics for at least ten days after harvest. Therefore the care with which the project is handled and the integrity of the cold chain through which the product moves after harvest is absolutely critical to its ability to meet quality and safety requirements.

Sub-optimal storage conditions can lead to increased quality and safety issues because the storage temperature will influence the rate

of respiration and the rate of microbial growth (Løkke et al., 2012; Alongi et al., 2019). With a longer supply chain, there is a greater potential for temperature abuse which can be detrimental to the product and increase the rate of deterioration. The longer the product takes to get to the retail shelf after packing, the less time the consumer has to enjoy the project before it reaches the end of shelf-life. Whilst there is encouragement to reduce the length of supply chains and grow more of the crop in the country where it is going to be consumed, e.g. through indoor farming, it will be many years before these initiatives can account for a significant portion of the ready to eat/ready to cook vegetables that are currently produced in Europe for consumption elsewhere. It is therefore valuable to continue to apply effort to improving cold chain management and to innovations in packaging that lead to increased quality of the product at the point of consumption.

2.5 Microbiology and shelf-life

With respect to SL, safety is the most important factor. The 'use-by' date, which is defined in relation to microbiological safety, is in

place to protect the consumer. It is an offence to sell any product past its stated 'use-by' date (SI 2014/1855, 2014). For leafy salads, the control of micro-organisms is one of the primary concerns; this is because of the relatively limited processing options available. Traditionally, salad vegetables do not carry any form of date as they are often unpackaged. However, with rising demand for convenience, leafy salads are increasingly being sold as RTE. Any product designated as RTE must carry a use-by date (EC 2073/2005). Often, a product that will be sold as RTE is further processed for added value – cut into portions, for example. Opposed to non-RTE products, RTE products are assumed not to be further processed by the consumer, therefore, they must be safe to eat within a stated time frame. There are very severe consequences, both financially and reputationally, for a business if there is a food poisoning outbreak from their product (Koukkidis and Freestone, 2018). As a consequence of having relatively few tools to ensure safety and severe consequences of injuring the consumer, the date on the pack is often a conservative estimate.

2.5.1 Causitive agents of microbial problems

At every stage in the supply chain, there is an opportunity for micro-organisms to contaminate food. Often the environment in which the food is produced, be it open-field or hydroponic for example, or the properties of the foodstuff itself are determinants of which micro-organisms will develop (Söderqvist, 2017). There are three micro-organisms that have specific regulations pertaining to the safety of leafy salads; these are *Escherichia coli (E. coli)* 0157:H7, *Listeria monocytogenes* (LM), and *Salmonella* (Table 2.1). *Salmonella* and LM have regulations that are in place while the product is on the shelves. In contrast, the law for *E. coli* is only applied during the manufacturing stage, as although it can be injurious to health, it is not known to grow on leafy salads under RTE conditions (Abdul-Raouf et al., 1993). Although there is evidence that LM and *Salmonella* can grow at chilled temperatures, these organisms are not generally considered to contribute to the spoilage of the salad product (Horev et al., 2012). These organisms are important with respect to SL. However, we are primarily focused on quality changes and therefore, they shall not be discussed in detail in this review.

Micro-organisms are part of the many factors that contribute to the spoilage of food. However, as with many processes in biology, no single factor is entirely responsible as physical, chemical and microbiological factors all contribute. Bacterial spoilage is often associated with slime and a watery appearance (Tournas, 2005) caused by the formation of biofilms and/or by breakdown of the underlying leaf material. In addition to producing mycelium and spores, fungi have also been associated with a watery appearance, therefore the causal organism of similar symptoms is not always straightforward to identify by appearance alone. Unsurprisingly, the species or micro-organisms that are able to survive and even replicate at refrigeration temperatures are most commonly associated with food-spoilage such as those belonging to the *Erwinia* species (Tournas, 2005).

Routine testing for food spoilage organisms is not standard practice. This may be due to the economics of administering these tests, the lack of guidance on testing the less frequently occurring organisms, lack of knowledge about the relationship between organism load and the prevalence of symptoms, or lack of knowledge about

the underpinning colonisation and disease development to provide informative predictive or actionable data.

2.5.2 Evaluation of microbial load

There are legally defined microbiological sampling and testing methodologies for establishing SL (EC 2073/2005, 2006). Because of this, microbiology is unique as a measure of quality in that the same criteria that establish the date on the pack are the same for every product that is sold within a particular jurisdiction. The standard methodology for assessing the microbiology of a product is defined in Commission Regulation 2073/2005 (2006), where the specific ISO method for testing is referred to. Aerobic Colony Count (ACC) is often used; thresholds vary for what is classed as unacceptable, but are usually in the range 10^5 – 10^7 colony forming units per gram (cfu/g) (Health Protection Agency, 2009; Calonica et al., 2019). Values in excess of this figure suggest the microbial flora is considered to have developed into one predominant organism (Health Protection Agency, 2009).

When measuring the microbiology over SL in RTE products, sam-

ples are taken at the start of production and at set points throughout the SL period. Organisms that are relevant to the safety of RTE salads are highlighted in (Table 2.1). Often the product is on the shelves before the results of the tests are known as the current testing methods usually require 48 hours of incubation time. So, if the results come back positive for pathogenic micro-organisms, products have to be removed or recalled depending on how far they have made it through the supply chain. A lot of research has been undertaken to try and develop novel non-destructive methods of quantifying micro-organims and the majority of these methods are based around imaging techniques (Pan et al., 2018; Herrero-Langreo, Scannell and Gowen, 2020).

For a method to be truly useful at assessing microbial accumulation during SL it has to enable measurements to occur while the product is still in its packaging, and for organisms related to spoilage there has to be some knowledge of what level of abundance should indicate a cause for concern (Fuertes et al., 2016). To the best of the authors' knowledge, there are no implementations of such a system. There are commercialised methods for the detection of various afla-

toxins in nuts and dried fruits, but there are yet to be similar methods in the fresh salad industry (Yanniotis et al., 2011; Wu, Xie and Xu, 2018). It is usual to see higher aerobic colony counts in products that have not been stored adequately. Due to the logistics of the supply chain, the retail environment, and the minimal processing options, leafy salads often have unsatisfactory numbers. Calonica et al. (2019) found that only 8.3 % of samples of salads taken from retailers were satisfactory (< 10^5 cfu/g) and by the end of shelf-life 80 % of samples were unsatisfactory (> 10^7 cfu/g). ACC gives an indication of the overall microbial status of the product and is not suitable as an indicator of specific organisms. As the microbial status of a leafy salad is often unsatisfactory, and that there are relatively limited options for controlling and monitoring micro-organisms, there is a large amount of work in research and development for discovering methods that can reduce microbial load and still deliver the quality of product that the consumer demands (Calonica et al., 2019).

Table 2.1: Microbial limits of safety and quality for precut fruit and vegetables (ready-to-eat).

Pertaining to safety			
Micro-organisms	**Absolute limit**	**Testing method reference**	**Stage at which the legislation applies**
E.coli[1]	1000 cfu/g	ISO 16649-1 or 2	Manafacturing process
Listeria monocytogenes[1]	Absence in 25 g	EN/ISO 11290-1	Before the food has left the food business operator
Listeria monocytogenes[1]	100 cfu/g	EN/ISO 11290-2	Products on the market during its shelf-life
Salmonella[1]	Absence in 25 g	EN/ISO 6579	Products on the market during its shelf-life
Pertaining to Quality			

Micro-organisms	**Class A Satisfac-tory**	**Class B accpetable**	**Class C Unsatisfactory**
Aerobic Colony Count[2]	$<10^4$	10^4 - $<10^5$ cfu/g	$\geq 10^5$ cfu/g
Aerobic Colony Count[3,4]			$\geq 10^5$ cfu/g
E.coli[2]	<20 cfu/g	20 - <100 cfu/g	$\geq 10^5$ cfu/g

1. (EC 2073/2005, 2006)

2. (Food and Environmental Hygiene Department, 2001)

3. (Calonica et al., 2019)

4. (Health Protection Agency., 2009)

2.5.3 Preventing microbial derived quality loss

Controlling micro-organisms on leafy salads affords far fewer technologies than most other food categories, since thermal treatments, which are well developed, are not feasible on salad leaves due to the perishability of the crop. There are numerous ways in which growth of micro-organisms can be controlled, and it is a highly active area of research, reflecting the economic importance of this problem (Costa et al., 2011; Mogren et al., 2018). There are broadly two different approaches to controlling micro-organisms, physical and chemical.

2.5.3.1 Physical methods of preserving fresh produce

Physical methods of controlling micro-organisms, apart from heat treatment, include treatments such as modified atmosphere packaging (MAP) and radiation-based techniques. Ultraviolet (UV) light has been studied in its application at reducing the microbial load on leafy salads, and has been found to be effective (Ignat et al., 2015); however, there is the possibility of damaging the leaves with high levels of exposure. The UV radiation disrupts DNA replication and

transcription in its germicidal action, but its action can also cause quality defects such as increased respiration, which is unfavourable as far as storage life is concerned, and in strong enough doses can physically degrade the leaves (Martínez-Hernández et al., 2015). Irradiation techniques using gamma radiation have been approved for use on lettuce and spinach in the USA by the FDA (Goodburn and Wallace, 2013), and have been shown to be effective in many studies (Chun et al., 2010; Olanya et al., 2015). However, there is conflicting evidence from RTE salads whether these types of treatment persist through shelf-life or just exert their effect as a one-time decontamination (Goodburn and Wallace, 2013). There does not seem to be a large take-up of this technology in the fresh produce industry, partly due to economic factors, but also due to consumer concerns over irradiated produce (Bearth and Siegrist, 2019).

Modifying or regulating the atmosphere inside the packaging of a product has been used extensively within the fresh produce industry, and there are many reviews on the topic (Caleb et al., 2013; Hussein et al., 2015). Typically, in MAP varying combinations of nitrogen, oxygen and carbon dioxide are used depending on the prod-

uct. Noble gases, which have low reactivity and no odour, have also been investigated in combination with 'traditional' gases and found to be effective in maintaining the quality of rocket (Char et al., 2012). However, in the same paper it was also reported that argon-enriched atmospheres increase respiration around 15%, which may reduce SL. The modified atmosphere is achieved either by gas flushing to displace the air inside the bag with a desirable composition of nitrogen (or other noble gas), oxygen and carbon dioxide (active MAP) or by using microperforations in the packaging to balance the respiration rate of the product with gas exchange between the internal headspace and the external environment (passive MAP). Passive MAP can take several days for equilibrium to be reached and, in both cases, the evolution of the internal atmosphere is dependent on factors controlling the respiration of the fresh product, e.g. temperature. If the permeability or environmental conditions are not optimised then the quality of the product will be severely compromised (Ares et al, 2008). There are many studies that show the attenuation of micro-organisms using modified atmospheres (Ioannidis et al., 2018; Kapetanakou et al., 2019). However, once the pack is opened the benefits of the MAP are lost. There are several pack-

aging parameters that affect the atmosphere within the bag, including film thickness, number of perforations, orientation of polymer chains and polymer type. For packaging of leafy salads polypropylene is the most common polymer, but the packaging parameters will vary depending on the product. The atmospheric conditions in MAP, which are usually low O_2, CO_2 and high nitrogen compared to atmospheric composition (Campbell-Platt, 2017), can give rise to negative quality aspects such as discolouration and off-odours (Nielsen et al., 2008; Tudela et al., 2013). However, in addition there are concerns over the sustainability of some of the materials used to package RTE salads, with recycling options severely limited. There is pressure to develop biodegradable, compostable or more easily recyclable packaging options that still retain the ability to control quality of the plant material within (Roohi et al., 2018).

2.5.3.2 Chemical methods of preserving fresh produce

Chemical methods of controlling micro-organisms are far more numerous, which may reflect the commercial viability of these methods for controlling micro-organisms (Goodburn and Wallace, 2013).

As vegetables tend to be washed to remove soil and debris, it makes practical and economic sense to use this stage to sanitise the produce for micro-organisms. Simply washing the produce in chlorinated water remains one of the most common practices when it comes to controlling micro-organisms on fresh produce. However, questions have been raised as to whether or not the results from chlorine washing are significantly different to washing with water alone (Luo et al., 2011) and there has been increasing pressure from regulatory authorities to reduce or remove chlorination from RTE products (Uhlig et al., 2017). There are many alternatives to chlorine, many of which are based on weak organic acids such as citric, malic and tartaric acid. The use of weak organic acids is based around overwhelming the ability of bacteria to remove protons from their cell interior and therefore not being able to effectively reproduce as they have to expend energy pumping out protons from their interior (Akbas and Ölmez, 2007). There are many examples of different chemical combinations in the literature, with different modes of action such as thymol or carvacrol, which are both thought to increase the membrane permeability of bacteria through interactions of the phenol group and its destabilised electrons with the cell mem-

brane (Zhou et al., 2007). Peroxyacetic acid produces reactive oxygen species which can damage DNA and lipids of bacteria; furthermore, it can denature proteins and enzymes by oxidising disulphide bonds which also increases membrane permeability (Vandekinderen et al., 2009). Cuggino et al., (2020) found that benzyl isothiocyanate (BITC) was synergistic when combined with chlorine to increase the effectiveness of decontamination over chlorine alone. Although they did state that the results may have been due to the change in the pH rather than the antimicrobial properties of the BITC.

Other plant-derived solutions such as aqueous *Origanum vulgare*, which is derived from oregano, has been shown to be effective in reducing *E. coli* O157:H7 in packed spinach and lettuce when combined with traditional sanitisers such as sodium hypochlorite (Poimenidou et al., 2016). Novel plant-derived compounds such as benzyl isothiocyanate BITC, oregano extract and organic acids are desirable not only for their effectiveness at decontaminating salad leaves, but also because they are not required to be stated on the label as they are generally regarded as safe (GRAS) and or classified as processing aids. GRAS products are an United States Food and Drug Admin-

stration designation and pertains to the safety of the substance that is used in a food product (Frestedt, 2018). This is an advantage as consumers are wary of decontaminants (Aoki et al., 2010). Ultimately it comes down to price and, if not already approved, getting the product approved by governing bodies; many of the alternatives to chlorine are not economically competitive (Calonica et al., 2019).

2.5.3.3 Nanotechnology and its role in food packaging

The incorporation of nanomaterials into food packaging is an area of research that is in the ascendency. Antimicrobial elements such as silver are being incorporated into packaging with success (Costa et al., 2011). However, as the technologies surrounding the use of nanomaterials is developing, the regulatory authorities have yet to form a consensus as to the efficacy and safety of many of the technologies and, therefore, few examples exist within the food industry (Eleftheriadou et al., 2017). This is particularly true of the use of heavy metals, such as silver, which can have detrimental effects on human health and the environment (Tóth et al., 2016). One of the concerns with incorporating sensors or nanomaterials into packag-

ing is the effect on the recyclability of the packaging; reducing food waste at the cost of increasing packaging waste is not a desirable trade-off.

2.5.4 The influence of seasonal and agronomic factors on microbial quality

One of the many reasons why it is hard to predict the SL of a product is due to the fluctuating environment in which the product is produced. The majority of leafy salads are grown in open-field; therefore, weather and seasonality play a role in determining the microbiological safety and the quality of the product. Caponigro et al., (2010) looked at six different RTE salad products from Italian supermarkets over two years and found that microbial loads peaked in the autumn months. It has been suggested that during periods of higher rainfall bacteria are better able to spread and be carried to different locations, which may be more of a factor than temperatures in accounting for the differences between seasons. However, the variability in bacterial loads is not consistently higher in the autumn/winter months. Rastogi et al., (2012) found that there was a

one-log decrease in culturable bacteria of lettuce grown in the winter season compared to the summer season. It is more likely that high rainfall leads to more soil splash onto the leaves and contamination through that more immediate route, rather than transfer in moisture-dense air between fields. Often it is atypical weather events such as high rainfall and flooding that are positively correlated with increased microbial contamination (Medina-Martínez et al., 2015), supporting the hypothesis that bacteria are transferred from the soil to the leaves. This is a particular concern when considering climate change and its potential for increased variability in weather conditions and the frequency of which extreme weather events occur (Liu et al., 2013).

Leafy salad crops that are field-grown have many more avenues for contamination than those that are grown in soil-less systems. Field-grown crops may also be exposed to contamination from livestock in surrounding fields, wild animals, standing water or manure fertiliser. In contrast, produce that is grown under-protected and/or soil-less systems, such as hydroponics, is able to be more tightly controlled. Manzocco et al., (2011) found that hydroponically grown lamb's let-

tuce did indeed result in a lower microbial count (Total Coliform and *Pseudomonas*) when compared with a soil-grown crop. However, there was no difference in Enterobacteriaceae, which hydroponically grown crops are also susceptible to as these organisms are typically found in contaminated water supply and can enter the plants via the roots (Lenzi et al., 2021).

As well as the variation from seasonal influences, and that of the growing environment, the plant maturity also has an impact on the SL of the product. A consistent finding is that immature leaves tend to have higher respiration rates than mature leaves. Higher respiration rates potentially reduce SL as the leaves may degrade quicker than those with lower respiration rates (Martínez-Sánchez et al., 2012; Hunter et al., 2017). It has also been observed that immature leaves have higher microbial counts than those that are at harvest maturity, which is a particular concern for baby leaf salads (Rastogi et al., 2012; Williams et al., 2013; Dees et al., 2015). It is has been suggested that as the plant matures, selective pressure on micro-organisms occurs which accounts for the decrease in micro-organisms present on mature leaves, but this has not been

proven, and often the seasonality effects are a confounding factor (Martínez-Sánchez et al., 2012). The many different factors that can influence the microbiology of salad leaves make forecasting how the safety and quality of a product will change throughout the year challenging. As it is difficult to predict how micro-organisms develop on salad crops from the growing stage, processing the leaves and storage in the consumers home, SL dates are often conservative to minimise the chance of 'injuring the consumer' at the expense of increasing waste.

2.5.5 Modeling and predicting microbial growth

The importance of keeping the consumer safe and meeting the quality standards that they expect are top priorities, because of this, predicting the growth of micro-organisms is a well-studied area (Tsironi et al., 2017). Typically, there are three classes of predictive modelling: primary modelling, where a few kinetic parameters are measured such as lag time or growth, and a growth rate with respect to time is calculated; secondary modelling, which incorporates environmental variables such as temperature (McKellar et al., 2012

;Tsironi et al., 2017) and their effect on the parameters from the primary model; tertiary modelling, which are consumer friendly packages designed for food business operators to be able to produce models of microbial growth, evaluate the safety of their products, and inform SL estimation (Koseki and Isobe, 2005). ComBase (https://www.combase.cc) provides links to many of these software packages. These models allow food businesses to estimate levels of micro-organisms at the time of consumption and factor in many different variables such as temperature, pH and preservatives (Psomas et al., 2011).

Often there are many different variables in the food supply chain that can affect the growth of micro-organisms, which are not captured within these models. The consequence of this is that companies will apply a conservative margin of error on the use-by date, of at least two days, which may reflect the lack of confidence in the underlying model. The length of time it takes for the product to reach the shelves after packaging is not always predictable and therefore providing for this also contributes to conservative labelling. There are always going to be errors in predictive modelling as it is not feasible to take all possible scenarios into account. The margin of error

is applied to avoid human disease, but as a consequence there may be more wastage (Wilson et al., 2017). With an increasing focus on waste and sustainability, and as more data are collected and models are further developed, margins of error may be reduced and potential wastage avoided. With growing research into dynamic methods of assessing micro-organisms and particularly the use of imaging methods, models will be produced that incorporate these measurements to provide more accurate predictions, or real-time measurements. Siripatrawan et al., (2011) found that they could detect *E.coli* using hyperspectral imaging (HI) on inoculated spinach leaves, and were able to predict the number of organisms from the imaging data using a neural network based model. Kang et al. (2011) were able to detect faecal contamination, which is a common route for pathogens to enter the food chain, using HI with romaine lettuce samples. However, there has yet to be any application of these methods and models in the retail environment.

When considering spoilage and quality, the underlying models which are used to implement a best-before date are far less developed, than those that predict use-by dates, if they are used at all. There is a lack

of research into markers that can be used to reliably predict quality, creating a barrier in negotiating an extension or reduction of the date on the pack as the supplier does not have sufficient evidence to back-up their perceived notion of quality. The consequence of this is that the date on the pack often does not change when the quality, and therefore shelf-life, does.

2.6 Human perception of quality

Often, the first and most significant parameter a consumer uses to decide if they will purchase or throw away a salad product is their visual perception (Paakki et al., 2019). The appearance is the first stimulus the consumer is faced with and is often used as a metric for acceptance or rejection of the product (Mielby et al., 2012). Therefore, having a good understanding and testing methodology for visual aspects of a product is important.

2.6.1 Visual disorders associated with leafy salads

With leafy salads, there is a plethora of different visual disorders that can occur (Figure 2.3). These include russet spotting, which is induced in iceberg lettuce by exposure to ethylene in the ppm range (López-Gálvez et al., 2015), or the yellowing of leaves due to chlorophyll degradation (Koukounaras et al., 2009). There are some disorders that are associated with discolouration in leafy salads that are typically induced by mechanical damage where internal cell structures are disrupted e.g. cutting. Pinking of iceberg lettuce is one such example where cell structures are disrupted allowing the interaction of compounds and enzymes that result in colour change that would not ordinarily occur if cells remained intact. Pinking is induced by the conversion of diphenols to quinones, and then melanin precipitates which produces pink and brown hues depending on subsequent reactions that are not yet fully understood (Saltveit, 2018).

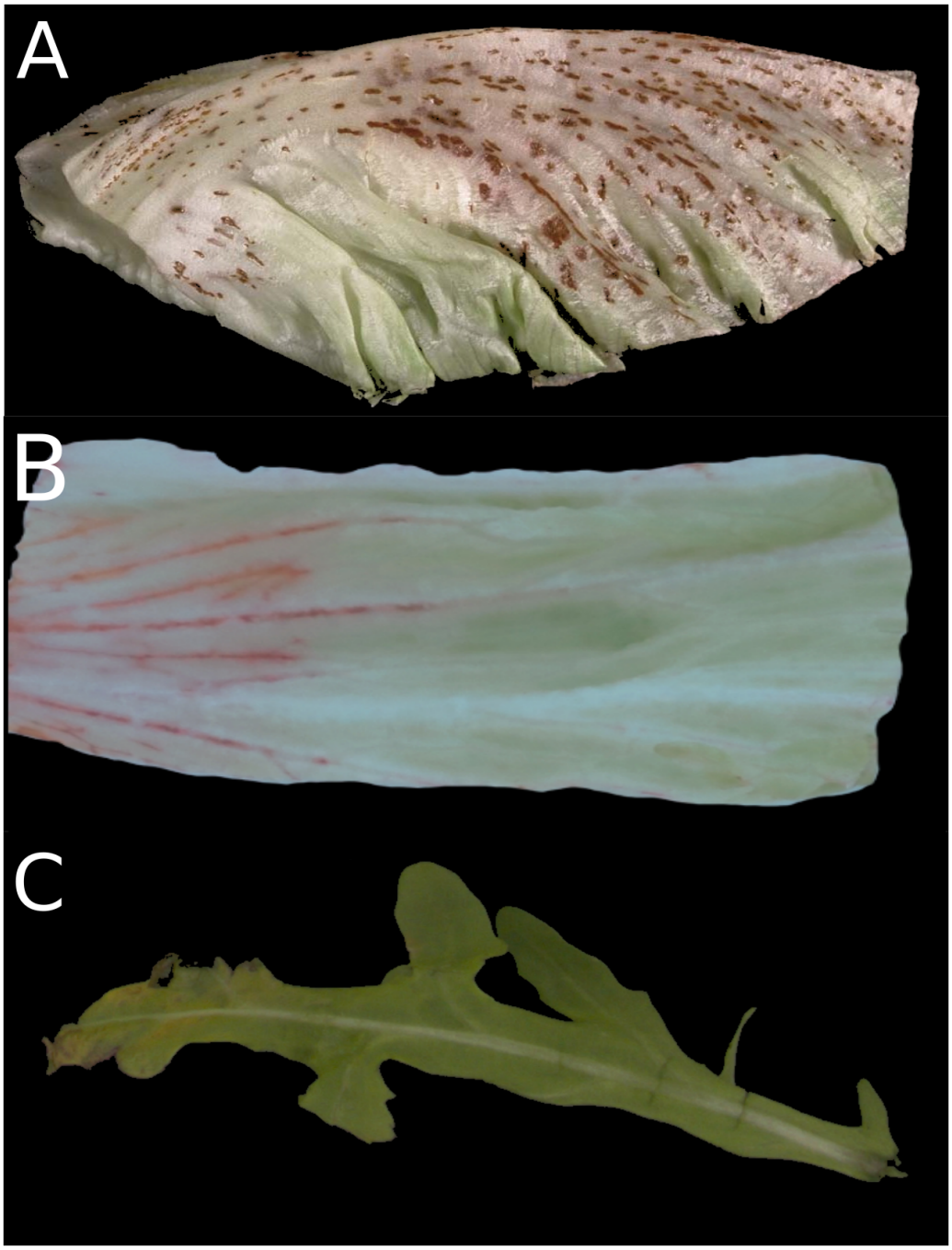

Figure 2.3: Example of visual disorders of salad leaves. Russet spotting on Iceberg lettuce (A), Pinking of cut tissue of Iceberg lettuce (B), and senescence of Rocket leaves (C) . Image (A) was taken from (Cantwell and Suslow, 2002)

As visual quality defects are instrumental in guiding the consumer's decision process, a lot of effort has been put into measuring and quantifying these disorders, both in academia and industry (Quested and Murphy, 2014; Manzocco et al., 2017). In contrast to microbiological assessment, which will often be outsourced, visual appearance will be determined within the business. Typically, visual appearance is assessed by a sensory panel or by a more objective approach involving the analysis of the emission spectra of the product. Depending on the equipment being used, this will typically be within the visible spectrum ($\sim$380 to 740 nm). Specification standards for each product will be defined and agreed upon by the supplier and retailer, and any product failing to meet the required standard will not be sold. Visual assessment by human assessors is perhaps the most common method utilised when considering the quality of a salad product over a shelf-life period due to its relative simplicity and low cost. The advantage of this approach, other than low cost, is that it is relatively quick and, when done with larger numbers of assessors, may align with the consumer perception of the product (Lee and Chandra, 2018; Nguyen et al., 2019; Sikora et al., 2020).

2.7 Instrumental assessment of quality

Objective assessment of visual quality has long been the goal of laboratory scientists studying postharvest changes. Only recently are these technologies being adapted for supply chain applications and the primary point at which they are implemented are in the packhouse. Often the use of image analysis, hyperspectral imaging or colorimetry (see sections 5.1-5.3 below) are used for automating sorting materials of very different visual qualities e.g. removing senescent spinach cotyledons from consignments of dark green baby leaf spinach leaves. Only recently has the possibility emerged of using such technologies to detect color/reflectance changes at an early stage that enable the prospect of some better prediction of shelf life.

Several technologies rely on the real-time detection of volatile aroma compounds that are produced as a consequence of senescence, tissue damage, degradation or microbiological proliferation on the leaves (Luca et al., 2017). Generally, the aroma is a tertiary consideration when consumers are assessing RTE salad leaves, since they cannot smell the product without damaging the packaging, although this may be a consideration for unpackaged leafy salads. Furthermore,

unless the salad leaves are particularly pungent or have a distinctive odour, such as rocket leaves, there is not much of an aroma to detect. From a food safety perspective, the aroma is not necessarily diagnostic of pathogens but off-odours are often associated with the presence of microorganisms (Goodburn and Wallace, 2013).

Identification of suitable volatile marker compounds has come from extensive work based on assessment by the human nose in the form of trained sensory panels or preference testing using untrained consumer panels. The human nose is, compared to current levels of technology, more sensitive than the equipment that is available for automated volatile sensing. As with visual appearance, there are several quantitative and qualitative methods for assessing aroma. Most often, a sensory panel is used to assess the aroma of a product; depending on the question being asked, a trained panel or untrained consumer panel will be used. Assessing a product using a panel can give both quantitative and subjective feedback in a real-world setting. Using a trained sensory panel to determine the descriptive characteristics of a product is common. Descriptive analysis can also be used for quality control, and often it is used to determine

consumer preference (Goularte et al., 2004; Murphy et al., 2011; Wieczyńska and Cavoski, 2018). There are many different methods for profiling a product with a sensory panel, such as quantitative descriptive analysis (QDA), and free-choice profiling (Murray et al., 2001). Typically, there are 8-16 trained panel members who produce an agreed vocabulary for attributes of the product. Descriptive characteristics, with rocket as an example, may pick up on aromas such as: peppery, green, mustard, sweet (Bell et al., 2016). The attributes of the product are then scored using an interval scale. However, without also identifying and quantifying the volatile organic compounds (VOCs), it is not possible to ascertain which compounds are responsible for which aromas, but research in this space has given rise to the identification of compounds which may be used to diagnose deteriorating quality (Dryahina et al., 2020), the potential of which is discussed in 5.4 and 5.5.

Different technologies have started to impact on the fresh produce market that give a real-time indication of freshness, or historical reporting of cold chain breaches. These typically rely on detection of respiratory gases and/or use chemistry to report changes in physi-

cal parameters such as temperature or humidity (Chen et al., 2013). These are covered in sections 5.6 and 5.7, together with a discussion of their potential and limitations.

2.7.1 Image analysis for assessing leafy salads

Image analysis (IA) is a more objective approach to assessing visual appearance and is becoming the predominant phenotyping method (Herrero-Langreo et al., 2020). Phenotyping refers to the observed characteristics of an organism, such as morphology, colour and biochemical properties. With IA, typically an RGB image is captured using anything from relatively inexpensive consumer devices such as mobile phone cameras (Tsaftaris and Noutsos, 2009); to more advanced dedicated equipment where spectral data in single nm bandwidths can be collected for each pixel (Lara et al., 2013). Once the images have been captured, features such as colour and size of the subject can be extracted using one of the many software packages dedicated to IA.

One website alone, www.quantitative-plant.org, has links to over 170 different tools for plant phenotyping and 28 open data sets that

can be used to train models (Lobet et al., 2013). With the use of machine learning algorithms for advanced feature extraction, the technology is progressing very quickly (Jiménez-Carvelo et al., 2019). IA is also much more applicable to industrial applications, as it can be automated, and is used in many different industries. Mo et al., (2017) developed a method for detecting foreign bodies on fresh-cut lettuce where a hyper-spectral scanner was placed above a moving conveyor belt. The analysis of the images captured by the camera was able to distinguish between lettuce and foreign bodies based on their absorbance in the range of (400-1000 nm), and reject samples accordingly.

The development of machine learning algorithms, that can enable leaf material to be imaged whilst still inside packaging, has been demonstrated, which is important if postharvest monitoring is to be achieved. In the paper of Cavallo et al. (2018), a convolutional neural network (CNN) was used to segment the images into three classes: plant, packaging and other. Currently, deep learning and CNNs are the go-to method for working with image data, as once the models are trained they can be very fast in their decision mak-

ing, allowing the possibility of live processing (Patrício and Rieder, 2018). There is no reason why this approach could not be applied to other leafy salads, and even be incorporated into consumer technology, such as smart phones.

2.7.2 Colorimetry for assessing leafy quality

Another method of classifying colour is with the use of chroma-meters (Mampholo et al., 2016). Chroma-meters are analytical instruments for measuring colour, which is typically presented in the LAB colour space. The advantage of this method is that it can be carried out with only one assessor, and objective data are obtained. The device measures a small area on the target ($\sim$ 1 cm^2) and therefore, depending on the target size and variability of colour, many measurements may need to be taken to accurately capture the colour of the target. One issue with this approach, particularly when it comes to salad leaves, is that there are sometimes large differences within individual leaves and between different leaves in the same pack. As the technique measures the leaf at different points, only average values are obtained, which makes it difficult to discriminate between

different manifestations of discolouration (Peiser et al., 1998). Prior to IA, this was the predominant method used; in recent years, the advantages that IA brings has meant that it has largely eclipsed the use of chroma-meters. Overall, considering the relative importance that the consumer places on the appearance of the product, there are few examples of methodologies for predicting colour change.

2.7.3 Quality assessment using hyper-spectral imaging

Looking outside the visible spectrum with hyperspectral imaging (HI), or reflectance data not detected by human vision, is currently providing more information about the state of the product (Mo et al., 2017). HI is much more expensive, both in the cost of equipment and the software and time needed for analysis. In comparison to spatial imaging where two-dimensional data is acquired, three-dimensional data are collected and each pixel has its own associated spectrum; the spectrum data (λ) in combination with spatial data (x, y) creates voxels in the form (x, y, λ). As different materials interact uniquely with different bands of the electromagnetic spectrum (EM), it is possible to gather data about the chemical composition of the material,

which is one of the major advantages of HI (Chaudhry et al., 2018). HI has been used to differentiate between rocket leaves stored at varying temperatures, and from this to infer quality. A random forest classifier was able to classify the reflectance data obtained from the imaging and correctly identify unseen samples 79 % of the time (Platias et al., 2018). Specific regions of the spectrum have been shown to be more informative than others. Diezma et al., (2013) found that 710 to 900 nm was particularly important for the degradation of spinach leaves. Simko, Jimenez-Berni and Furbank, (2015) found similar results with lettuce, with 744 nm being the most informative wavelength for determining the quality difference between fresh and decayed lettuce. This is not particularly surprising as this portion of the electromagnetic (EM) spectrum is used for the basis of the normalised difference vegetation index (NDVI). NDVI distinguishes between 'healthy' and 'stressed' plants by the difference in reflection of the near-infrared (NIR) region of the EM spectrum, and has been used for a relatively long time for this purpose (Gitelson and Merzlyak, 1996).

Typically, when one method such as HI, is used alone with no further

analysis, the results tend to heavily weight chlorophyll senescence, as with NDVI, as the primary factor with respect to change (Beghi et al., 2016). The measured values for colour change in packaged salad leaves are not always linear; often there is an initial change over the first few days and then a reversal (Løkke et al., 2013). The colour change and then reversal, has been theorised to be related to the accumulation of liquid inside the pack, causing some areas to degrade to a greater extent and making the leaf appear darker (Løkke et al., 2013; Volpe et al., 2018). The change of colour and subsequent reversal makes classifying quality based on colour alone difficult, and the technology is not suitable for implementation in the retail or consumer part of the supply chain (Jedermann et al., 2009). The image/colour/spectral analysis described in these preceding sections does have potential for automating shelf life quality assessment that is performed by packers and consequently to provide a more consistent objective analysis than currently occurs between different assessors. However, the pack houses are assessing shelf life quality in the same time frame as the consumer, so the real gains in this area would be for methods to be developed that could predict quality loss in a particular consignment ahead of when the consumer becomes

aware of it.

2.7.4 Detecting and identifying volatile compounds emitted from leafy salad crops

Challenges remain to identify compounds which are reliably associated with quality and depending on how detection is implemented, specific to the salad leaves in question (Spadafora et al., 2016; Ioannidis et al., 2018). Typically, gas chromatography with mass spectrometry is the analytical method of choice, preferably using the same samples for chemical and sensory panel analysis to provide comparable results. The media used to capture the VOCs before measurement on a GC system are selected based on the compounds that are expected to be in the subject material. Solid-phase micro extraction (SPME) is a method often used for capturing volatile compounds that are emitted in the headspace of a leafy salad (Spietelun et al., 2010). A fibre coated in an adsorbent material is placed inside the headspace until an equilibrium has been reached between the fibre, the sample and the headspace. After the equilibrium has been reached the fibre is then placed in the GC system where the VOCs

are desorbed and detected.

Recently, a number of researchers have focused their studies on VOCs emitted from rocket leaves. Spadafora et al. (2016) found that sulphur-containing VOCs tended to increase over shelf-life; it was noted that the increase was correlated with an increase in numbers of micro-organisms isolated from the leaves. In this case, the volatiles were extracted from the headspace of the pack and captured on Tenax traps then measured using GC-MS. Similar results have also been obtained by Bell et al., (2016) using thermal desorption with gas chromatography-time-of-flight mass spectrometry (TD-GC-TOF-MS) with a comparable extraction protocol. Typically, GC-MS methods cannot quantify the abundance of VOCs over time. This is because VOC compounds are often unknown or uncommon, meaning generating standards to quantify the absolute abundance of them are cost-prohibitive. Because of this, the appearance or disappearance of specific VOCs is often used as a marker of shelf-life (Lonchamp et al., 2009; Luca et al., 2017; Ioannidis et al., 2018). For leafy salads, it is only rocket that has had more than a couple of papers identifying compounds associated with qual-

ity. The lack of informative VOCs from other salad leaves may be due to rocket being particularly pungent or conversely the lack of VOCs emitted from other leafy salad crops. The appearance of compounds such as pentane, 2-ethylfuran and dimethyl sulphide, have been identified as markers of microbial activity (Luca et al., 2017), and have been associated with degradation of quality during storage in rocket salads (Dryahina et al., 2020). VOCs arising from cellular senescence or degradation induced by the presence of microorganisms are hard to distinguish from each other. Therefore, it is challenging to ascribe particular compounds to microbial or cellular origin.

There are many research examples (Lonchamp et al., 2009; Spadafora et al., 2016; Raffo et al., 2018) illustrating the value of detecting volatile compounds in packaged salad that claim to be diagnostic of SL. However, it is a huge leap to move from volatile detection on sophisticated laboratory equipment to a technology that is commercially viable and implemented within industry. The challenges for this technology are currently threefold: Firstly the appropriate volatile markers need to be identified for each crop; this is perhaps

the most difficult step as there are many variables, e.g., cultivar, growing environment, that influence plant metabolism and therefore the volatiles released from a plant (Bell et al., 2017). The detected volatiles also need to be reliably associated with quality degradation that would be predictive of consumer rejection of the product. Furthermore, technologies for detecting the identified VOCs need to be developed that are cost-effective commercially and can work in real-time to monitor quality.

2.7.5 Electronic noses for automated odour sensing

Gas sensor devices or 'e-noses' can be tuned to specific VOCs, therefore once the critical compounds concerning quality are established, devices for their detection can be built at relatively low cost. E-noses are non-specific detectors and are calibrated to detect a group of compounds rather than specific ones (Cortellino et al., 2018). 'E-noses' are relatively new, and the technology is developing rapidly. One of the issues with e-nose devices is that they are quite variable, both in manufacturing consistency and that they can degrade in their performance over time, depending on their environment, which

has adverse effects on the quality of the data they generate. There has been much effort to develop algorithms that correct any variance relative to a master device (Yan and Zhang, 2016). The issue of consistency between devices could be a significant barrier to incorporating sensors into a retail or domestic setting. For a method to be non-destructive, the sensor must either be incorporated into the packaging, which provides many challenges such as increased price, increased difficulty of recycling and specificity of compounds detected. Still, it may be successful at diagnosing quality deterioration by measuring generic markers of degradation such as dimethyl sulphide. Alternatively, there needs to be an external sensor that is placed within the vicinity of the subject. However, the external sensor may detect aroma from a variety of origins, and therefore, needs to monitor specific compounds and is unlikely to work for bagged leafy salads or vegetables since volatiles will be contained within the package (Cortellino et al., 2018).

2.7.6 Quality sensors within "intelligent packaging"

Sensors have been developed that can be incorporated into the packaging of a product, and therefore allow real-time feedback about the condition of the product within (Torri et al., 2008; Fuertes et al., 2016). A recent review by Beshai et al (2020) categorised intelligent packaging sensors into four types: optical, biosensors, gas, and humidity sensors. Optical sensors rely on the techniques discussed in the sub-sections above and it remains difficult to see how these can easily be incorporated into packaging in a format that can inform the consumer, although the potential for screening at an earlier stage in the supply chain is possible by linking sensors to radio frequency identification (RFID) tags to collate information and ensure data transmission throughout a supply chain.

Attention has inevitably turned towards technologies that have potential to detect foodborne pathogens, given the seriousness of the consequences if these proliferate on food destined for human consumption. Zhang et al. (2017) have made the best progress towards developing a system with a low detection threshold, through using a Janus emulsion assay which they demonstrated would sensitively

and selectively bind to *E. coli* at 10^4 cfu/mL and which could be read via a smartphone app. However, this still relies on a liquid medium and, crucially, that the bacteria come into direct contact with the sensor. These are substantial assumptions and therefore there is an attraction towards sensor technologies that monitor gaseous compounds. López-Carballo et al. (2019) developed a sensor that can be incorporated within flexible packaging, of samples containing infant milk formula, utilising the redox reaction of methylene blue to signify changes in quality. As the sensor was monitoring O_2, it would only be suitable for MAP as the bag is hermetically sealed. Carbon dioxide may be a better target for gas sensors incorporated into packaging, since its atmospheric concentration is only 0.04 % whereas in MAP it tends to be in the 4-10 % range. Borchert (2013) described an optochemical CO_2 sensor which uses a phosphorescent reporter dye and a colourimetric pH indicator incorporated in plastic matrix. The sensor retained its sensitivity to CO_2 for 21 days at 4 °C and could detect concentrations accurately within a minute of exposure, reporting them using a colour change requiring simple instrumentation, with a four minute recovery time. Despite the potential offered by VOCs that are specific to particular crops or that are produced as

a result of microbiological contamination, to date no monitoring or detection systems have been developed that could be incorporated into packaging. Beshai et al. (2020) review current monitors for respiratory gases and humidity, but the only 'freshness' monitors that use non-respiratory gases depend on the sensor being in direct contact with the food which is not the case for packed vegetables and salads or wrapped wholehead lettuce.

2.7.7 Time Temperature Indicators

Maintaining an unbroken cold chain is key to preserving quality and safety of fresh produce (Cantwell and Suslow, 2002) with short breaks in cold temperature less severe than prolonged periods above the optimal temperature. Even within a single cold chain variability exists, for example, depending on the proximity of a pallet to the cooling system in the lorry or the location of a crate within a pallet. Two classes of Time Temperature Indicators (TTI) exist: those that are data driven and those that display a colour change based on a physio-chemical reaction (Taoukis and Labuza, 1989; Maciel et al., 2012).

Data loggers or labels such as Radio-frequency identification (RFID) tags that report temperature, humidity etc have been used commercially for some time, but often as stand-alone units that have to be incorporated within the packs in a crate which then need manual recovery and interpretation. There is considerable commercial attraction to the development of time-temperature indicators that can be incorporated into packaging or crate labelling systems, especially those which offer instant visual means of interpretation rather than plugging into a computer (Costa et al., 2013). RFID tags do offer this possibility, but they are limited by battery life, the need to be in close proximity to the reader, and their own lifespan. Torres-Sánchez et al. (2020) report the development of a multiple non-linear regression (MNLR) model that relates the temperature to the maximum shelf life in a predictive manner, but at present this relies on the integration of sensory and physico-chemical quality attributes. The best data-driven solutions therefore remain RFID tags that can integrate multiple signals from temperature, humidity and ammonia and which are sufficiently sophisticated to interpret the relationship between these parameters (Quintero et al., 2016).

Visual indicators have a great deal of appeal commercially, particularly if they report the full history that the product has experienced through the supply chain and if they can be incorporated into the packaging. At present, chemical colour change is usually reliant on the speed of an enzymic reaction linked to a pH change, polymer state changes linked to colour change, or the growth rate of bioindicator microorganisms (Lee and Rahman, 2014). They tend to only be able to report sub-optimally high temperatures, since they all work on the principal that raised temperatures lead to a faster response of the target reaction. They are therefore unsuitable for detecting when temperatures have been lower than optimal, for example, if basil has been chilled below 12 ºC. An additional practical problem is that the indicators have to be stored at low temperature before they are deployed to prevent the colour change happening before the tag has been attached to the package. However, a number of TTI products are used very successfully in a commercial setting, particularly for frozen or chilled food products. Considering that leafy salads have a relatively short SL, incorporating sensors into the packing of RTE products may not offer a reasonable return on investment, especially when considering implications the sensor may have

on recyclability of the product. It remains to be seen if detection and monitoring of VOCs can provide data to the consumer that allows for real-time monitoring of the health and remaining longevity of a product that they purchase.

2.8 Concluding remarks

Previous technological advances within the food ecosystem, particularly with respect to imaging, have been implemented at the processing stage where cameras detect out-of-specification leaves and reject them. However, as was remarked when date labels were being introduced: distribution and storage conditions are important to the longevity of a product. There is currently no way for the retailer or consumer to update their expectations of shelf-life once the date on the pack has been set. The dates placed on the packaging, if any, are the only guide the consumer has as to the quality or safety of the product. Although some methods for non-destructively measuring quality postharvest have been explored, none have yet to be implemented in a consumer study to measure the impact such technologies could provide with regards to reducing waste. High-end consumer

refrigerators are now being produced with integrated computers and cameras that are able to monitor the contents, and give real-time feedback to the consumer by network-connected devices. However, there are currently no devices on the market offering product specific monitoring or giving real-time feedback to the consumer regarding quality or safety.

The economic benefit of increasing the accuracy of SL estimations has been estimated at 55 $\pm$ 15 million pounds per day of savings, per day of increased SL from UK households for leafy salads (Lee et al., 2015). Furthermore, it is estimated that retailers would save 2720 tonnes of leafy salads from waste per day of increased SL. There is a clear case for providing the consumer with more accurate information about the state of the product. However, although the technology for sensing quality and safety is progressing, there is still a long way to go in order to be able to reduce the amount of waste, whilst maintaining safety and quality.

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Chapter 3

Growth temperature influences postharvest glucosinolate concentrations and hydrolysis product formation in first and second cuts of rocket salad

3.1 Background

It is clear that conditions under which a product is stored will affect its decline over shelf life. The pre-harvest factors will shape

the potential of the product's shelf life. There is a wealth of knowledge mapping the relationships between agronomic factors and subsequent nutrient content or shelf life quality (Proietti et al., 2004; Luna et al., 2012; Ntsoane et al., 2016). However, there is little information combining genetic and environmental data to understand the impact of nature versus nurture on subsequent post harvest life. This chapter investigates how the environmental variable of growth temperature affects the growth and subsequent shelf life of different cultivars and species of rocket salad.

Rocket leaves have a distinctive taste and aroma that is characterised by glucosinolates and subsequent glucosinolate hydrolysis products. As well as this, there have been health benefits associated with this class of compounds (Hayes et al., 2008) and particularly sulforaphane (Mazarakis et al., 2020). Therefore, understanding how these compounds change over shelf life would explain how the quality, from a nutritional and flavour context, change over shelf life and to what extent the growing environment may be predictive of this change.

3.1.1 limitations

Due to the lower number of plants that were successfully grown (RW2, Table 3.1), and re-grown (RW2/3) after the second cut, the conclusions drawn from the statistical analysis involving these plants could potentially be overinterpreted. Although there is no technical reason why an ANOVA cannot be run with low sample numbers, the underlying assumptions (robustness of the equal variance assumption) can be difficult to verify (Wilcox, 1995). In theory, the low sample numbers should reduce the power of the model. As a result, significant differences between samples with low numbers would not arise unless the differences between the mean were very large (Lantz, 2013). As a result, when evaluating the results shown in this chapter with respect to RW2/3 the low sample size should be considered as the results may be returned based on a general linear model rather than ANOVA (Shaw and Mitchell-Olds, 1993).

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3.2 Contribution to the project

The original idea for the project was proposed by Luke Bell and following a meeting with Luke and myself we came up with a project idea that would be mutually beneficial. *CRediT authorship contribution statement as appeared in the original article*

Jake Jasper: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review and editing. **Carol Wagstaff**: Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review and editing. **Luke Bell**: Conceptualization, Data curation, Formal analysis,

Investigation, Methodology, Supervision, Validation, Visualization, Writing - original draft, Writing - review and editing.Contribution breakdown percentage by each author respectively [45, 10, 45]

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3.3 Abstract

Rocket salad species (*Diplotaxis tenuifolia* and *Eruca sativa*; also known as *E. vesicaria*) are known for their high concentrations of health-related isothiocyanates, which are derived from secondary metabolites called glucosinolates. Increases in temperature due to climate change and extreme weather event frequency over the coming decades are likely to influence not only the growth of leafy vegetables, but also their nutritional density. It is therefore essential to

determine the impacts of these in order to mitigate crop losses and nutritional decline in future. Our data show there is a strong influence of pre-harvest growth temperatures on glucosinolate biosynthesis and formation of glucosinolate hydrolysis products postharvest, and that this is genotype dependent. High growth temperature (40 °C) severely retarded germination, growth, regrowth, and survival of rocket plants. Highest glucosinolate concentrations were observed in first and second cuts at 40 °C, but did not correspond to highest isothiocyanate concentrations (observed at 30 °C, second cut). These data indicate that high growth temperatures increase glucosinolate accumulation, but growth and productivity is significantly reduced. Much greater emphasis is needed for breeding cultivars tolerant to high growth temperatures in order to maximise nutritional benefits imparted by temperature stress.

3.4 Introduction

Rocket salads are a popular group of leafy vegetables belonging to the Brassicaceae family. *Diplotaxis tenuifolia* and *Eruca sativa* comprise the majority of global rocket production, and are well known

for pungent aromas and flavours. Each species has distinct morphological characteristics, though *E. sativa* is much more varied in this regard (Bennett et al., 2006). Over the last 20 years there has been a surge in interest in the crops for their phytochemical content, particularly for glucosinolates (GSLs) and their hydrolysis products (GHPs; Bell and Wagstaff, 2019). Foremost of these are isothiocyanates (ITCs), and specifically sulforaphane (SF); consumption of which has been linked with a reduced risk of developing some cancers (Fimognari and Hrelia, 2007).

GSL profiles are notoriously variable across growth environments in many Brassicaceae species, and the formation of GHPs even more so (Bell and Wagstaff, 2019). GSL biosynthesis is inherently tied to the stress responses of all Brassicales plants (Mostafa et al., 2016), and as such, concentrations within tissues can vary markedly according to growth temperature (Kask et al., 2016), light quality (Schreiner et al., 2009), and salinity (Cocetta et al., 2018); as well as biotic factors from pests and disease (Schlaeppi et al., 2010). Climate change is leading to more extreme temperatures in places used to cultivate horticultural crops, and consumer demand is leading to the adoption

of more land and more protected cultivation practices to meet the yield and quality expectations.

A large amount of work has been done to determine the glucosinolate (GSL) profiles of rocket within first harvest (or cut) leaves (Cataldi et al., 2007; Chun et al., 2015; Force et al., 2007; Toledo-Martín et al., 2017), and only one study has assessed second cut composition (Nitz and Schnitzler, 2002), but only looked at three compounds. Second cuts are primarily favoured by growers and processors for their perceived increased pungency and overall quality (Bell and Wagstaff, 2019), yet the scientific literature has thus far failed to consider this common horticultural practice in experimental designs. It is speculated that multiple cuts increase the abundance of glucosinolates and isothiocyanates in rocket species. The increase of secondary metabolites in response to mechanical wounding is well known in other horticultural species (Jahangir et al., 2009). This has clear implications for taste, flavour, safety, and health-related properties of leaves.

The impact of different growing environments, such as temperature variation, on crop nutritional composition is also poorly studied in

rocket; adding an additional influencing factor on these traits. It is therefore possible that variable growing temperatures will also affect (positively or negatively) the consumer eating experience. The exact genetic mechanisms that regulate GSL biosynthesis under high or low temperatures are unknown, partly due to interacting and co-occurring stresses, such as drought. It is likely however that the imposition of high/low temperature stress promotes activity of transcription factors such as MYC2 and MYB28, which promote GSL biosynthesis (Gigolashvili et al., 2009).

Temperature effects upon GSL synthesis and GHP formation are poorly understood in rocket species, and have important implications for the synthesis of specific health-associated compounds. Rocket crops are grown on every inhabited continent, and are exposed to a huge range of cultivation temperatures (Bell et al., 2015). They can be grown under mild, temperate conditions, such as in southern England, (summer maximum temperatures averaging 20.4 °C; Met Office 1981-2010 data), to hot Mediterranean temperatures (such as the Bay of Naples, Italy, summer maximum temperatures averaging 29.5 °C; World Meteorological Organization). In addition, crops are

commonly cultivated under glass or polytunnel in summer months, where internal daytime temperatures can rise to over 35 °C (Di Gioia et al., 2018). In growing regions such as Lazio (Italy) and New South Wales (Australia), outdoor summer daytime temperatures can regularly exceed 40 °C, and therefore have significant impacts on the growth of leafy vegetables. Crops under protection are therefore doubly affected, as internal temperatures may exceed 50 °C without adequate ventilation. By the end of the 21st century, atmospheric CO_2 concentrations are projected to rise to between 730 and 1000 ppm. This will lead to average global temperature increases of between 1-3.7 °C (Gray and Brady, 2016). Combined with an increased likelihood of extreme weather events (such as heat waves and drought), protected leafy crops such as rocket are especially vulnerable to losses and or changes in growth rate (Kron et al., 2019).

The effects of such growing extremes are presently unknown, and it is likewise unstudied how growth temperature affects regrowth, phytochemical content, or shelf life retention of health-associated compounds. Postharvest work has already demonstrated that these compounds are subject to fluctuation (Bell et al., 2017c; Yahya et al.,

2019), but it is unknown to what degree growth temperature and cut influence this process. In light of climate change and global warming effects in future, it is also likely that extremes in temperature will become more common, and therefore it is important to understand how crop growth and quality may be affected.

This study presents phytochemical data relating to the growth of two *D. tenuifolia* and two *E. sativa* rocket cultivars under different growth temperatures. This study is the first to examine the impact of cultivation temperatures on secondary metabolite formation that has consequences for nutrition and flavour qualities of rocket crops. We hypothesised that each species would see increases in GSL concentrations at the higher cultivation temperatures, but that each cultivar would produce differing relative concentrations according to genotype, as has been highlighted in a previous study (Bell et al., 2015). We speculated that the initial concentrations of GSLs postharvest would influence the degree of biosynthesis and retention during the cold storage period, and in turn impact the abundance of GHPs formed.

3.5 Materials and methods

3.5.1 Plant material

Two *D. tenuifolia* and two *E. sativa* pre-commercial cultivars were supplied by Elsoms Seeds Ltd. (Spalding, UK). For reasons of commercial sensitivity specific details regarding the genetic origin of these will not be given. *E. sativa* cultivars were designated RS4 and RS8, and *D. tenuifolia* cultivars RW2 and RW3.

3.5.2 Growing conditions, simulated processing, and shelf life storage sampling

Forty seeds of each cultivar were sown into module trays containing peat-based seedling compost, and germinated under three temperature conditions in Saxcil growth cabinets. The three temperature conditions were as follows: 20 °C (daytime; 15 °C night), 30 °C (daytime; 25 °C night), and 40 °C (daytime; 30 °C night). Lighting conditions were consistent between each chamber and set to a long-day cycle (16 h light, 8 h dark). Light intensity was set to 380 µmol

Table 3.1: Numbers of biological replicates under each temperature condition and cut per temperature treatment.

	Temperature and condition					
Cultivar	**20 °C**		**30 °C**		**40 °C**	
	1st	**2**nd	**1**st	**2**nd	**1**st	**2**nd
RS4	12	12	12	7	16	dns
RS8	11	11	15	8	13	dns
RW2	5	3	9	3	dns	dns
RW3	10	7	10	7	2	dns

Abbreviations: RS = salad rocket; RW = wild rocket; dns = did not survive

m^{-2} s^{-1}. Humidity was ambient. Healthy seedlings were transplanted into 1 l pots (containing peat-based compost) on an individual basis, upon the development and expansion of two true leaves. Pots were watered daily, as required, to field capacity.

Plants were harvested on an individual basis, and were considered of commercial maturity once 10–15 leaves were developed. See Table 3.1 for numbers of biological replicates harvested for each cultivar under each respective condition. Upon reaching this point, plants were harvested as close to the soil as practical by hand using sterile scissors and left to regrow. It should be noted that not all plants survived the first cut, and that the 40 °C treatment severely impaired growth and survival.

All plants were harvested between 10 a.m. and 12 p.m to minimise

the effects of diurnal fluctuations in secondary metabolites (Huseby et al., 2013). The harvested leaves were initially placed in Ziploc bags and then transferred to the laboratory. Upon arrival, plants were hand processed individually by turbulent washing in mildly chlorinated water (30 ppm, sodium hypochlorite; Suslow, 2000) for one minute, followed by gentle rinsing in non-chlorinated water for one minute. Finally leaves were placed in a hand operated salad spinner and dried for another minute to simulate factory processing conditions. Leaves were divided into equal amounts and designated D0, D4, and D7 according to the beginning of shelf life storage in order to replicate the industry shelf life dating procedure. D0 samples were placed immediately into a -80 °C freezer. D4 and D7 samples were placed in laser perforated bags and closed with an electric heat-sealer, then stored in the dark at 4 °C (Bell et al., 2016). On respective sampling days, each of the bagged leaves were frozen at -80 °C. All sampling took place between 10 a.m. and 12 p.m, as per the initial time of harvest. This entire process was repeated for the second cut of regrown leaves until all plants were either harvested or had died.

3.5.3 Leaf material preparation, and extraction

Frozen leaf material was lyophilized in batches for three days. Leaves were ground into a fine powder using a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ, USA) and stored in tubes until extraction and analysis.

GSL extraction was performed as per the protocol presented by (Bell et al., 2015) with modifications. Briefly, 40 mg of dried leaf powder was placed into Eppendorf tubes and put into a heat block (80 °C for ten minutes). Afterwards, 1 ml of preheated methanol water (70 % v/v) was added to dried powder, vortexed vigorously, and placed in a water bath (75 °C) for 20 min. Samples were cooled to halt the extraction and then centrifuged for five minutes (16,050 x g, ambient temperature), the supernatant collected, and filtered (0.22 µm PVDF Acrodisc syringe filters; VWR, Lutterworth, UK). Crude extracts were stored at -80 °C before dilution (5x) and analysis conducted by LC–MS.

GHPs were extracted by GC-MS according to the protocol published by Ku et al. (2016) with modifications. The extraction du-

ration was optimized for maximum yields of GHPs by comparison of extractions for three hours incubation at 30 °C with immediate dichloromethane (DCM) extraction, and three, nine, and 21 h post incubation with DCM.

50 mg of sample was hydrolysed in 1 ml of distilled H_2O for three hours at 30 °C, before subsequent extraction in DCM overnight (21 h). The DCM layer was then collected and transferred to glass vials and stored at -80 °C until analysis by GC–MS.

3.5.4 LC–MS and GC–MS analyses

For LC–MS, samples were analyzed in a random sequence with standards and QC samples. External standards of progoitrin (PRO; 99.07 %, HPLC), glucoraphanin (GRA; 99.86 %, HPLC), glucoerucin (GER; 99.68 %, HPLC), glucobrassicin (GBR; 99.38 %, HPLC), and gluconasturtiin (GNAS; 98.38 %, HPLC) were prepared for quantification of GSL compounds according to the method presented by Jin et al. (2009). GER was used to quantify glucorucolamine (GRM), diglucothiobeinin (DGTB), glucosativin (GSV), and DMB, as no standards are presently available for these compounds. GBR

was used to quantify the indole GSLs 4-methoxyglucobrassicin (4MOB) and neoglucobrassicin (NGB; Table 3.2). All standards were purchased from PhytoPlan (Heidelberg, Germany). Recovery of extracted GSLs was calculated by spiking six random samples with sinigrin upon the addition of pre-heated methanol (Merck, Gillingham, UK). The average recovery of sinigrin was 104.8 %. Limits of detection (LOD) and quantification (LOQ) were established for the method by running serial dilutions of sinigrin (LOD = 5.38 µmol L^{-1}; LOQ = 16.3 µmol L^{-1}).

Table 3.2: Glucosinolates and glucosinolate hydrolysis products identified in *Eruca sativa* and *Diplotaxis tenuifolia* cultivars. a = Quantified using authentic standards, b = Quantified using glucoerucin standard. c = Quantified using glucobrassicin standard. d = Quantified using sulforaphane standard.

	Glucosinolates				
	Trivial name	**Abbreviation**	**R-group name**	**Retention time**	**Identifying *m/z* [M-H]$^-$**
1	Glucorucolamine[b]	GRM	4-(cysteine-S-yl)butyl	4.7	4.93
2	Progoitrin[a]	PRO	(2R)-2-hydroxybut-3-enyl	5.9	388
3	Glucoraphanin[a]	GRA	4-methyl-sulfinyl-butyl	6.0	436
4	Diglucothiobeinin[b]	DGTB	4-(-d-gluco-pyranosyl-disulfanyl)	12.7	600
5	Glucosativin[b]	GSV	4-mercap-tobutyl	16.4	406
6	Glucoerucin[a]	GER	4-methylthio-butyl	22.7	420
7	-	DMB	Dimeric 4-mercap-tobutyl	23.0	405 (811, 731)
8	Glucobrassicin[a]	GBR	Indol-3-ylmethyl	23.5	447
9	Gluconasturtiin[a]	GNAS	2-phenethyl	24.0	422
10	4-methoxy-glucobrassicin[c]	4MOB	4-methoxy-3-indolylmethyl	24.2	477
11	Neogluco-brassicin[c]	NGB	1-methoxy-3-indolylmethyl	25.6	477

	Glucosinolate hydrolysis products		
	Trivial name	**Hydrolysis product of**	**MS spectra m/z**
1	Sativin[d]	Glucosativin	147, 114, 87, 72, 60
2	Erucin[d]	Glucoerucin	161, 115, 72, 61
3	Sulforaphane[a]	Glucoraphanin	177, 160, 114, 72, 55
4	Bis-(4-isothiocyanatobutyl)-disulfide[a]	DMB	292, 146, 114, 87, 72, 55

LC–MS analysis was performed in the negative ion mode on an Agilent 1260 Infinity Series LC system (Agilent, Stockport, UK) equipped with a binary pump, degasser, auto-sampler, column heater and diode array detector; coupled to an Agilent 6120 Series single quadrupole mass spectrometer. Separation of samples was achieved on a Gemini 3 µm C18 110 Å (150 × 4.6 mm) column (with Security Guard column, C18; 4 mm x 3 mm; Phenomenex, Macclesfield, UK). GSLs were separated during a 40 min chromatographic run, with a 5 min post-run sequence. Mobile phases consisted of ammonium formate (0.1 %; A) and acetonitrile (B) with the following gradient timetable: (i) 0 min (A–B, 95:5, v/v); (ii) 0–13 min s (A–B, 95:5, v/v); (iii) 13–22 min s (A–B, 40:60, v/v); (iv) 22−30 mins (A–B, 40:60, v/v); 30−35 mins (A–B, 95:5, v/v); (v) 35−40 mins (A–B, 95:5, v/v). The flow rate was optimized for the system at 0.4 ml min^{-1}, with a column temperature of 30 °C; 20 µl of sample was injected into the system. Quantification was conducted at a wavelength of 229 nm.

MS analysis settings were as follows: Atmospheric pressure electrospray ionization was carried out in negative ion mode (scan range

m/z 100–1000 Da. Nebulizer pressure was set at 50 psi, gas-drying temperature at 350 °C, and capillary voltage at 2000 V. Compounds were identified using their primary ion mass [M-H]- (Cataldi et al., 2007) and by comparing relative retention times with those of Lelario et a. (2012; Table 3.2). Data were analyzed using Agilent OpenLAB CDS ChemStation Edition for LC–MS (vA.02.10). GSL concentrations from each time point were averaged; see Table 3.2 for all n per treatment. This approach was also conducted for GHP analysis.

GHPs were identified and analysed according to the method presented by Bell et al. (2017) with the following modification. Extracts were separated on a Zebron ZB-AAA (10 m, 0.25 mm i.d.; Phenomenex) capillary during a seven minute run. GC conditions were as follows: split 1:20 at 250 °C, with a 2.5 µl injection. Helium was used as the carrier gas at a flow rate of 1.5 mL.min^{-1}. The oven program was: 30 °C min from 110 °C to 320 °C, with a one minute hold at 320 °C. Concentrations of all GHPs were calculated as equivalents of SF standard (Sigma).

All concentrations quoted within the text are on a dry weight basis for both GSLs and GHPs.

3.5.5 Statistical analysis

ANOVA analyses of all data were performed using XL Stat (Addinsoft, Paris, France). Each respective analysis was conducted with a protected post hoc Tukey's Honest Significance Difference (HSD) test ($P < 0.05$). In some cases, due to the unbalanced sample numbers the underlying assumptions of the model are difficult to assess. Therefore, comparisons between involving RW2 and the 2nd cut for RW3 should be interpreted with extra caution.

Principal Component Analysis (PCA) was performed using XL Stat with Pearson correlation analysis (n-1), Varimax rotation, and the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy. The KMO value for the analysis was 0.71, indicating a satisfactory level of sampling. The analysis produced four informative Principal Components (PCs) with Eigenvalues >1.0. The cumulative explained variability within these components totalled 78.1 %. After Varimax rotation, PCs 1, 2, and 8 produced the highest degree of explanatory spatial separation.

3.6 Results and discussion

3.6.1 The effects of growth temperature on germination and time to first and second cuts

3.6.1.1 Germination

Germination time was shortest under the 30 °C condition, and *E. sativa* cultivars showed a clear trend for earlier establishment than *D. tenuifolia* (Figure 3.1 a), although it was not statistically significant. RW2 and RW3 had slow germination and growth at both 20 °C and 40 °C, with RW2 not germinating at all under the latter condition. As rocket species have a Mediterranean origin, it is not entirely surprising that germination is optimal at 30 °C, however our data do suggest that *E. sativa* is better adapted to temperature extremes. This could be of particular relevance to growers cultivating rocket under glass or polytunnel where temperatures may regularly exceed 35 °C in the summer. Conversely, growers in cool or temperate regions, cultivating rocket in open field, may find *E. sativa* quicker to establish.

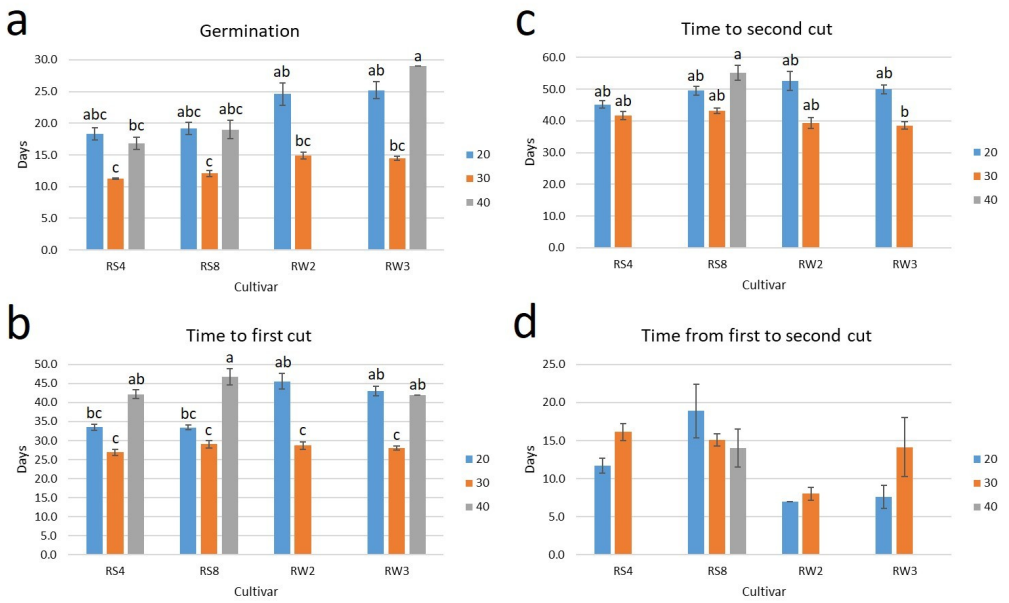

Figure 3.1: The number of days taken for *Diplotaxis tenuifolia* (RW) and *Eruca sativa* (RS) cultivars to germinate (a), reach first cut maturity (10 – 15 true leaves; b), reach second cut maturity (10 – 15 regrowth leaves; c), and from first to second cut (d). Error bars represent standard error of the mean. The legend on each subplot refers to the temperature in degrees Celsius. Becuase of the low sample size for the second cut (Table 3.1), there were no significant difference between samples in subplot d, and therefore no letters are shown.

3.6.1.2 Time to first cut

Harvest age of rocket is highly variable between growth environments (Hall et al., 2015), and as such we selected a physiological growth phase as a benchmark for harvest between each temperature condition (development of 10-15 leaves). At 40 °C all plant growth was severely retarded (Figure 3.1 b) although not always statistically significant, and no plants were ready for first harvest before 40

days. This suggests that adverse high temperature conditions may have a large impact on the productivity of rocket crops, regardless of species. However, for RW, particularly RW2 the sample size was small and as a result the effect of temperature is less reliable.

Exposure to temperatures $> 37\,°C$ for prolonged periods of time can be lethal in many plants without acclimatisation. This is due to the inactivation or denaturation of proteins (Schöffl and Panikulangara, 2018). Without adequate time to adjust to heat shock (such as through expression of heat shock proteins) growth is slowed or even halted; ultimately leading to plant death. It is therefore remarkable that the rocket plants (particularly RS8) tested in this experiment were able to tolerate these conditions.

3.6.1.3 Time to second cut

The differences between temperature conditions for the second harvest were less pronounced (Figure 3.1. c). At 40 °C however there was no regrowth for RS4, RW2, or RW3. Plants of these cultivars typically senesced a few days after first cut and died. As mentioned previously, given that the 40 °C temperature was extreme (Schöffl

and Panikulangara, 2018), *E. sativa* in particular displayed a high tolerance.

Such conditions are not unheard of in protected environments, and may become more common under protected conditions in future due to global warming. It is interesting therefore that RS8 showed little adverse effects to the extreme temperature condition, and regrew within approximately 15 days. While rocket is not bred for temperature tolerance at this time, it does suggest that if climatic conditions become more challenging in future there are cultivars capable of withstanding such high temperature extremes. As will be discussed in subsequent sections, this may also have important implications for biosynthesis of health-related compounds in rocket.

3.6.1.4 Time from first to second cut

Regrowth of RW2 was extremely fast, taking only seven days to regrow $>$ 10 leaves after the first cut at 30 °C (Figure 3.1. d). These data suggest that *D. tenuifolia* second cuts may be much more productive than *E. sativa* under 20–30 °C conditions, although due to low sample sizes this was not statistically significant. While initial

establishment and growth rate is slow in the first cut, this is offset by a much quicker subsequent regrowth rate.

3.6.2 The effects of growth temperature on glucosinolate concentrations and hydrolysis product formation during shelf life storage

3.6.2.1 First cut at 20 °C

There was a clear trend in first cut *E. sativa* cultivars for higher GSL accumulation compared with *D. tenuifolia* (Figure 3.2). At D0, ANOVA pairwise comparisons of these samples were non-significant between cultivars, with the exception of 4-methoxy-glucobrassicin (4MOB) ($P<0.0001$; Table 3.3). At 20 °C, RS4 and RS8 both showed a clear trend for increased GSL concentrations over the seven day shelf life period, peaking at the final time point (D7). This is in agreement with observations made by Bell et al. (2017) in a field grown UK *E. sativa* crop. RW2 and RW3 by comparison peaked on D4, but contained almost half the concentrations of RS4 and RS8. These trends were only followed by RS8 and RW2 for hydrolysis

product formation, and concentrations were generally low across all time points (Figure 3.3). This has implications for consumers, and suggests that health-related benefits are cultivar and time-dependent postharvest. The variability of isothiocyanates and other GHPs in rocket postharvest is documented (Bell and Wagstaff, 2019). The timing of consumption is therefore a critical consideration for determining the efficacy of rocket cultivars against disease and chronic illness, and should be included as a more prominent factor for consideration in clinical investigations.

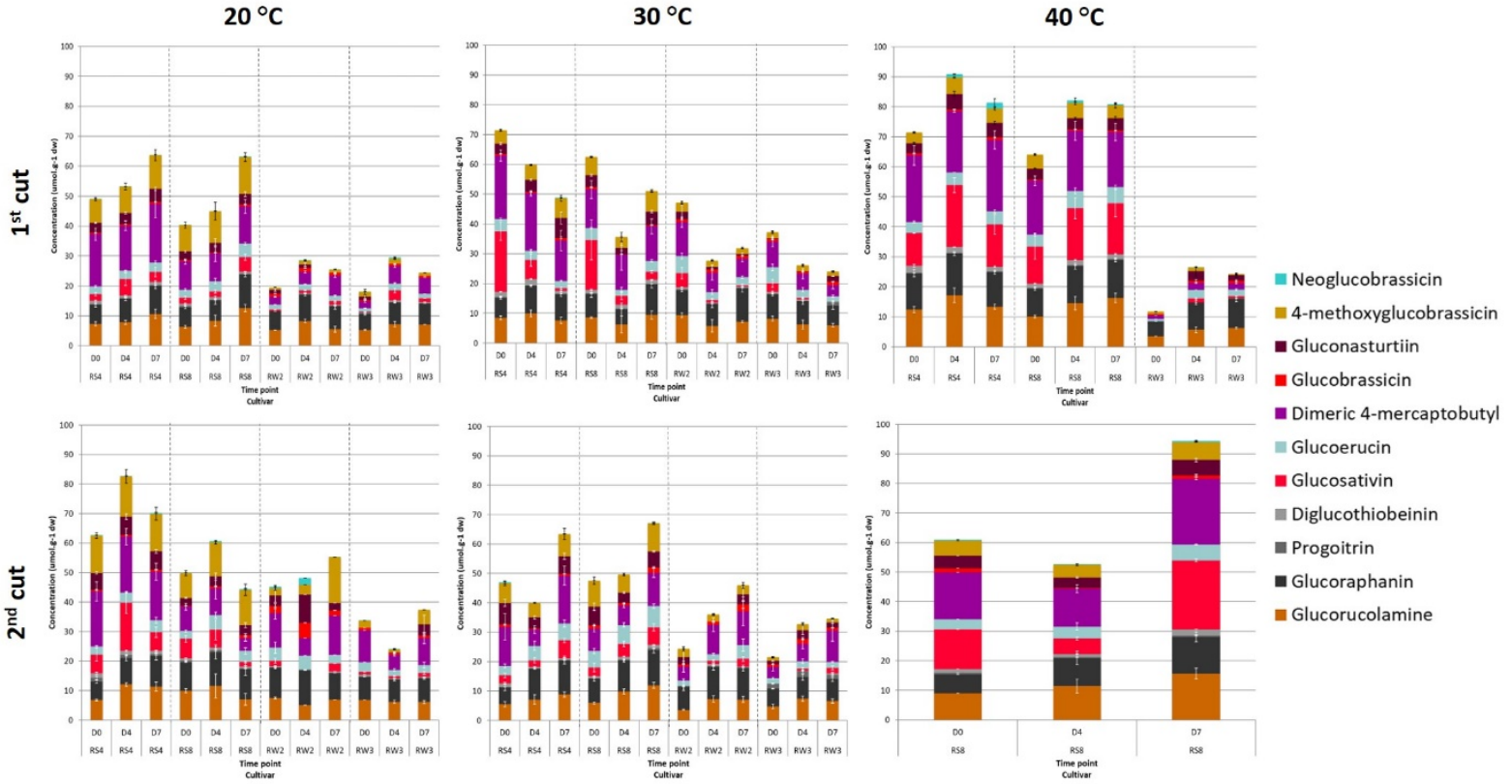

Figure 2

Figure 3.2: Glucosinolate concentrations in first and second cut *Diplotaxis tenuifolia* (RW) and *Eruca sativa (RS)* grown at four different temperatures. See inset for glucosinolate colour coding. Abbreviations: D0, start of shelf life; D4, fourth day of shelf life; D7, seventh day and end of commercial shelf life. Error bars represent standard error of the mean for each respective compound. Note that sample RW3 at 40 °C consists of <3 biological replicates due to plant death.

Table 3.3: Results of Analysis of Variance type 3 sums of squares significance values for four rocket cultivars grown under three environmental temperature conditions, at three postharvest time points

		Glucosinolates												Hydrolysis products			
Factors & interactions	Time point / cultivar	Glucoraphanin	Progoitrin	Gluconasturtiin	4-methoxyglucobrassicin	Total Glucosinolates	Sativin	Erucin	Sulforaphane	Total glucosinolate hydrolysis products
Cultivar	D0	***	*	ns	***	***	ns	***	ns	***	***	ns	***	***	ns	***	***
	D4	**	ns	ns	***	***	**	***	***	***	***	ns	***	***	***	***	***
	D7	***	***	***	***	***	***	***	ns	**	***	ns	***	*	**	***	**
Growth temperature	D0	ns	ns	ns	ns	ns	*	ns	ns	ns	**	ns	ns	ns	ns	ns	*
	D4	ns	ns	ns	ns	ns	ns	ns	***	ns	**	ns	ns	ns	ns	ns	ns
	D7	ns	ns	***	ns	***	ns	ns	ns	ns	***	ns	ns	ns	ns	*	ns
Cut number	D0	**	ns	*	ns	ns	ns	ns	**	ns	ns	*	ns	ns	ns	ns	ns
	D4	ns	ns	ns	ns	ns	ns	ns	***	ns	ns	ns	ns	***	ns	ns	***
	D7	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	ns	***
Cultivar x temperature	D0	**	ns	ns	ns	ns	ns	ns	*	ns	**	ns	ns	ns	ns	**	ns
	D4	ns	ns	**	ns	ns	ns	ns	***	*	*	ns	ns	ns	ns	**	ns
	D7	*	ns	**	**	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cultivar x cut number	D0	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	*
	D4	ns	ns	ns	ns	ns	ns	ns	***	ns	ns	ns	ns	ns	ns	***	*
	D7	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cultivar x temperature x cut number	D0	*	ns	*	ns	ns	**	ns	*	ns	ns	ns	ns	ns	ns	ns	ns
	D4	ns	ns	***	ns	ns	ns	ns	***	**	ns	ns	ns	ns	ns	ns	ns
	D7	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Temperature x cut number	RS4	*	ns	*	**	***	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns
	RS8	ns	*	ns	ns	ns	**	ns	**	ns	ns	ns	ns	**	ns	**	ns
	RW2	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	**	ns	ns	ns	ns	ns
	RW3	ns	**	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Temperature x time point	RS4	ns	ns	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	RS8	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*
	RW2	ns	ns	*	ns	ns	ns	ns	ns	ns	***	**	ns	ns	ns	ns	ns
	RW3	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cut number x time point	RS4	ns	ns	ns	ns	**	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	RS8	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	RW2	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	**	ns	ns	ns	ns	ns
	RW3	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Temperature x cut number x time point	RS4	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns
	RS8	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	RW2	**	ns	ns	**	*	ns	ns	ns	ns	***	**	ns	ns	ns	**	ns
	RW3	ns	*	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Abbreviations: D0 = day 0 shelf life; D4 = day 4 shelf life; D7 = day 7 self life; RS = salad rocket, Eruca sativa; RW = wild rocket, Diplotaxis tenuifolia.

Significance values: *** = P<0.001; ** = P<0.01; * = P<0.05; ns = not significant. See Supplementary Data File S1 for detailed P-values and Tukey's Honest Significant Difference pairwise comparisons.

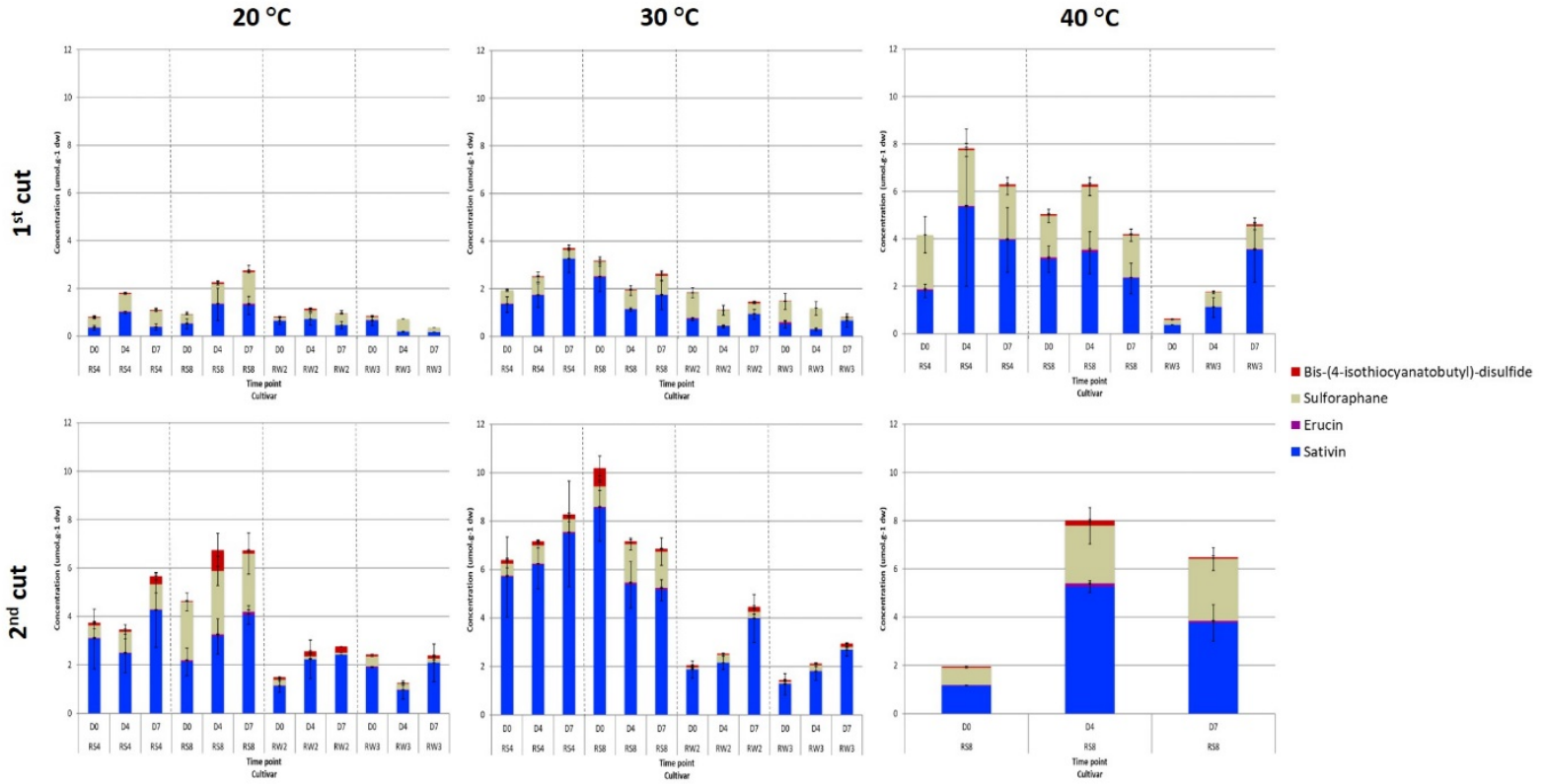

Figure 3

Figure 3.3: Glucosinolate hydrolysis products concentrations produced by first and second cut *Diplotaxis tenuifolia* (RW) and *Eruca sativa* (RS) grown at four different temperatures. See inset for hydrolysis product colour coding. Abbreviations: D0, start of shelf life; D4, fourth day of shelf life; D7, seventh day and end of commercial shelf life. Error bars represent standard error of the mean for each respective compound. Note that sample RW3 at 40 °C consists of <3 biological replicates due to plant death.

3.6.2.2 Second cut at 20 °C

In the second cut at 20 °C the trend between species was reversed: RS4 and RS8 peaked on D4, and RW2 and RW3 peaked on D7 for GSLs (Figure 3.2). Again, indolic GSL concentrations were significantly different at D0. RW2 contained significantly higher abundance of GBR than RS4 and RS8 ($P = 0.003$), and conversely, RS4 had higher amounts of 4MOB compared with each of the *D. tenuifolia* cultivars (12.7 ± 0.9 µmol g^{-1}; $P < 0.0001$). These differences were repeated in D4 samples (GBR and 4MOB, $P < 0.0001$, Table 3.3) and suggest a distinct difference in indolic GSL metabolism between the two species; something that has not been previously observed. Indolics are linked with chemopreventative properties, such as promoting cancer cell cycle arrest (Hayes et al., 2008) and selection for improved indolic profiles of rocket could lead to increased health benefits.

Temporal changes in 4MOB were most pronounced in RW2, with a large and significant increase in abundance at D7 (15.6 µmol g^{-1}) compared to D0 (2.5 ± 0.7 µmol g^{-1}) and D4 (3.3 µmol g^{-1}). Indolic GSLs are involved with abscisic acid (ABA) metabolism and synthe-

sis (Malka and Cheng, 2017), and therefore such large increases over the course of shelf life may be indicative of increased senescence induced by plant hormone activity. This may be of significance when selecting rocket cultivars for improved shelf life traits; especially in second cut *D. tenuifolia*, which is the most common rocket product on global supermarket shelves.

Second cuts of each cultivar produced large average increases in GHP formation (Figure 3.3). The patterns of change over shelf life did not match those of GSLs, suggesting that GSL content is not an accurate proxy for the abundance and ratios of GHPs that are formed. Importantly SF, which has been linked with anticarcinogenic effects in vivo (Liang et al., 2008) saw large increases in the *E. sativa* cultivars, but not in *D. tenuifolia*; concentrations were significantly higher in RS8 ($P < 0.0001$). This was also repeated at D4 (RS8, 2.6 ± 0.6 µmol g^{-1}; $P < 0.0001$, Table 3.3) and indicates that second cut *E. sativa* may be better suited for formation of health related SF at lower growth temperatures than *D. tenuifolia*.

3.6.2.3 First cut at 30 °C

In RS4 the decrease from D0 to D7 (Figure 3.2) was predominantly due to a significant reduction in GSV (from 20.4 ± 3.0 µmol g^{-1} to 0.9 ± 0.2 µmol g^{-1}; $P < 0.0001$, Table 3.3). This trend was not reflected in the GHP profile of RS4 however, where concentrations were highest at D7 (3.7 ± 0.6 µmol g^{-1}). Although no significant differences were found between cultivars or growth temperatures, these trends again suggest that GSL content is not an accurate predictor of GHPs.

3.6.2.4 Second cut at 30 °C

Second cuts showed no significant differences from the first at 30 °C for GSL content, indicating more consistent biosynthesis. This is a desirable characteristic for growers and processors as it in turn may contribute to improved consistency in taste and flavour between cuts.

In terms of individual GSL differences between cultivars, RS8 again contained significantly higher concentrations of 4MOB (8.9 ± 1.2 µmol g^{-1}) than RW3 at D0 (1.2 ± 0.2 µmol g_{-1}; $P < 0.0001$). On D4 of

shelf life, RW3 by contrast contained significantly more PRO than the other cultivars (2.0 $\pm$ 0.5 µmol g^{-1}; $P < 0.0001$; Table 3.3). This compound is known to impart extreme bitterness and is a target for reduction through breeding (Ishida et al., 2014). While concentrations may not be high at the point of harvest, breeding selections should also take into account such possible increases in synthesis postharvest to reduce consumer rejection. PRO is also associated with anti-nutritional properties (Mithen et al., 2000) and efforts should be made to reduce concentrations in rocket cultivars through breeding.

In terms of temporal changes for each cultivar over the shelf life duration, one significant difference was of note: compared to the D0 sample point, D4 and D7 samples of RS4 contained significantly greater concentrations of GRA (5.7 ± 1.1 µmol g_{-1}, 10.6 ± 0.2 µmol g_{-1}, and 11.7 ± 1.3 µmol g_{-1}, respectively; $P < 0.0001$, Table 3.3). This matches observations made by Bell et al. (2017) where shelf life increases in this GSL were also observed for some cultivars.

Despite negligible changes in GSL content between cuts, GHPs saw large and significant increases compared to the first cut. The exact

regulatory mechanisms for GHP formation in rocket are largely unknown, but evidence is mounting that it is not purely a spontaneous process of 1:1 conversion of GSLs. It may be that although GSL concentrations may be lower, myrosinase activity can remain higher, and/or actively promote the formation of GHPs; such as through the action of epithiospecifier modifier 1 (ESM1) genes. Myrosinase(s), are a family of enzymes that are responsible for the hydrolysis of glucosinolates to many different compounds such as isothiocyanates, thiocyanates and nitriles. The resulting product from myrosinase activity is dependent on the composition and activity of associated proteins (Kliebenstein et al., 2005). After seeds, myrosinase and glucosinolates concentrations are highest in the leaf tissue, in the "defence theory" having high concentrations in these locations is indicative of the relative importance in defending these areas of the plant (Martin and Müller, 2007). ESM1 has a role in isothiocyanate formation (Angelino and Jeffery, 2014). Although it is not currently clear as to the exact functioning (Zhang et al., 2006), it does seem to suppress nitrile formation by myrosinase in favour of isothiocyanates. It is important to understand how this is controlled under abiotic stress conditions, as it will likely influence the nutri-

tional benefits obtained from leaves.

With the exception of RS8, all cultivar concentrations peaked on D7 further supporting previous reports of this (Bell et al., 2017c). At D0, RS8 produced significantly more SAT (8.5 ± 1.4 µmol g_{-1}; $P < 0.0001$) than the *D. tenuifolia* cultivars; and the highest concentrations overall of any tested sample (10.2 ± 2.0 µmol g_{-1}; $P < 0.0001$, Table 3.3). It is possible that such high concentrations of this compound would greatly increase the pungency of a cultivar, and support the anecdotal observations often made by growers.

3.6.2.5 First cut at 40 °C

RW3 contained significantly less GRM (3.5 µmol g^{-1}; $P < 0.0001$), GRA (5 µmol g^{-1}; $P < 0.0001$), DGTB (not detected; $P < 0.0001$), and DMB (1.3 µmol g^{-1}; $P < 0.0001$) than the *E. sativa* cultivars at D0. In D4 samples, concentrations increased in RW3, however this was significantly lower than RS4 and RS8 for accumulation of GSV (1.3 ± 0.3 µmol g^{-1}; $P < 0.0001$). At D7, concentrations in RW3 declined, with RS4 and RS8 containing significantly more GRM, DGTB, and GSV (all $P < 0.0001$, Table 3.3).

Relative concentrations of GHPs were higher in RW3, particularly at D7, than the relative amounts of GSLs. The trend for the two *E. sativa* cultivars to contain higher abundances was however similar (Figure 3.2). At D4 these contained significantly greater concentrations of SF than both RW2/RW3 ($P < 0.0001$, Table 3.3), but by D7 there were no significant differences. The retention of SF throughout the shelf life period is an important finding that suggests that potent health-related effects (Sivapalan et al., 2018) may be present in rocket leaves up to a week postharvest, even after the imposition of severe abiotic stress.

3.6.2.6 Second cut at 40 °C

RS8 was the only cultivar tested that survived and regrew under the 40 °C treatment, and it also contained the highest observed GSL concentrations of any condition or cut (Figure 3.2). While no significant differences between each time point were observed, there is a clear trend for concentrations to increase at D7. This is one of the highest concentrations reported to-date for *E. sativa*, and it is clear that temperature response combined with cut in this cultivar

results in extremely high GSL concentrations postharvest. GHPs however were relatively low (Figure 3.3) with highest abundance at D4 (8.0 ± 1.1 µmol g^{-1}). This disparity between GSL and GHP abundances may be suggestive of myrosinase impairment or reduced activity.

3.6.2.7 Cultivar differences between growth temperature treatments

The disparity between rocket species GSL accumulations and GHPs is also evidenced when comparing broadly between growth temperature conditions. Table 3.3 contains the Type III Sum of Squares analysis results and reveals there are fewer significances between growth temperature and cut treatments for *D. tenuifolia* than *E. sativa* cultivars. This suggests that the former species is much less variable in terms of GSLs and GHPs, however (based on the two cultivars tested) is unable to achieve significant changes in health and flavour-related compounds between growth temperatures. This attribute is important however, and better for (potentially) maintaining uniformity of taste and flavour traits between temperature extremes. If the goal is to make rocket species more nutritionally dense, *E. sativa*

possesses a degree of environmental plasticity in response to different growth temperatures that lends itself well to synthesis of GSLs such as GRA and GER. RS4 and RS8 also produced greater concentrations of SF under each condition and cut, therefore making cultivars more efficacious against chronic diseases than the more commonly consumed *D. tenuifolia*.

Considering the factors contributing to differences in concentrations, *E. sativa* is significantly influenced by temperature, with relatively few compounds affected by the respective interactions between temperature, harvest (cut) and sample point. For RW2 indolic GSL concentrations (4MOB and NGB) were most significantly affected by each factor and their interactions. RW2 by contrast had the most variability for GRA and PRO concentrations. GHPs by contrast (regardless of species) were most significantly influenced by the cut number. This indicates that while total GSLs may not be significantly changed after second cut, GHPs are. This may reflect a change in the expression of respective genes and enzymes regulating hydrolysis rather than those involved in GSL biosynthesis per se, and may give rise to improvements in nutritional quality.

3.6.2.8 Effects of growth temperature on postharvest concentrations

Irrespective of cultivar or species, several significant associations between growth temperature and shelf life concentrations of GSL compounds were found. At D0 GER concentrations were significantly affected by growth temperature ($P = 0.012$), as well as total GHPs ($P = 0.027$). At D4, two significances were observed for the indolic GSLs GBR ($P = 0.001$) and 4MOB ($P = 0.002$). By D7 there were several GSLs and one ITC significantly associated with growth temperature; these were PRO ($P < 0.0001$), GSV ($P = 0.000$), 4MOB ($P = 0.000$), and SF ($P = 0.034$, Table 3.3). These data are of particular interest for two reasons: the first is that PRO and GSV are thought to contribute significantly to the taste and flavour profile of rocket leaves (Pasini et al., 2011; Raffo et al., 2018). Their relative increases/decreases over the course of shelf life may therefore alter sensory properties, and conceivably consumer preference (Bell et al., 2017b). The second is that SF is associated with health-related benefits, and therefore cultivars could be improved by selecting for plants able to form greater concentrations later into shelf life (e.g. RS8; Bell, Yahya, et al., 2017).

As presented in (Table 3.3), there were numerous significant interactions between cultivar, cut, and growth temperature, making exact predictions of postharvest concentrations and profiles difficult. It is clear however that growth temperature influences the potential nutritional and sensory status of cultivars, and goes some way to explain the inconsistencies observed by growers and processors between growing regions and cuts of the same cultivar.

3.6.3 Principal component analysis

Figure 3.4 shows PCs 1 and 2 of the PCA analysis and explain 40.87 % of the observed variation within the data. PC1 separates predominantly for total GSL content, as well as DGBT, GSV and DMB. PC2 by comparison separates strongly for SAT and total GHP formation. This is of note because it indicates that a high GSL concentration does not necessarily correlate with high GHP formation. To give two examples; first cut D0 RS4 plants grown at 30 °C contained relatively reduced concentrations of GHPs compared to the observed GSLs. Conversely, second cut plants of the same cultivar and temperature saw marked increases in GHP formation relative to GSL

concentration, which was largely unchanged between cuts at this temperature.

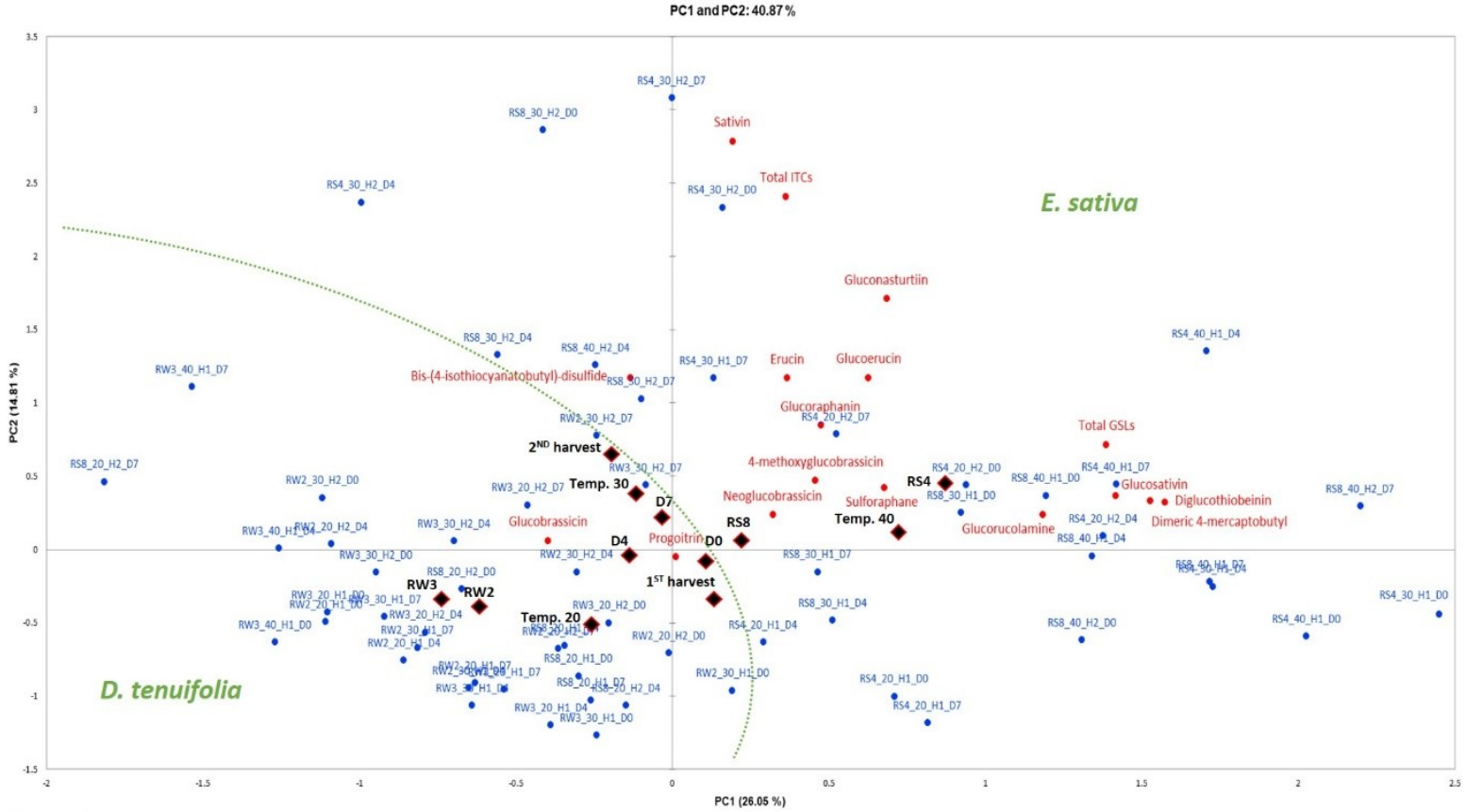

Figure 3.4: Principal Component Analysis biplot of glucosinolate and hydrolysis product concentrations or rocket cultivars grown at four different growth temperatures. Components PC1 and PC2 are presented and represent 40.87 % of total variation. Blue data points = sample loadings; red data points = glucosinolate and hydrolysis product scores; black diamonds = cultivar, cut, shelf life time point, and temperature centroids. The dotted green ellipse emphasises the clustering of RW2/3 from the salad leaves RS4/8.

PC1 also separates for species (Figure 3.4), as it is clear that the *D. tenuifolia* cultivars tested are generally low accumulators of GSLs, and form relatively few GHPs compared to RS4 and RS8. As a proportion of the overall GSL profile, RW2 and RW3 contained greater concentrations of indolic GSLs (such as GBR) as well as PRO (Table 3.2).

As highlighted in previous studies however, concentrations of GSLs/GHPs are not in and of themselves indicators of perceived sensory traits such as pungency; so it may be that the stark differences in the species' profiles may not be reflected in their taste and flavour attributes (Bell et al., 2017a). Other modulating influences such as sugar concentrations may also affect this, so it is therefore important to note that pungency is not indicative of health-related benefits and vice versa. A salad rocket may, for example, be very mild tasting but still potentially contain many fold-higher amounts of GHPs which are masked by other compounds.

3.7 Conclusions

This paper has demonstrated the effects of cultivation temperature and multiple harvests on postharvest GSL and GHP concentrations in rocket species. While it has been anecdotally accepted by growers that pungency increases according to the number of cuts a crop receives, very few previous studies have accounted for this common horticultural practice.

Temperate grown crops ($\sim$20 °C average outdoor summer temperatures) are often noted for their less pungent aroma and flavour than those from hotter countries (such as Italy, Portugal, and Morocco). Our data show that total GSL concentrations between growth temperatures are not significantly affected, but that it is the abundance of GHPs produced which differs (Figure 3.1). It is clear and unsurprising that growth at 40 °C is detrimental to plant development and regrowth, however it is also apparent that there is a significant increase in GSL biosynthesis, and also SF formation postharvest. Our data also highlight that some *E. sativa* cultivars may be better adapted to growth under extreme temperatures, as RS8 showed remarkable tolerance to the 40 °C treatment, and a propensity for in-

creased SF productions under these conditions. More research will be required to determine if this tolerance is indicative of the species more widely when compared with *D. tenuifolia*.

Rocket crop growth under protected conditions can routinely reach or even exceed 40 °C, especially in summer months in countries such as Italy. With such extremes in temperature likely to increase in future due to climate change, it is important to determine the effects on nutritionally dense crops such as rocket. It is likely under such conditions that yields and production will be reduced, but that the nutritional density of crops may actually increase.

The GSL data presented are in agreement with previous studies of other Brassicaceae species (see Bell and Wagstaff, 2017 for a summary) however few other studies have also analysed GHPs in tandem. Our data suggest that it is incorrect to assume that GHP profiles and abundances are affected in a similar fashion to GSLs under different growth temperatures. Fluctuations in GHP abundance and conversion from GSLs is related to both environment and genotype. This is consistent with observations found in Brassica vegetables, where the concentrations of hydrolysis products is typically much

less than the total concentration of the GSL precursors (Hanschen and Schreiner, 2017). GRA conversion to SF varied from 0.9 % (RW2, second cut, D7, 20 °C) to 25.1 % (RS8, second cut, D4, 40 °C); and GSV/DMB conversion to SAT (Fechner et al., 2018) from 1.7 % (RS4, first cut, D7, 20 °C) to 100 % (RW3, first cut, D7, 40 °C) (Table 3.2).

The relative changes in the formation of these compounds between growth temperatures indicates that there is an environmental effect upon myrosinase. This may be in terms of total plant content and/or activity, but the differences observed here between genotypes suggests that this is also as a result of genetic variation. The focus of breeding should therefore shift from selecting cultivars with high GSL concentrations, and more towards those that convert them to GHPs most efficiently. This may involve selection for different myrosinase genotypes, but could also feasibly extend to epithiospecifier modifier proteins, such as ESM1, which promote ITC formation (Angelino and Jeffery, 2014).

There are many factors regulating and inhibiting hydrolysis of GSLs other than nascent myrosinase abundance and activity; such as pH,

temperature, ascorbic acid concentration, and enzyme co-factor presence/absence. While the kinetics of isolated compounds and myrosinase are well understood, it is still unclear how regulatory mechanisms within plant matrices control GHP formation and abundance. It is important to better understand the postharvest hydrolysis of GSLs since any health-related impacts of consuming Brassicaceae foods (such as rocket) will depend greatly upon pre-harvest environment, rather than postharvest storage conditions alone.

3.8 References

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Chapter 4

Postharvest changes that occur in ready-to-eat wild rocket in the domestic environment

4.1 Background

This paper aims to expand on the work carried out in chapter 3, which examined the preharvest environmental factors of species/cultivar and temperature on the subsequent growth and GSL/GHP content over a typical postharvest storage period. The GSL/GHP content of rocket is an important quality factor as these compounds are critical

to the characteristic flavour and aroma of rocket (Bell et al., 2017). Therefore, understanding how these compounds change over time is an primary factor in understanding how rocket quality changes postharvest. By examining several different potential markers of postharvest longevity, a greater understanding of the postharvest changes within rocket leaves was obtained. An intention of this research paper was to develop a method for the automated analysis of visual quality of rocket leaves. Unlike iceberg lettuce, rocket leaves do not have a distinctive postharvest visual disorder that is easily quantifiable, such as enzymatic browning/pinking. There are common disorders such as senescence due to loss of chlorophyll, but this is typically manifested during the growing stage (Koukounaras et al., 2007). However, compared to other crops such as wholehead lettuce or kale, where the leaves are mechanically stronger, baby leaf salads often have textural issues (Saini et al., 2017). Baby leaves, such as rocket, have a relatively high respiration rate that can manifest as excess moisture build-up in the packaging. The "sogginess" is a common complaint from consumers regarding rocket leaves (Chapter 6, Figure 6.3). The result of excess moisture does contribute to the degradation of visual quality but does not necessarily manifest

in a form that is quantifiable in terms of colour, e.g. darkening of leaf tissue.

The method developed to try and overcome these issues involves taking the probability distributions of hue angles for two images that were to be compared (e.g. an image of fresh leaves versus an image on day 20 postharvest) and calculates the distance between these distributions to assess similarity, with the greater the deviation from the score for the fresh image the lower the visual quality. Compared with many older methods of assessing visual appearance by using mean values of point measurements, this method more closely represents how a human assessor views an image as all information is taken into account. One of the key aims of this paper was to evaluate the variation of various postharvest indicators over multiple seasons, as this is often lacking from papers studying markers of shelf life. As food production changes location throughout the year to maintain a steady supply, there are many different changes in how the crop is produced. Furthermore, growing conditions throughout and between seasons can be highly variable. Therefore, for any given marker to be reliable as an indicator or postharvest longevity, it must

not be unique to any given growing environment or method.

4.2 Contribution to the project

Jake Jasper: Data curation, Method Development, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing.

Carol Wagstaff: Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Martin Chadwick Writing - original draft.

Stephen Elmore Supervision, Writing - original draft.

Contribution breakdown percentage by each author respectively [70, 20, 5, 5]

The following sections in this chapter have been submitted to Post Harvest Biology and Technology

4.3 Abstract

On-pack dates for RTE rocket salads are by law given as “use-by” in order to protect the consumer from pathogenic microorganisms that may otherwise cause harm to health. These dates are chronologically consistent, usually seven days from the date of packing, and do not reflect any information about the biology of the crop in the pack. A consequence of the current date-labelling approach is that food waste is increased because consumers discard food that is either out of date, or which degrades in appearance before the on-pack date is reached. Discovering markers that can dynamically monitor the in-pack condition of fresh produce such as rocket is, therefore, desirable to reduce food waste, as would be a means of more accurately assessing the potential shelf life of the crop at the point of intake to the packing factory. We assessed quality markers based on imaging, microbiology, sugars, ammonia, nitrates, glucosinolates and their breakdown products throughout an extended shelf life of three independent trials of bagged rocket salad. The trials illustrated the considerable variability that exists between different consignments of the same crop, with the second trial lasting half

as long as the other two. Our results demonstrate the potential of absolute concentration of sugars, nitrates and ammonia at intake as potential predictors of shelf life and the potential of ammonia concentration changes to provide real-time dynamic information on the quality of leaf inside the pack. Both approaches will help suppliers, retailers and consumers to accurately assess and predict shelf life, thereby potentially reducing food waste.

4.4 Introduction

In the UK, rocket salads are generally consumed as a ready-to-eat (RTE) product and typically have a post-pack life of seven days. The given shelf life (SL) of a product is set to avoid type II errors (false negative) where food is sold that is "injurious to health," or that does not meet the quality expected but is indicated to be otherwise. The date set is a compromise between giving the consumer enough time to eat the product, not exposing the consumer to any microbiological harm, and providing the quality they expect. Type I (false positive) and type II errors are inversely related, as type II errors are minimised, there is a higher chance of food that is accept-

able to be consumed being discarded.

As with all biology, the organism is the product of its genetics and environment, and therefore, many different factors can affect the SL of a product (Chapter 3 3; Siomos and Koukounaras, 2007). It might therefore be expected that the SL would change accordingly, but in spite of the inherent variability of SL the on-pack dates are remarkably consistent and are rarely adjusted irrespective of quality assessment at intake to the packhouse. One of the issues is that processors do not currently have the data required to more accurately predict the "quality" of the crop and, therefore, must be relatively conservative. One of the primary challenges with defining more accurate models of SL is to find robust markers that can be used to measure and predict what the SL for any given consignment should be. With the advance in agricultural sensing technologies, packaging sensors and Internet-of-things devices, far more data are being collected in all parts of the supply chain (Jasper et al., 2021). It is becoming possible for data to inform real-time feedback to, and from, the consumer about the condition of any individual product (Wang et al., 2015). Furthermore, these methods may be useful to retailers who

could implement dynamic pricing, which can also reduce food waste (Buisman et al., 2019). When referring to food waste, we are particularly concerned with food available to the consumer that is fit for consumption and does not consider losses in production or typically unconsumed elements such as peel.

Specific data for leafy salads is hard to come by as it is usually incorporated into a larger category when statistics are published – usually fruit and vegetables. However, in 2012, it was estimated that around 20 % of leafy salad purchases ended up as avoidable waste (Quested and Murphy, 2014). Overall, food waste from retailers is estimated to be around 5 % (Welch et al., 2018).

The objective of this study was to identify potential biological markers that could be used to define SL of rocket salad more accurately, to study the variation in markers between seasons and between individual bags, and to use the non-destructive method of RGB imaging to analyse quality. This may enable us to find markers of quality which allow for the continuous monitoring of product throughout SL, providing real time feedback to the consumer which in turn may help to reduce food waste.

4.5 Materials and Methods

4.5.1 Plant material

Growing information for each trial is shown in Table 4.1. All plant material (*Eruca Sativa*) were obtained from the central distribution centre within 24 hours of arrival and transported in ambient conditions to The University of Reading. Upon arrival the samples were immediately stored in a walk-in refrigerator at 4 °C in the dark, until analysis. Sixteen replicates were analysed at each timepoint. After sampling the leaves were transferred immediately into a -20 °C freezer.

Frozen leaves were lyophilized in batches for four days. Leaves were then ground into a fine powder using a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ, USA) and sieved.

Table 4.1: Environmental Variables for rocket leaves grown in Italy (IT) and the United Kingdom (UK)

ID	Product	Season	Location	Growing conditions	Time of harvest to packing date (days)	Pack to use-by date on pack (days)	Average temperature in month to harvest (°C)
R1	"Wild Rocket" 90g	Nov 18	Battipaglia - IT	Tunnel	6	7	21
R2	"Wild Rocket" 90g	May 19	Chioggoa, Battipaglia, Taglio - IT	Tunnel	6	7	25
R3	"Wild Rocket" 90g	Sep 19	Warwickshire - UK	Tunnel	3	6	18

4.5.2 Imaging and colour analysis

4.5.2.1 Image capture

Images were captured from an RGB camera (Table 4.2) mounted on a tripod inside a custom built light-box. The light-box contained four tube-lights (Phillips TL-D 58W/835). An Xrite Color Passport was placed in shot to allow for colour correction. Images were captured in the raw format. Leaves were placed on the background with two centimetres around of space between leaves to aid with background extraction, and the leaves were arranged so they filled the camera's field of view which was typically around 20 leaves (Appendix Figure 4.13).

Table 4.2: Camera settings

Variable	Value
Camera	Canon G9x mk2
Shooting mode	Manual
ISO	200
Picture mode	Neutral
Aperture size	5.6
Exposure time	1/80
Focus range	Macro
White balance	Custom

4.5.2.2 Image processing and colour statistics

Raw images were processed in RawTherapee (v5.7). Each image contained an x-rite ColorChecker Passport Photo 2 (Michigan, USA), which was used to adjust the colour of the images with reference to a master image to ensure colour constancy between images. Adjusted images were then exported with no compression as png file type.

Colour statistics were calculated from individual leaves within each

image by a custom Python (v3.6) script utilising the packages PlantCV (v3.0) and scikit-image (v0.16.2).

4.5.3 Extraction and quantification of ammonia

Fifty milligrams of milled lyophilized plant material was added to 1 mL of deionised water. The mixture was then sonicated at ambient temperature (22 °C) for 30 minutes prior to centrifugation for 15 minutes (16,050 x g, ambient temperature). The supernatant was then filtered through a 0.45 μm syringe filter (Cole-Parmer) before ammonia analysis. The same extract was used for nitrate and nitrite determination (section 4.5.7).

Ammonia was quantified by enzymatic determination through reaction with α-ketoglutaric acid (KGA) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of L-glutamate dehydrogenase (GDH) (Merck). GDH reacts specifically with ammonia to form L-glutamate and oxidized nicotinamide adenine dinucleotide phosphate (NADP+) products. The oxidation of NADPH, which is proportional to the ammonia concentration was measured by absorbance in triplicate at 340 nm using a Jenway 6315 spec-

trometer (Stone, UK).

4.5.4 Enumeration of aerobic bacteria

Samples for microbial analysis were taken and analysed immediately after imaging. Enumeration of aerobic bacteria was carried out using 3M's Petrifilm Aerobic count plates (3M, St Paul, MN, USA). Ten grams of leaves were added to 90 mL of sterile 0.85% w/w Maximum Recovery Diluent (Merck) and then homogenized in a Stomacher 400 (Seaward, UK) for 1 minute at 260 rpm as presented by Wood et al., (2015). Ten-fold serial dilutions of the homogenate were plated onto Petrifilm plates then allowed to incubate for 48 hours at 30 °C before the colonies were counted.

4.5.5 Extraction and quantification of chlorophyll A, B and total carotenoids

Fifty milligrams of milled lyophilized material was added to 1 mL of Dimethylformamide (DMF) anhydrous (Merck) and stored in the dark at 4 °C for 48 hours before filtering through a syringe filter

(0.45 μm) into fresh Eppendorf tubes. Samples were diluted by combining 4 mL of DMF with 1 mL of sample and pipetted into BRAND UV semi-micro cuvettes (Merck) prior to measurement. Each samples absorbance were measured at wavelengths: 480, 647, 664 nm using a Jenway 6315 spectrometer, each sample was measured in triplicate. Quantification was carried out using formula presented by Wellburn (1994).

4.5.6 Determination of Glucose, Fructose and Sucrose

Free monosaccharides were extracted according to the method presented by Bell et al. (2017), with the exception that 20 mg of lyophilized leaf powder was extracted. Extracts were analysed on an Agilent 1100 series HPLC system equipped with a binary pump, degasser, and auto-sampler, with an external column heater (30 °C). A Bio-Rad Aminex HPX-87H (300 $\times$ 7.8 mm, 9 μm particle size) column with a Micro-Guard Cation H guard column (Bio-Rad, Watford, United Kingdom) was used to achieve separation with an isocratic gradient of 5 mM sulfuric acid, and a flow rate of 0.3 mL per min. A Polymer Laboratories ERC-7515 refractive index detector

(Church Stretton, United Kingdom) was used to detect monosaccharides. Compounds were quantified using authentic standards and analyzed with Agilent ChemStation software (Santa Clara, CA, United States).

4.5.7 Determination of Nitrite and Nitrate

Extractions were prepared as described in section 4.5.3 above. Samples were diluted 1/100 prior to analysis for nitrate and nitrite concentration. The analysis of samples was carried out as described in Kramkowski et al. (2017). Briefly, the method uses HPLC to separate nitrate and nitrite from the sample on a NO-PAK column (Eicom). Nitrate is then reduced to nitrite on a NO-RED column (Eicom), after which the nitrite reacts with a Greiss reagent to form a light absorbing compound which is then detected at 540 nm using a DAD detector. The HPLC system used Eicom's ENO-30. The flow rate of the carrier solution was 0.33 mL.min^{-1} and 0.1 mL.min^{-1} for the reactor solution. The column temp was set to 35 °C and the run time was 8 minutes. Compounds were quantified using authentic standards.

4.5.8 Determination of Glucosinolates and Isothiocyanates

The extraction and analysis was identical to that of Jasper et al., (2020). Only compounds for which external standards were obtained are reported.

4.5.9 Visual Quality

Visual quality was determined by visually assessing each image and categorising each image as good, marginal or poor quality based on the interpretation of the researcher (Appendix Figure 4.10), where good quality exhibited no yellowing and less than 5 % damage. Discoloured tissue was determined visually with reference to the HSV (hue, saturation, value) colour space, damaged tissue values around [90,0:100,20]. The visual assessment did not take place on the same day as sampling, rather the images were assessed during the analysis. Marginal quality exhibited minimal yellowing, less than 10 % leaves damaged and minimal moisture build-up on leaves. Unsatisfactory quality was anything in excess of marginal quality in terms of yellowing and moisture accumulation.

4.5.10 Statistical analysis

Ordinary least Squares analyses of all data were performed using statsmodels v0.12.2 (Seabold and Perktold, 2010). Each respective analysis was conducted with a post hoc Tukey's Honest Significance Difference (HSD) test ($p < 0.05$). To compare the differences between the hue distributions of each image, the Hellinger distance was used, which compares probability distributions. The formula used is shown in equation 4.1

$$h(image1, image2) = \frac{1}{\sqrt{2}}.||\sqrt{image1} - \sqrt{image2}||_2 \qquad (4.1)$$

Where $image1$ and $image2$ are the hue distributions of the respective images

4.6 Results and Discussion

Three independent trials were conducted R1 (Italy), R2 (Italy) and R3 (UK), all using protected tunnels (Table 4.1). For both R1 and R3, the trials continued to the end of the planned three weeks. However, R2 deteriorated very quickly after the first week and at 14 days the leaves had severely degraded and became impractical to image and therefore the trial was stopped.

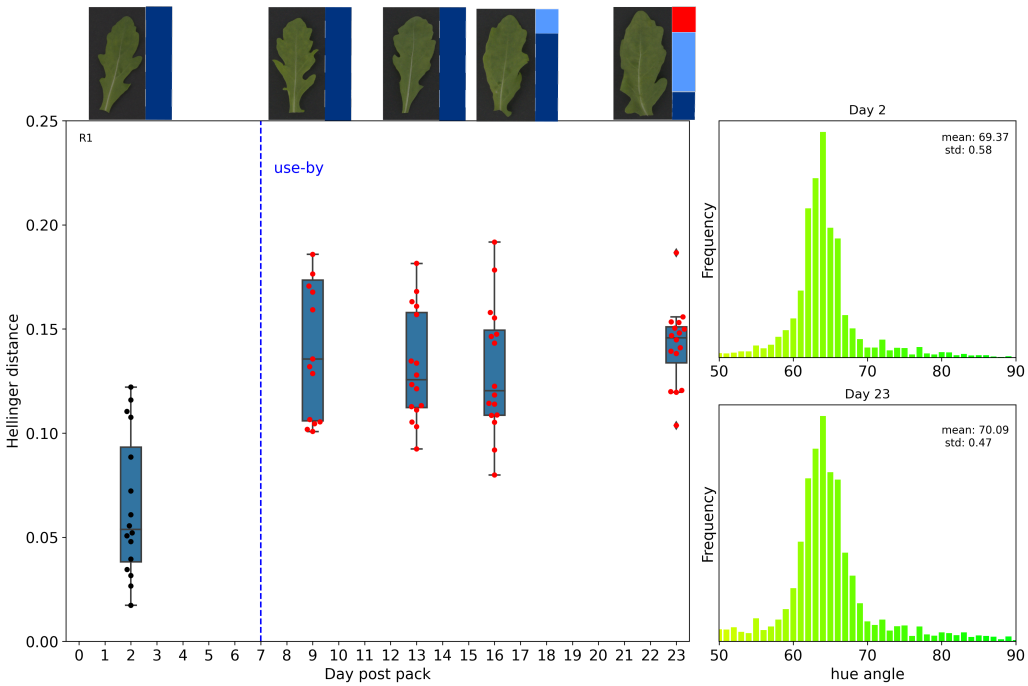
Table 4.3: The proportion of samples designated as good, marginal or poor for all rocket leaves asssessed visually. R1, R2 and R3 are three seperate batches of commercial RTE ricket grown in Italy (Nov 18), Italy (May 19) and the UK (Sep 19). For each batch there were 96 samples in total with 16 randomlly selected samples for analysis at each time point.

Trial	Day post-pack	Visual Quality (Good:Marginal:Poor)%
R1	2	100:0:0
	6	100:0:0
	9	100:0:0
	13	100:0:0
	16	94:6:0
	23	25:56:19
R2	2	100:0:0
	5	87:13:0
	9	87:13:0
	12	0:0:100
R3	1	94:6:0
	4	94:6:0
	8	100:0:0
	11	81:19:0
	15	63:37:0
	21	13:25:62

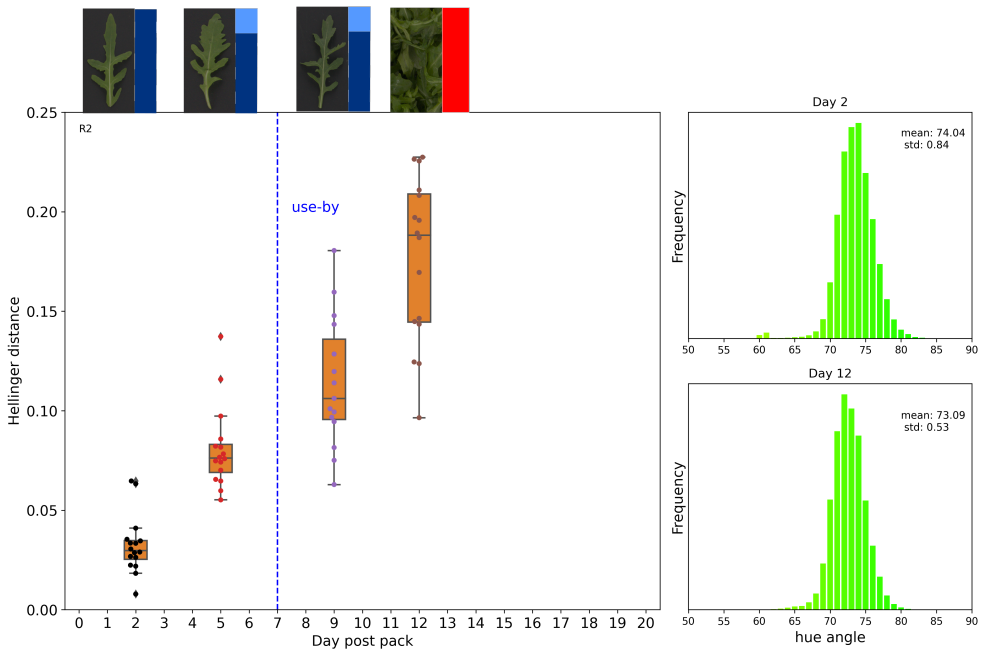
From visual inspection (Table 4.3), the first trial R1 did not reach the point where the majority of the samples were classified as unsatisfactory. Both R2 and R3 had the majority of samples classified as unsatisfactory at the final time-point. Within the time-period that is typical for a RTE rocket salad ($\sim$ 7 days) there were no samples that were classified as poor. However, it is known many consumers

(Lyndhurst, 2008) do not rely solely on use-by dates in their decision making process for consuming or discarding a product. It is known that many consumers use visual appearance as their primary consideration when considering the quality of leafy salad (van der Laan et al., 2011) and therefore will potentially consume a product after the manufactures use-by date.

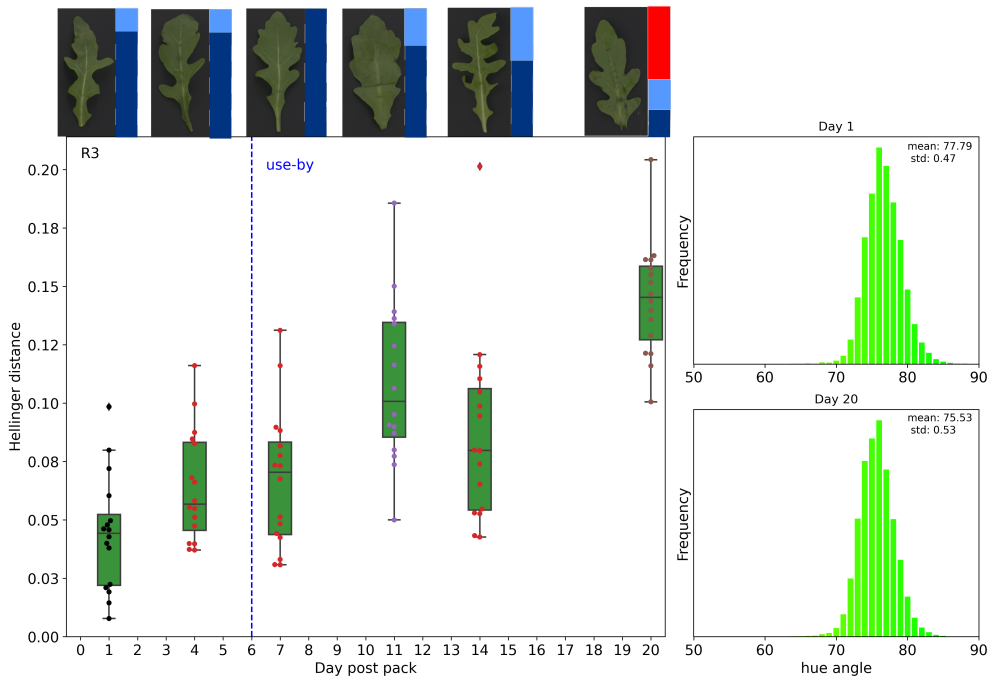
4.6.1 Visual quality by analysis of images



(a) R1


R2
use-by
Hellinger distance
Day post pack
Day 2
mean: 74.04
std: 0.84
Frequency
Day 12
mean: 73.09
std: 0.53
Frequency
hue angle

(b) R2



(c) R3

Figure 4.1: Histogram comparisons of normalised spectral data using Hellinger distance. Plots a, b and c are three independent trials grown in Italy (November 2018), Italy (May 2019) and the UK (September 2019). On the primary axis each boxplot represents the analysis of images at different time points. The average of each image taken throughout each trial was compared with the average image taken at intake to calculate the difference between the extracted hue histograms. The greater the distance, the bigger the difference in colour between two images. The coloured point markers within each plot indicate a significant difference in the mean distance of the hue histograms measured at each time point where $p < 0.05$. The secondary plots for each a,b,c are examples of the hue histograms at the indicated timepoints. For example, in R1, from day nine onwards all the point markers are red which means there are no significant difference between these timepoints, but they are all different from day two which has black point markers. For each time point in each plot n = 16. The leaves above the primary axis are representative images from each time point with the visual quality score represented by the dark-blue (good), light-blue (marginal), red (poor) bars next to the image.

There were no significant changes in the mean hue values for any trial (R1-R3) at any time point. When measuring every pixel in each leaf, the overwhelming majority of hues were the same value, as can be seen by the relatively narrow range of the histograms in (Figure 4.1). Given that there was also no difference over SL for chlorophyll measurements (Figure 4.2) it followed that there would not be a significant change in the mean colour of the leaves. Areas where there was clear deterioration only represented a small number of pixels, and therefore, have a small or no impact on the mean (Appendix Figure 4.12). Upon visual inspection (Table 4.3) it is clear that degradation did occur, but this was not captured by the mean or median values.

Comparing the histograms of hue values allows for smaller differences in colour to be accounted for. Histogram comparisons have been used in plant imaging for the detection of disease (Ali et al., 2017), water stress (Kim et al., 2015) and general leaf recognition (Munisami et al., 2015), but not for quality comparison in SL studies. The results show that over the postharvest period the colour of the leaves does change significantly from intake as distance between

the hue histograms increase, this is most notable in (Figure 4.3, b) which had the most severe degradation.

These results demonstrate that given a reference image for comparison, the change in quality can be monitored non-destructively. Given enough data regarding the pre-harvest conditions and consumer preferences this method may be used to model the rate of deterioration and predict when the consumer will reject the product.

4.6.2 Chlorophyll

It is chlorophyll or the degradation of, that is primarily responsible for the colour of rocket leaves. Therefore, if there were to be any significant changes in the visual appearance, it may be expected that there would be a change in the concentration of chlorophyll over postharvest storage, which could be quantified by measuring green pixels with image analysis.

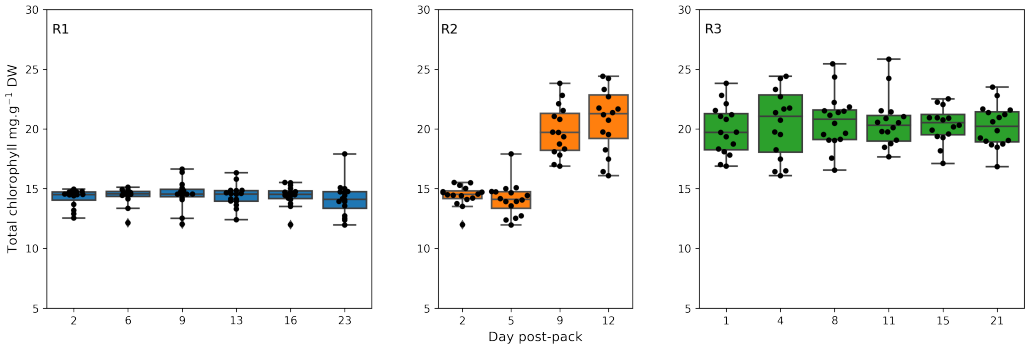


Figure 4.2: The changes in total chlorophyll over time for rocket leaves stored at 4 °C. There were no significant differences between any time points for any trial ($p < 0.05$) represented by no differences in the colour of point markers within each box plot. For each time point n=16.

There was no difference between the chlorophyll concentration of samples at any point for any trial. These results show that the visible differences were not due to the degradation of chlorophyll which is often cited as a reason for colour change (Yamauchi and Watada, 1991; Koukounaras et al., 2009). Although the concentration of chlorophyll within leaves did not change throughout SL, it can be observed that samples with the highest concentration (R3) also had the highest mean hue value (Figure 4.1, c). There are many factors that can influence chlorophyll concentration including genetics, growing and storage conditions. However, from these data the chlorophyll content measured in each trial was relatively similar with R3 having a slightly higher concentration than R1 which was also reflected in the mean hue angle (Figure 4.1, a). It is notable that

the chlorophyll concentration is much less variable in R1 than both R2 and R3. . There were no changes in the abundance of chlorophyll over the post harvest period from these trials, but the variance of chlorophyll between samples may be a proxy indicator of the growing conditions and subsequent postharvest longevity. The trial with the least variation in chlorophyll (R1) also had the least change in visual quality, and the inverse was true for R2.

4.6.3 Aerobic Colony Count and shelf life

Aerobic Colony Count (ACC) is a relatively general microbial test and, as such, is carried out for many different food items. Although it is not a measure of product safety, it is helpful to give an indication of the overall contamination of the product. It is often used as an indicator of quality, whereby an unsatisfactory result (10^6 – 10^7 cfu.g^{-1}) may indicate that there are sub-optimal conditions in the supply chain (Health Protection Agency., 2009). Given that microorganisms can be responsible for the spoilage of leafy salads and affect visual quality, ACC may be a useful early predictor. Without a measure of microbial status, it is not possible to fully appreciate

the extent of any given indicator of postharvest life.

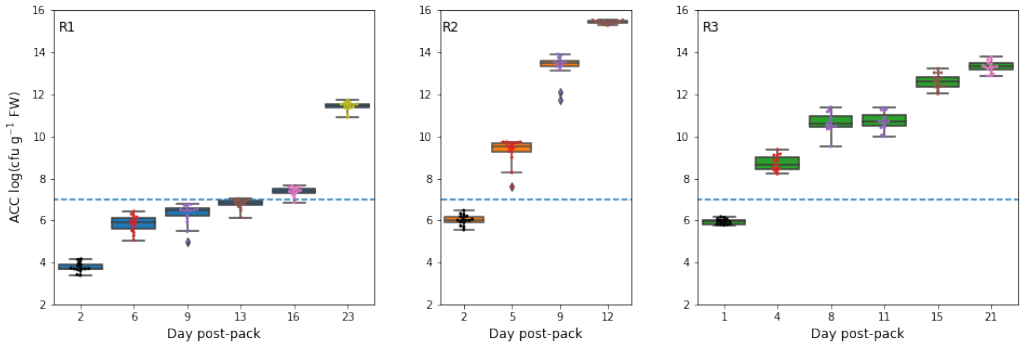


Figure 4.3: The changes in Aerobic Colony Count measured in rocket leaves throughout shelf life stored at 4 °C. Differences in colour for points represent statistical significance for the mean at each time point where p <0.05. The dotted horizontal lines represents 10^7 cfu.g^{-1} which is typically the upper threshold when referring to satisfactory and unsatisfactory quality. For all time points n=16.

Upon intake, all samples were in a range that would be considered satisfactory for ACC (Figure 4.3), which depending on the product and specification, is typically less than 10^7 cfu.g^{-1} (Health Protection Agency, 2009; Calonica et al., 2019). From visual inspection, samples did not become visually poor until the ACC was greater than 10^{13} cfu.g^{-1}, which is far higher than is recommended as a quality standard. With the exception of R2, it was typically one week after the products given use-by date that leaves became visually poor (Table 4.3). However, with R2, which arrived with a similar mean ACC as R3, the deterioration was much faster leading to the trial being stopped earlier than planned. These data show that the initial

bacterial load is not sufficient to predict subsequent growth, and the preharvest, along with supply chain conditions need to be taken into consideration for predicting microbial load.

4.6.4 Nitrates

Rocket is well known as a hyper-accumulator of nitrate, and there are legally defined limits for the concentration of nitrates in EU legislation (1258/2011, 2011). Along with glucosinolates, nitrates are a primary consideration when considering the nutritional quality of rocket leaves (Cavaiuolo and Ferrante, 2014). Furthermore, it has been reported that under high nitrogen availability, cell wall stability declines and cell nitrate content increases (Darlison et al., 2019). Therefore cell nitrate content may give an indication of the postharvest robustness of rocket leaves. Although there are many studies evaluating nitrates from a pre-harvest perspective (Kim et al., 2015; Signore et al., 2020) there are very few that look at the changes postharvest.

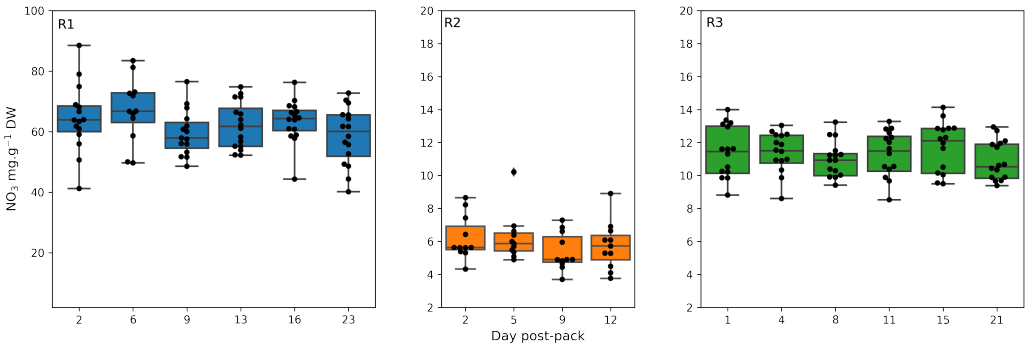


Figure 4.4: The changes in nitrate over time for rocket leaves stored at 4 °C. Differences in colour for points represent statistical significance for the mean at each time point where p <0.05. For all time points n=16.

Nitrate concentrations were highly variable between trials, but were stable over storage. These results are in agreement with (Kyriacou et al., 2019) where samples that were grown in the winter (R1) and Autumn (R3) had higher levels of nitrate than those grown in the summer (R2) and they also found that nitrate did not significantly change over SL. The data from this study and Kyriacou et al. (2019) suggest that preharvest conditions are the primary concern when determining the nitrate content of rocket leaves. As R2 had the lowest overall nitrate content, it is less likely that the cause of the severe degredation is due to cell wall degredation as a result of high osmotic pressure, if this was the case, R1 may be expected to exhibit the most degredation. However, the relatively low nitrate concentration may be an indicator of sub-optimal growing conditions. Although,

as the chlorophyll levels were not affected by the relatively low nitrate, which is a commonly attributed defect with low nitrate crops (Miras-Moreno et al., 2020), the mechanism as to why a low nitrate crop would degrade quicker is difficult to identify without further research.

4.6.5 Sugars

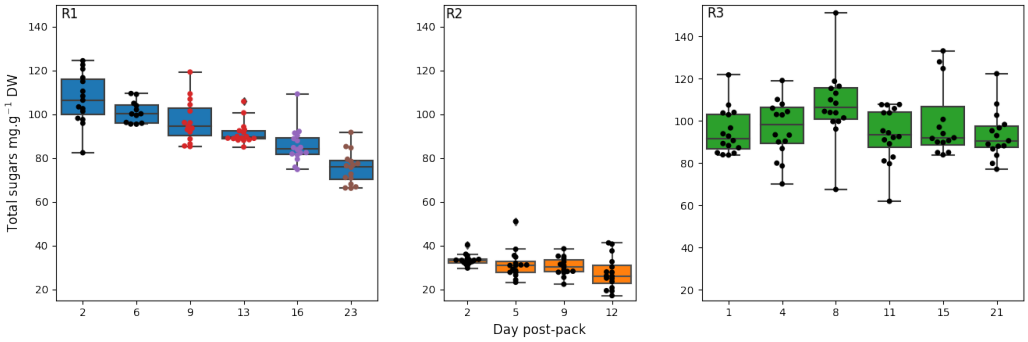


Figure 4.5: The changes in total sugars (Glucose, Fructose and Sucrose) over time for rocket leaves stored at 4 °C. Differences in colour for points represent statistical significance for the mean at each time point where p <0.05. For all time points n=16.

Glucose, Fructose, and Sucrose were very highly correlated (Appendix Tables: 4.4, 4.5, 4.6), and therefore the sum (Total sugars) will only be addressed (Figure 4.5). R1 was the only trial with a significant change over SL, where there was a loss of 1.7 ± 0.53 mg per day of storage. The further trials (R2 and R3) were stable over the storage period. The data suggest that monitoring the change in con-

centration of sugars or total sugars is not a good indicator of quality, since a change in sugar concentration does not always correlate with a change in leaf quality on the basis of appearance. However, the absolute concentration of total sugars at intake may well be indicative of future postharvest performance. In our experiments, R2, which had the lowest concentrations at the start of the experiment, deteriorated far quicker than R1 and R3, which had comparable sugar concentrations approximately three fold higher than R2 at the start of the experiment. Even at the final timepoint R1 sugar concentration was two times higher than the first R2 timepoint and R3 remained three times higher throughout. The relatively low total sugar concentrations in R2 may be diagnostic of poor storage conditions in the prior supply chain, whereby the crop has been held at sub-optimal temperatures, increasing respiration (Koukounaras et al., 2007). These data indicate that the leaves may have a minimum threshold of total sugars required to maintain postharvest quality throughout shelf life. Although dynamic changes in total sugars concentrations may not a useful marker of quality, it may be that sugars give an indication of pre harvest nutrition which, in turn, may be useful in assessing the SL potential of the crop.

4.6.6 Ammonia

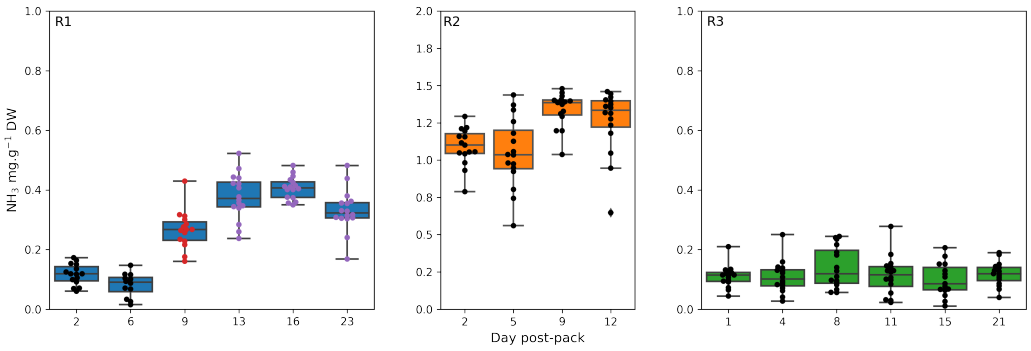


Figure 4.6: The changes in ammonia over time for rocket leaves stored at 4 °C. Differences in colour for points represent statistical significance for the mean at each time point where p <0.05. For all time points n=16.

Ammonia levels in R1 significantly increased over the first two weeks of SL, and then plateaued, whereas in both subsequent trials concentrations were constant throughout SL. Ammonia, has been associated with changes in colour (Mastrandrea et al., 2016), although the results were more compelling at temperatures greater than 5 °C. The data here do not show any relationship between any measure of visual quality and ammonia. There was a significant (p <0.0001) negative correlation (R = -0.58) between total sugars (Figure 4.5) and ammonia (Appendix Figure 4.9). It has been reported that an increase in ammonia in rocket leaves is due to a decrease in activity of glutamine synthetase, an enzyme which assimilates ammonia in protein metabolism (Mastrandrea, Amodio and Cantwell, 2016). Pre-

cursors for glutamine come from the Citric Acid Cycle (Sanderson et al., 2010), so it follows that an increase in sugar metabolism may have a subsequent increase in ammonia when the regulatory pathways for its removal have been disrupted by harvest (Commichau, Forchhammer and Stülke, 2006). However, no previous studies have considered a link between ammonia and sugar content with respect to postharvest disorders. Of the three trials R2 had the highest concentration of ammonia, the lowest concentration of nitrate, and was the fastest to deteriorate. These data add further evidence that ammonia may be a viable marker for quality when considering the overall concentration.

4.6.7 Glucosinolates (GSLs) and Glucosinolate hydrolysis products (GHPs)

Between all three trials, there was a high level of variance between GSLs, with a fortyfold difference between R1 and R2. However, similar results have been found in previous studies (Bennett et al., 2007; Hall et al., 2015). Many different variables are known to influence GSL concentrations, including environmental conditions, agro-

nomic practice, cut and stress (Villarreal-García et al., 2016). As the GSLs and subsequent breakdown products are desirable from a nutritional perspective, there has been a great deal of effort to increase these compounds (Del Carmen Martínez-Ballesta et al., 2013; Yang et al., 2015) and inducing a stress effect is often effective, although it does seem to depend on which GSL is measured as to whether the stress increases or decreases the GSL (Cocetta et al., 2018). The relatively large difference between R2 and R1/R3 may be influenced by increased stress, consistent with the severely reduced postharvest life compared with the other trials and may further indicate sub-optimal supply chain conditions.

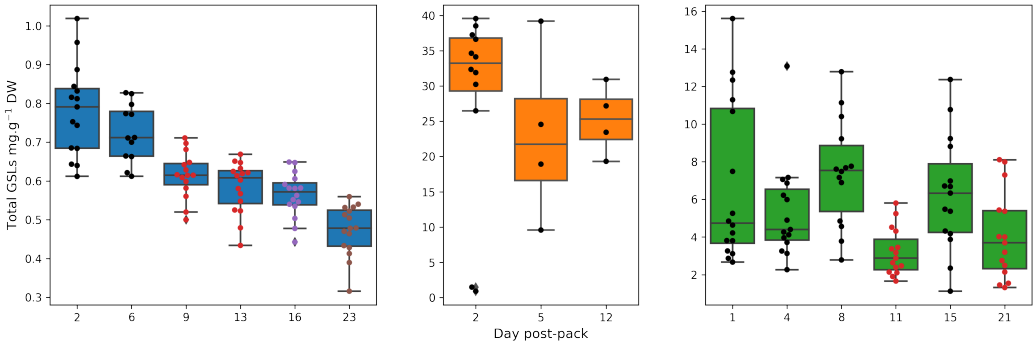


Figure 4.7: The changes in total glucosinolates over time for rocket leaves stored at 4 °C. Differences in colour for points represent statistical significance for the mean at each time point where p <0.05. For R1 and R3 at all time points n=16, For R2 n=[16,4,4].

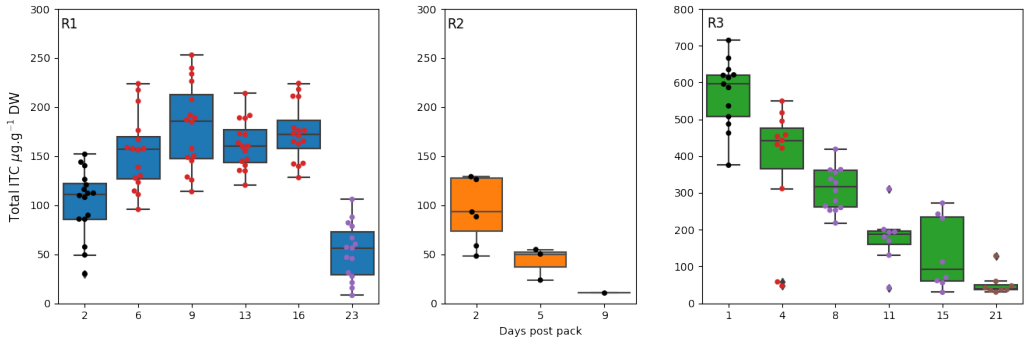


Figure 4.8: The changes in total Isothiocyanates over time for rocket leaves stored at 4 °C. Differences in colour for points represent statistical significance for the mean at each time point where p <0.05. For R1 and R3 at all time points n=16, For R2 n=[6,3,1].

Between all three trials, there was a high level of variance between GSLs, with a fortyfold difference between R1 and R2. However, similar results have been found in previous studies (Bennett et al., 2007; Hall et al., 2015). Many different variables are known to influence GSL concentrations, including environmental conditions, agronomic practice, cut and stress (Villarreal-García et al., 2016). As the GSLs and subsequent breakdown products are desirable from a nutritional perspective, there has been a great deal of effort to increase these compounds (Del Carmen Martínez-Ballesta et al., 2013; Yang et al., 2015) and inducing a stress effect is often effective, although it does seem to depend on which GSL is measured as to whether the stress increases or decreases the GSL (Cocetta et al., 2018). The relatively large difference between R2 and R1/R3 may

be influenced by increased stress, consistent with the severely reduced postharvest life compared with the other trials and may further indicate sub-optimal supply chain conditions. Although there were large differences in GSL concentrations between trials, there was no clear correlation with the subsequent breakdown products. It may be expected that there would be a high correlation between the concentration of precursor (GSL) and subsequent breakdown product (GHP), although with a time delay and the degree of conversion dependent on myrosinase. However, R2 had 40x more GSL than R1 but had comparable concentrations of GHP. The disparity between GSL and GHP concentration was also found by (Jasper et al., 2020), where crops grown at a higher temperature accumulated a higher concentration of GSLs but had a relatively low GHP concentration compared to crops grown at lower temperatures. The discrepancy between GSLs and GHP may be indicative of impairment to myrosinase, particularly when considering R2. The ratio of GSL to GHP may add further evidence that the crop had sustained damage, possible elevated temperatures prior to arrival at the retailer.

In all three trials, of the GSLs detected, glucoraphanin (GRP) was

predominant and represented 65-97 % of the total GSLs by weight. Sulforaphane was the only glucosinolate breakdown product to be detected in all samples. This was not particularly suprising considering that GRP was the most abundant GSL, accounting for 70-100 % of total GHPs detected. GRP is the direct precursor to sulforphane and is produced by the enzyme myrosinase upon disruption of the cellular structure (Gu, Guo and Gu, 2012). Sativin was also detected in trials R2 and R3, as with sulforphane, there were only significant differences in R3 where there was a decline over the first three time points ($p < 0.001$) whereafter there was no significant change until the final time point.

The composition of compounds within salad leaves are highly variable, and GSLs in brassicas are no exception, depending on many different factors such as, cultivar (Bell et al., 2017) and preharvest conditions (Hall et al., 2015; Jasper et al., 2020). The overall trend of the data is that glucosinolates decrease over SL (Figure 4.8), these findings are in contrast to those observed by (Bell et al., 2017; Hall et al., 2015). However, we did observe instances of GSLs increasing over SL such as progoitrin, epiprogoitrin, 4-hydroxyglucobrassicin

and glucotropaeolin (data not included), but as they are in relatively low abundances they had little impact on the overall trend.

Although, there were large differences between the abundance of [GSLs/GRA] between trials, this was not reflected in the corresponding sulforaphane concentrations which were relatively consistent with a decline over the postharvest period. The implication of this is that sulforophane may be a reliable marker of SL and quality. GSLs, and their subsequent breakdown products, are of often remarked for their health benefits (Traka and Mithen, 2009). The data presented here show that these health promoting compounds are reduced over SL. However, as the data also show, the change over SL is much lower than the variance between trials, and as such the preharvest factors are more important in determining the abundances of potential health promoting compounds.

4.7 Conclusion

Many different compounds, have been tested for potential markers of quality, but as we have demonstrated, almost all of these markers

are more variable between trials than over SL within a trial. Of all the variables measured, ACC had the highest correlation with visual quality (Appendix Figure 4.11) which has been determined previously (Martínez-Sánchez et al., 2006; Wieczynska et al., 2016). It was clear that R2 provided a better growth environment for aerobic microorganisms, evidenced by the growth rate. However, for R2, there were no significant changes in any other variables than ACC over the postharvest period providing no evidence that microorganisms influenced any of the variables for this trial. It is well known that micro-organisms are related to the spoilage of leafy vegetables and contribute to a slimy/watery appearance (Jasper et al., 2021). However, from these results, it is unclear as to the direct impact of microorganisms on any of the biochemicals assessed.

In the trial that had the most severe degradation (R2), there were relatively low sugars and relatively high ammonia concentrations, when compared with other trials , which may be indicative of a crop with a low SL potential. It is known that a more stressed plant will produce less chlorophyll (Helena et al., 2017) which may explain help to explain some of the findings for R2, where it is suspected

the growing conditions may have been sub-optimal. Whilst it is not clear if any single marker, or combination of markers will reliably be able to predict the time a product will take to degrade. There are measurements that may have given a clue as to the longevity of the product. R2, which had a relatively low abundance of sugars and nitrate and a high concentration of ammonia, may have given a predictive indication that the crop would degrade quicker than average, which would allow retailers and suppliers to adjust their SL accordingly providing there were no safety implications. However, further repetitions and data collection are required to confirm these findings and once reliable predictions can be made, with respect to SL of product under static conditions. To understand how the dynamic conditions of the supply chain and consumer handling of the product affects SL data need to be obtained dynamically which will allow the assumptions of models to be updated and more accurate SL predictions to be given and waste caused by on-pack dates to be reduced.

4.8 Appendix

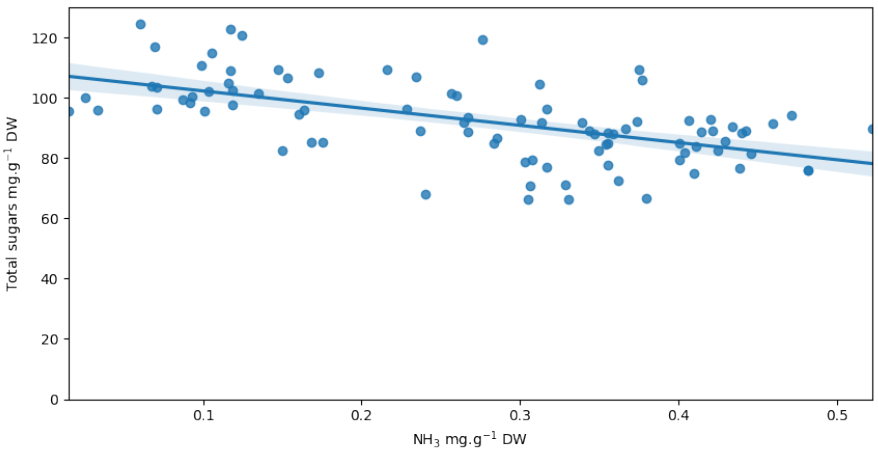


Figure 4.9: A scatterplot showing the relationship between Total soluble sugars and ammonia in ready-to-eat rocket leaves stored at 4 °C. The shaded area represents the 95 % confidence interval. These data were taken R3 and n=96.

No sign of yellowing, and
< 5% leaves damaged.

Minimal yellowing,
and < 10% leaves damaged.
Minimal
moisture on leaves

Clearly unnaceptable.
Lot's of decay;moisture on the leaves.
Greater than 10% leaves damaged

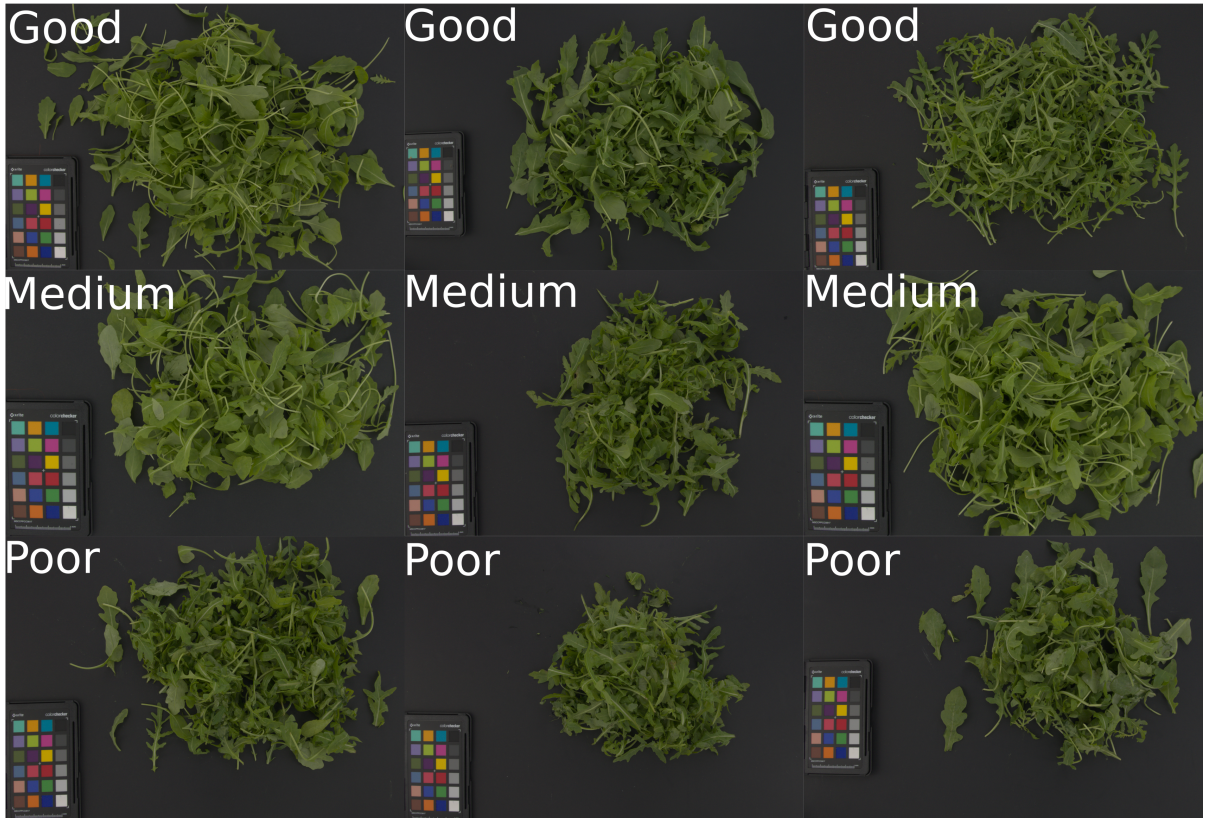


Figure 4.10: Quality guide used to visually assess rocket leaves for trials R1, R2 and R3.

Table 4.4: Pearson correlation and p-value for sugars extracted from rocket leaves (R1).

Trial 1 (R1)	Glucose	Fructose	Sucrose	Total
Glucose		0.63, <0.001	0.5, <0.001	0.87, <0.001
Fructose	0.63, <0.001		0.6, <0.001	0.87, <0.001
Sucrose	0.5, <0.001	0.6, <0.001		0.76, <0.001

Table 4.5: Pearson correlation and p-value for sugars extracted from rocket leaves (R2).

Trial 2 (R2)	Glucose	Fructose	Sucrose	Total
Glucose		0.68, <0.001	0.76, <0.001	0.93, <0.001
Fructose	0.68, <0.001		0.41, <0.02	0.87, <0.001
Sucrose	0.76, <0.001	0.41, <0.02		0.76, <0.001

Table 4.6: Pearson correlation and p-value for sugars extracted from rocket leaves (R3).

Trial 3 (R3)	Glucose	Fructose	Sucrose	Total
Glucose		0.78, <0.001	0.3, <0.003	0.85, <0.001
Fructose	0.78, <0.001		0.3, <0.003	0.89, <0.001
Sucrose	0.3, <0.003	0.3, <0.003		0.61, <0.001

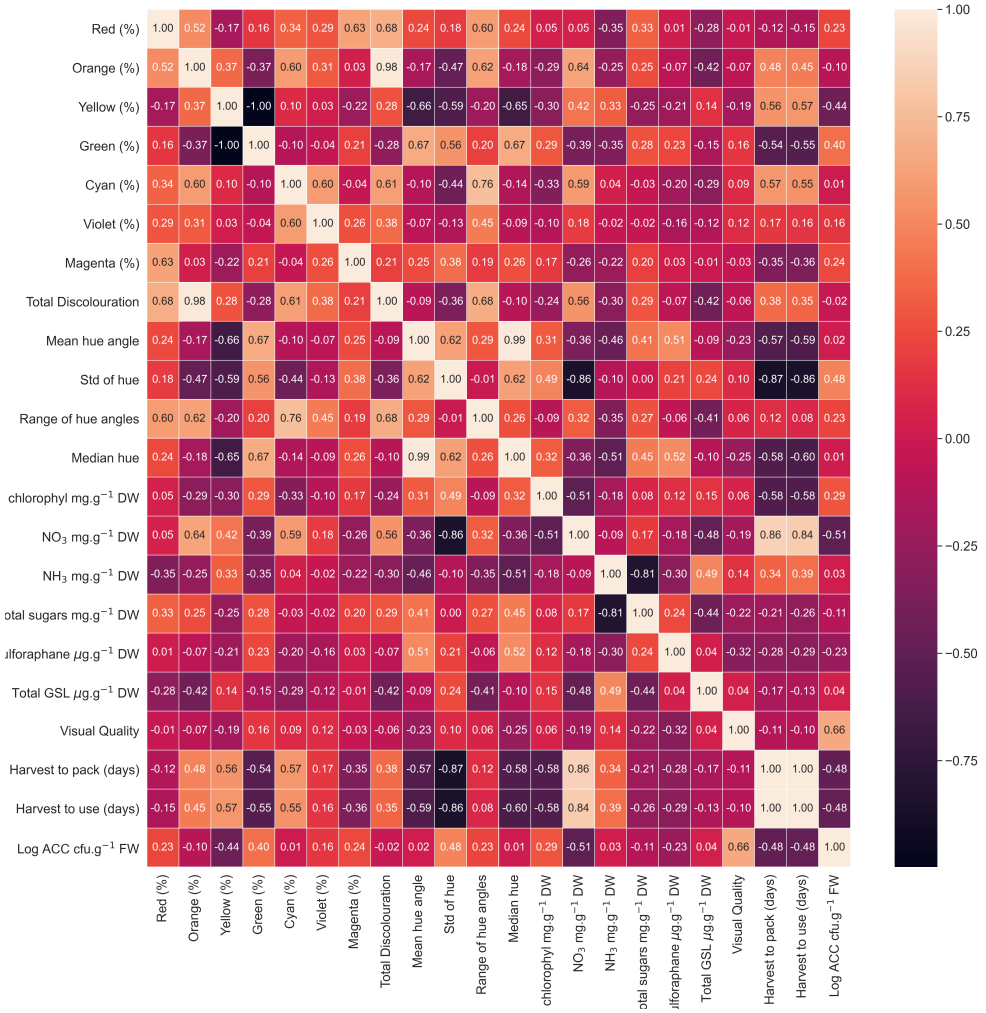


Figure 4.11: A heatmap showing the correlation of pooled data across the three trials - R1, R2, and R3

Figure 4.12: A comparison of the top 20 colours (by percentage) of two representative leaves from R3 taken at different time points. The mean hue for each is 77.7 and 75.5 for days 1 and 20 respectively

Figure 4.13: [
An example of the image capture of rocket leaves.]An example of the image capture of rocket leaves.

4.9 References

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Chapter 5

Postharvest changes that occur in ready-to-eat iceberg lettuce in the domestic environment

5.1 Background

The estimation of shelf life dates is inherently a stochastic process, given the variable nature of growing conditions and the handling of the product by the consumer. Where there is high variability around an estimation, to avoid overestimating the "true" use-by date and potentially harming the consumer, a conservative estimate is imple-

mented to avoid injury by consuming harmful products (De Corato, 2020). One of the reasons why date estimation has to be conservative is the lack of markers of the biological condition of the produce within the pack that could aid in the accurate setting of on-pack dates. Furthermore, once the consumer has purchased the product, unless the consumer follows the directions on the pack exactly, any shelf life estimate would become less accurate depending on the consumer's actions. Iceberg lettuce is a relatively robust crop and does not suffer as much as other leafy salads with textural defects such as wilting. However, fresh cut iceberg lettuce does have a particularly prominent visual disorder known as enzymatic browning, which manifests as brown/pink discolouration on the rib section of the leaves (Saltveit, 2018) and which often appears before the stated pack use-by date is reached. Given the relative importance of discolouration and its influence on rejection by the consumer, lettuce is a prime candidate for non-destructive testing using imaging equipment and subsequent image analysis. Developing a robust methodology for evaluating the rate of discolouration that could provide up to date information to the consumer may provide more reliable estimates of shelf life (Jasper et al., 2021). A positive unintended

consequence of developing a method to analyse the colour of images for this paper was that a function was developed that was later incorporated into the software package PlantCV, a software package that now encompasses many aspects of plant phenotyping. The functions extract all colour data from an image in the HSV colour space and calculate several different statistics such as circular mean, median and range (Fahlgren et al., 2019).

In Chapter 3, the differences in postharvest life as a consequence of variable growing conditions were highlighted. Whilst the crops were grown in homogenous conditions within each growth cabinet, variability in each condition was due to the inherent inter-plant variability. Due to the economics of commercial horticulture, many leafy vegetables are grown in open fields (Koukounaras et al., 2020). As opposed to controlled environments, growing crops in open fields introduces many environmental variables that influence the final product, including weather and pests. This chapter investigates the physiological, biological and microbiological changes in commercially grown lettuce across three independent field trials to identify key markers that can be used to predict shelf life more accurately.

5.2 Contribution to the project

Jake Jasper: Conception of study, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing.

Carol Wagstaff: Conception of study, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Martin Chadwick Writing - original draft, Writing - review & editing

Stephen Elmore Supervision, Writing - original draft, Writing - review & editing

Contribution breakdown percentage by each author respectively [70, 20, 5, 5]

The remaining sections have been submitted to Post Harvest Biology and Technology.

5.3 Abstract

Lettuce is one of the most common leafy vegetables sold at supermarkets in the UK. Over the past couple of decades, ready-to-eat (RTE) pre-cut lettuce products have become more popular, and other than the physical nature of the RTE products, there is also a legislative difference. Any product sold as RTE has to be stored <8 °C and must have a use-by date to protect the consumer from potentially pathogenic microorganisms. On-pack dates intend to reflect the status of the product to the consumer, and in theory, should change with seasonal variation as the growing environment impacts the quality of the crop. However, the implementation of on-pack dates are often static (seven days after packaging) as there is a general lack of empirical measurements that can be used to predict how a product will change with a reasonable degree of accuracy. One of the downstream effects of biologically inaccurate date labelling is that they contribute to food waste, where leafy salads are a product category that has a relatively high level of avoidable food waste from the consumer compared to other food product categories. Discovering markers that can be used to predict more accurately or dynamically

monitor a product's condition, and/or to dynamically monitor it in real time is desirable as a method to reduce food waste.

5.4 Introduction

In the United Kingdom (UK), iceberg lettuce is a commonly consumed leafy salad, it is sold both in wholehead form and as a RTE with a typical post-pack life of around seven days. The post-pack life for any ready-to-eat product is denoted by a use-by date. The use-by date, reasonably, is a conservative estimate to account for the variability the product may incur during its life. The primary concern when setting a shelf life is avoiding type II errors (false negative) where food is sold that may be "injurious to health" or that may not meet the quality expected by the consumer. Therefore, the on-pack date is a balance of providing the consumer with enough time to consume the product, protecting the consumer from harm, and providing the quality of product the consumer expects. As type I (false positive) and type II errors are inversely related; as the potential harm to the consumer is reduced, the potential for waste is increased.

With waste reduction becoming a more considerable concern in light of environmental and economic pressure, the usefulness of on-pack dates has come into question (Quested and Murphy, 2014; Lee et al., 2015; Eriksson et al., 2016). Previously, it has been estimated that ~ 20 % of leafy salads purchased by the consumer end up as "avoidable waste" (Quested and Murphy, 2014). In comparison, the total figure from retailers is around five percent (Welch et al., 2018). The usefulness of on-pack dates especially the best-before is not a new concern, back in the 1960's when they were first being considered there were objections:

"Much depends on the quality and freshness of ingredients and on distribution and storage conditions." (ALINORM 65/22, 1965).

Due to the innumerable variables that are contributing to the development of any given leafy salad, it is difficult to produce accurate estimates as to how the physiology of the product will change over time given the inconsistency of production methods and variability of plant genotypes, environmental conditions and postharvest handling. In turn, this leads to more conservative estimates of shelf life, and therefore, type II errors are minimised at the expense of type I

leading to waste among consumers who consider such dates. The only way to avoid having to make estimates of shelf life is to introduce a dynamic approach of monitoring or predicting the status of the product.

For the supplier, a marker that is consistent with a particular measure of quality/safety would allow them to reduce the margin of error that is applied to a product. From the point at which the consumer has the product, the uncertainty of shelf life is increased since differences in storage habits will affect the product in multiple ways. To account for this, a dynamic approach in the consumer environment may provide a significant reduction in waste. With the increasing harvesting of consumer data, internet-of-things devices and advances in machine learning, it is becoming possible for real-time monitoring of products to give feedback about the condition of the product directly to the consumer.

The objective of this study was to investigate biological and physiological markers that could be used to more accurately implement on pack dates. We investigated the variance in markers over an elongated SL period and between seasons with the use of the non-

destructive method of imaging to analyse quality. This may enable us to find reliable markers of quality and markers that can be continuously monitored to provide up-to-date information about the condition of a product.

5.5 Materials and Methods

5.5.1 Plant materials

Growing information for each trial is shown in Table 5.1. All plant materials were obtained from the central distribution centre within 24 hours of arrival and transported in ambient conditions to The University of Reading. Upon arrival the samples were immediately stored in a walk-in refrigerator at 4 °C in the dark, until analysis. Sixteen replicates were analysed at each time-point. The length of each trial was 23 days post-pack to evaluate the crop for an extended shelf life period of approximately three times the length of the commerical pack date to use-by date. After sampling the leaves were transferred immediately into a -20 °C freezer. Frozen leaves were lyophilized in batches for four days. Leaves were then ground into

a fine powder using a Wiley MiniMill (Thomas Scientific, Swedesboro, NJ, USA) and sieved.

Table 5.1: Environmental variables for iceberg lettuce grown in the United Kingdom (UK) and Spain (ES)

ID	Product	Season	Location	Growing conditions	Time of harvest to packaging date (days)	Pack to use-by date on pack (days)	Average temperature in month to harvest (°C)	Average rainfall in month to harvest (mm)
IL1	"Iceberg Lettuce" 300g	Feb 19	ES	open field	6	9	13	1
IL2	"Iceberg Lettuce" 300g	Jul 19	UK	open field	3	8	18	221
IL3	"Iceberg Lettuce" 300g	Nov 20	UK	open field	3	6	6	24.5

5.5.2 Imaging and colour analysis

5.5.2.1 Image capture

Images were captured from an RGB camera (Table 5.2) mounted on a tripod inside a custom built light-box. The light-box contained four tube-lights (Philips TL-D 58W/835). Images were captured in the raw format. Leaves were placed on the background with two centimetres around of space between leaves to aid with background extraction, and the leaves were arranged so they filled the camera's field of view.

Table 5.2: Camera settings

Variable	Value
Camera	Canon G9x mk2
Shooting mode	Manual
ISO	200
Picture mode	Neutral
Aperture size	5.6
Exposure time	1/80
Focus range	Macro
White balance	Custom

5.5.2.2 Image processing and colour statistics

Raw images were processed in RawTherapee (v5.7). Each image contained an x-rite ColorChecker Passport Photo 2 (Michigan, USA), which was used to adjust the colour of the images with reference to a master image to ensure colour constancy between images. Adjusted images were then exported with no compression as png file type.

Colour statistics were calculated from individual leaves within each

image by a custom Python (v3.6) script utilising the packages PlantCV (v3.0) and scikit-image (v0.16.2). Firstly, the images were converted to HSV format and then the leaves were masked using a threshold on the saturation chanel so the background could be removed. Then the hue channel was extracted and from this the range of hues and mean hue were calculated as well as the percentage of discolouration.

5.5.2.3 Visual Quality

Visual quality was determined by visually assessing each image and categorising each image as if good, marginal or poor quality based on the interpretation of the researcher where good quality exhibited minimal perceptible damage or discolouration. Marginal quality exhibited less than 2% overall discolouration for which the hue values were anything outside of the HSV values [34-148,0-100,0-100] and had less than 5 % tissue damage. The analysis of discolouration was not done at intake, but of the images during the analysis, so that the discolouration could be empirically measured. Poor quality was any deterioration in excess of marginal quality. An example of the images in each classification is shown in (Appendix Figure 5.8).

5.5.3 Extraction and quantification of ammonia

Fifty milligrams of milled lyophilized plant material was added to 1 mL of deionised water. The mixture was then sonicated at ambient temperature (22 °C) for 30 minutes prior to centrifugation for 15 minutes (16,050 x g, ambient temperature). The supernatant was then filtered through a 0.45 μm syringe filter (Cole-Parmer) before ammonia analysis. The same extract was used for nitrate and nitrite determination (section 5.5.7 below). Ammonia was quantified by enzymatic determination through reaction with α-ketoglutaric acid (KGA) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of L-glutamate dehydrogenase (GDH) (Merck). GDH reacts specifically with ammonia to form L-glutamate and oxidized nicotinamide adenine dinucleotide phosphate (NADP+) products. The oxidation of NADPH, which is proportional to the ammonia concentration was measured by absorbance in triplicate at 340 nm using a Jenway 6315 spectrometer (Stone, UK).

5.5.4 Enumeration of aerobic bacteria

Samples for microbial analysis were taken and analysed immediately after imaging. Enumeration of aerobic bacteria was carried out using 3M's Petrifilm Aerobic count plates (3M, St Paul, MN, USA). Ten grams of leaves were added to 90 mL of sterile 0.85% w/w Maximum Recovery Diluent (Merck) and then homogenized in a Stomacher 400 (Seaward, UK) for 1 minute at 260 rpm as presented by (Wood et al., 2015). Ten-fold serial dilutions of the homogenate were plated onto Petrifilm plates then allowed to incubate for 48 hours at 30 °C before the colonies were counted.

5.5.5 Extraction and quantification of chlorophyll A, B and total carotenoids

Fifty milligrams of milled lyophilized material was added to 1 mL of Dimethylformamide (DMF) anhydrous (Merck) and stored in the dark at 4 °C for 48 hours before filtering through a syringe filter (0.45 μm) into fresh Eppendorf tubes. Samples were diluted by combining 4 mL of DMF with 1 mL of sample and pipetted into

BRAND UV semi-micro cuvettes (Merck) prior to measurement. Each samples absorbance were measured at wavelengths: 480, 647, 664 nm using a Jenyway 6315 spectrometer, each sample was measured in triplicate. Quantification was carried out using formula presented by Wellburn (1994).

5.5.6 Determination of Glucose, Fructose and Sucrose

Free monosaccharides were extracted according to the method presented by Bell et al. (2017), with the exception that 0.02 g of lyophilized leaf powder was extracted. Extracts were analyzed on an Agilent 1100 series HPLC system equipped with a binary pump, degasser, and auto-sampler, with an external column heater (30 °C). A Bio-Rad Aminex HPX-87H (300 $\times$ 7.8 mm, 9 μm particle size) column with a Micro-Guard Cation H guard column (Bio-Rad, Watford, United Kingdom) was used to achieve separation with an isocratic gradient of 5 mM sulfuric acid, and a flow rate of 0.3 mL.min^{-1}. A Polymer Laboratories ERC-7515 refractive index detector (Church Stretton, United Kingdom) was used to detect monosaccharides. Compounds were quantified using authentic standards and analyzed with

Agilent ChemStation software (Santa Clara, CA, United States).

5.5.7 Determination of Nitrite and Nitrate

Extractions were prepared as described in section 5.5.3 above. Samples were diluted 1/100 prior to analysis for nitrate and nitrite concentration. The analysis of samples were carried out as described in Kramkowski et al. (2017). Briefly, the method uses HPLC to separate nitrate and nitrite from the sample on a NO-PAK column (Eicom). Nitrate is then reduced to nitrite on a NO-RED column (Eicom), after which the nitrite reacts with a Greiss reagent to form a light absorbing compound which is then detected at 540 nm using a DAD detector. The HPLC system used Eicom's ENO-30. The flow rate of the carrier solution was 0.33 mL.min^{-1} and 0.1 mL.min^{-1} for the reactor solution. The column temperature was set to 35 °C and the run time was 8 minutes. Compounds were quantified using authentic standards.

5.5.8 Statistical analysis

Ordinary least Squares analyses of all data were performed using statsmodels (Seabold and Perktold, 2010). Each respective analysis was conducted with a post hoc Tukey's Honest Significance Difference (HSD) test ($p < 0.05$).

5.6 Results and Discussion

Three independent trials were conducted IL1 (Spain), IL2 (UK) and IL3 (UK), all grown in open field (Table 5.1).

5.6.1 Visual quality

Table 5.3: The proportion of samples designated as good marginal or poor for all iceberg lettuce samples assessed visually. IL1, IL2 and IL3 are three seperate batches of commercial RTE iceberg lettuce grown in Spain (Feb 19), UK (Jul 19), UK (Nov 20) respectively. For each batch there were 96 samples, with 16 randomlly selected samples assessed at each time point.

Trial	Day post-pack	Visual Quality (Good:Marginal:Poor)%
IL1	4	100:0:0
	7	100:0:0
	11	92:8:0
	14	100:0:0
	18	92:8:0
	25	86:8:8
IL2	4	100:0:0
	7	100:0:0
	11	94:0:6
	14	100:0:0
	18	81:19:0
	25	44:56:0
IL3	2	100:0:0
	6	100:0:0
	9	100:0:0
	13	100:0:0
	19	81:19:0
	23	75:25:0

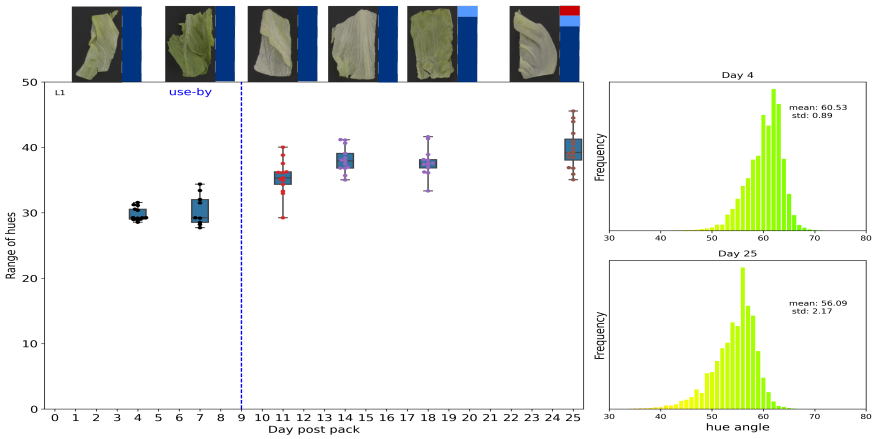
From visual inspection (Table 5.3), there were only two time points (Day 25, IL1) and (Day 11, IL2) which had samples which were classified as poor. Within the time-period that is typical of a ready-

to-eat lettuce product ($\sim$ 7 days post pack) there were no samples that were classified as poor. From visual inspection, it was clear that in all cases that the crops were of high quality and exhibited none of the common visual disorders such as enzymatic browning or russet spotting that are known to be unfavoured by consumers (Saltveit, 2018). While the use-by date is an indication of safety, many consumers do not solely rely on these dates in their decision making to reject a product. It is known that many consumers use visual appearance as their primary consideration when considering the quality of leafy salad (van der Laan et al., 2011) and therefore will consume a product after the manufactures use-by date. For the samples in this study, given the lack of visual degradation, it may be expected that consumers may consume the product after the use-by date.

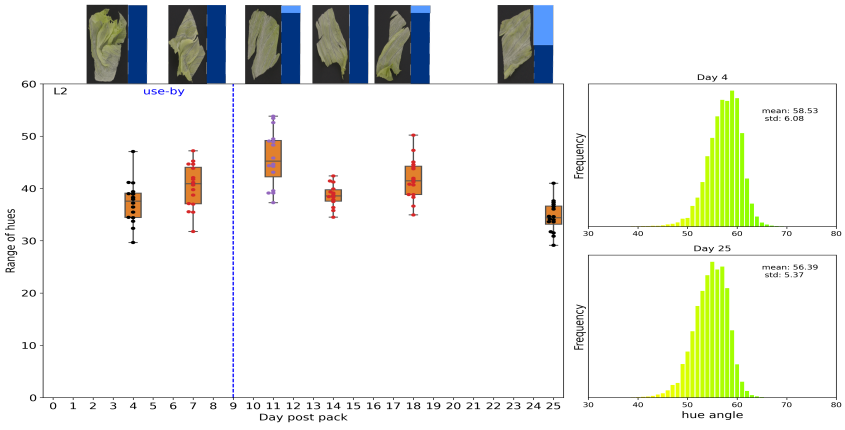
5.6.2 Visual quality and analysis of images

With the exception of the first trial (IL1), where there was a significant difference ($p < 0.001$) in the mean hue value between intake and the end of shelf life (Appendix table 5.4), the mean hue values

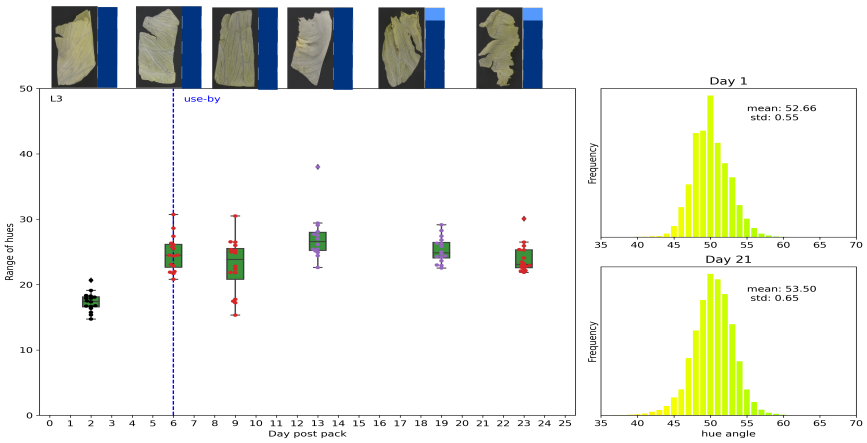
remained stable throughout testing. These results are not particularly surprising given that there were no changes in chlorophyll at any time point in any trial (Figure 5.2). If there were any changes in chlorophyll measurements, it may be expected that there would be a hue shift away from green (Agüero et al., 2008). Furthermore, from visual inspection (Table 5.3), there was minimal discolouration, such as pinking (Saltveit, 2018), throughout all trials.



(a) IL1



(b) IL2



(c) IL3

Figure 5.1: The range of hues extracted from images of iceberg lettuce leaves stored at 4 °C. Plots a, b and c are three independent trials grown in Spain (February 2019), UK (July 2019) and the UK (November 2020). Differences in colour within a plot for point markers (e.g. black on day 1 versus Red on day 4 of IL3 (c)) represent significant differences between shelf life days, where $p < 0.05$. For each time point in each plot n=16. The leaves above the primary axis are representative images from each time point with the visual quality score represented by the red, amber, green bars next to the image.

Where visual defects were observed, and there was an evident change in the colour of the tissue, subsequent analysis of the corresponding image showed no significant change in the mean hue value when compared with the values obtained from images at intake. This is because the visual defects often represented a tiny portion of the leaf, and although visible by eye, only represented a small portion of the image (Appendix Figure 5.9). Therefore, using the mean hue is not a particulalrly useful measure of visual quality in RTE iceberg lettuce. However, when analysing the range of hues, there is

a significant positive correlation (R^2= 0.75, p <0.0001) with shelf life. Therefore, in comparison to mean hue values, the range better represents the quality of the lettuce and may be used to model deterioration.

As the leaves senesce and degrade, defects such as dark necrotic tissue or a glassy appearance due to mechanical damage increase the number of unique hues. In all three trials, there were significant differences in the range of hues over the first two week period (Figure 5.1). Given that the typical SL of a salad product is usually no more than 7-10 days (Bell et al., 2017; Koukounaras et al., 2020), monitoring the change in the hue profile may be a valuable indicator for quality. However, as with any analytical system there needs to be a reference for comparison which has its own issues, such as which image to use as the base "fresh" image. Over a long time period, individual consumer preferences could be stored and used for reference, where comparisons could be made to previous stages when a product is discarded.

Typically, when evaluating iceberg lettuce over SL, a browning index is used as this is a well-recognised phenomenon that consumers

reject (Alongi et al., 2019). However, browning or other forms of discolouration are not a guaranteed outcome, as was the case in these three trials, whereby there were no significant changes in hues associated with discolouration (Appendix Table 5.4). Therefore, other measures of visual quality are required if the consumer is to be informed. Furthermore, there are other lettuce varieties such as lamb's lettuce (Ferrante et al., 2009) and romaine (López-Gálvez et al., 1996) that are less affected by browning, that a more generalisable method would be suited.

There were no significant associations between the change in colour and any other variables measured in this study. However, others have found that changes in colour are associated with increases in ammonia (Pace et al., 2014; Mastrandrea et al., 2016). The results of these trials do not corroborate the findings of others, finding no relationship between colour and ammonia concentrations. The results from these trials demonstrate that, given a reference image, the change in quality may be monitored non-destructively in the absence of apparent discolouration. With a large dataset, that would be possible to collect by a grower or retailer, and knowledge of consumer prefer-

ences, this method may be used to model the rate of deterioration and predict when the consumer will dismiss the product.

5.6.3 Chlorophyll

Chlorophyll is responsible for the majority of the colour of iceberg lettuce, but it is less prominent than in other leafy salads such as rocket or spinach due to the "rib" section, which is typically white. Furthermore, due to how lettuce grows with the more mature outer leaves having greater chlorophyll content than the younger inner leaves (Henriques and Park, 1976), depending on how the lettuce is processed, the number of outer more mature leaves may vary, and as a consequence, the colour. As it is highly unlikely that a product would reach the shelves without any green leaf material, and as such, the green hue resulting from chlorophyll is likely to dominate other hues. Therefore, if there were any changes in colour over the postharvest period, it may be expected that chlorophyll plays a role in the change, which makes it a candidate marker for quality.

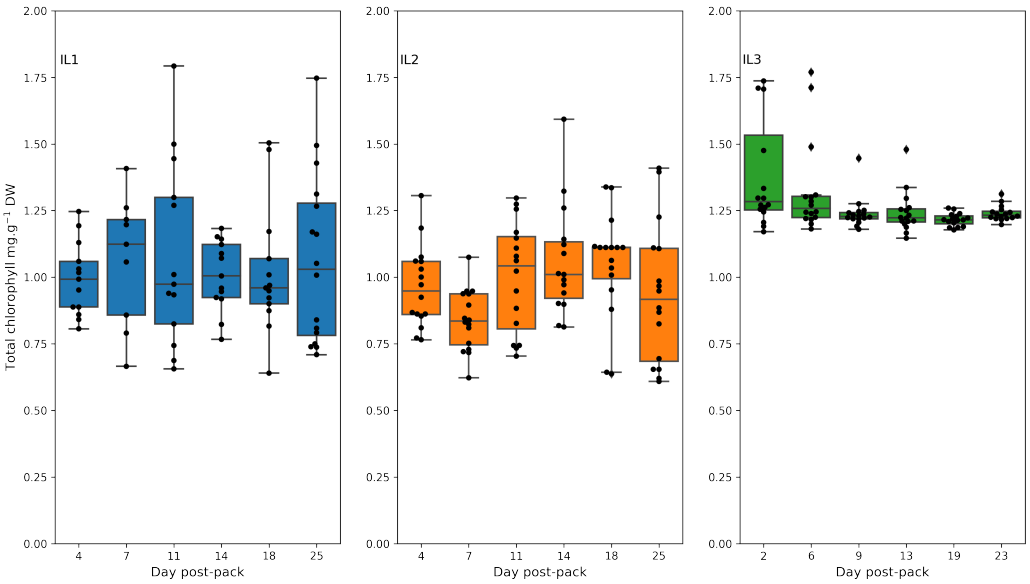


Figure 5.2: The changes in total chlorophyll over time for ready to eat lettuce leaves stored at 4 °C. Differences in colour for points represent statistical significance for the mean at each time point where $p < 0.05$. For each time point n=16.

Chlorophyll had the least variation of all the measurements taken and exhibited no significant change over SL or between trials. The data show that chlorophyll is stable over a typical SL period and is therefore not a marker that can be used to differentiate product over SL. Although chlorophyll degradation has previously been cited (Yamauchi and Watada, 1991; Koukounaras et al., 2009) as factor in the colour change of a salad product, neither these measurements or the colour measurements provided any evidence that chlorophyll content is a postharvest marker of quality. However, this discrepancy may be due to pre-prepared salads, which could have

had the outer leaves discarded. It has been reported that the outer leaves are more susceptible to degradation (Agüero et al., 2008), which may account for the difference between these results and the literature. Although there was no significant change in chlorophyll levels in these trials, IL3 was less variable overall, suggesting that the leaves were less variable due to fewer rib sections in the sample. One of the issues with using chlorophyll as a marker of quality for RTE iceberg lettuce is that there is a processing step that, depending on the processor and the product specification, certain leaves that do not meet specification may be removed and therefore, the remaining leaves may not accurately represent the postharvest potential of the product.

5.6.4 Aerobic Colony Count (ACC) and shelf life

ACC is a general microbial test with no legal precedence in leafy salads but is often used to give an indication of the hygiene of a product. It is often used as an indicator of quality, whereby an unsatisfactory result (10^6 – 10^7 cfu/g) may indicate that there are sub-optimal conditions in the supply chain (Health Protection Agency., 2009).

Under domestic storage conditions typically recommended for leafy salads (4 °C), microbial growth is not attenuated but slowed significantly. Therefore, it can be expected that there will be an increase in ACC over the postharvest period. As microorganisms are known to be associated with product spoilage (Calonica et al., 2019), the omission of a measure of microbial status would potentially diminish the confidence of any given postharvest indicator if not included in the analysis.

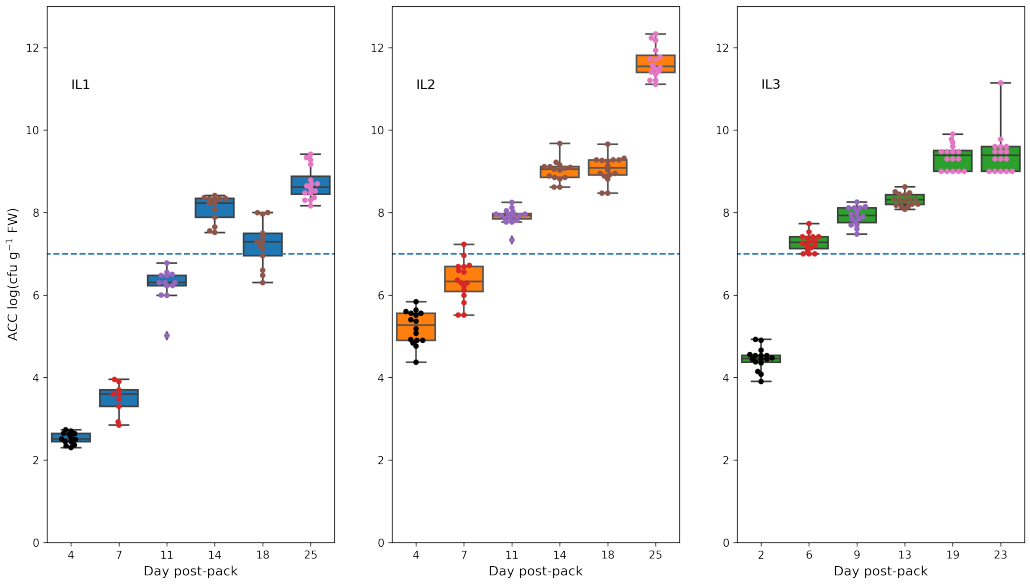


Figure 5.3: The changes in Aerobic Colony Count in measured in lettuce leaves throughout shelf life stored at 4 °C. Differences in colour for points represent statistical significance for the mean at each time point where p <0.05. The horizontal dashed lines reperesent 10^7 cfu.g $^{-1}$ which is typically the upper threshold when referring to satisfactory and unsatisfactory quality. At each timepoint n=16.

At intake, all samples were well within the range that would typi-

cally be classed as satisfactory for aerobic colony count (Figure 5.3). The figure varies slightly depending on the product and specification but is usually less than 10^7 cfu.g^{-1} (Health Protection Agency., 2009; Calonica et al., 2019). From visual inspection, it was not until the ACC count was greater than 10^{10} cfu.g^{-1} that a change in visual quality was discernible. The visual changes did not start to manifest until around two weeks into the trial, which is $\sim$ ten days after the use-by date; even at this point, the visual changes were relatively minor. Although ACC is not diagnostic of pathogens, relying on visual heuristics is potentially dangerous given that visual defects appear after a time period in which pathogens could reach a harmfull level.

The change in ACC was fairly similar in all trials (Figure 5.3); however, the trial that started with the lowest ACC also had the lowest overall final ACC (Figure 5.3, IL1) as well as the least decline in visual quality. The initial ACC may be a useful indicator of quality in the first instance, with a relatively high count may indicate that the decontamination of the produce may not have been sufficient. Under optimal storage conditions (4 °C) and over a time scale that is likely longer (>7 days) than the consumer would normally keep

a salad, there was no indication that microorganisms affected the appearance of the salad. While many consumers often use visual appearance to decide when to discard a product (van der Laan et al., 2011), it is clear that with ready-to-eat iceberg lettuce, using visual appearance alone may lead to harm for the consumer, assuming the use-by date is accurate.

There was a weak positive correlation ($R^2 = 0.34$) between the number of rainy days in the growing period and the ACC with IL1 the lowest (1 day), and IL3 the highest with (26 days in which more than 1 mm of rain fell). The inverse was true for temperature, with IL1 having the lowest average growing temperature (13 °C) and IL3 having the highest with 18 °C. Therefore, the combination of a moist environment and a hospitable growing temperature for microorganisms may be the reason that IL3 had the highest ACC. However, the data were not conclusive and to confirm these findings, more repetitions would be required. Furthermore, these data show that the initial bacterial load is not sufficient to predict subsequent growth, and the preharvest, along with the supply chain conditions need to be taken into consideration for predicting microbial load.

5.6.5 Sugars

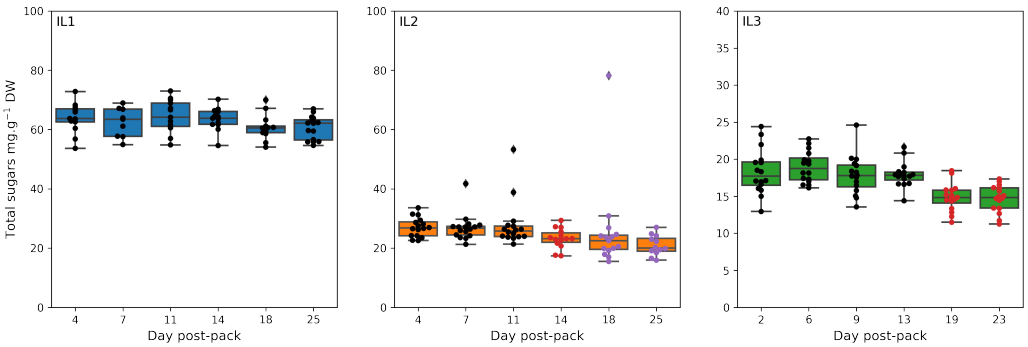


Figure 5.4: The changes in total sugars (Glucose, Fructose and Sucrose) over time for ready to eat leattuce leaves stored at 4 °C. Differences in colour for points represent statistical significance for the mean at each time point where p <0.05. At each time point n=16.

Although data were collected for sucrose, glucose and fructose, these three sugars were highly correlated (R^2 = 0.91) and therefore only the sum of these "Total sugars" will be discussed (Figure 5.4). Overall, it can be observed that there is a slight decline in total sugars over the shelf life period for (Figure 5.4, IL1) and (Figure 5.4, IL3) the decline was 0.13 and 0.15 $mg.g^{-1}.day^{-1}$ whereas (Figure 5.4, IL2) was 2.5 $mg.g^{-1}.day^{-1}$. The measurement of sugars is often used a marker of respiration, as the leaf metabolism continues postharvest its sugars are depleted (Zhan et al., 2013; Mbong et al., 2017). If it was known what the sugar concentration was at the start of the shelf life period any subsequent measurement might give an indication

as to how it has been stored. With a high rate of decline showing greater respiration, but this is not something that would be considered as pragmatic marker applicable for the consumer, but may be usefull for prediciting longevity at the processing stage.

Even though the data exhibited an overall decline over the entire shelf life period, it was not until two weeks postharvest that there were any significant changes. These data suggest that sugars are not a particularly useful indicator of postharvest longevity as a measurement by themselves, but may be useful when taken into consideration with other measurements such as nitrates. Given these results, it is suspected that all three of the sample batches tested were of high quality.

5.6.6 Nitrates

In comparison to some other leafy vegetables such as rocket or spinach, lettuce is not known to be a particularly high accumulator of nitrates. There are legal limits for the concentration of nitrates within iceberg lettuce (1258/2011, 2011), but unlike rocket, there is no seasonal aspect to the limits whereby “iceberg type” lettuce grown in open

air can contain a maximum of 2 g of $NO_3.kg^{-1}$ fresh weight. Nitrate content is known to be related to the preharvest use of nitrogen (Liu et al., 2014), and therefore, the nitrate content may give an indication as to the preharvest nutrition and potential postharvest longevity. Furthermore, it is known that high nitrate content can increase leaf size (surface area) at the expense of decreased thickness (Hoque et al., 2010), and the relative decrease in thickness can lead to greater potential for decreased postharvest longevity as it is more susceptible to damage (Garrido et al., 2014).

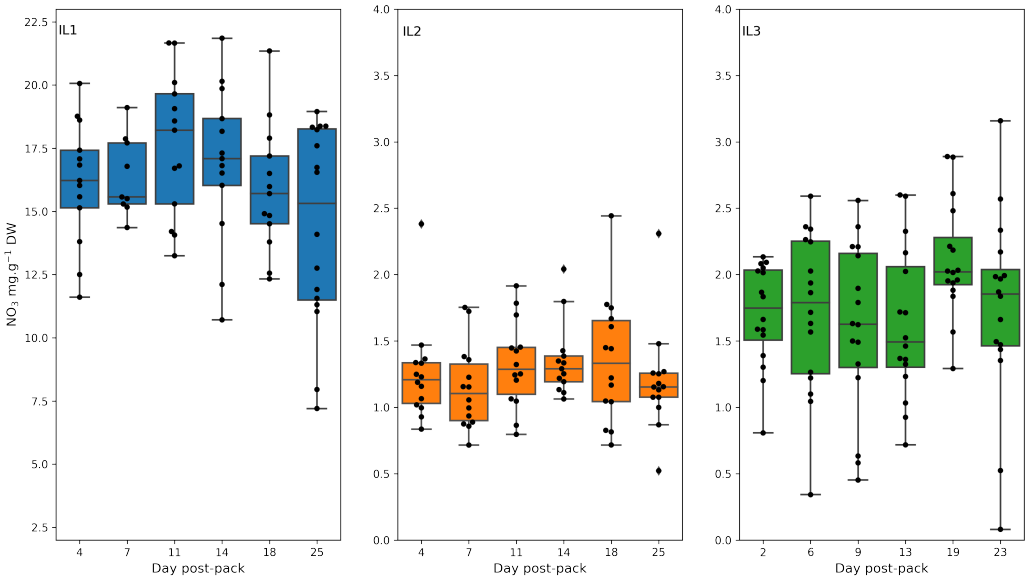


Figure 5.5: The changes in nitrate over time for ready to eat iceberg lettuce leaves stored at 4 °C. Differences in colour for points represent statistical significance for the mean at each time point where p <0.05. At all times points n=16.

Throughout all three trials, NO_3 concentrations were stable over the

shelf life period. The trial grown in Spain during the winter (Figure 5.5, IL1) had the highest concentrations which were greater than 10x of those grown in the UK. It has been reported that, lettuce grown during winter months accumulate more NO_3 than summer grown crops (Konstantopoulou et al., 2010), this is also reflected in EU law where crops grown in the colder months (October - March) have a higher threshold than those grown outside this period (1258/2011, 2011). Although lettuce does not currently have any seasonal variation in the legislation. However, from the two trials grown in the UK, there were no significant differences between crops grown in the summer and winter, therefore, it is clear that environmental variables such as sunlight are not the only factor in determining tissue nitrate concentrations. IL1 had a tenfold higher concentration of NO_3 than both IL2 and IL3. Large variations in nitrate concentration are not uncommon (Kyriacou et al., 2019), so the values reported here are well within typical values. The tenfold higher concentration of IL1 did not indicate any difference in the other measured variables except sugars, which were also highest in IL1. The ratio of nitrate and sugars has been previously studied in butterhead lettuce by Behr and Wiebe (1992). However, it was found that they were inversely

correlated, contradicting what we have found.

As there were no changes over shelf life, which supports the findings of Konstantopoulou et al., (2010), NO_3 does not seem to be a valuable marker of quality during SL. However, IL1 had the highest tissue concentrations of both nitrate and total sugars and the lowest numbers of samples that were classified as poor after visual inspection. Therefore the assessment of these variables may be useful in assessing the potential postharvest longevity of the crop.

5.6.7 Ammonia

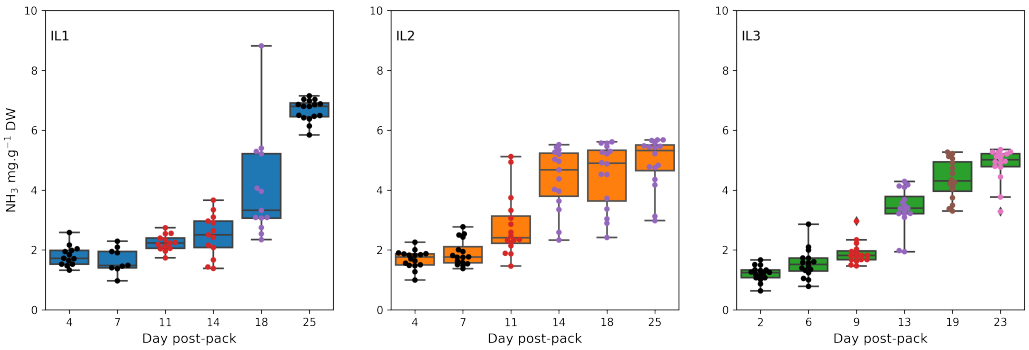


Figure 5.6: The changes in ammonia over time for ready to eat lettuce leaves stored at 4 °C. Differences in colour for points represent statistical significance for the mean at each time point where p <0.05. At each time point n=16.

Of all the markers assessed in this study, ammonia had the most significant changes over shelf life. In all trials, there was an increase in

ammonia from the "fresh" samples to the oldest (Figure 5.6). However, it was not until around day eleven, where there were significant differences ($p < 0.05$, for R1-R3). Pace et al., (2014) also found similar results with RTE lettuce, and they also reported a relationship between ammonia and changes in visual quality.

It has previously been reported that the accumulation of ammonia is related to senescence by way of protein catabolism (Toivonen, 1997). The relatively few studies that have measured ammonia in leafy salads over shelf life also report a correlation with a change in leaf colour (Pace et al., 2014; Mastrandrea et al., 2016). We did not find any evidence that ammonia influenced the colour of the leaves for iceberg lettuce. However, there was a significant ($p = 1.9 * 10^{-35}$, $R^2 = 0.46$) correlation between ammonia and ACC (Figure 5.7). There are a few potential sources for the increase in ammonia, such as ammonifying bacteria, e.g. *E. coli* metabolising nitrate or nitrite and producing ammonia (Wang et al., 2019), and as the bacteria become more numerous over shelf life, the ammonia increases (Stremińska et al., 2012). Or, as a by-product of disrupted protein metabolism where glutamine synthetase, an enzyme that assimilates

ammonia in protein metabolism, is inhibited, and therefore ammonia accumulates (Chandra et al., 2006; Mastrandrea, Amodio and Cantwell, 2016).

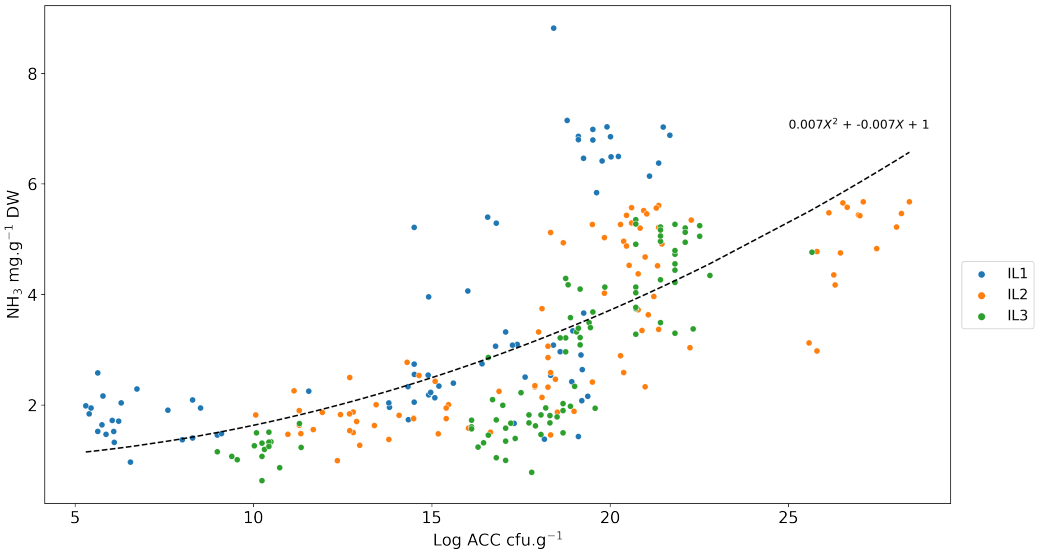


Figure 5.7: The relationship between changes in ammonia and aerobic colony count for trials IL1, IL2 , IL3, R^2 = 0.46. n=288.

These data warrant further exploration of increases in ammonia in lettuce over shelf life. Although it was not until after the manufacturer's use-by date that ammonia concentrations were significantly different from the "fresh" samples, there are potential quality and or safety implications related to ammonia depending on if it is derived from microorganisms (safety) or protein catabolism (quality). Furthermore, ammonia is volatile and, therefore, could be detected by a gas sensor allowing for non-destructive sensing. Moreover, produc-

ing a sensor that can detect ammonia is a relatively well devleoped process (Zhai et al., 2020), and can be incorporated into the packaging but is yet to be tried with a leafy salad product.

5.7 Conclusion

Of the traits assessed, ammonia concentration has the greatest potential as a marker for shelf life quality. Ammonia had a strong positive correlation (R^2 = 0.85, p = $3.5 * 10^{-77}$) with shelf life (Figure 5.6) and aerobic colony count (Figure 5.7). Furthermore, significant changes in ammonia were detected within a time frame representing a typical shelf life period. From the pooled 268 replicates there was a significant (p = $1.97 * 10^{-35}$) strong correlation (R^2 = 0.46) between ammonia and aerobic colony count, which sugests that ammonia may be an indicator of microbial quality. However, contrary to previous work (Cantwell et al., 2010; Mastrandrea et al., 2016), there was no clear relationship between ammonia concentrations and measurements of visual quality. Further research is needed to establish the cause of ammonia accumulation in iceberg lettuce and to distinguish whether ammonia is marker of microbial accumulation,

metabolic disruption, or both.

Given the present situation, where there are limited markers that a producer/processor is able to use to define the on-pack date accurately, the date on the pack tends to include a relatively large margin of error. The effect of giving a relatively large margin of safety is that those people who rely on on-pack dates may be more wasteful. Developing more accurate estimates of shelf life would help to alleviate some of the food waste in this category. To be as accurate as possible dynamic monitoring is required, which can be achieved by either volatile sensing or by the use of cameras.

Ammonia was the most indicative marker of quality from all the variables analysed in this study (Figure 5.6). Furthermore, it may be possible to monitor its accumulation with imaging methods. Seeing as refrigerators with integrated cameras are coming into the market, this method may be commercially viable within the near future. It is also possible to continuously monitor ammonia with a gas sensor device, although this approach has one distinct disadvantage. Given that it is unlikely that ammonia accumulation is unique to iceberg lettuce when stored in typical domestic conditions, a gas sensor may

pick up ammonia from another source or there may be a challenge with sensing something that is inside a pack. Tissue ammonia accumulations have also been reported in spinach and kale (Cantwell et al., 2019). To give real-time information about the condition of a product and therefore more accurately represent the quality and safety of a product, imaging may be more useful than volatile sensing.

It is still unclear as to if any variable, or combinations of variables, can be used to reliably represent the status of a product and tailor that to consumers' preferences and ultimately reduce waste. These results add further evidence to suggest that tissue ammonia concentration may be an important marker for the quality of iceberg lettuce. Furthermore, when analysing the colour, rather than taking average values, which may miss small but important changes, computer vision methods that take into account all available information may be more useful when assessing quality.

5.8 Appendix

Table 5.4: The summary statistics from the imaging data for icebrg lettuce trials L1-3. Significant differences are only within trial and column where p <0.05.

Trial	Day post pack	mean hue ± std	range of hues	total discolouration %
L1	4	60.6 ± 5.2 a	29.8 a	0.2 a
	7	64.6 ± 4.8 b	30.5 a	0.1 a
	11	57.2 ± 6.0 c	35.4 b	0.7 a
	14	56.8 ± 6.2 c	38.1 bc	0.8 a
	18	56.6 ± 6.2 c	37.7 bc	0.8 a
	25	56.8 ± 6.6 c	39.8 c	1.3 b
L2	4	58.8 ± 6.0 a	37.3 ac	0.4 a
	7	58.8 ± 5.8 a	40.4 a	0.4 a
	11	57.6 ± 7.4 a	45.8 b	1.5 b
	14	57.0 ± 5.4 b	38.7 a	0.4 a
	18	58.0 ± 6.0 a	41.8 b	0.4 a
	25	56.4 ± 5.4 b	34.6 c	0.4 a
L3	2	52.6 ± 4.0 a	17.3 a	0.1 a
	6	58.0 ± 7.6 a	30.8 b	0.6 b
	9	53.0 ± 5.4 a	22.9 b	0.5 b
	13	52.6 ± 6.2 a	27.2 c	0.6 b
	19	53.0 ± 6.0 a	25.3 bc	0.5 b
	23	53.6 ± 5.8 a	24.0 b	0.4 b

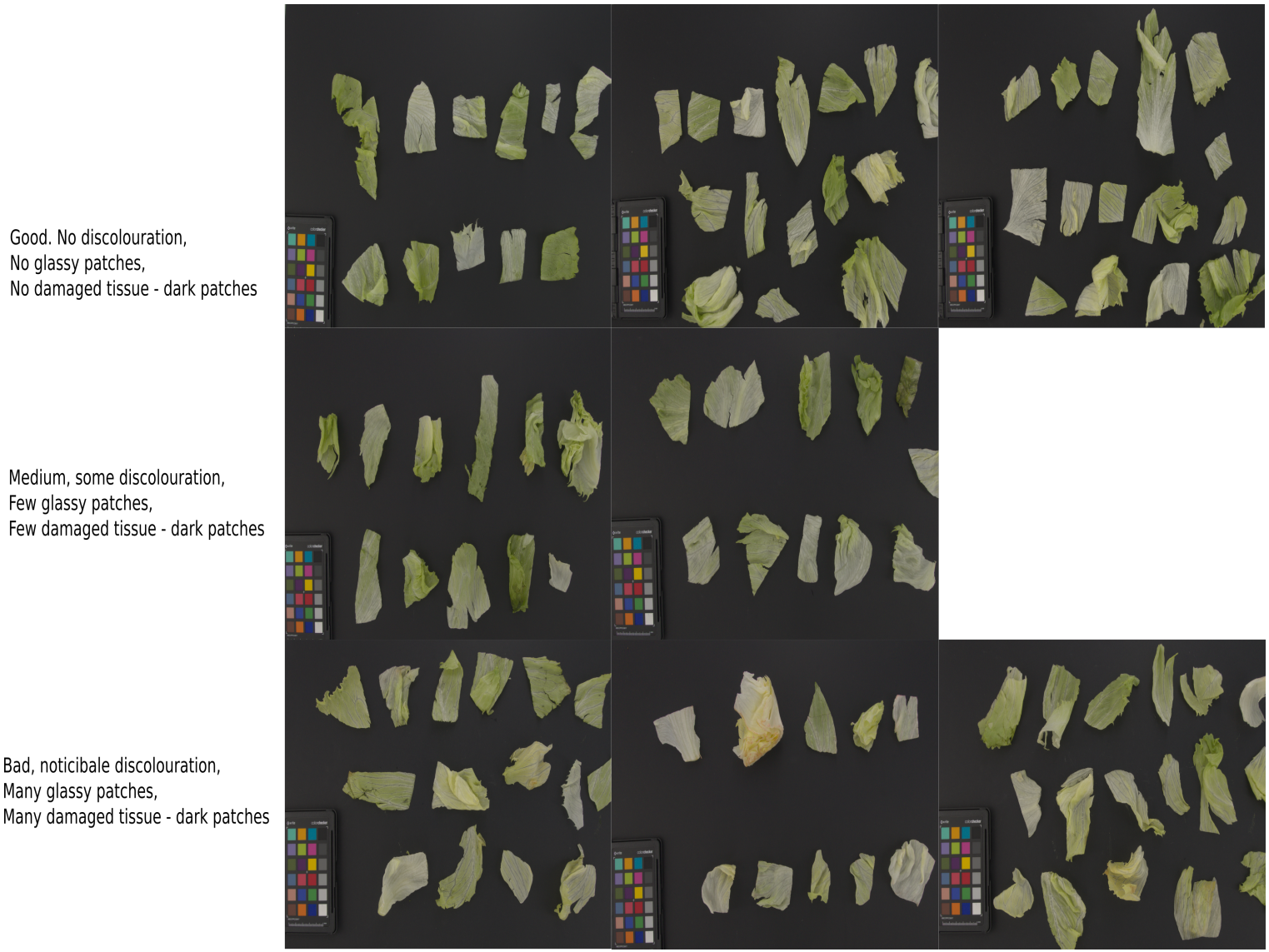


Figure 5.8: The decision making criteria for visual quality for Iceberg lettuce

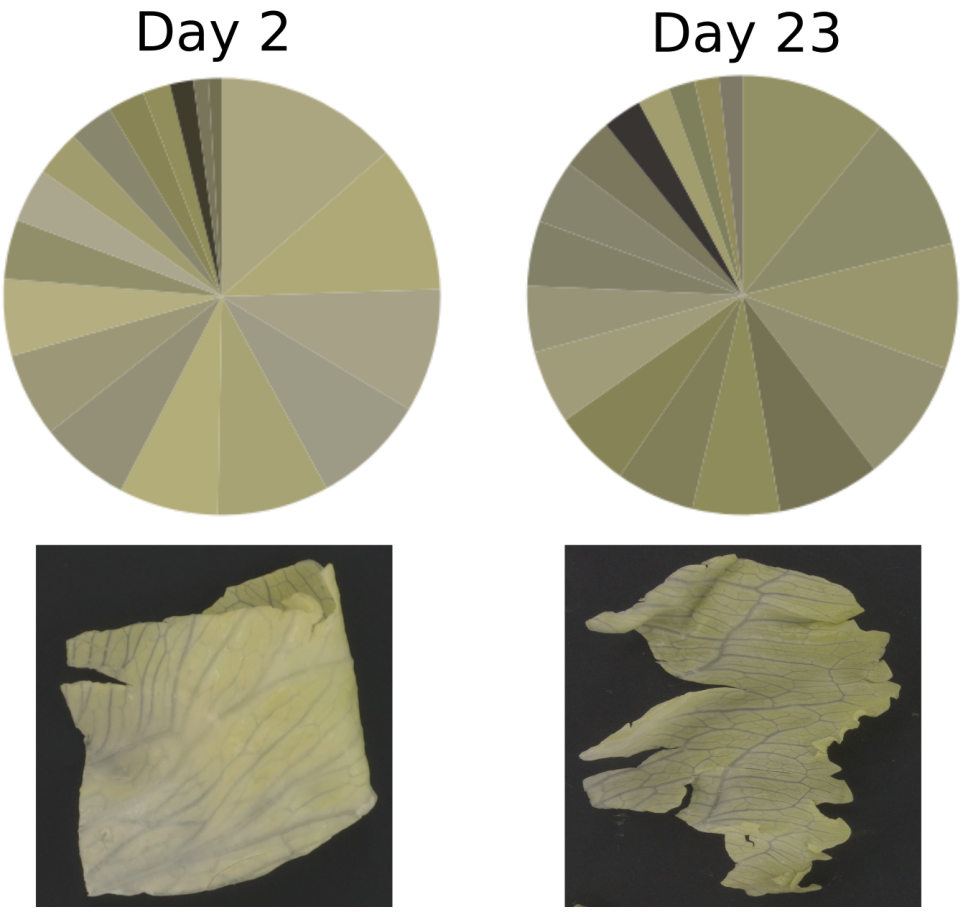


Figure 5.9: A comparison of the 20 most common (by %) hues for two iceberg lettuce leaf portions from trial R3 on days 2 and 23 respectively.

5.9 References

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Chapter 6

Assessing consumer attitudes towards leafy salads using time lapse video and data mining of supermarket online comments.

6.1 Background

Whilst the previous Chapters (4-5) have evaluated postharvest longevity from a purely technical perspective, this chapter considers the consumers' perspective of visual appearance and general attitude toward

leafy salads. Without understanding the variation in the consumers' rejection of leafy salads, it is difficult to put shelf-life dating in the context of how consumers use them. When providing information to the consumer to satisfy their demands, it serves to have a good understanding of what they desire. It is clear that many different factors contribute to the perception of a product, but the visual appearance is typically the most important (van der Laan et al., 2011). Although with respect to leafy salads, there is not a large volume of literature on the subject, it is extensively studied for food in general. This chapter investigates how the consumer assesses visual quality over an extended shelf life period and how changes in visual quality correspond with the rejection of two leafy salad products, lettuce and rocket. The author wrote an ethics application to the School of Chemistry, Food and Pharmacy at the University of Reading. Ethical approval was granted for the experiment, and participants were recruited online from Amazon's MTurk platform. A novel method, in the context of the leafy salads and visual perception, was developed whereby a camera and lighting system was incorporated into a domestic refrigerator to capture timelapse video of salad leaves during postharvest storage, which would later be shown to consumers. This

research was conducted during a Covid19 lockdown period, which helped drive the innovation in conducting research remotely.

One particularly novel aspect of this research is the use of consumer comments taken from supermarket websites to assess quality perception regarding leafy salads. Analysis of consumer reviews has been studied more extensively in business literature, but not typically for a specific product category (Pavlou and Dimoka, 2006). Online retailers see these comments similar to word-of-mouth marketing and are aware of the potential for positive influences on sales (Floyd et al., 2014). Compared to traditional consumer research, where participants are often paid to participate in a study, online comments are not biased by this incentive. Furthermore, the commenter is likely to have some investment in the product, having already purchased it. However, it may be that consumers who write online reviews, either positive or negative, may be less representative of general society (Yan and Wang, 2018) and may be biased towards leaving negative or postive comments. Furthermore, it is not particularly transparent as to how the online comments are moderated as they are not moderated by a third party and are shown on

the website of each particular retailer; unfavourable comments could be removed. The analysis of these consumer reviews could provide a unique insight into reasons for product liking or rejection that has not been previously utilised.

Developing an understanding of how the consumer assesses quality from a visual perspective and particularly what colour profile is seen as unfavourable to the consumer will help guide shelf life estimates. Furthermore, the rejection rate of a salad product may give insight into the proportion of waste that could be expected with any given shelf life date.

6.2 Contribution to the project

Jake Jasper: Conception of study, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing.

Carol Wagstaff: Conception of study, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Stephen Elmore Supervision, Writing - original draft, Writing - review & editing.

Lisa Methven Writing - original draft, Formal analysis, Writing - review & editing.

Contribution breakdown percentage by each author respectively [70, 10, 5, 15]

The following section has been submitted to Food Quality and Preference.

6.3 Abstract

On average, in the United Kingdom, only 30-40 % of people consume the currently recommended intake of five portions of fruit and vegetables per day. Furthermore, there is a relatively large amount of waste from fruits and vegetables compared to other food product categories. This is particularly true of leafy salads, where approximately 20 % of edible product is thrown away. With the government targets of both increasing fruit and vegetable consumption and re-

ducing carbon emissions, leafy vegetables represent a key area for progress. Although with leafy salads there are always on pack dates in the form of a use-by or best-before date, consumers tend to rely on visual assessment when deciding if a salad is consumable. Our data show that younger consumers use on-pack dates more than older more experienced consumers, who tend to rely more on visual perception. Furthermore, this paper explores consumers rejection of the leafy salads rocket and iceberg lettuce when presented with only visual information and conclude that when there are minimal changes in the visual quality over shelf life (<2 %), most consumers ($\sim$ 60 %) reject the product long after the manufacturers use-by date . Where there are minimal quantifiable changes in visual appearance, as was the case with rocket leaves, the majority of consumers indicated that they would consume the product 1-2 weeks after the use-by date. With lettuce, which is more prone to visual disorders, the range was 2-6 days after the use-by date. With an increasing uptake in online grocery shopping we also evaluate the consumers' attitudes toward online shopping for leafy salads by analysing public-facing reviews published on the website of major retailers. This work combines primary research and data mined consumer comments from online

retailers' websites to understand the expectations of the consumer when it comes to visual quality.

6.4 Introduction

Visual quality is often cited as the most important parameter for the consumer when considering purchasing or discarding a food item, and is particularly true of the fresh-produce category and leafy salads (Chonpracha et al., 2020). For all pre-prepared leafy salads that are RTE, manufacturers will provide an indication of when the product is no longer fit for consumption in the form of a use-by date. However, the consumer still values their own visual perception of a product as an important factor in determining whether or not to purchase or consume a product (Lyndhurst, 2008).

In the UK, the use of online shopping has been steadily increasing since the turn of the millennium. Alongside the increase in online shopping, the importance of product images has also risen for both the consumer and the retailer. For the retailer, the images differentiate the products within their own ranges, premium versus home-

brand, for example, and between competitors (Singh and Söderlund, 2020). Whereas for the consumer, the images help inform the consumer about the attributes of the product they are potentially going to purchase (Park et al., 2021). Around 13 % of people regularly get home delivery, and a further 10 % use online or "e-commerce" for their groceries occasionally (Hood et al., 2020). There are many advantages to the consumer for shopping online, convenience being the primary advantage. It is also more environmentally friendly to have one vehicle make many deliveries rather than many vehicles driving to stores to collect food (Shahmohammadi et al, 2020). During the Covid pandemic in the UK, online shopping increased by 200 % (Emarsys, 2020), and when consumers were asked if they would continue online shopping post-pandemic only 18 % of respondents said they would move back to in-store only (City Food Lecture, 2021). However, one significant disadvantage to online food shopping is the inability for the consumer to physically assess the product, which is often cited as a reason as to why consumers do not like to shop online and is particularly true of fresh produce (Hackney et al., 2006).

To help the consumer evaluate a product whilst shopping online, the retailer will provide information about the product, such as the nutritional declaration, and reviews from consumers who have previously purchased it. There will also be images of the product, and it is well known that product packaging can influence the consumers' intention to purchase a product (Simmonds and Spence, 2017). It is generally understood that images are often modified with post-processing techniques to make the images more appealing to consumers (Lazard et al., 2018). However, outside of packaging design, there is little research as to how the colour profile of an image influences the consumers decision making (Spence and Velasco, 2018). A leafy salad product that has been manipulated to look more green may be more appealing to the consumer (Spack et al., 2012). Pre-prepared salads have become a common option for consumers who are looking for convenience, which is also cited benefit for shopping online (Mallinson et al., 2016). However, one issue with pre-prepared lettuce is enzymatic browning which can be exacerbated by wounding, which is inherent in pre-prepared or RTE lettuce (Saltveit, 2018). As consumers do not get to view their products when shopping online and therefore cannot reject a product if

it does not meet their standards, RTE salads may be more susceptible to losses by a decline in visual quality for consumers shopping online. Although, the consumer does have the option of rejecting the product upon arrival, which then shifts the wastage back to the retailer, providing it does not go on to another consumer. This is clearly a situation that the retailer has an incentive to minimise as depending on the fate of the rejected product; it can represent a double loss, where the consumer does not have the product, and it is then not possible to sell on.

Many researchers have focused on measuring visual quality, mainly by assessing colour, and as a result, the visual quality is often defined by empirical colour measurements such as hue angle or red, green and blue (RGB) pixel values (MartínezSánchez et al., 2012; Alongi et al., 2019; Chapters 4 and 5). Although colour values are often used as a proxy for visual quality, it is clear that in the cases where there is little perceptible colour change over a typical seven-day shelf life period of fresh produce, consumers will use other visual cues to form their opinion of the quality of the product. In a study of rocket leaves, physical changes, such as the wilting of

leaves, or a deviation in the morphology from the consumers' expectation, led to a different perception of visual quality even though the colour may have remained the same (Løkke et al., 2012). The build-up of moisture in the packaging also can also have the same effect and does not necessarily affect the colour. While it is accepted that the consumer assesses visual quality prior to any other factor (Wadhera and Capaldi-Phillips, 2014), the relative importance of the features that make up visual quality, such as colour and shape, remains unknown.

Image analysis, also known as computer vision, as a method for analysing visual appearance, has become the predominant method for quantifying visual quality, largely due to declining costs in equipment driven by consumer electronics. Refrigerators can now be purchased with integrated cameras, which could allow for the real-time monitoring of groceries in situ. Furthermore, there are many freely available tools for the analysis of images with one website alone, www.quantitative-plant.org, having links to over 170 different tools for plant phenotyping and 28 open data sets that can be used to train models (Lobet et al., 2013). The advantage of image analysis over

a sensory panel is extensibility, whereby the methods can be automated and integrated into other processes, such as refrigerators (Zhang et al., 2018).

In this paper, we evaluate the visual parameters that equate to the quality of RTE iceberg lettuce and rocket leaves from the consumer perspective. Firstly, we do this by analysing online shopping reviews and subsequently evaluating consumer acceptance of product images presented as time-lapse videos. We further quantified changes in visual parameters over shelf life and related the changes to consumer acceptance. Finally, to understand how the change in the colour of images affects visual quality from the consumer's perspective, we extracted the colour from the leaf images using image analysis software and compared the colour data with the rejection of the product by the consumer. We hypothesize that the greater the deviation of the image colour from the initial image seen by the consumer, the greater the rejection will be.

6.5 Methods

6.5.1 Consumer comments of bagged lettuce and rocket from UK retail websites

Consumer comments were collected from the public facing websites of supermarkets operating in the UK: Asda, Sainsbury's, Tesco, Waitrose and Morrisons. The comments were collected by searching for bagged lettuce and rocket products within each website and all available comments were taken. For Waitrose and Morrisons the earliest comment was from 2014; Asda, Tesco and Sainsbury's the earliest comment was 2018. For analysis good reviews were those where the consumer rated the product greater than three stars and the bad reviews were those less than three stars. Neutral reviews were those that equalled three, as the scale was 1-5 in all cases, there were no instances of rounding.

6.5.2 Analysis consumer comments collected from UK supermarket websites

Sentiment analysis was conducted using the TfidfVectorizer from the scikit-learn package for python (Buitinck et al., 2013). The stop words were from NLTK (Bird et al., 2009) English corpus, with additional words that are related to the subject e.g. lettuce.

The sentiment analysis approach multiplies the occurrences of each word in a document (term frequency), here document refers to each individual comment, by the inverse frequency of the same word in the collection of documents (inverse document frequency). The result of this is that for words that are very common and therefore assumed to be less important, approach 0, whereas words that are less common and therefore more informative have a higher value.

For sentiment classification, comments that had greater than three stars were consider "good" and those that were lower than three were considered "bad".

6.5.3 Plant material and the production and analysis of images and time-lapse videos.

For each trial, samples of pre-cut bags of iceberg lettuce and wild rocket were collected from the local supermarket (Waitrose, Caversham, UK) and were then sub-sampled (30 g of material) into clear packaging which was suitable for salad leaves: Amcor P-Plus 30μ oPP with a permeability of 49300cc/O_2/linear meter/day/atm (Amcor, Ledbury Flexibles, Bristol, UK) and immediately placed in the sampling refrigerator. The samples were purchased prior to the start of each trial, with the first lettuce samples purchased in January 2020 (ILA), the first rocket samples in February 2020 (RA), the second lettuce sample purchased in March 2020 (ILB) and the second rocket sample purchased in April 2020 (RB). The length of the time lapses were chosen so that there was a video of a typical post harvest life ($\sim$ 14 days) and also a video that was taken to an extreme point ($\sim$ 25 days post harvest) where there was strong off-odours and excessive moisture build-up in the packaging.

A Raspberry Pi camera board v2.1 (The Raspberry Pi Foundation, UK) was placed inside a domestic refrigerator set to 5 °C with two

Adafruit NeoPixel (NY, USA) sticks (warm white - 4500K) in line with the sample platform, and were activated 10 seconds prior to image capture. Images were taken every five minutes over the trial period.

All camera settings that could be modified were set to manual to limit changes in colour, this included: exposure mode, automatic white balance gain, ISO (the standard used to relate digital images to brightness standards used in film images), brightness.

The captured images were made into a timelapse video using kdenlive (kdenlive.org, v20.08.1). Videos were rendered at 60 frames per second at 1080p resolution and exported in the lossless H.264 format which as the highest possible quality option, so that the minimal image quality was lost during editing. Each second in the final video was five hours in real-time.

There were two videos produced (A and B) for both salad types, iceberg lettuce (ILA and ILB), rocket (RA and RB). The videos designated as "A" were the shorter shelf life representing a realistic time frame in which a consumer may keep a leafy salad product. In comparison, those designated "B" were the longer videos taken to a point

where the product was clearly unacceptable. The thumbnail of each video was a blank black image, so it was not possible to see what the video contained until the play button was clicked.

For the test image (Figure 6.1) the distribution of the image on the left was shifted +20 degrees making the image appear more green, and on the right the hues were shifted -20 degrees to give a redder appearance. The hue shift in either direction was approximately 10 %, which was deemed significant enough to be noticeable, by the author's, but not so extreme as to be unrealistic.

6.5.4 Recruitment of participants

Participants were recruited by Amazon's crowdsourcing MTurk platform. Participants could access the survey if they were living in the UK and had an historical acceptance rate on the MTurk platfrom greater than 95 % as recommended by (Keith et al., 2017) for data quality. For completing the survey, which took an average of eight minutes and thirty seconds, participants were paid £1.02. Each participant was only allowed to complete the survey once. The survey was carried out in July and August of 2020. The survey had ethics

approval by the school of Chemistry, Food and Pharmacy of the University of Reading (ref: 31/2020). Prior to starting the survey, participants were informed as to reason why the research was being undertaken and how the data was going to be used. The participants had to confirm that they understood the conditions of participation before they could undertake the survey.

6.5.5 Consumer survey

The survey was created with Google Forms and consisted of three sections; background questions, evaluation of the lettuce and rocket images that had been colour modified, evaluation of the time-lapse videos of lettuce over shelf-life.

6.5.5.1 Background questions

Participants were also asked some background questions related to their demographic, purchasing and consumption habits. Participants were asked three demographic questions, all multiple choice (gender, age and the highest level of education achieved). They were also

asked to record details about the device they were using, including screen brand and model, operating system and device model. We also embedded the short Ishihara test within the questionnaire to test for colour blindness. Three questions were asked relating to the purchase and consumption of leafy salads: How many portions of leafy salads do you consume per week? (select from: less than one, 1, 2, 3, 4, 5, more than 5); Where do you normally purchase your leafy salad products? (select from: in-store or online) and how do you normally consume your salad (select from: as a standalone meal, as part of another meal, or other). Two questions regarding quality were asked: when purchasing leafy salads what is the most important factor? (Price, Date on pack, visual appearance or other) and what is your most common reason for discarding a leafy salad? (Not used in time, passed the date on the pack, became visually unappealing, smelled-off or other). Where the option was given for "other", the participants could enter a short comment. Aside from the demographic questions, the questions were presented in a random order as were the selectable answers to the questions.

6.5.5.2 Evaluation of colour modified lettuce images

Participants were shown an image which comprised of the same lettuce leaves with different hue distributions (Figure 6.1) where the original image was in the center with modified images on the left and right. The participants were then asked to indicate which image they would consider for purchase in the form of tick all that apply. The participants did not see the histograms with the image only the portion of Figure 6.1 encapsulated with a black background. Each participant only saw the image once. The image was presented with the three versions of the lettuce in the same order to each participant to mimic side by side product images on a web page of similar products. As there is no legislation regarding online product images, it could be assumed that the colour of the images may be altered to influence consumer behaviour.

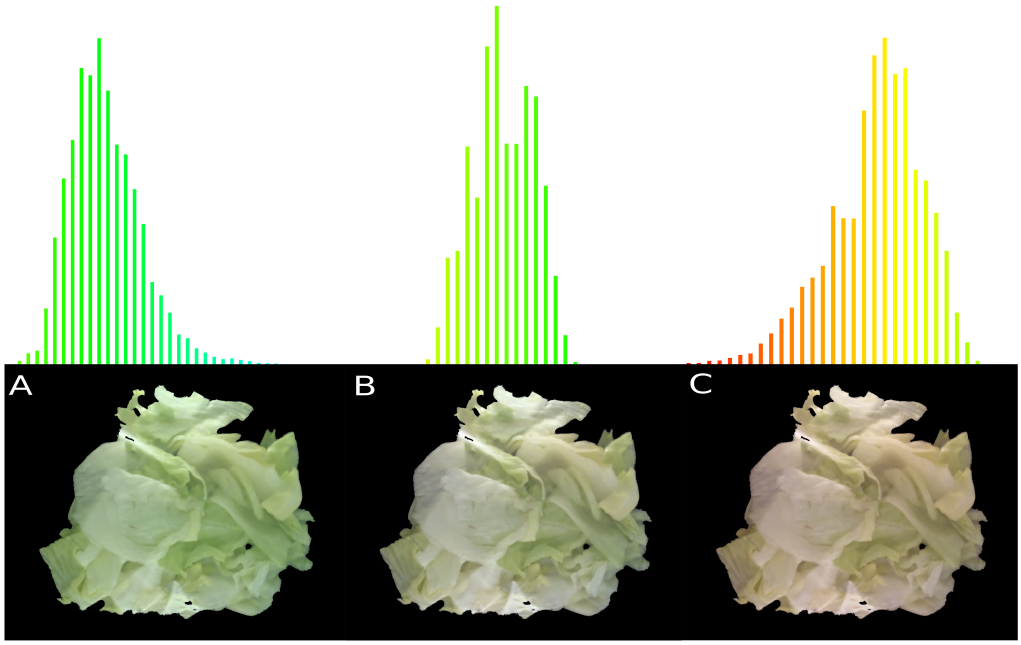

Figure 6.1: The test image shown to participants with added hue histograms of the image. A) Hue angle range 70-130, B) 25-100, C) 0-60.

6.5.5.3 Evaluation of the time-lapse videos

Participants were presented fours videos in a set order: the iceberg shorter shelf life video (ILA), followed by the rocket shorter shelf life video (RA), then the longer shelf life video of the lettuce (ILB) and rocket video (RB). They were asked to record the time point at which they would no longer consume the product.

6.5.5.4 Image analysis from time-lapse videos

Each individual image that made up the time-lapse video contained an x-rite ColorChecker Passport Photo 2 (Michigan, USA), which was used to adjust the colour of the images with reference to a master image to ensure colour constancy between images. The master image was taken from the first day of the first trial for each lettuce and rocket. Adjusted images were then exported with no compression as png file type. When analysing the colour of a time-lapse video at any given time-point, the images corresponding to the time-point indicated by the consumer was analysed. Colour statistics (mean hue angle, hue angle distribution, discolouration) were calculated from individual leaves within each image by a custom Python (v3.8) script utilising the packages PlantCV v3.0 (https://plantcv.readthedocs.io) and scikit-image v0.16.2 (https://scikit-image.org) v0.16.2. The discolouration was defined as any hue values outside of the HSV ranges [34-148,0-100,0-100], which are the red/brown hues.

6.5.6 Statistical analysis of survey data

Contingency tables were produced from response data for demographic and consumer habit questions, and associations were tested using chi-square tests using the scikit-learn (v 0.22.1) module for Python (Pedregosa et al., 2011). Results were considered significant if $p \leq 0.05$.

6.6 Results and discussion

6.6.1 Consumer comments from online food shoppers

For rocket there was a large amount of variation in the number of comments left on each retailers website, with the highest having 101 comments (Waitrose), the second most having 24 (Tesco), with the lowest only with five (Sainsbury's). In comparison, RTE iceberg lettuce was much less variable, where the highest number of comments on any website was 32 (Morrisons), the second most having 18 (Asda) and the lowest seven (Sainsbury's). Furthermore, the retailer who had the majority of comments for rocket was only the

third-highest for lettuce. This difference suggests that it is not necessarily retailer-specific as to whether a consumer will leave feedback but rather the specific product. There were approximately twice as many comments for rocket than there were for iceberg-lettuce, however, this was driven by one supermarket for rocket. Otherwise, the number of reviews for each product was comparable.

6.6.1.1 Lettuce

The clearest difference between the consumer comments for lettuce is that in the negative reviews, discolouration, specifically browning, is clearly an important factor (Figure 6.2). In contrast, the good reviews are more concerned with textural attributes such as “crisp”, and the abstract concept of “fresh”. Interestingly, the good reviews do not mention that the colour is good, and the bad reviews do not mention that the “freshness” or “crispness” is bad, rather they focus on colour aspects. A skew towards positivity is not unusual with online reviews (Engler et al., 2015). However, the opposite was true of the lettuce reviews; it may be that where there is a clear and obvious perceived defect, such as discolouration, consumers are more likely

to complain. Furthermore, it is noted that in the negative reviews, the use-by date is clearly something consumers appreciate when the product is not perceived to last as long as the date on the pack suggests. Reciprocally, when there is a positive perception of the product, the idea that the product meets or exceeds the date on the pack is not a particularly high concern. In the context of shelf-life dating, consumers still reject products prior to them becoming potentially dangerous if the perceived quality is not as expected, which further highlights the visual importance of the product in the consumer's decision making.

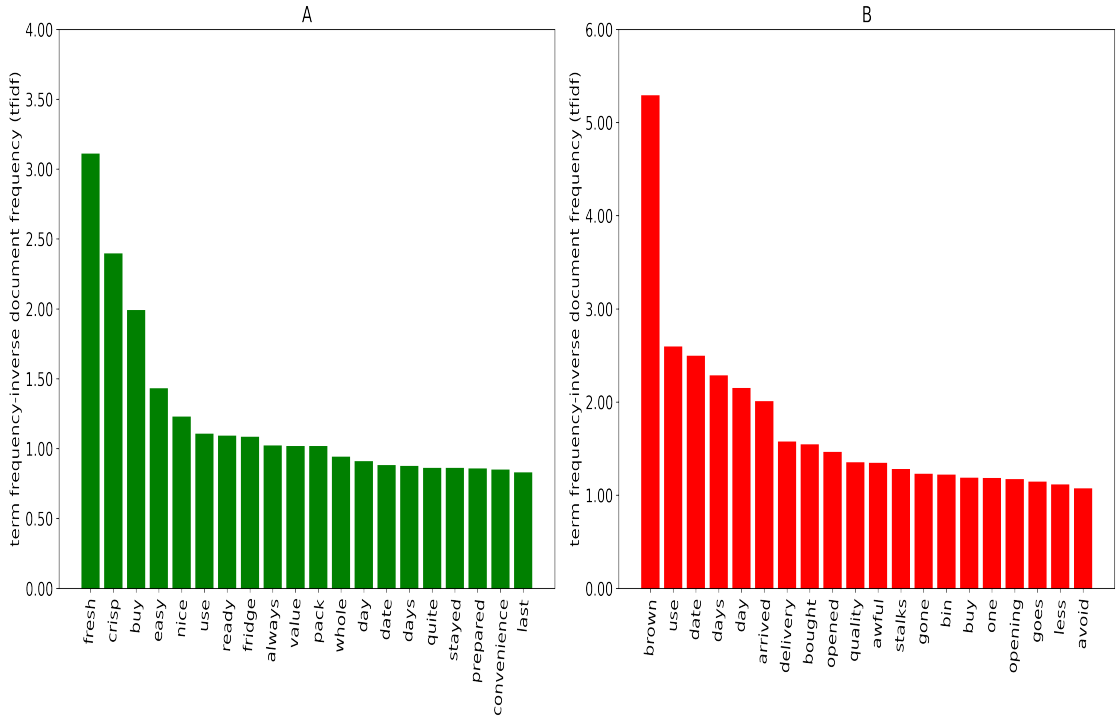

Figure 6.2: The 20 most highly weighted words from the good and bad reviews for RTE iceberg lettuce. There are 29 good (> three-star) reviews, 47 bad (< three-star) reviews and six three-star (neutral) reviews.

6.6.1.2 Rocket

It is evident that for rocket, colour is not something that the consumers consider to be positive or negative (Figure 6.3) evidenced by no words relating to colour appearing in the top 20. Flavour and taste are clearly important attributes that are expressed in positive reviews, whereas negative reviews tend to focus on dates, also seen in lettuce, and moisture build-up in the packaging. Even more so

than with lettuce, there is a skew towards positive reviews (118 versus 30 bad). Overall, there are far more reviews of rocket salads than of RTE iceberg lettuce. This may be because rocket leaves have a higher price than lettuce and may potentially be valued more (Mastrobuoni et al., 2014).

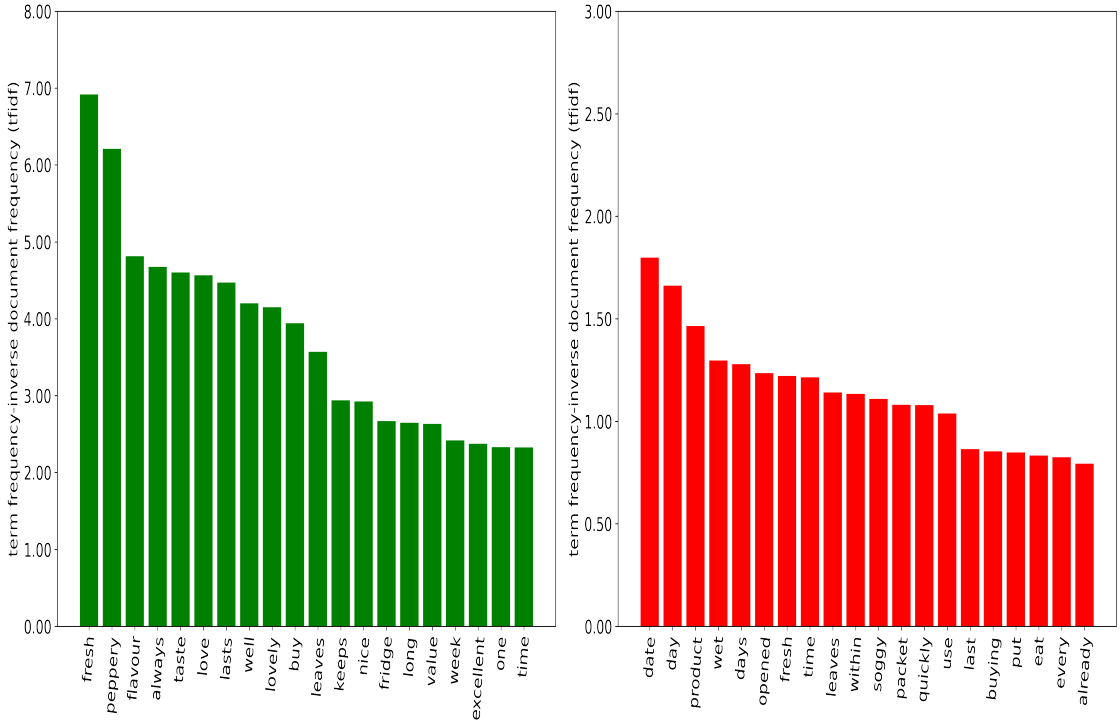

Figure 6.3: The 20 most highly weighted words from the good and bad reviews for RTE rocket. There are 118 good (> three-star) reviews, 30 bad (< three-star) reviews and eight three-star (neutral) reviews.

6.6.2 Demographics, shopping and consumption habits for consumers that took part in the survey

There were 346 participants that took part in the survey. The age and gender distribution of the participants are shown in (Figure 6.4).

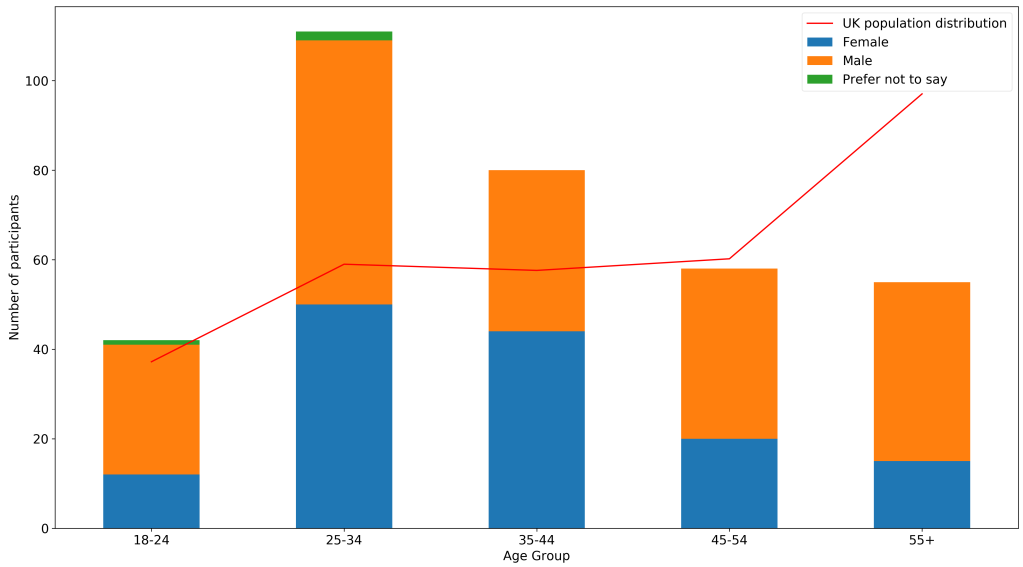

Figure 6.4: The age and gender distribution of participants who completed the survey. The "UK population distribution" is the sum of male and females in that age bracket divided by two and expressed as a percentage of adults. (ONS, 2019). The UK population distribution shows the number of participants expected to be in the survey if the online cohort reflected the population of the UK. N = 346.

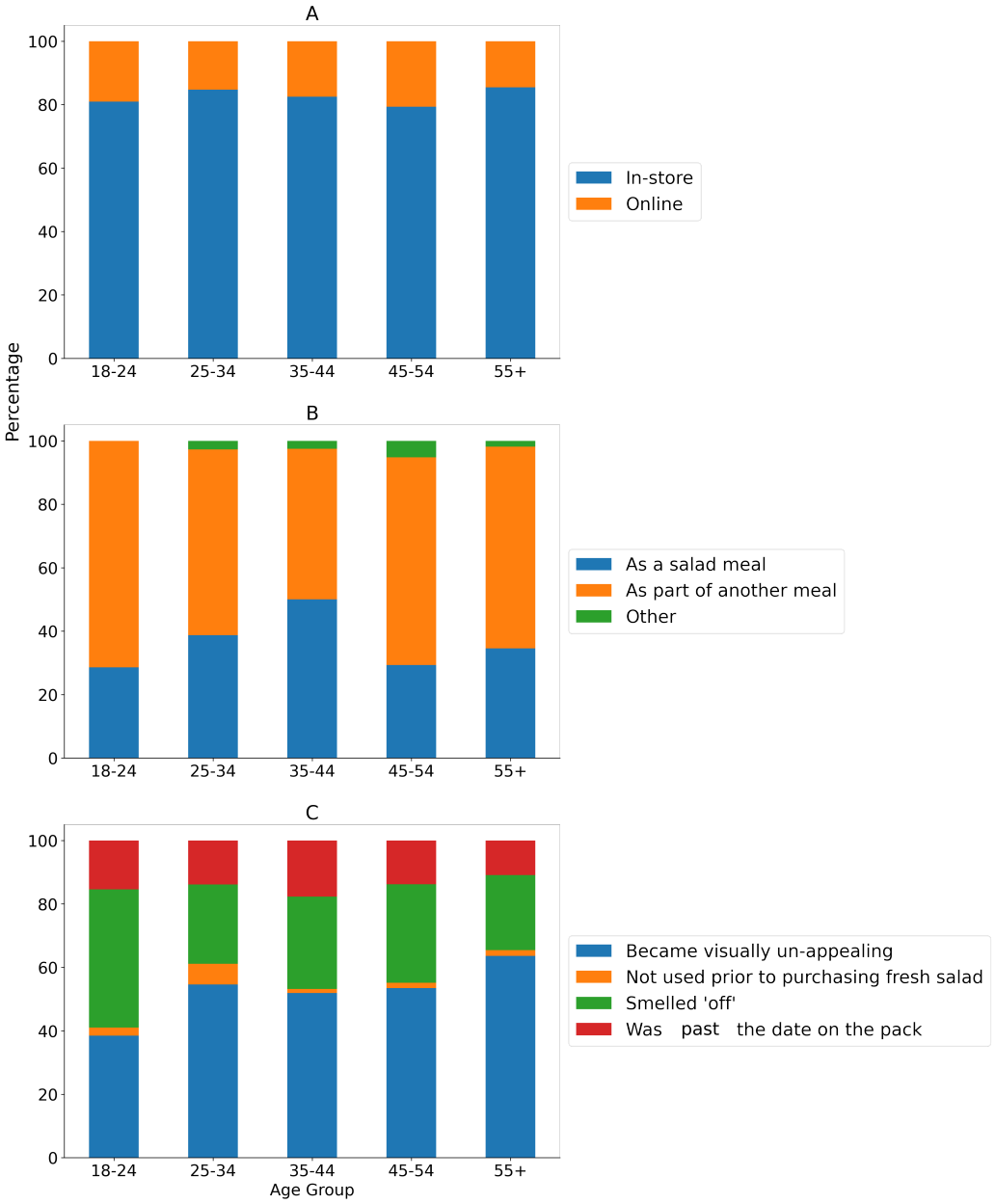

Figure 6.5: The proportion of consumer responses to the questions: A) Where do you normally purchase your leafy salads? B) How do you normally consume your leafy vegetables? C) What is your most common reason for discarding a leafy salad? (Number of consumers in each age group: n = (39, 108, 79, 58, 55).

6.6.2.1 Purchasing online versus in-store

From the sample population it is clear that the majority ($\sim$ 80 %) of people did their shopping in-store (Figure 6.5, A). This survey was coincided with the 1st COVID-19 "lockdown" which may have changed people's shopping habits, potentially over representing online shopping. Furthermore, those who were more willing to participate in an online survey may be more likely to also shop online (Hernández et al., 2011). Previously it has been estimated that the number of shoppers purchasing groceries online in the UK is 15 % (Munson, Tiropanis and Lowe, 2017). From the survey, there was no-significant difference in consumption or waste habits between participants that shopped online compared to those that shopped in store ($p= 0.99$ and $p= 0.95$ respectively). Typically, when asked why people choose to shop in store or online, those who prefer to shop in store often cite the ability to pick up and look at the product a reason as to why it is preferred (Hackney et al., 2006). Therefore, it may be expected that those who purchase online would be less interested in the visual quality of the product, but this is not supported by the data from this survey.

6.6.2.2 Consumption habits

The majority of respondents tended to consume their salad as part of another meal with 36 % typically consuming their salad as a standalone meal (Figure 6.5, B). There was no significant difference (p = 0.15) in the importance of visual quality between those who mainly consumed salad as a meal on its own and those who typically incorporate salad leaves into a larger meal.

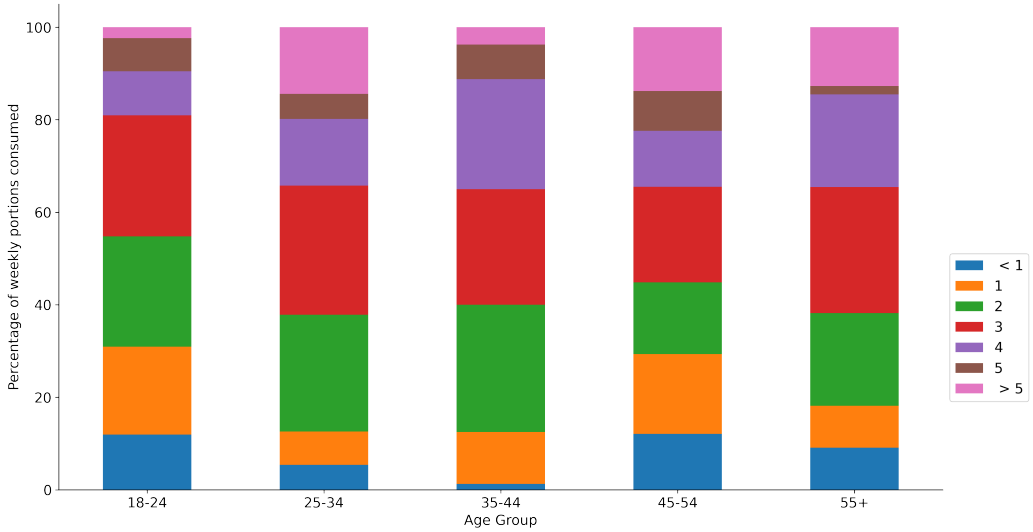

Figure 6.6: The number of leafy salad portions consumed per week by respondents to the survey grouped by age. N of age groups = (39, 108, 79, 58, 55).

On average, the 25-34 age group reported consuming the most portions of leafy salads per week of 3.2 $\pm$ 1.4, with 18-24 consuming the lowest 2.5 $\pm$ 1.3, there was a significant difference between

these two groups (p = 0.004). There were no significant differences between any other group with the 35-44 age group consuming on average 2.9 $\pm$ 1.2, 45-54 consuming 2.8 $\pm$ 1.5 and 55+ consuming 2.9 $\pm$ 1.3 portions per week. These data are consistent with historical data for the UK. The 18-24 age group does consume the fewest portions of fruits and vegetables per week, with all over age groups having similar consumption habits (ONS, 2020).

6.6.2.3 Reasons for discard and purchase

The majority (57.5 %) of respondents selected "Visual appearance" as the primary decision factor when purchasing a leafy salad product. After visual appearance, the date on the pack was the second most selected option (23 %) followed by price (17 %). Fewer than 5 % of participants gave another reason as to their purchase factor, and the majority of those were re-wordings of the options provided. For discarding the product, "Became visually un-appealing" was the most common reason with the exception of the 18-24 age group where it was equal to "smelled off" (Figure 6.5, C). There was a trend in the data that suggested that the older the respondent

was, the more often they would rely on a visual assessment (or less on smell and dates) of the leafy salad although this result was not significant ($p = 0.35$). However, it is also noted that smell sensitivity declines with age (Zhang and Wang, 2017), which may partly explain this data. There was an association between portions consumed per week and reasons for discarding the product (Figure 6.7), whereby those who consumed one or fewer portions were significantly more likely to discard when the product passed an on-pack date than those who consumed four portions ($p = 0.0021$) and more than five portions ($p = 0.05$) per week. These data suggest that those who are less familiar with a product are more likely to use the information provided by the manafacturer in the form of on-pack dates. The same result was not true for the decision to purchase the product where all groups considered the visual quality to be the most important, with date on the pack second and price third. Similar results to these were discovered by Lyndhurst (2008) who emphasized the importance of leafy salads' visual component from the consumer perspective. However, any decision to discard or purchase a product is based around a multitude of factors such as previous experience, emotional state and the composition of the meal being prepared (van

der Laan et al., 2011).

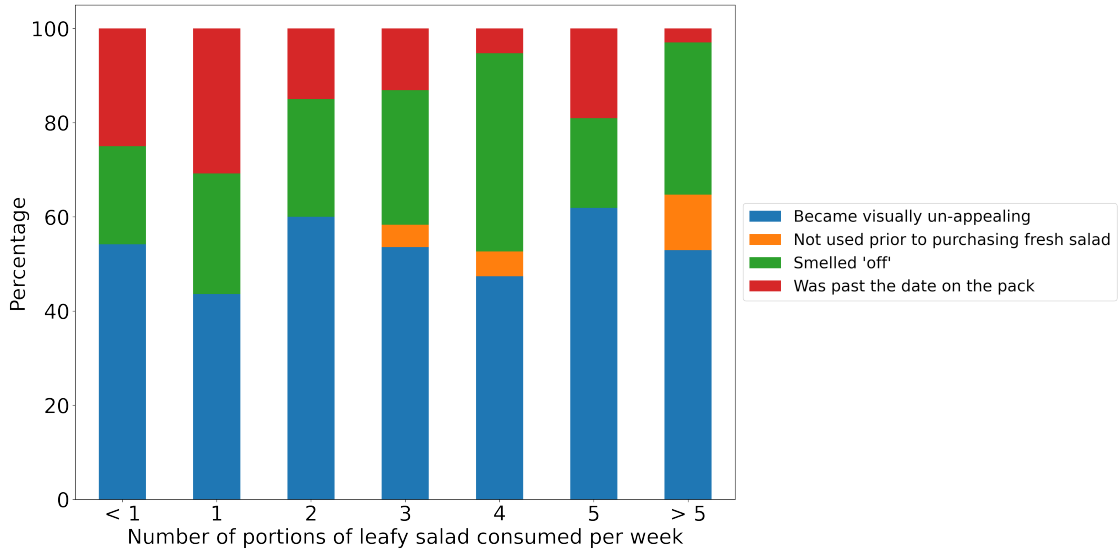

Figure 6.7: Relationship between portions of leafy salad consumer per week and reason for rejection. The proportion of consumers within each consumption category were n = (24, 39, 80, 76, 52, 21, 30).

6.6.3 Consumer acceptance responses to colour altered images of pre-cut lettuce

From the colour manipulated image that was shown of iceberg lettuce (Figure 6.1), the overwhelming majority of consumers (66 %) declared that they would only choose the lettuce depicted in the "greened" image ($p < 0.001$). A further 30 % indicated they would only purchase lettuce depicted by the greened image or the original, whereas only two percent chose the image that had increased

red hues. Given that the images were identical, with the exception of colour, it can only be the colour that influenced the consumers' decision. It has previously been shown that consumers prefer darker green leaves in comparison to paler leaves (Chonpracha et al., 2020) as we have also found.

It is unclear how influenced consumers are by image colour in their decision to purchase groceries online, but it is cited as one of the primary reasons consumers prefer to shop in store (Hackney et al., 2006). As leafy salads are influenced by seasonal and geographical growing environments, it is expected that the visual appearance will differ throughout the year. However, online images do not reflect this change and remain the same, and it is unclear as to how online images are regulated. It is conceivable that the images may be produced in a way that is more appealing to a consumer, as many facets of the consumer experience are designed to maximise consumer spending (Guthrie and Parikh, 2020).

6.6.4 Discolouration and consumer rejection of lettuce and rocket over storage time; presented by time-lapse video

6.6.4.1 Lettuce

Over the standard shelf life of lettuce, captured by video ILA, there was a relatively low level of discolouration of less than 2 % of the total leaf area over 14 days (Figure 6.8, ILA). However, over the extended shelf life (Figure 6.8, ILB), the discolouration reached 12 % in 16 days. Although greater discolouration is likely to occur over a longer shelf life period, the differences observed between ILA and ILB are most likely attributed to differences in growing conditions. Where there is a relatively low level of discolouration present, such as in (Figure 6.8, ILA) which shows a minimal linear increase in discolouration over time, the corresponding proportion of consumers rejecting the product was also linear. With relatively low discolouration, it was not until six days after the use-by that 50 % of the consumers would no longer consume the product. The packaging did not have a date label, and the point at which most of the consumers rejected the product was inferred from curve fit to (Figure 6.8, ILA). When there are minimal perceivable changes in

the product, the shelf life seems to be over-estimated by the consumer. Similar results were obtained by (Manzocco et al., 2017) with Lamb's lettuce, but instead of time-lapse videos, the participants were shown still images.

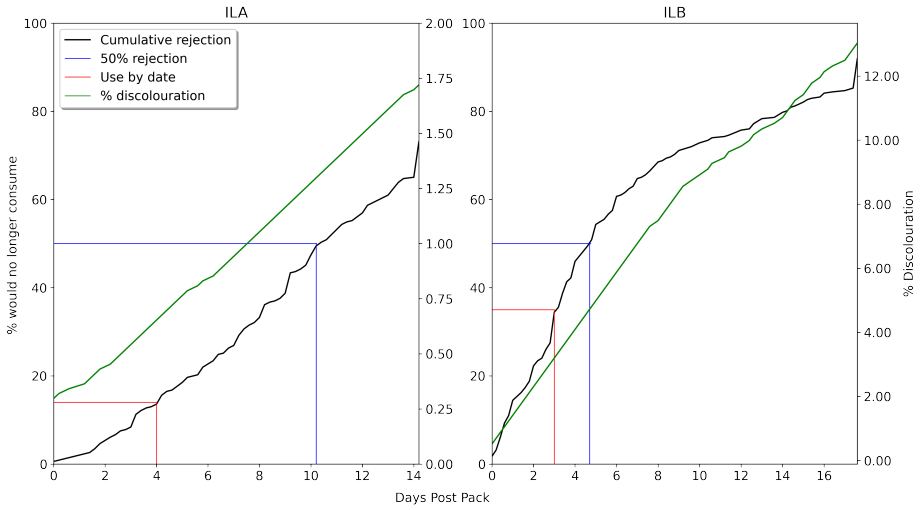

Figure 6.8: Consumer rejection of iceberg lettuce time lapse videos stored at 4 °C. ILA was the first video shown to consumers and ILB was the third. The use-by date was not included on the packaging or indicated to the consumers. For each video n = 346.

Where discolouration, in both rate and magnitude, was more apparent the sooner the majority of consumers rejected the product (Figure 6.8, ILB). There was a ten percent increase in discolouration and rejection by the consumer per day up to day eight, whereafter the rate of change started to reduce. As time passed, those who are less influenced by discolouration are the remaining participants, and

therefore, the rate of rejection slows down. The video that contained the leaves with the most discolouration (Figure 6.8, ILB) had a 50 % rejection rate that was within two days of the use-by date of the original product where the discolouration was around 5 %. From this, it is clear that the colour was a significant factor in the consumer rejecting the product. Across both videos, there were only 25 % and 5 % of people, respectively, who would consume the product at any stage shown in the videos (Figure 6.8, ILA, ILB).

It is clear that the colour of the product is highly important when the consumer is assessing the product, particularly with lettuce, where there is often clear pink/brown discolouration (Saltveit, 2018). However, when the product does not deteriorate or discolour to a noticeable amount, consumers are less discriminatory and therefore would estimate a shelf life that is longer than the date shown on the pack (Chonpracha et al., 2020).

6.6.4.2 Rocket

The rocket leaves exhibited no evident colour change that could be expressed in terms of changes in aggregate pixels of that colour e.g.

yellow. The phenomenon of no clear change in measurable colour, yet an observable decline in visual quality, is evident in the literature with many papers reporting no colour change when measured empirically (Gutierrez and Rodriguez, 2017; Giannoglou et al., 2020) contrasted with those that have asked consumers/sensory panels to assess colour (Amodio et al., 2015). Our work demonstrates that analysing individual colours does not describe rocket leaves' visual changes and that more complex method was required. There were clear changes in the rocket leaves videos, such as water droplet formation and wilting that were not present in the lettuce videos. Respiration and moisture build-up in packaging are known issues with leafy salads and are unappealing to the consumer (Løkke et al., 2012; Martínez-Sánchez et al., 2012) and may explain a proportion of the rejection by the consumer for the videos they were shown, given the minimal colour changes. For both of the videos shown, 50 % of the consumers rejected the products seven (Figure 6.9, RA) and fifteen (Figure 6.9, RB) days after the use-by date on the original packaging. Similar to the lettuce video (Figure 6.8, ILA) where there was minimal visual change, the majority of the consumers rejected the product far later than the on-pack date suggested - based

on visual information alone. These data suggest that when visual appearance alone is considered, the point at which the consumer is willing to consume the product is perceived to be much longer than the safety margin applied by the retailer from the consumer's perspective. In this study, the consumer could not see the manufacturer's on-pack date, undoubtedly affecting the consumer's decision to reject a product. Furthermore, other factors such as the time until the next shop is due, or the time since the last shop, as well as the potential meal planning considerations could not influence the consumer's decision which would be factors if the consumer had the product in their own home.

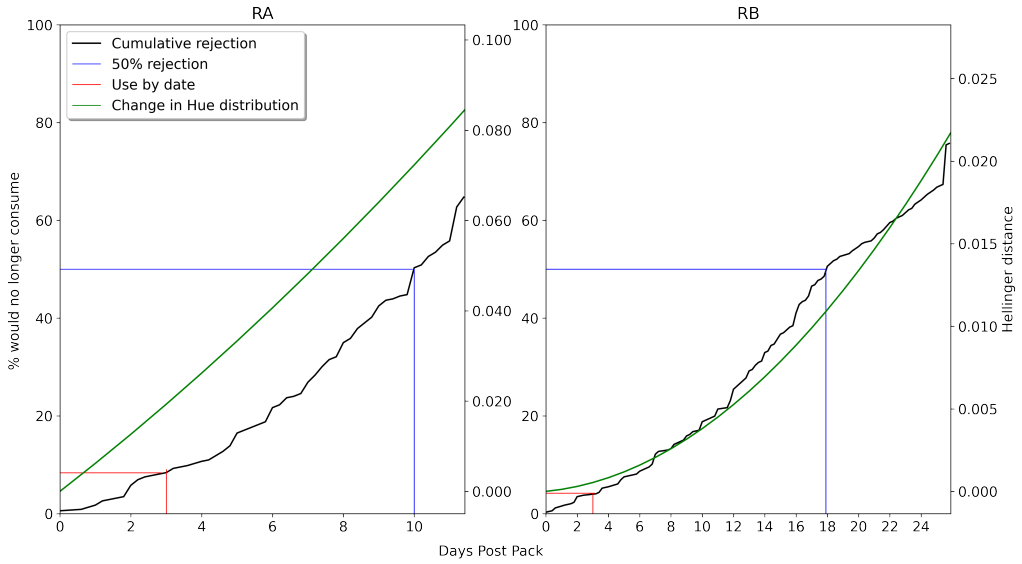

Figure 6.9: Consumer rejection of rocket leaves time lapse videos stored at 4 °C. RA was the second video shown to consumers and RB was the fourth. The use-by date was not included on the packaging or indicated to the consumers. For each video n = 346.

Although there were no differences in individual colours, there were differences in the images that made up the video. The hue channel was extracted from the images, and histograms were then constructed of the hues to compare these differences. The distance between histograms different days was calculated using the hellinger distance.

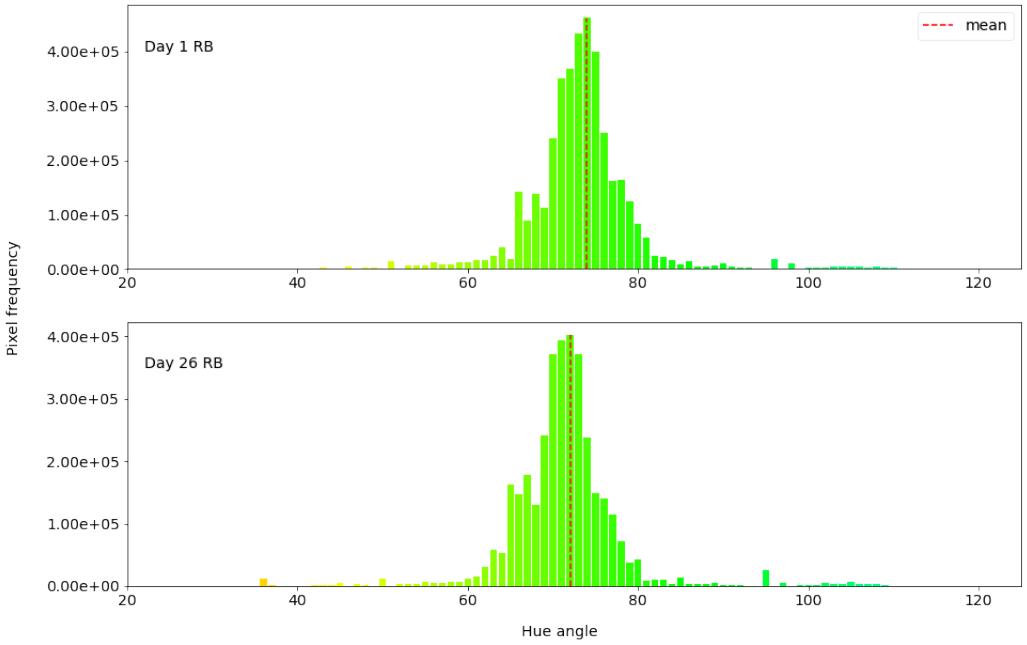

Figure 6.10: A comparison of the hue histograms for the first and last image of the second rocket time lapse video RB.

Figure 6.10 shows the difference between the hue histograms extracted from the first and last images of time lapse video RB. As the leaves are mostly green, the number of pixels that are green far exceed those of another hue. When there are clear visual defects they often make up only a small percentage of pixels and therfore don't affect the mean hue. Taking the entire distribution into account using a histogram comparions metric (in this case hellinger distance) allows for smaller changes in the image to be realised.

In general, the distances increased over time. Therefore, the differ-

ences in the hue distributions increased over time. Using distance metrics to compare images is a relatively common technique for computer vision tasks (Liu and Yang, 2013; Font et al., 2014); however, it has not been previously used to compare images in a shelf life trial.

The primary concern with expressing changes in visual quality as a distance metric between hue distributions is that it is reliant on a reference image. Whereas, where there are clear, quantifiable colour changes, such as in lettuce, consumer rejection can be expressed in terms of specific colours. It is apparent that factors other than the colour change, such as liquid accumulation, are important for visual quality and are picked up by the consumer but may have an insignificant impact on colour.

6.7 Conclusions - what do consumers want from their salads?

The results from this study have shown that prior experience is important in how consumers evaluate a product. The results of this

study conclude that, in the context of salad leaves, consumers with greater experience tended to rely less on on-pack dates and more on there own visual perception (Figure 6.5, C).

Where colour change can be observed, consumers will use this a proxy for quality; however, it is clear that other visual features are used when a colour change is not perceived. After purchasing a product, the consumer has an image of what a fresh salad should look like and is anchored to that image (Lieder et al., 2018). The further the product deviates from this, the less likely someone is to consume the product. However, in this study there was a proportion of the population $\sim$ 20 % for which discolouration was not a primary concern (Figures 6.8-6.9).

Over storage of lettuce there was a clear and obvious increase in discolouration (Figure 6.8, ILB) of leaf area by approximately 0.8 % /day). The point at which 50 % of the consumers rejected the product was within two days of the manufacturer's use-by date. Where there was minimal visual change (for example, 0.1 % discolouration of lettuce or overall the rocket samples where the video concluded negligible visual change), the time at which the majority of the con-

sumer rejected the product was typically more than one week after the expiry date (Figures 6.8-6.9). It may be expected that if consumers only relied on visual quality then there may be much higher incidences of food poisoning, assuming that use-by dates are accurate and that people would report such incidences. The results from this study are in-line with a recent survey by the Food Standards Agency (FSA, 2021), where 76 % of adults had knowingly consumed food past its use-by date, and 44 % of respondents considered the use-by date as a "usefull" guide. The findings of this study do not support the basis for extending use-by dates, without accompanying verification that the microbial safety would not be compromised.

Of the tangible reasons for discarding a leafy salad product, visual appearance is the primary factor, but it is only a portion of the multiple factors that go into decision making. Prior experience is important as to what extent a consumer will take into account the visual appearance, with the less experienced tending to incorporate shelf life dates to a larger extent in their decision making (Figure 6.5, C). Considering the visual appearance, colour has a large influence on

the decision making progress, but only when the product has a significantly different colour profile than is expected. Otherwise, visual features, such as moisture build-up or wilting, will be more important.

6.8 Appendix

6.8.1 Video Links

The time-lapse videos used in this study can be accessed via the following links:

- ILA - https://youtu.be/O_2gDxuoK3Y
- ILB - https://youtu.be/qc2vjYlA9bo
- RA - https://youtu.be/657dCPqfO4o
- RB - https://youtu.be/Gcx25aGrNE0

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Chapter 7

General Discussion

This thesis has sought to understand further how freshness is defined for leafy salads, how this changes in the consumer environment and how waste from this particular resource can be reduced. Food waste has always been a secondary concern to the under-production of food (Facchini et al., 2018). However, with increasing concern over population growth and climate change, the ability to consistently increase available food supplies is not a given (Reisch et al., 2021). The sustainable use of resources is a rising priority and using water, energy and inorganic chemicals to produce food that never gets consumed contributes to the unsustainability of our food system. Reducing the amount of food that is wasted from all parts of

the supply chain and, more importantly in developed countries, from the consumer is vital (Reynolds et al., 2014). With the exception of cultural change, which is notoriously hard to define and effect (e.g. consumers knowing exactly what they need to purchase and actually consuming all of it), several options can be taken to minimise food waste (Reutter et al., 2017). Preventing the overproduction of food and over-purchasing of food by the consumer is the optimal solution (Facchini et al., 2018); after this point, everything is waste management. From the consumer perspective, only purchasing the required amount of food is the easiest way to reduce food waste, although this is not always an option, and particularly with leafy salads, the pack size is predetermined. Secondary to only purchasing what is required is the optimisation of on-pack dates to more accurately reflect the condition of the product (Quested et al., 2011). In Chapter 6, it was found that on-pack dates were not the primary decision-driver as to whether a product was discarded, suggesting that consumers are not entirely confident that the dates represent the true condition of the product (Figure 6.5, C).

One by-product of the centralisation of the food supply by supermar-

kets is that stock control became more complex with large volumes of products. As a consequence, dates were placed on the packs of products to aid supermarket workers. Slowly these on-pack dates mutated to also provide information to the consumer about the condition of the product (Figure 2.2). The condition of any given product is variable, but the way in which this is communicated to the consumer is static. Knowing the condition of the product allows communication to the consumer about the safety, in the form of "use-by", or quality as a "best-before" date. Typically, when safety is concerned a conservative margin is added, at the expense of potential waste, to the date on the pack. A consumer consuming food that is harmful to them is a type I error whereas, throwing away food that is acceptable to eat whilst believing it to be harmful is a type II error. As type I and type II errors are inversely related, minimising one increases the other proportionally (Akobeng, 2016). With perfect information, it would be possible to predict the precise time point at which a product would no longer be consumable. However, this is unlikely to happen any time soon, hence the margin of error. To provide the consumer with as accurate at possible information about the condition of a product, continuous monitor-

ing would be required (Brizio and Prentice, 2015). This is currently an aspiration for the majority of food products, rather than a reality. Those systems that do exist, such as time-temperature indicators or CO_2 monitors, are limited to monitoring just one aspect of safety or freshness, whereas this thesis has demonstrated that freshness and shelf life is a complexity that depends on many interlinked biological events. As described in more detail in the following section, Chapter three evaluated several different factors that influence the postharvest phytochemical composition (Table 3.1) of rocket leaves including: growing temperature, harvest, species and cultivar (Figures 3.1 and 3.2).

Whilst it is acknowledged that the research described in this thesis is limited to the leafy vegetables iceberg lettuce, and rocket and the waste from these products are relatively minor in comparison to other food products such as red meat, it is hoped that some of the fundamental ideas which have been studied will apply to other fresh produce and aid with the reduction of waste.

The main aims of this thesis were to:

- Understand how shelf life is defined in the context of leafy salads, both in terms of legislation and by quality attributes.
- To monitor and evaluate potential physiological, biological and microbiological markers of shelf life.
- Assess the consumers perception of shelf life and how this aligns with previously identified markers.

Chapter two discussed the current system for defining how leafy salad products change over time and highlights the historical context in which the current system of shelf life dating came to exist (Figure 2.2). Furthermore, we considered the primary areas in which shelf life dates may be assessed, such as microbiology (Table 2.2), visual appearance, and aroma. This chapter suggested that while the process of shelf life dating is many decades old at this point (Shottenfeld, 1973), there is a significant degree of variation around the date given on the pack due to the the difficulty in accurately predicting the condition of any given product weeks in the future. The duality of inaccurate shelf life dates is a wasted product on one side, and food being sold that could be injurious to health or not of the quality expected by the consumer may be sold on the other. One of

the main conclusions from this chapter was that to minimise food waste, a dynamic system for monitoring food and updating the estimate of shelf life would be required to reduce the margin of error that is currently applied to salads. This led to further examination of pre-harvest factors that influence the variability over subsequent shelf life in Chapter two.

Chapter three evaluated the two different species of rocket leaves (*Diplotaxis tenuifolia* and *Eruca sativa*) grown at different temperatures (Table 3.1) and the effect these different factors had on the postharvest quality, as measured by key characteristic compounds (Figures 3.1 and 3.2). One aspect that was previously missing from the body of literature is the influence of multiple cuts of the same plant on its phytochemical composition, which is common practice among farmers but has previously been absent from the literature. It was found that the glucosinolate and glucosinolate breakdown products were higher in the second cut of rocket leaves, which highlights that when assessing rocket leaves for quality the cut is a factor that should be considered. In this chapter Jasper et al., (2020) found that there was a strong influence of genotype and growth temperature

on the postharvest glucosinolate hydrolysis products. Several characteristic compounds, such as progoitrin and glucosativin (Figure 4.7) were found to increase/decrease over shelf life. Although these are not solely responsible for the taste/aroma of the product, they do contribute to it significantly (Bell et al., 2017). Recent publication of the IPCC report on Climate Change (2021) has demonstrated unequivocally that the temperature of the earth is rising due to human activity. The consequence of this is increased extreme weather events and fluctuations in the environmental conditions that we will need to produce food crops under. Jasper et al (2020) demonstrated that a variable preharvest environment will lead to less consistent postharvest duration and performance. Suppose the quality of leafy salads do become more variable due to a more unpredictable environment. In that case, it is likely that the on-pack dates will become shorter to account for this, unless a more accurate method for monitoring and predicting shelf life is implemented. Chapters three and four investigate several potential and established markers for quality in iceberg lettuce and rocket salads.

Chapters four and five extended the work undertaken in Chapter two,

focusing on the postharvest assessment of commercially available rocket salads and iceberg lettuce, respectively. From the research carried out in the previous chapters, several potential and known markers were chosen to be analysed through the extended shelf life trials. Furthermore, as it was postulated that a dynamic approach to assessing a particular product would be required to reduce the margin of error associated with on-pack dates, an imaging system and analysis method were developed. One serendipitous outcome from this research was the development and inclusion of a function for analysing the mean hue of an image, which functioned by extracting the hue histogram from an image and calculating the circular mean and standard deviation. This function has been in the open-source software "PlantCV", which was developed by the Danforth centre (https://www.danforthcenter.org), since 2018 and the package has been used by many researchers since.

Chapters four and five followed similar methodologies as each other, but with the addition of measuring glucosinolates and glucosinolate breakdown products for rocket leaves. For rocket, of all the variables measured, aerobic colony count had the highest correlation with vi-

sual quality (Figure 5.7; R^2 = 0.46, p = $3.1 * 10^{-28}$). It was also found that using conventional metrics for quantifying visual quality (mean hue values) did not represent the degradation of the product particularly well. As a consequence, a more advanced approach had to be undertaken where rather than using aggregate statistics revolving around measuring the mean hue, it was found that using a distance function for the comparison of hue histograms in the HSV colour space was more effective in quantifying the visual quality of the rocket leaves (Figure 6.10). As far as we are aware, this method has not been used for this purpose before. This methodology was later used in Chapter six for the analysis of time-lapse video in response to consumer rejection (Figures 6.8 and 6.9). In the time-lapse video, it was clear that there were changes in visual quality as there were signs of wilting and moisture build-up in the packaging. However, when analysing the mean hues, there were no clear differences over the video. It was even difficult for lab members to tell if the video was being played in reverse, so analysing the hue histograms was found to be a useful method, particularly when visual differences between images were hard to distinguish. Whilst none of the phytochemical variables measured correlated with postharvest stor-

age, the trial that deteriorated the quickest R2 (Figure 4.5) also had the lowest levels of total soluble sugars and the highest ammonia content (a good quality marker in lettuce (Figure 4.6)) as well as the highest level of glucosinolates (Figure 4.7). The relative levels of sugars, ammonia and glucosinolates suggest that the leaves were previously in a stressed state, most likely due to suboptimal storage conditions (Witkowska and Woltering, 2014).

The work from Chapters 4 and 5 were compiled with work carried out in Chapter 6 to produce a model of consumer rejection in response to changes in measured parameters. For both rocket and lettuce, Figures 7.1 and 7.2 were produced by taking the average from each of the consumer rejection curves (Figures 6.8 and 6.9) and then regressing the averaged curve with the combined data for (R1-3, IL1-3) for each of the reported parameters. The consumer rejection curves were compared with the discolouration of the respective lettuce/rocket leaves, which could then be used to model the change in the other reported parameters, as discolouration was measured in both datasets. Therefore, the change in total sugars (for example) could be compared with the rejection of iceberg lettuce

from Figure 6.8 to estimate the proportion of consumers that would reject that product at a given time point. These compositional figures are intended to emphasise that using a chronological date to represent the safety/quality of a product is inferior to using biological measurements. Although it is recognised that as the data was not collected at the same time, there are limitations as to how accurate the data is and the predictions that can be made from it.

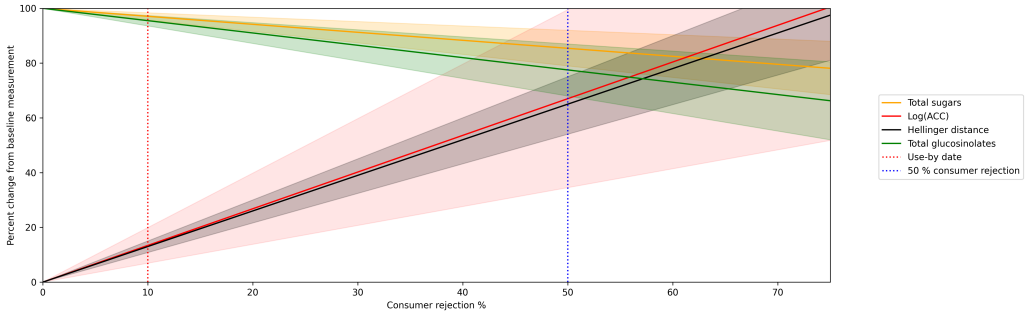

Figure 7.1: Plot showing the average percent change with respect to consumer rejection across all rocket trials, for any variable that exhibited a significant change $p < 0.05$. The consumer rejection was calculated from the averaged results of the rocket time-lapse videos in Chapter 6. The shaded area represents the standard deviation for each paramter displayed.

From the compositional data in (Figure 7.1), the four variables (total sugars, total glucosinolates, aerobic colony count and hellinger distance) that exhibited a significant change over the postharvest period are shown. From these data it is the hellinger distance and aerobic colony count that varied the most with respect to consumer rejection. As with the lettuce results and the results from the time-lapse

video found in Chapter 6, the consumer rejection rate predicts that the majority of consumers would reject the product long after the use-by date when only considering visual appearance (Figures 6.9 and 6.8). Using a time-based system to communicate the product condition to the consumer doesn't necessarily reflect the condition of the product as, within a bag of salad leaves, the rate at which different leaves degrade is variable. The process of prediction for any stochastic system will likely produce variable results and the only way to overcome this is to continually update priors based on new information (Wang et al., 2014) if as high accuracy as possible is required. Therefore, if the waste that arises from on-pack dates is to be reduced to as low as possible, a dynamic approach is required. This work warrants further research into variables that could be dynamically monitored and to those that may indicate the rate of subsequent degradation of a product from measurements taken at harvest.

Chapter five found that ammonia has the greatest potential as an indicator of iceberg lettuce quality over shelf life (Figure 5.6) and beyond. Although the mechanism as to the increase in ammonia over shelf life is not formally established, previous studies have suggested

that the increase in ammonia is associated with protein catabolism (Cantwell et al., 2010). However, we also found that there was a significant association with micro-organisms (R^2 = 0.46, p = 1.97 $*$ 10^{-35}) which had not been a variable taken into account in previous studies. With the exception of soluble sugars, which declined throughout the postharvest period, none of the other variables exhibited any consistent trend over the three trials.

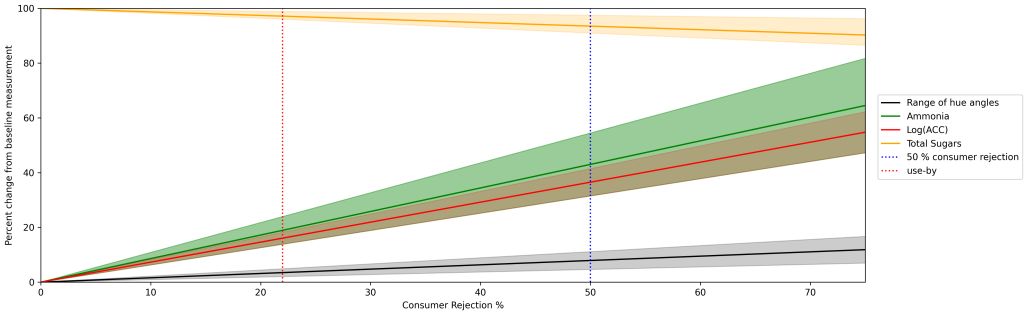

Figure 7.2: Plot showing the average percent change with respect to consumer rejection across all lettuce trials, for any variable that exhibited a significant change $p < 0.05$. The consumer rejection was calculated from the averaged results of the lettuce time-lapse videos in Chapter 6. The shaded area represents the standard deviation of each parameter displayed.

From the compositional data in (Figure 7.2), the four variables (total sugars, ammonia, aerobic colony count and range of hues) that exhibited a significant change over the postharvest period are shown. From these data, ammonia varied the greatest over the postharvest period, followed by aerobic colony count. Similar to the results found in Chapter 6, the consumer rejection rate predicts that the ma-

jority of consumers would reject the product around one week after the use-by date when only considering visual appearance (Figure 6.8). These data further highlight the potential importance of ammonia as an indicator of postharvest status. Although from the three trials conducted in this thesis, there was a relatively low level of discolouration in all trials, had this been higher (as was the case in the ILB in Chapter 6), the rate of change for range of hues would have been much higher, and the point at which most consumers would reject the product would be much closer to the use-by date. The results from this chapter suggests that future work looks at the possible pathways, be it microbial or metabolic, by which ammonia is produced to gain greater insight into the biology underpinning postharvest senescence of iceberg lettuce. Furthermore, the chapter identified that remote sensing of ammonia is currently possible (Kuswandi et al., 2012) and should be trialled with leafy salads.

Finally, Chapter 6 built on the methodologies developed in the previous chapters and assessed the consumer's attitudes and preferences towards the leafy salads, rocket and iceberg lettuce. From an online survey of 346 consumers, it was found that when there were no

clear visual defects, such as enzymatic browning or dark necrotic tissue, and no on-pack date, most consumers would consume the packed product around ten days after the use-by date had expired. However, when there were clear changes in visual quality (Figure 6.10), the majority of consumers rejected the product within a couple of days of the on-pack date. Furthermore, it was found that prior experience was an important factor in how consumers assessed a product, with younger, less experienced consumers more often considering the use-by date when discarding a product (Figure 6.5). It is clear that visual appearance is the primary factor consumers will use when purchasing or discarding a leafy salad. However, approximately 20 % of the consumers indicated they would consume the product at any time point that they were shown in any of the videos which were up to three times the stated use-by date (Figure 6.9). It is clear that decision making does not just hinge on one factor (Chen and Antonelli, 2020), and although it is seen that visual appearance is highest on the list, it is not the sole factor in food preference. As far as the authors are aware, the analysis of time-lapse videos for the visual quality of leafy salads has not been reported before in the academic literature.

Another novel aspect of this chapter is the analysis of online consumer comments left on supermarket websites to understand how consumers describe products they like and dislike. The consumer comment data found that in positive comments, attributes such as flavour and the concept of "fresh" were often cited, whereas, for poor products, the mention of a date was likely to be mentioned indicating that the shelf life was not as expected (Figure 6.3). For lettuce, the textural attribute of "crisp" ranked highest in the positive consumer comments, whereas the colour attribute "brown" was the word that ranked highest in the negative comments, followed by mentions of on-pack dates (Figure 6.2). For comments left on rocket products, "fresh" also ranked highest, followed by flavour and taste-related phrases, whereas date related words ranked highest in the negative reviews with words such as "wet" and "soggy" also ranking highly (Figure 6.3). As no words related to colour ranked highly in the analysis of consumer comments for rocket adding weight to the results that were found in Chapter four, whereby it was found that the mean hue of the image did not change significantly (Figure 4.1) and more advanced methods of analysis were required. The analysis carried out in this chapter was novel, and it is hoped that

the methodologies used here will help researchers and industry alike guide their efforts in exploring the factors consumers consider most important.

7.1 Future work

7.1.1 Large scale data acquisition and further development of computer vision and analysis system

Several further experiments could be undertaken to develop the work on image analysis for the measurement of leafy salads in the consumer environment. From a technological perspective, things have progressed rapidly since this work began, whereby cameras have become available which are higher resolution for similar cost to those used in this project. For the imaging in Chapters 4-6, the addition of infrared imaging would allow changes in the crop that are imperceptible to the human visual system to be evaluated (Brito et al., 2015). Combining the approach of incorporating the consumers perspective carried out in Chapter 6 with large scale data collection would allow predictions to be made as to the shelf life of a particular crop much

further in advance. Using the methodology that was established in Chapter 6 over many more harvests in combination with data gathering for several biological variables that may indicate postharvest longevity, such as soluble sugars, ammonia and nitrate, would allow better prediction of when a consumer would no longer consume the product. The prediction of when a consumer will discard a product is a big-data problem (Lusk, 2017), which is difficult for any individual researcher to solve alone. However, as commercial refrigerators with integrated cameras have been launched within the last few years, tracking a product from the growth stage to disposal is becoming more feasible. Linking up the entire supply chain for large scale data collection would allow for much greater predictive power, which could be used to forecast losses from the consumer more accurately (Wang et al., 2020). Furthermore, with more data gathering, it would be possible to provide the consumer with more information about the product they intend to purchase, particularly with online shopping, where the information provided to the consumer is not limited to the size of the packaging. With online shopping increasing, it is feasible that consumers may demand more information about the condition of the produce prior to purchase. Con-

sumers are often reluctant to purchase products online as they prefer to see the product they are purchasing (Munson et al., 2017). Online grocery shopping is in its relative infancy, and there is only a representative image of what the product may look like. Currently, there is no specific legislation governing the product images on websites (Park et al., 2021). It is generally accepted that the image will differ slightly from the product delivered as the image is not the actual image of the product. However, with advances in infrastructure, it is possible to dynamically update websites with up to date images that are more representative. Experiments that integrate data capture from all parts of the supply should be investigated, enabling more accurate predictions about the condition of any given product to be conveyed to the consumer and possibly reducing waste derived from misaligned expectations from the consumers and delivery of the product (Aschemann-Witzel et al., 2018). This would require collaboration between all parts of the supply chain, including the grower, processor, retailer, consumer and researchers.

7.1.2 Develop an accurate understanding of current shelf life dating via microbial testing to understand how variable current use-by dates are.

Given the conservative nature of on-pack dates, quantifying the margin of error of use-by dates would provide significant insight into the economic and environmental impact of such conservatism. Retrieving samples from retailers and testing them for the legally defined pathogen criteria, including salmonella, listeria, and *E. coli* (Table 2.1), would allow for the use-by date's accuracy to be quantified. Ostensibly the difference between a use-by and a best-before is of liability (section 2.4.2). A conservative estimate of a use-by date reduces the liability of the retailer. For example, with lettuce, the difference between an RTE lettuce product and a non-RTE is essentially decontamination and liability. The same pathogens can eventually develop on washed and unwashed lettuce it is merely a matter of time as decontaminant solutions only reduce the bacterial load (Uhlig et al., 2017). Further study into the accuracy of on-pack dates is required if there is to be any change in the associated waste that is influenced by them (section 2.8).

7.1.3 Conduct further research to develop a causal model of the increase in tissue ammonia over the postharvest life of iceberg lettuce.

From the research in Chapter 5, it was clear that ammonia concentration was the best indicator of quality in iceberg lettuce. Previous studies have also found ammonia to be an indicator of quality, but the origin of ammonia is yet to be established. The previous studies theorised that the origin is from disrupted protein metabolism (Cantwell et al., 2010) and in (Chapter 5) that it may be from microorganisms (Flythe, 2009). It is important to distinguish the primary source from which ammonia is derived as it is potentially the difference between quality and safety, or best-before and use-by (section 2.4.2). An experiment with sterilised versus non sterilised leaves may isolate the source of ammonia or culturing the microorganisms from leaves at a stage during shelf life that ammonia is elevated. It may then be possible to verify if any of them were known to produce ammonia from the cultured microorganisms. Furthermore, as has previously been discussed (Figure 5.6), it is possible to integrate sensors to detect ammonia within flexible packaging, which

makes it an excellent candidate for a marker of quality.

7.1.4 Further develop the work into online consumer reviews and extending it to include all food products.

From the insights gained from the online consumer comments in Chapter 6, it was shown that novel insights could be gathered with respect to consumer preferences (Figures 6.2, 6.3). This process could be easily expanded to include all leafy salad products. The resulting database could be invaluable in identifying consumer preferences for minimal cost. This information would be able to further direct consumer research and plant breeding of fresh produce that is more desirable to the consumer. The decisions based upon the dataset could lead to increased consumption by consumers to meet nutritional targets set out by the government. The proposed dataset could then be replicated for all food products.

7.2 Conclusion

Food waste has a variety of influences, including socio-political, technological, situational and personal factors (Quested and Murphy, 2014). From one perspective, having enough food where the waste generated is of minor concern on an individual level is a somewhat privileged position, and on a societal level may indicate a prosperous society. However, even in one of the wealthiest nations, the distribution of food between all citizens is not so developed that everyone has equal freedom to waste food, which was highlighted during the COVID-19 pandemic (Power et al., 2020). With a growing global population that will add increasing pressure on agriculture, and the increase in global temperature that is likely to make farming more challenging in many respects, alongside the sustained stringency of supermarket specifications, the sustainable use of resources and how food is produced and distributed is more in focus than ever before.

The National Food Strategy (https://www.nationalfoodstrategy.org) released a report in July 2021 with 14 recommendations relating to health and food security in the UK. Recommendation two suggests

that all large food companies should publish annual food data on their sales and waste. This proposed data would likely be a useful resource to researchers and analysts and may filter down to the consumer. In the agri-food supply chain, it is clear that retailers hold the majority of the power (Hingley, 2005). Although supermarkets often claim that they are simply responding to consumer demand, it is also clear that actions taken by the retailers influence the wants and needs of the consumer (Dawson, 2013). Furthermore, due to the need for growers of food to have their produce on the shelves of the major retailers, the retailers have asymmetrical influence over food production and rather than being a conduit of food distribution, they also have a strong influence on how and what food is grown. Given that fresh produce and leafy salads are particularly wasteful due to their short shelf life , such a system would need to be implemented in a way as not to dissuade the production of these relatively high-waste food products (Wilson et al., 2017). Given that leafy salads are highly variable depending on the environment (Chapters 3-5), food waste statistics for these products are likely to be relatively variable from year to year.

It has been mentioned several times throughout this thesis that the consumer generates the majority of avoidable food waste from leafy salads, which at best is a sign of a healthy supply chain. In Chapter 6, it was reported that experience is a key component in food waste, where older, more experienced consumers tended to rely on their experience over the shelf life dates on the packaging (Figure 6.5). It is positive to see that many of the recommendations in the National Food Strategy report are related to educating the consumer, and trying to get people to be more responsible consumers. Advances in technology have given us many advantages that have undoubtedly enhanced the lives of many people. Some of the methodologies highlighted in this thesis may well contribute to a reduction in food waste and increased consumption of leafy salads, improving the population's overall health. However, some of the advances in technology may have resulted in a less informed consumer from a paradoxical information overload (Desai, 2019) that has detached the consumer from production, and with increasing online shopping and automated fulfilment centres, the detachment may increase (Moss, 2021). The responsibility for food waste is incumbent on everyone, and engaging consumers with all aspects of the food system

should not be overlooked.

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Chapter 8

Published papers

8.1 Paper 1: Determining the quality of leafy salads: Past, present and future

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Review

Determining the quality of leafy salads: Past, present and future

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ABSTRACT

The relatively high proportion of avoidable waste from leafy salads and the under-consumption of fruits and vegetables generally is contributing toward renewed interest in the value of on-pack dates, particularly those that indicate quality. Current methods of predicting shelf-life in fresh vegetables and salad are relatively conservative due to the high variability of the product and few reliable markers that can be used to predict shelf-life. This is evidenced by the proportion of wastage in this category where fresh vegetables and salad account for almost a quarter of all avoidable food waste by weight. We have looked at the historical context in which date markings have been derived, how they function currently and look at how the current system could be improved. We review the three primary factors that influence the quality of a product – microbiology, visual quality, aroma – and suggest that if more accurate predictions of shelf-life are to be obtained non-destructive methods of testing need to be developed in order to provide the consumer with accurate information about the current state of the product.

1. Introduction

1.1. The fresh produce industry

Fresh produce is a category that encompasses farmed horticultural products, most commonly fruits and vegetables. Globally the yield and value of this sector has been increasing steadily over the last decade, and this trend is set to continue. From 2008–2018 global vegetable production increased from 4.4×10^8 to 6.4×10^8 tonnes and was forecast to maintain this growth (Euromonitor International, 2019). In Europe, 2.2 million hectares of land were used to produce fresh vegetables with nearly half coming from just three countries: Italy, Spain and Poland. Within that, approximately 17.8 % of the land is used for leafy and stalked vegetable production (De Cicco, 2016). The United Kingdom (UK) dedicates 78,000 ha to vegetable and salad production (DEFRA, 2018a).

In the UK, which historically has one of the highest consumptions of fruit and vegetables in Europe (Eurostat, 2018), 46 g of leafy salads were purchased per person per week (DEFRA, 2018b). In the last decade, the number of prepared leafy salad items purchased has doubled in the UK from a spend of 519–1100 million pounds showing an increase in the desire to consume more conveniently prepared leafy vegetables as part of a balanced diet (Kantar World Panel, 2018). The desire for more leafy vegetables, along with increases in population, has resulted in a significant increase in importing leafy vegetables to the UK over the last couple of decades (Fig. 1).

1.2. Challenges facing the fresh produce industry

There is a mounting pressure on the entire global food system to increase sustainable food production, to cope with the growth in population numbers and the dietary changes that occur as populations become more affluent (Gerbens-Leenes et al., 2010). It is estimated that food production will have to increase by 70 % by the year 2050; not only will it have to increase in volume, but also in safety and nutrition (SEC (2010)379, 2010).

Alongside pressure from an increasing population, there are guidelines from governments and health organisations to increase consumption of fruits and vegetables. The World Health Organisation (WHO) recommends that people consume 400 g of fruits and vegetables per day to improve overall health. However, this goal is not commonly achieved (EUFIC, 2012). Increased production of fruits and vegetables is one part of the solution, another is increasing the consumption of those which have been grown, harvested and purchased. The majority of food waste in countries with highly developed food chains occurs with consumers, and the longer the consumer keeps the food after purchase the less likely they are to consume it (Porat et al., 2018). As the produce ages the consumer views it as less valuable, due to its perceived decline in quality

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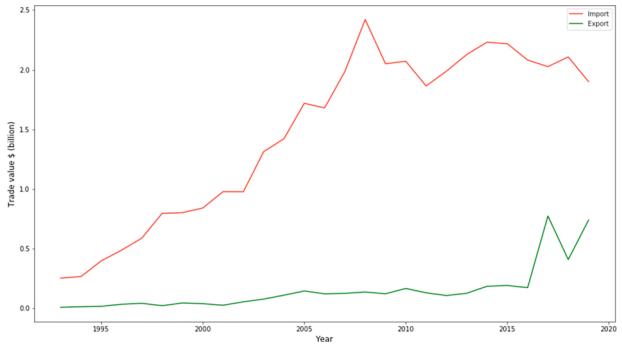

Fig. 1. The UK trade balance of leafy vegetables from the Comtrade database comprised of lettuce, spinach and chicory (https://comtrade.un.org/).

and safety. Often food that is acceptable to eat is wasted; in the UK in 2012 it was estimated that 37.8 % of leafy salad purchases ended up as avoidable waste (Quested and Murphy, 2014).

Food waste is a multifactorial problem and losses are not always avoidable. However, there are many aspects to improve on and these are covered by Sustainable Development Goal 12.3 (FAO, 2019). One particularly important area is on-pack dates. In the majority of cases, where a date is present on the pack (best-before or use-by) it is indicating either safety or quality of the pack contents. With respect to safety there are robust scientific methods that are used to define the date, although a margin of error is usually applied, which itself may increase waste. With quality the consequences of errors are less serious for consumer health and, as such, the ways in which the dates are derived are often quite rudimentary. This leaves larger margins for error and can potentially mislead the consumer, causing them to discard the salad when it is still safe to consume. Approximately 70 % of the time consumers use on-pack dates to decide whether or not a salad is 'okay' to consume. Similarly, the appearance is also cited as a deciding factor 70 % of the time; in contrast, less than 10 % of respondents said that smell was used (Lyndhurst, 2008). This highlights the importance of providing accurate information to the consumer and that consumers often rely on visual cues when evaluating a product. The situation is further complicated by the fact that consumers often open the bag and consume some of the product immediately afterwards, but then often keep the remainder for another day. The combination of changing the gaseous atmosphere inside the bag and manual handling of the leaves often renders the 'use-by' date aspirational, to the extent that some suppliers advise that bags are guaranteed until the 'use-by' date or 24 h after opening the bag, whichever is soonest. Educating sustainability-minded consumers about what constitutes real deterioration may help to alleviate some of the waste that occurs when consumers throw away product prior to the end date on the pack. Equally, encouraging disposal of waste salad into compost rather than landfill will have benefits for sustainability in the home. Retail waste can be on a much larger scale, for example when shelves are stacked with salad products in anticipation of good weather, only to find that unseasonable rain and cold weather (a common feature of a UK summer) drives consumers away from salad purchase. In these cases developing better systems for collection and valorization of wasted leaves and packaging are needed to improve sustainability goals.

One of the biggest barriers the industry has to being able to provide accurate information to the consumer is the lack of reliable tests for markers of quality (Spadafora et al., 2016; Tsironi et al., 2017), and those that do exist measure the current status of the product rather than providing any predictive information relating to shelf-life (SL). As a consequence, the quality indication given by use-by dates is often tenuous; furthermore, when it is suspected that quality will be diminished and a shorter SL is required, there is little evidence to back this up and the date on pack often stays the same regardless of what quality assessments were made at harvest or at factory intake.

This review will explore the options available to suppliers and retailers that would help reduce the volume of food loss and waste that occurs in the ready-to-eat salad industry. This will include an evaluation of the technologies available for predicting shelf life of the leaves before they are packed, ways of dynamically assessing quality loss during shelf life, and advice that may be given to consumers that would help prevent food waste from bagged salads in the home.

2. Shelf-life: brief history and definitions

2.1. A history of shelf-life legislation

As long as there has been trade there have been rules and customs. Early food law was primarily concerned with food adulteration (Sophia, 2014). With the rise of centralised distribution in the food supply network starting in the 1970's more advanced methods of stock control were required (Moore, 1991). Marks and Spencer introduced sell-by dates in the UK in 1973 to keep track of stock (Marks and Spencer, 2020), but that was not intended to convey information to the consumer. It was not until 1980 that there was a statute requiring dates to be included on packaging informing consumers of quality. A date of 'minimum durability', now commonly known as 'best before', was introduced in the UK (SI1980/1849, 1981SI/, 1981SI1980/1849, 1981) soon after similar legislation (79/112/EEC, 1978) was introduced to the European Economic Community (EEC). Use-before dates were introduced in the same document, and later revised to the wording 'use-by' (89/395/EEC, 1989). A year later the UK introduced use-by dates into its own legislation in an amendment to the Food Labelling Regulations (SI 1984/1305, 1984). The introduction of a date of minimum durability was first discussed by Codex Alimentarius in 1965, where the committee agreed with a statement from the UK delegates (ALINORM 65/22, 1965):

> 'Much depends on the quality and freshness of ingredients and on distribution and storage conditions.'

The next mention of a date of minimum durability was in 1972 when a standard list of date markings was discussed, to consolidate the markings being used (ALINORM 72/22, 1972). The first appearance of the definition in a similar form as it is today was presented by the Federal Republic of Germany:

"*If the minimum durability date was applied in such a manner so that foods exceeding the date and which are still in good condition were not removed from the market, then both the producer and the consumer would benefit, the latter in terms of possibly lower priced foods.*" (ALINORM 74/22, 1974).

They also stated that: "*without such an application of this type of date marking provision, the risk existed of restricting distribution to the larger, higher volume retailers.*"

However, in the UK the attitude was still of the view that the date of minimum durability was unnecessary other than for stock control purposes, and that minimum durability was 'open to interpretation', and argued that the SL would be variable depending on the storage conditions used by the consumer (Sawyer et al., 1980). The best-before date remains controversial (Neff et al., 2019) and the definition is still being discussed (REP18/FL, 2018) (Fig. 2).

2.2. Legal definitions relating to shelf-life

The European Union set out two different formats for on-pack date labels; the first, 'use by', is for products that are likely to be injurious to health at a certain point in time. The second date label is the 'date of minimum durability', or 'best before' which is the 'date until which the food retains its specific properties when properly stored' (1169/2011, 2011). These static dates on the packs of fresh produce can be considered the SL of the produce within. However, 'shelf-life' is not specifically used

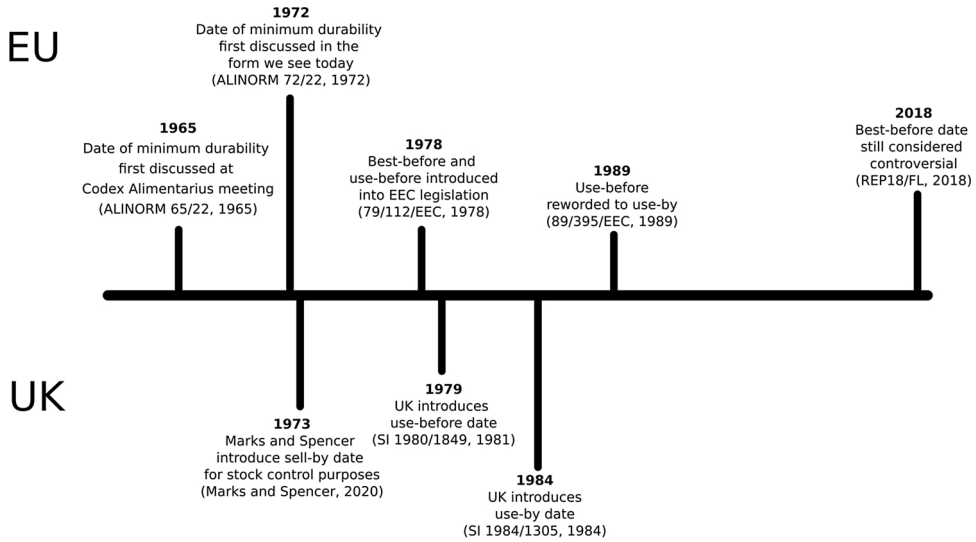

Fig. 2. A timeline showing key milestones in the formation of on-pack date labelling that we see today in the UK and EU.

in EU labelling legislation, but it does appear in (2073/2005) related to the microbiological criteria of food (Article 2,f):

> "'Shelf-life' means either the period corresponding to the period preceding the 'use by' or the minimum durability date, as defined respectively in Articles 9 and 10 of Directive 2000/13/EC."

The words 'Shelf-life' do appear in a statuary instrument in the UK, but the definition refers to 'use-by' and 'best-before' definitions in EU legislation (SI 2014/1855, 2014SI /, 2014SI 2014/1855, 2014).

Unlike other categories in the food industry, the fresh produce industry has limited options when it comes to food processing and preservation. Because of this, the life of fresh produce is particularly short post-harvest. This is certainly true of leafy salads, where products are not expected to last longer than two-weeks post-harvest (Tsironi et al., 2017; Bell et al., 2017). The most significant reason for accurate communication of SL by a use-by date is ensuring microbiological safety. Any product designated as RTE must carry a use-by date (EC 2073/2005, 2006EC /, 2006EC 2073/2005, 2006) and, since bagged salad leaves are usually in this category, the suppliers do not have a choice but to impose a use-by date rather than a best-before date. It is a criminal offence to sell food that has passed its use-by, but this is not true for food that is past its best-before date (178/200, 2002) although retailers often do not sell food past its best-before date.

Although leafy salads are required to have use-by dates, some products carry a best-before date where it is assumed further processing, e.g. cooking, will occur in the home – for example with products such as sliced kale or spinach. This leads to some anomalies in the current retail system: spinach sold as a single line bagged salad is classed as RTE and is subject to a 'use-by' date. The same type of leaf is sold as a different line with other leafy green vegetables that are marketed for cooking and therefore has a 'best-before' date on pack. Since there is nothing to stop a consumer using a vegetable spinach in a salad, or blending sliced kale into a smoothie, it is clear that the distinction between use-by and best-before is a somewhat artificial construction that doesn't necessarily protect consumers who are consuming them raw from microbiological safety breaches. Best-before dates are set to give the consumer an indication of the decline in the quality of the product. As the decline in quality is a result of decay and senescence, which are biological processes, there are many different inputs and pressures that influence the decline. Variance in salad crops are attributed to differences in growing conditions such as light intensity (Fu et al., 2012), and irrigation strategy (Luna et al., 2012; Allende and Monaghan, 2015). As well as the agronomic inputs, the genetic factors such as species and cultivar, influence the variability in the post-harvest longevity of the product (Ntsoane et al., 2016; Bell et al., 2017; Jasper et al., 2020). Furthermore, as the leaves of the plant mature at different rates, there will be significant differences in the quality of leaves from the same plant. Because of this, the quality of the leaves within the individual bags may be highly variable. All of the pre- and post-harvest factors make accurately assessing a product's SL difficult, and interplant variability is one of the biggest challenges.

Commercially, all produce within a particular plot of land is planted and harvested at the same time. Within the plot there will inevitably be some variation in rates of growth and development, plus there will be leaves of different developmental stages within the same plant. Therefore, leaves of different maturities are harvested together, meaning that the physiology and chemical composition will be different between leaves in the same bag. Whenever a ready-to-eat (RTE) salad bag is assessed the SL will be based on the average for all the leaves within the pack. This variation increases the difficulty in defining SL, and as to why the date on packs is set conservatively.

Suppliers of fresh produce will choose a use by date that is a set number of days after the produce is packed. This date has a margin of error, perhaps two days, and this interval will stay static throughout the year, with the occasional adjustment downwards if the crop is known to have a significant reduction in quality. Therefore, the date on the pack is set to cover the worst-case scenario, which is good for ensuring public health, but sub-optimal for minimising waste (Lee et al., 2015). Food waste associated with these products could be mitigated by retail and domestic purchasers with better planning and logistical tools (for retailers) to improve the relationship between supply and demand. However, with current supply chains requiring several days between harvest and point of sale, and with rapidly fluctuating weather conditions driving very short-term fluctuations in what consumers choose to eat with a crop that takes several weeks to develop from sowing to harvest, managing supply to consumption patterns is challenging. Alternative supply chains are discussed in the section below that may enable shortened crop cycles and more localised supply chains that may both improve quality and reduce waste.

There is a vast amount of literature assessing different facets of fresh-produce physiology and biochemistry over SL (e.g. Wagstaff et al., 2007; De Corato, 2020). However, there is a disconnect between the information gathered in academia, and the dates that are placed on the packs of consumer goods. This is often because the advanced methods used in academia do not translate to industry, due to practical, economic and technological constraints. Moreover, studies are rarely repeated across the seasons and varieties appropriate for an individual type of crop.

Hence, markers may be indicative of SL under a certain set of conditions but as conditions change these markers often do not produce generalised values in a way that is useful for industry to adopt.

There are many ways of quantitatively assessing quality attributes that are linked to SL. The challenge for those wishing to implement such measures is that the underpinning biology that regulates leaf degradation and quality loss is highly variable depending on factors linked to plant development, agronomy and post-harvest handling. The following sections explore quality attributes linked to SL, providing information on the biological factors underpinning the measurable symptoms, methods for quantitatively analysing each factor or its symptom, and a review of available technologies that can currently predict the development of a quality marker.

2.3. The supply chain of RTE leafy salads

The food supply chain for ready to eat or ready to cook cut fresh vegetables can be rather long, given the delicate nature and cellular vulnerability of these plant products. For example, if a product is grown in southern Spain for consumption in the UK it can be 24 h between harvest and starting its journey, during which time it is imperative to remove the field heat from the crop as rapidly as possible (Bell et al., 2017) and to thereafter keep it at optimal storage temperature so that metabolic processes are arrested without causing chill damage. It can take three days to transport the crop by road to the UK, with temperatures often highly variable between different parts of the lorry. On arrival in the UK the crop may spend another 24–48 hours being washed, processed and packed before it is distributed to a retail outlet. Typically a use by date on pack can be five to seven days after packing, meaning that the product has to meet quality threshold criteria relating to appearance, safety and organoleptic characteristics for at least ten days after harvest. Therefore, the care with which the product is handled and the integrity of the cold chain through which the product moves after harvest is absolutely critical to its ability to meet quality and safety requirements.

Sub-optimal storage conditions can lead to increased quality and safety issues because the storage temperature will influence the rate of respiration and the rate of microbial growth (Løkke et al., 2012; Alongi et al., 2019). With a longer supply chain, there is a greater potential for temperature abuse which can be detrimental to the product and increase the rate of deterioration. The longer the product takes to get to the retail shelf after packing, the less time the consumer has to enjoy the project before it reaches the end of shelf-life. Whilst there is encouragement to reduce the length of supply chains and grow more of the crop in the country where it is going to be consumed, e.g. through indoor farming, it will be many years before these initiatives can account for a significant portion of the ready to eat/ready to cook vegetables that are currently produced in Europe for consumption elsewhere. It is therefore valuable to continue to apply effort to improving cold chain management and to innovations in packaging that lead to increased quality of the product at the point of consumption.

3. Microbiology and shelf-life

With respect to SL, safety is the most important factor. The 'use-by' date, which is defined in relation to microbiological safety, is in place to protect the consumer. It is an offence to sell any product past its stated 'use-by' date. For leafy salads, the control of micro-organisms is one of the primary concerns; this is because of the relatively limited processing options available. Traditionally, salad vegetables do not carry any form of date as they are often unpackaged. However, with rising demand for convenience, leafy salads are increasingly being sold as RTE. Any product designated as RTE must carry a use-by date (EC 2073/2005, 2006EC /, 2006EC 2073/2005, 2006). Often, a product that will be sold as RTE is further processed for added value – cut into portions, for example. As RTE products are not going to be further processed by the consumer, they must be safe to eat within a stated timeframe. There are very severe consequences, both financially and reputationally, for a business if there is a food poisoning outbreak from their product (Koukkidis, Giannis, Freestone, Primrose, 2018). As a consequence of having relatively few tools to ensure safety and severe consequences of injuring the consumer, the date on the pack is often a conservative estimate.

3.1. Causative agents of microbial problems

At every stage in the supply chain, there is an opportunity for micro-organisms to contaminate food. Often the environment in which the food is produced, be it open-field or hydroponic for example, or the properties of the foodstuff itself are determinants of which micro-organisms will develop (Söderqvist, 2017). There are three micro-organisms that have specific regulations pertaining to the safety of leafy salads; these are *E. coli* 0157:H7, *Listeria monocytogenes* (LM), and *Salmonella* (Table 1). *Salmonella* and LM have regulations that are in place while the product is on the shelves. In contrast, the law for *E. coli* is only applied during the manufacturing stage, as although it can be injurious to health, it is not known to grow on leafy salads under RTE conditions (Abdul-Raouf et al., 1993). Although there is evidence that LM and *Salmonella* can grow at chilled temperatures, these organisms are not generally considered to contribute to the spoilage of the salad product (Horev et al., 2012). These organisms are important with respect to SL. However, we are primarily focused on quality changes and therefore, they shall not be discussed in detail in this review.

Micro-organisms are part of the many factors that contribute to the spoilage of food. However, as with many processes in biology, no single factor is entirely responsible as physical, chemical and microbiological factors all contribute. Bacterial spoilage is often associated with slime and a watery appearance (Tournas, 2005) caused by the formation of biofilms and/or by breakdown of the underlying leaf material. In addition to producing mycelium and spores, fungi have also been associated with a watery appearance, therefore the causal organism of similar symptoms is not always straightforward to identify by appearance alone. Unsurprisingly, the species or micro-organisms that are able to survive and even replicate at refrigeration temperatures are most commonly associated with food-spoilage such as those belonging to the *Erwinia* species.

Routine testing for food spoilage organisms is not standard practice.

Table 1
Microbial limits of safety and quality for precut fruit and vegetables (ready-to-eat).

Pertaining to safety			
Micro-organisms	Absolute limit	Testing method reference	Stage at which the legislation applies
E. coli[1]	1000 cfu/g	ISO 16649–1 or 2	Manufacturing process
Listeria monocytogenes[1]	Absence in 25 g	EN/ISO 11290–1	Before the food as left the food business operator
	100 cfu/g	EN/ISO 11290–2	Products on the market during its shelf-life
Salmonella[1]	Absence in 25 g	EN/ISO 6579	Products on the market during its shelf-life

Pertaining to Quality			
Micro-organisms	Class A Satisfactory	Class B Acceptable	Class C Unsatisfactory
Aerobic Colony Count[2]	$< 10^4$ cfu/g	10^4 - $< 10^5$ cfu/g	$\geq 10^5$ cfu/g
Aerobic Colony Count[3,4]			$> 10^7$ cfu/g
E. coli[2]	< 20 cfu/g	20 - < 100 cfu/g	≥ 100 cfu/g

[1] (EC 2073/2005, 2006EC /, 2006EC 2073/2005, 2006).
[2] (Food and Environmental Hygiene Department, 2001).
[3] (Calonica et al., 2019).
[4] (Health Protection Agency, 2009).

This may be due to the economics of administering these tests, the lack of guidance on testing the less frequently occurring organisms, lack of knowledge about the relationship between organism load and the prevalence of symptoms, or lack of knowledge about the underpinning colonisation and disease development to provide informative predictive or actionable data.

3.2. Evaluation of microbial load

There are legally defined microbiological sampling and testing methodologies for establishing SL (EC 2073/2005, 2006EC /, 2006EC 2073/2005, 2006). Because of this, microbiology is unique as a measure of quality in that the same criteria that establish the date on the pack are the same for every product that is sold within a particular jurisdiction. The standard methodology for assessing the microbiology of a product is defined in Commission Regulation 2073/2005 (2006), where the specific ISO method for testing is referred to. Aerobic Colony Count (ACC) is often used; thresholds vary for what is classed as unacceptable, but are usually in the range 10^5 – 10^7 colony forming units per gram (cfu/g) (Health Protection Agency, 2009; Calonica et al., 2019). Values in excess of this figure suggest the microbial flora is considered to be from one predominant organism (Health Protection Agency, 2009).

When measuring the microbiology over SL in RTE products, samples are taken at the start of production and at set points throughout the SL period. Organisms that are relevant to the safety of RTE salads are highlighted in Table 1. Often the product is on the shelves before the results of the tests are known as the current testing methods usually require 48 h of incubation time. So, if the results come back positive for pathogenic micro-organisms, products have to be removed or recalled depending on how far they have made it through the supply chain. A lot of research has been undertaken to try and develop novel non-destructive methods of quantifying micro-organisms and the majority of these methods are based around imaging techniques (Pan et al., 2018; Herrero-Langreo et al., 2020).

For a method to be truly useful at assessing microbial accumulation during SL it has to enable measurements to occur while the product is still in its packaging, and for organisms related to spoilage there has to be some knowledge of what level of abundance should indicate a cause for concern. To the best of the authors' knowledge, there are no implementations of such as system. There are commercialised methods for the detection of various aflatoxins in nuts and dried fruits, but there are yet to be similar methods in the fresh salad industry (Yanniotis et al., 2011; Wu et al., 2018). It is usual to see higher aerobic colony counts in products that have not been stored adequately. Due to the logistics of the supply chain, the retail environment, and the minimal processing options, leafy salads often have unsatisfactory numbers. Calonica et al. (2019) found that only 8.3 % of samples of salads taken from retailers were satisfactory ($< 10^5$ cfu/g) and by the end of shelf-life 80 % of samples were unsatisfactory ($> 10^7$ cfu/g). ACC gives an indication of the overall microbial status of the product and is not suitable as an indicator of specific organisms. As the microbial status of a leafy salad is often unsatisfactory, and that there are relatively limited options for controlling and monitoring micro-organisms, there is a large amount of work in research and development for discovering methods that can reduce microbial load and still deliver the quality of product that the consumer demands.

3.3. Preventing microbe-derived quality loss

Controlling micro-organisms on leafy salads affords far fewer technologies than most other food categories, since thermal treatments, which are well developed, are not feasible on salad leaves due to the perishability of the crop. There are numerous ways in which growth of micro-organisms can be controlled, and it is a highly active area of research, reflecting the economic importance of this problem (Costa et al., 2011; Mogren et al., 2018). There are broadly two different approaches to controlling micro-organisms, physical and chemical.

3.3.1. Physical methods of preserving fresh produce

Physical methods of controlling micro-organisms, apart from heat treatment, include treatments such as modified atmosphere packaging (MAP) and radiation-based techniques. Ultraviolet (UV) light has been studied in its application at reducing the microbial load on leafy salads, and has been found to be effective (Ignat et al., 2015); however, there is the possibility of damaging the leaves with high levels of exposure. The UV radiation disrupts DNA replication and transcription in its germicidal action, but its action can also cause quality defects such as increased respiration, which is unfavourable as far as storage life is concerned, and in strong enough doses can physically degrade the leaves (Martínez-Hernández et al., 2015). Irradiation techniques using gamma radiation have been approved for use on lettuce and spinach in the USA by the FDA (Goodburn and Wallace, 2013), and have been shown to be effective in many studies (Chun et al., 2010; Olanya et al., 2015). However, there is conflicting evidence from RTE salads whether these types of treatment persist through shelf-life or just exert their effect as a one-time decontamination (Goodburn and Wallace, 2013). There does not seem to be a large take-up of this technology in the fresh produce industry, partly due to economic factors, but also due to consumer concerns over irradiated produce (Bearth and Siegrist, 2019).

Modifying or regulating the atmosphere inside the packaging of a product has been used extensively within the fresh produce industry, and there are many reviews on the topic (Caleb et al., 2013; Hussein et al., 2015). Typically, in MAP varying combinations of nitrogen, oxygen and carbon dioxide are used depending on the product. Noble gases, which have low reactivity and no odour, have also been investigated in combination with 'traditional' gases and found to be effective in maintaining the quality of rocket (Char et al., 2012). However, in the same paper it was also reported that argon-enriched atmospheres increase respiration around 15 %, which may reduce SL. The modified atmosphere is achieved either by gas flushing to displace the air inside the bag with a desirable composition of nitrogen (or other noble gas), oxygen and carbon dioxide (active MAP) or by using microperforations in the packaging to balance the respiration rate of the product with gas exchange between the internal headspace and the external environment (passive MAP). Passive MAP can take several days for equilibrium to be reached and, in both cases, the evolution of the internal atmosphere is dependent on factors controlling the respiration of the fresh product, e.g. temperature. If the permeability or environmental conditions are not optimised then the quality of the product will be severely compromised (Ares et al., 2008). There are many studies that show the attenuation of micro-organisms using modified atmospheres (Ioannidis et al., 2018; Kapetanakou et al., 2019). However, once the pack is opened the benefits of the MAP are lost. There are several packaging parameters that affect the atmosphere within the bag, including film thickness, number of perforations, orientation of polymer chains and polymer type. For packaging of leafy salads polypropylene is the most common polymer, but the packaging parameters will vary depending on the product. The atmospheric conditions in MAP, which are usually low O_2, CO_2 and high nitrogen compared to atmospheric composition (Campbell-Platt, 2017) can give rise to negative quality aspects such as discolouration and off-odours (Nielsen et al., 2008; Tudela et al., 2013). However, there are concerns over the sustainability of some of the materials used to package RTE salads, with recycling options severely limited. There is pressure to develop biodegradable, compostable or more easily recyclable packaging options that still retain the ability to control quality of the plant material within (Roohi et al., 2018).

3.3.2. Chemical methods of preserving fresh produce

Chemical methods of controlling micro-organisms are far more numerous, which may reflect the commercial viability of these methods for controlling micro-organisms. As vegetables tend to be washed to remove soil and debris, it makes practical and economic sense to use this

stage to sanitise the produce for micro-organisms. Simply washing the produce in chlorinated water remains one of the most common practices when it comes to controlling micro-organisms on fresh produce. However, questions have been raised as to whether or not the results from chlorine washing are significantly different to washing with water alone (Luo et al., 2011) and there has been increasing pressure from regulatory authorities to reduce or remove chlorination from RTE products (Uhlig et al., 2017). There are many alternatives to chlorine, many of which are based on weak organic acids such as citric, malic and tartaric acid. The use of weak organic acids is based around overwhelming the ability of bacteria to remove protons from their cell interior and therefore not being able to effectively reproduce as they have to expend energy pumping out protons from their interior (Akbas and Ölmez, 2007). There are many examples of different chemical combinations in the literature, with different modes of action such as thymol or carvacrol, which are both thought to increase the membrane permeability of bacteria through interactions of the phenol group and its destabilised electrons with the cell membrane (Zhou et al., 2007). Peroxyacetic acid produces reactive oxygen species which can damage DNA and lipids of bacteria; furthermore, it can denature proteins and enzymes by oxidising disulphide bonds which also increases membrane permeability (Vandekinderen et al., 2009). Cuggino et al. (2020) found that benzyl isothiocyanate (BITC) was synergistic when combined with chlorine to increase the effectiveness of decontamination over chlorine alone. Although they did state that the results may have been due to the change in the pH rather than the antimicrobial properties of the BITC. Other plant-derived compounds such as Origanum vulgare, which is derived from oregano, has been shown to be effective in reducing E. coli O157: H7 packed spinach and lettuce when combined with traditional sanitisers such as sodium hypochlorite (Poimenidou et al., 2016). Novel plant-derived compounds such as BITC, oregano extract and organic acids are desirable not only for their effectiveness at decontaminating salad leaves but also because they are not required to be stated on the label as they are generally regarded as safe (GRAS) and or classified as processing aids. This is an advantage as consumers are wary of decontaminants (Aoki et al., 2010). Ultimately it comes down to price and, if not already approved, getting the product approved by governing bodies; many of the alternatives to chlorine are not economically competitive.

3.3.3. Nanotechnology and its role in food packaging

The incorporation of nanomaterials into food packaging is an area of research that is in the ascendency. Antimicrobial elements such as silver are being incorporated into packaging with success (Costa et al., 2011). However, as the technologies surrounding the use of nanomaterials is developing, the regulatory authorities have yet to form a consensus as to the efficacy and safety of many of the technologies and, therefore, few examples exist within the food industry (Eleftheriadou et al., 2017). This is particularly true of the use of heavy metals, such as silver, which can have detrimental effects on human health and the environment (Tóth et al., 2016). One of the concerns with incorporating sensors or nanomaterials into packaging is the effect on the recyclability of the packaging; reducing food waste at the cost of increasing packaging waste is not a desirable trade-off.

3.4. The influence of seasonal and agronomic factors on microbial quality

One of the many reasons why it is hard to predict the SL of a product is due to the fluctuating environment in which the product is produced. The majority of leafy salads are grown in open-field; therefore, weather and seasonality play a role in determining the microbiological safety and the quality of the product. Caponigro et al. (2010) looked at six different RTE salad products from Italian supermarkets over two years and found that microbial loads peaked in the autumn months. It has been suggested that during periods of higher rainfall bacteria are better able to spread and be carried to different locations which may be a more of a factor than temperatures in accounting for the differences between seasons. However, the variability in bacterial loads is not consistently higher in the autumn/winter months. Rastogi et al. (2012) found that there was a one-log decrease in culturable bacteria of lettuce grown in the winter season compared to the summer season. It is more likely that high rainfall leads to more soil splash onto the leaves and contamination through that more immediate route, rather than transfer in moisture-dense air between fields. Often it is atypical weather events such as high rainfall and flooding that are positively correlated with increased microbial contamination (Medina-Martínez et al., 2015), supporting the hypothesis that bacteria are transferred from the soil to the leaves. This is a particular concern when considering climate change and its potential for increased variability in weather conditions and the frequency of which extreme weather events occur (Liu et al., 2013).

Leafy salad crops that are field-grown have many more avenues for contamination than those that are grown in soil-less systems. Field-grown crops may also be exposed to contamination from livestock in surrounding fields, wild animals, standing water or manure fertiliser. In contrast, produce that is grown under-protected and/or soil-less systems, such as hydroponics, is able to be more tightly controlled. Manzocco et al. (2011) found that hydroponically grown lamb's lettuce did indeed result in a lower microbial count (Total Coliform and Pseudomonas) when compared with a soil-grown crop. However, there was no difference in Enterobacteriaceae, which hydroponically grown crops are also susceptible to as these organisms are typically found in contaminated water supply and can enter the plants via the roots (Lenzi et al., 2021).

As well as the variation from seasonal influences, and that of the growing environment, the plant maturity also has an impact on the SL of the product. A consistent finding is that immature leaves tend to have higher respiration rates than mature leaves. Higher respiration rates potentially reduce SL as the leaves may degrade quicker than those with lower respiration rates (Martínez-Sánchez et al., 2012; Hunter et al., 2017). It has also been observed that immature leaves have higher microbial counts than those that are at harvest maturity (Rastogi et al., 2012; Williams et al., 2013; Dees et al., 2015). It is has been suggested that as the plant matures, selective pressure on micro-organisms occurs which accounts for the decrease in micro-organisms present on mature leaves, but this has not been proven, and often the seasonality effects are a confounding factor. The many different factors that can influence the microbiology of salad leaves make forecasting how the safety and quality of a product will change throughout the year challenging. As it is difficult to predict how micro-organisms develop on salad crops from the growing stage, processing the leaves and storage in the consumers home, SL dates are often conservative to minimise the chance of 'injuring the consumer' at the expense of increasing waste.

3.5. Modelling and predicting microbial growth

The importance of keeping the consumer safe and meeting the quality standards that they expect are top priorities, because of this, predicting the growth of micro-organisms is a well-studied area. Typically, there are three classes of predictive modelling: primary modelling, where a few kinetic parameters are measured such as lag time or growth, and a growth rate with respect to time is calculated; secondary modelling, which incorporates environmental variables such as temperature and their effect on the parameters from the primary model; tertiary modelling, which are consumer friendly packages designed for food business operators to be able to produce models of microbial growth, evaluate the safety of their products, and inform SL estimation. ComBase (https://www.combase.cc) provides links to many of these software packages. These models allow food businesses to estimate levels of micro-organisms at the time of consumption and factor in many different variables such as temperature, pH and preservatives (Psomas et al., 2011).

Often there are many different variables in the food supply chain that

can affect the growth of micro-organisms, which are not captured within these models. The consequence of this is that companies will apply a conservative margin of error on the use-by date, of at least two days, which may reflect the lack of confidence in the underlying model. The length of time it takes for the product to reach the shelves after packaging is not always predictable and therefore providing for this also contributes to conservative labelling. There are always going to be errors in predictive modelling as it is not feasible to take all possible scenarios into account. The margin of error is applied to avoid human disease, but as a consequence there may be more wastage (Wilson et al., 2017). With an increasing focus on waste and sustainability, and as more data are collected and models are further developed, margins of error may be reduced and potential wastage avoided. With growing research into dynamic methods of assessing micro-organisms and particularly the use of imaging methods, models will be produced that incorporate these measurements to provide more accurate predictions, or real-time measurements. Siripatrawan et al. (2011) found that they could detect E.coli using hyperspectral imaging (HI) on inoculated spinach leaves, and were able to predict the number of organisms from the imaging data using a neural network based model. Kang et al. (2011) were able to detect faecal contamination, which is a common route for pathogens to enter the food chain, using HI with romaine lettuce samples. However, there has yet to be any application of these methods and models in the retail environment.

When considering spoilage and quality, the underlying models which are used to implement a best-before date are far less developed, than those that predict use-by dates, if they are used at all. There is a lack of research into markers that can be used to reliably predict quality, creating a barrier in negotiating an extension or reduction of the date on the pack as the supplier does not have sufficient evidence to back-up their perceived notion of quality. The consequence of this is that the date on the pack often does not change when the quality, and therefore shelf-life, does.

4. Human perception of quality

Often, the first and most significant parameter a consumer uses to decide if they will purchase or throw away a salad product is their visual perception (Paakki et al., 2019). The appearance is the first stimulus the consumer is faced with and is often used as a metric for acceptance or rejection of the product (Mielby et al., 2012). Therefore, having a good understanding and testing methodology for visual aspects of a product is important.

4.1. Visual disorders associated with leafy salads

With leafy salads, there is a plethora of different visual disorders that can occur (Fig. 3). These include russet spotting, which is induced in iceberg lettuce by exposure to ethylene in the ppm range (López-Gálvez et al., 2015), or the yellowing of leaves due to chlorophyll degradation (Koukounaras et al., 2009). There are some disorders that are associated with discolouration in leafy salads that are typically induced by mechanical damage where internal cell structures are disrupted e.g. cutting. Pinking of iceberg lettuce is one such example where cell structures are disrupted allowing the interaction of compounds and enzymes that result in colour change that would not ordinarily occur if cells remained intact. Pinking is induced by the conversion of diphenols to quinones, and then melanin precipitates which produces pink and brown hues depending on subsequent reactions that are not yet fully understood (Saltveit, 2018).

As visual quality defects are instrumental in guiding the consumer's decision process, a lot of effort has been put into measuring and quantifying these disorders, both in academia and industry (Quested and Murphy, 2014; Manzocco et al., 2017). In contrast to microbiological assessment, which will often be outsourced, visual appearance will be determined within the business. Typically, visual appearance is assessed

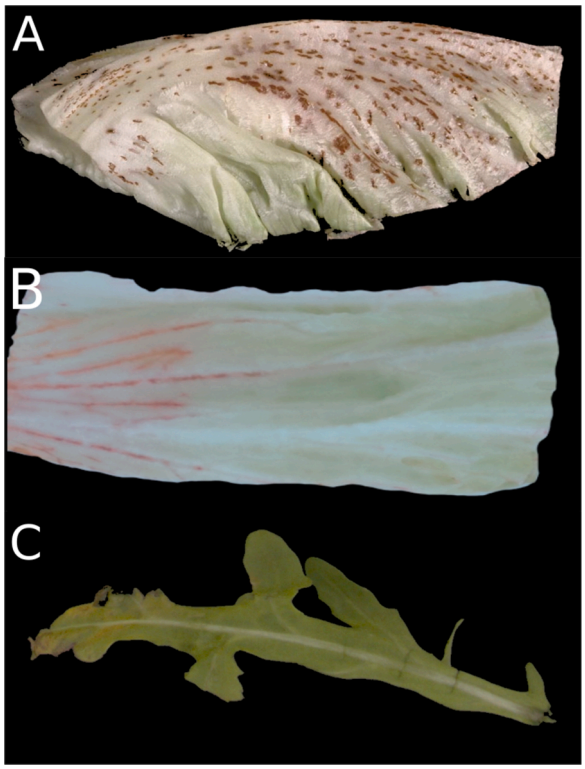

Fig. 3. Example visual disorders of salad leaves. Russet spotting on Iceberg lettuce (A), Pinking of cut tissue of Iceberg lettuce (B), and senescence of Rocket leaves (C). Image (A) was taken from (Cantwell and Suslow, 2002).

by a sensory panel or by a more objective approach involving the analysis of the emission spectra of the product. Depending on the equipment being used, this will typically be within the visible spectrum (~380 to 740 nm). Specification standards for each product will be defined and agreed upon by the supplier and retailer, and any product failing to meet the required standard will not be sold.

Visual assessment by human assessors is perhaps the most common method utilised when considering the quality of a salad product over a shelf-life period due to its relative simplicity and low cost. The advantage of this approach, other than low cost, is that it is relatively quick and, when done with larger numbers of assessors, may align with the consumer perception of the product (Lee and Chandra, 2018; Nguyen et al., 2019; Sikora et al., 2020).

5. Instrumental assessment of quality

Objective assessment of visual quality has long been the goal of laboratory scientists studying postharvest changes. Only recently are these technologies being adapted for supply chain applications and the primary point at which they are implemented are in the packhouse. Often the use of image analysis, hyperspectral imaging or colorimetry (see sections 5.1-5.3 below) are used for automating sorting materials of very different visual qualities e.g. removing senescent spinach cotyledons from consignments of dark green baby leaf spinach leaves. Only recently has the possibility emerged of using such technologies to detect color/reflectance changes at an early stage that enable the prospect of some better prediction of shelf life.

Several technologies rely on the real-time detection of volatile aroma compounds that are produced as a consequence of senescence, tissue damage, degradation or microbiological proliferation on the leaves (Luca et al., 2017). Generally, the aroma is a tertiary consideration when consumers are assessing salad leaves, since they cannot smell the product without damaging the packaging. Furthermore, unless the salad

leaves are particularly pungent or have a distinctive odour, such as rocket leaves, there is not much of an aroma to detect. From a food safety perspective, the aroma is not necessarily diagnostic of pathogens but off-odours are often associated with the presence of microorganisms.

Identification of suitable volatile marker compounds has come from extensive work based on assessment by the human nose in the form of trained sensory panels or preference testing using untrained consumer panels. The human nose is, compared to current levels of technology, more sensitive than the equipment that is available for automated volatile sensing. As with visual appearance, there are several quantitative and qualitative methods for assessing aroma. Most often, a sensory panel is used to assess the aroma of a product; depending on the question being asked, a trained panel or untrained consumer panel will be used. Assessing a product using a panel can give both quantitative and subjective feedback in a real-world setting. Using a trained sensory panel to determine the descriptive characteristics of a product is common. Descriptive analysis can also be used for quality control, and often it is used to determine consumer preference (Goularte et al., 2004; Murphy et al., 2011; Wieczyńska and Cavoski, 2018). There are many different methods for profiling a product with a sensory panel, such as quantitative descriptive analysis (QDA), and free-choice profiling (Murray et al., 2001). Typically, there are 8–16 trained panel members who produce an agreed vocabulary for attributes of the product. Descriptive characteristics, with rocket as an example, may pick up on aromas such as: peppery, green, mustard, sweet (Bell et al., 2016). The attributes of the product are then scored using an interval scale. However, without also identifying and quantifying the volatile organic compounds (VOCs), it is not possible to ascertain which compounds are responsible for which aromas, but research in this space has given rise to the identification of compounds which may be used to diagnose deteriorating quality (Dryahina et al., 2020), the potential of which is discussed in 5.4 and 5.5.

Different technologies have started to impact on the fresh produce market that give a real-time indication of freshness, or historical reporting of cold chain breaches. These typically rely on detection of respiratory gases and/or use chemistry to report changes in physical parameters such as temperature or humidity. These are covered in sections 5.6 and 5.7, together with a discussion of their potential and limitations.

5.1. Image analysis for assessing leafy salads

Image analysis (IA) is a more objective approach to assessing visual appearance and is becoming the predominant phenotyping method. Phenotyping refers to the observed characteristics of an organism, such as morphology, colour and biochemical properties. With IA, typically an RGB image is captured using anything from relatively inexpensive consumer devices such as mobile phone cameras (Tsaftaris and Noutsos, 2009); to more advanced dedicated equipment where spectral data in single nm bandwidths can be collected for each pixel (Lara et al., 2013). Once the images have been captured, features such as colour and size of the subject can be extracted using one of the many software packages dedicated to IA.

One website alone, www.quantitative-plant.org, has links to over 170 different tools for plant phenotyping and 28 open data sets that can be used to train models (Lobet et al., 2013). With the use of machine learning algorithms for advanced feature extraction, the technology is progressing very quickly (Jiménez-Carvelo et al., 2019). IA is also much more applicable to industrial applications, as it can be automated, and is used in many different industries. Mo et al. (2017) developed a method for detecting foreign bodies on fresh-cut lettuce where a hyper-spectral scanner was placed above a moving conveyor belt. The analysis of the images captured by the camera was able to distinguish between lettuce and foreign bodies based on their absorbance in the range of (400−1000 nm), and reject samples accordingly.

The development of machine learning algorithms, that can enable leaf material to be imaged whilst still inside packaging, has been demonstrated, which is important if post-harvest monitoring is to be achieved. In the paper of Cavallo et al. (2018), a convolutional neural network (CNN) was used to segment the images into three classes: plant, packaging and other. Currently, deep learning and CNNs are the go-to method for working with image data as, once the models are trained, they can be very fast in their decision making, allowing the possibility of live processing (Patrício and Rieder, 2018). There is no reason why this approach could not be applied to other leafy salads, and even be incorporated into consumer technology, such as smart phones.

5.2. Colorimetry for assessing leafy quality

Another method of classifying colour is with the use of chroma-meters (Mampholo et al., 2016). Chroma-meters are analytical instruments for measuring colour, which is typically presented in the LAB colour space. The advantage of this method is that it can be carried out with only one assessor, and objective data are obtained. The device measures a small area on the target (~1 cm^2) and therefore, depending on the target size and variability of colour, many measurements may need to be taken to accurately capture the colour of the target. One issue with this approach, particularly when it comes to salad leaves, is that there are sometimes large differences within individual leaves and between different leaves in the same pack. As the technique measures the leaf at different points, only average values are obtained, which makes it difficult to discriminate between different manifestations of discolouration (Peiser et al., 1998). Prior to IA, this was the predominant method used; in recent years, the advantages that IA brings has meant that it has largely eclipsed the use of chroma-meters.

Overall, considering the relative importance that the consumer places on the appearance of the product, there are few examples of methodologies for predicting colour change.

5.3. Quality assessment using hyper-spectral imaging

Looking outside the visible spectrum with hyperspectral imaging (HI), or reflectance data not detected by human vision, is currently providing more information about the state of the product. HI is much more expensive, both in the cost of equipment and the software and time needed for analysis. In comparison to spatial imaging where two-dimensional data is acquired, three-dimensional data are collected and each pixel has its own associated spectrum; the spectrum data (λ) in combination with spatial data (x, y) creates voxels in the form (x, y, λ). As different materials interact uniquely with different bands of the electromagnetic spectrum (EM), it is possible to gather data about the chemical composition of the material, which is one of the major advantages of HI (Chaudhry et al., 2018). HI has been used to differentiate between rocket leaves stored at varying temperatures, and from this to infer quality. A random forest classifier was able to classify the reflectance data obtained from the imaging and correctly identify unseen samples 79 % of the time (Platias et al., 2018). Specific regions of the spectrum have been shown to be more informative than others. Diezma et al., (2013) found that 710–900 nm was particularly important for the degradation of spinach leaves. Simko, Jimenez-Berni and Furbank, (2015) found similar results with lettuce, with 744 nm being the most informative wavelength for determining the quality difference between fresh and decayed lettuce. This is not particularly surprising as this portion of the electromagnetic (EM) spectrum is used for the basis of the normalised difference vegetation index (NDVI). NDVI distinguishes between 'healthy' and 'stressed' plants by the difference in reflection of the near-infrared (NIR) region of the EM spectrum, and has been used for a relatively long time for this purpose (Gitelson and Merzlyak, 1996).

Typically, when one method such as HI, is used alone with no further analysis, the results tend to heavily weight chlorophyll senescence, as with NDVI, as the primary factor with respect to change (Beghi et al., 2016). The measured values for colour change in packaged salad leaves

are not always linear; often there is an initial change over the first few days and then a reversal (Løkke et al., 2013). The colour change and then reversal, has been theorised to be related to the accumulation of liquid inside the pack, causing some areas to degrade to a greater extent and making the leaf appear darker. The change of colour and subsequent reversal makes classifying quality based on colour alone difficult, and the technology is not suitable for implementation in the retail or consumer part of the supply chain. The image/colour/spectral analysis described in these preceding sections does have potential for automating shelf life quality assessment that is performed by packers and consequently to provide a more consistent objective analysis than currently occurs between different assessors. However, the pack houses are assessing shelf life quality in the same time frame as the consumer, so the real gains in this area would be for methods to be developed that could predict quality loss in a particular consignment ahead of when the consumer becomes aware of it.

5.4. Detecting and identifying volatile compounds emitted from leafy salad crops

Challenges remain to identify compounds which are reliably associated with quality and depending on how detection is implemented, specific to the salad leaves in question. Typically, gas chromatography with mass spectrometry is the analytical method of choice, preferably using the same samples for chemical and sensory panel analysis to provide comparable results. The media used to capture the VOCs before measurement on a GC system are selected based on the compounds that are expected to be in the subject material. Solid-phase micro extraction (SPME) is a method often used for capturing volatile compounds that are emitted in the headspace of a leafy salad. A fibre coated in an adsorbent material is placed inside the headspace until an equilibrium has been reached between the fibre, the sample and the headspace. After the equilibrium has been reached the fibre is then placed in the GC system where the VOCs are desorbed and detected.

Recently, a number of researchers have focused their studies on VOCs emitted from rocket leaves. Spadafora et al. (2016) found that sulphur-containing VOCs tended to increase over shelf-life; it was noted that the increase was correlated with an increase in numbers of micro-organisms isolated from the leaves. In this case, the volatiles were extracted from the headspace of the pack and captured on Tenax traps then measured using GC–MS. Similar results have also been obtained by Bell et al. (2016) using thermal desorption with gas chromatography-time-of-flight mass spectrometry (TD-GC-TOF-MS) with a comparable extraction protocol. Typically, GC–MS methods cannot quantify the abundance of VOCs over time. This is because VOC compounds are often unknown or uncommon, meaning generating standards to quantify the absolute abundance of them are cost-prohibitive. Because of this, the appearance or disappearance of specific VOCs is often used as a marker of shelf-life (Lonchamp et al., 2009; Luca et al., 2017; Ioannidis et al., 2018). For leafy salads, it is only rocket that has had more than a couple of papers identifying compounds associated with quality. The lack of informative VOCs from other salad leaves may be due to rocket being particularly pungent or conversely the lack of VOCs emitted from other leafy salad crops. The appearance of compounds such as pentane, 2-ethylfuran and dimethyl sulphide, have been identified as markers of microbial activity (Luca et al., 2017), and have been associated with degradation of quality during storage in rocket salads (Dryahina et al., 2020). VOCs arising from cellular senescence or degradation induced by the presence of micro-organisms are hard to distinguish from each other. Therefore, it is challenging to ascribe particular compounds to microbial or cellular origin.

There are many research examples (Lonchamp et al., 2009; Spadafora et al., 2016; Raffo et al., 2018) illustrating the value of detecting volatile compounds in packaged salad that claim to be diagnostic of SL. However, it is a huge leap to move from volatile detection on sophisticated laboratory equipment to a technology that is commercially viable and implemented within industry. The challenges for this technology are currently threefold: Firstly the appropriate volatile markers need to be identified for each crop; this is perhaps the most difficult step as there are many variables, e.g., cultivar, growing environment, that influence plant metabolism and therefore the volatiles released from a plant (Bell et al., 2017). The detected volatiles also need to be reliably associated with quality degradation that would be predictive of consumer rejection of the product. Furthermore, technologies for detecting the identified VOCs need to be developed that are cost-effective commercially and can work in real-time to monitor quality.

5.5. Electronic noses for automated odour sensing

Gas sensor devices or ‘e-noses’ can be tuned to specific VOCs, therefore once the critical compounds concerning quality are established, devices for their detection can be built at relatively low cost. E-noses are non-specific detectors and are calibrated to detect a group of compounds rather than specific ones (Cortellino et al., 2018). ‘E-noses’ are relatively new, and the technology is developing rapidly. One of the issues with e-nose devices is that they are quite variable, both in manufacturing consistency and that they can degrade in their performance over time, depending on their environment, which has adverse effects on the quality of the data they generate. There has been much effort to develop algorithms that correct any variance relative to a master device (Yan and Zhang, 2016). The issue of consistency between devices could be a significant barrier to incorporating sensors into a retail or domestic setting. For a method to be non-destructive, the sensor must either be incorporated with the packaging, which provides many challenges, but may be successful at diagnosing quality deterioration measuring generic markers of degradation such as dimethyl sulphide. Alternatively, there needs to be an external sensor that is placed within the vicinity of the subject. However, the external sensor may detect aroma from a variety of origins, and therefore, needs to monitor specific compounds and is unlikely to work for bagged leafy salads or vegetables since volatiles will be contained within the package.

5.6. Quality sensors within “intelligent packaging”

Sensors have been developed that can be incorporated into the packaging of a product, and therefore allow real-time feedback about the condition of the product within (Torri et al., 2008; Fuertes et al., 2016). A recent review by Beshai et al. (2020) categorised intelligent packaging sensors into four types: optical, biosensors, gas, and humidity sensors. Optical sensors rely on the techniques discussed in the sub-sections above and it remains difficult to see how these can easily be incorporated into packaging in a format that can inform the consumer, although the potential for screening at an earlier stage in the supply chain is possible by linking sensors to radio frequency identification (RFID) tags to collate information and ensure data transmission throughout a supply chain.

Attention has inevitably turned towards technologies that have potential to detect foodborne pathogens, given the seriousness of the consequences if these proliferate on food destined for human consumption. Zhang et al. (2017) have made the best progress towards developing a system with a low detection threshold, through using a Janus emulsion assay which they demonstrated would sensitively and selectively binds to *E. coli* at 10^4 cfu/mL and which could be read via a smartphone app. However, this still relies on a liquid medium and, crucially, that the bacteria come into direct contact with the sensor. These are substantial assumptions and therefore there is an attraction towards sensor technologies that monitor gaseous compounds. López-Carballo et al., 2019 developed a sensor that can be incorporated within flexible packaging, of samples containing infant milk formula, utilising the redox reaction of methylene blue to signify changes in quality. As the sensor was monitoring O_2, it would only be suitable for MAP as the bag is hermetically sealed. Carbon dioxide may be a better

target for gas sensors incorporated into packaging, since its atmospheric concentration is only 0.04 % whereas in MAP it tends to be in the 4–10 % range. Borchert et al., 2013 described an optochemical CO2 sensor which uses a phosphorescent reporter dye and a colourimetric pH indicator incorporated in plastic matrix. The sensor retained its sensitivity to CO_2 for 21 days at 4 °C and could detect concentrations accurately within a minute of exposure, reporting them using a colour change requiring simple instrumentation, with a four minute recovery time. Despite the potential offered by VOCs that are specific to particular crops or that are produced as a result of microbiological contamination, to date no monitoring or detection systems have been developed that could be incorporated into packaging. Beshai et al. (2020) review current monitors for respiratory gases and humidity, but the only 'freshness' monitors that use non-respiratory gases depend on the sensor being in direct contact with the food which is not the case for packed vegetables and salads.

5.7. Time temperature indicators

Maintaining an unbroken cold chain is key to preserving quality and safety of fresh produce (Cantwell and Suslow, 2002) with short breaks in cold temperature less severe than prolonged periods above the optimal temperature. Even within a single cold chain variability exists, for example depending on the proximity of a pallet to the cooling system in the lorry or the location of a crate within a pallet. Two classes of Time Temperature Indicators exist: those that are data driven and those that display a colour change based on a physio-chemical reaction.

Data loggers or labels such as RFID tags that report temperature, humidity etc have been used commercially for some time, but often as stand-alone units that have to be incorporated within the packs in a crate which then need manual recovery and interpretation. There is considerable commercial attraction to the development of time-temperature indicators that can be incorporated into packaging or crate labelling systems, especially those which offer instant visual means of interpretation rather than plugging into a computer. RFID tags do offer this possibility, but they are limited by battery life, the need to be in close proximity to the reader, and their own lifespan. Torres-Sánchez et al. (2020) report the development of a multiple non-linear regression (MNLR) model that relates the temperature to the maximum shelf life in a predictive manner, but at present this relies on the integration of sensory and physico-chemical quality attributes. The best data-driven solutions therefore remain RFID tags that can integrate multiple signals from temperature, humidity and ammonia and which are sufficiently sophisticated to interpret the relationship between these parameters (Quintero et al., 2016).

Visual indicators have a great deal of appeal commercially, particularly if they report the full history that the product has experienced through the supply chain and if they can be incorporated into the packaging. At present, chemical colour change is usually reliant on the speed of an enzymic reaction linked to a pH change, polymer state changes linked to colour change, or the growth rate of bioindicator microorganisms (Lee and Rahman, 2014). They tend to only be able to report sub-optimally high temperatures, since they all work on the principal that raised temperatures lead to a faster response of the target reaction. They are therefore unsuitable for detecting when temperatures have been lower than optimal, for example if basil has been chilled below 12 °C. An additional practical problem is that the indicators have to be stored at low temperature before they are deployed to prevent the colour change happening before the tag has been attached to the package. However, a number of TTI products are used very successfully in a commercial setting, particularly for frozen or chilled food products. Considering that leafy salads have a relatively short SL, incorporating sensors into the packing of RTE products may not offer a reasonable return on investment, especially when considering implications the sensor may have on recyclability of the product. It remains to be seen if detection and monitoring of VOCs can provide data to the consumer that allows for real-time monitoring of the health and remaining longevity of a product that they purchase.

6. Concluding remarks

Previous technological advances within the food ecosystem, particularly with respect to imaging, have been implemented at the processing stage where cameras detect out-of-specification leaves and reject them. However, as was remarked when date labels were being introduced: distribution and storage conditions are important to the longevity of a product. There is currently no way for the retailer or consumer to update their expectations of shelf-life once the date on the pack has been set. The dates placed on the packaging, if any, are the only guide the consumer has as to the quality or safety of the product. Although some methods for non-destructively measuring quality post-harvest have been explored, none have yet to be implemented in a consumer study to measure the impact such technologies could provide with regards to reducing waste. High-end consumer refrigerators are now being produced with integrated computers and cameras that are able to monitor the contents, and give real-time feedback to the consumer by network-connected devices. However, there are currently no devices on the market offering product-specific monitoring or giving real-time feedback to the consumer regarding quality or safety, and certainly not for complex products such as leafy salads that are packaged in their current format.

The economic benefit of increasing the accuracy of SL estimations has been estimated at 55 ± 15 million pounds per day of savings, per day of increased SL from UK households for leafy salads (Lee et al., 2015). Furthermore, it is estimated that retailers would save 2720 tonnes of leafy salads from waste per day of increased shelf-life. There is a clear case for providing the consumer with more accurate information about the state of the product. However, although the technology for sensing quality and safety is progressing, there is still a long way to go in order to be able to reduce the amount of waste, whilst maintaining safety and quality.

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Declaration of Competing Interest

The authors report no declarations of interest.

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8.2 Paper 2: Growth temperature influences postharvest glucosinolate concentrations and hydrolysis product formation in first and second cuts of rocket salad

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Growth temperature influences postharvest glucosinolate concentrations and hydrolysis product formation in first and second cuts of rocket salad

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ABSTRACT

Rocket salad species (*Diplotaxis tenuifolia* and *Eruca sativa*; also known as *E. vesicaria*) are known for their high concentrations of health-related isothiocyanates, which are derived from secondary metabolites called glucosinolates. Increases in temperature due to climate change and extreme weather event frequencies over the coming decades are likely to influence not only the growth of leafy vegetables, but also their nutritional density. It is therefore essential to determine the impacts of these in order to mitigate crop losses and nutritional decline in future. Our data show there is a strong influence of pre-harvest growth temperatures on glucosinolate biosynthesis and formation of glucosinolate hydrolysis products postharvest, and that this is genotype dependent. High growth temperature (40 °C) severely retarded germination, growth, regrowth, and survival of rocket plants. Highest glucosinolate concentrations were observed in first and second cuts at 40 °C, but did not correspond to highest isothiocyanate concentrations (observed at 30 °C, second cut). Hydrolysis product formation is proportionately not as great as glucosinolate increases at 40 °C, possibly due to inhibition of enzyme function(s) at higher temperatures. These data indicate that high growth temperatures increase glucosinolate accumulation, but growth and productivity is significantly reduced. Much greater emphasis is needed for breeding cultivars tolerant to high growth temperatures in order to maximise nutritional benefits imparted by temperature stress.

1. Introduction

Rocket salads are a popular group of leafy vegetables belonging to the Brassicaceae family. *Diplotaxis tenuifolia* and *Eruca sativa* comprise the majority of global rocket production, and are well known for pungent aromas and flavours. Each species has distinct morphological characteristics, though *E. sativa* is much more varied in this regard. Over the last 20 years there has been a surge in interest in the crops for their phytochemical content, particularly for glucosinolates (GSLs) and their hydrolysis products (GHPs; Bell and Wagstaff, 2019). Foremost of these are isothiocyanates (ITCs), and specifically sulforaphane (SF); consumption of which has been linked with a reduced risk of developing some cancers (Fimognari and Hrelia, 2007).

GSL profiles are notoriously variable across growth environments in many Brassicaceae species, and the formation of GHPs even more so (Bell and Wagstaff, 2019). GSL biosynthesis is inherently tied to the stress responses of all Brassicales plants (Mostafa et al., 2016), and as such, concentrations within tissues can vary markedly according to growth temperature (Kask et al., 2016), light quality (Schreiner et al., 2009), and salinity (Cocetta et al., 2018); as well as biotic factors from pests and disease (Schlaeppi et al., 2010). Climate change is leading to more extreme temperatures in places used to cultivate horticultural crops, and consumer demand is leading to the adoption of more land and more protected cultivation practices to meet the yield and quality expectations.

A large amount of work has been done to determine the glucosinolate (GSL) profiles of rocket within first harvest (or cut) leaves (Cataldi et al., 2007; Chun et al., 2015; Force et al., 2007; Toledo-Martín et al., 2017), and only one obscure study has assessed second cut composition (Nitz and Schnitzler, 2002), but only looked at three compounds. Second cuts are primarily favoured by growers and processors for their perceived increased pungency and overall quality (Bell and Wagstaff, 2019), yet the scientific literature has thus far failed to consider this common horticultural practice in experimental designs. It is speculated that multiple cuts increase the abundance of glucosinolates and isothiocyanates in rocket species. The increase of secondary metabolites in response to mechanical wounding is well known in other horticultural species (Jahangir et al., 2009). This has clear implications for taste, flavour, and health-related properties of leaves.

The impact of different growing environments, such as temperature

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variation, on crop nutritional composition is also poorly studied in rocket; adding an additional influencing factor on these traits. It is therefore possible that variable growing temperatures will also affect (positively or negatively) the consumer eating experience. The exact genetic mechanisms that regulate GSL biosynthesis under high or low temperatures are unknown, partly due to interacting and co-occurring stresses, such as drought. It is likely however that the imposition of high/low temperature stress promotes activity of transcription factors such as *MYC2* and *MYB28*, which promote GSL biosynthesis (Gigolashvili et al., 2009).

Temperature effects upon GSL synthesis and GHP formation are poorly understood in rocket species, and have important implications for the synthesis of specific health-associated compounds. Rocket crops are grown on every inhabited continent, and are exposed to a huge range of cultivation temperatures. They can be grown under mild, temperate conditions, such as in southern England, (summer maximum temperatures averaging 20.4 °C; Met Office 1981–2010 data), to hot Mediterranean temperatures (such as the Bay of Naples, Italy, summer maximum temperatures averaging 29.5 °C; World Meteorological Organization). In addition, crops are commonly cultivated under glass or polytunnel in summer months, where internal daytime temperatures can rise to over 35 °C (Di Gioia et al., 2018). In growing regions such as Lazio (Italy) and New South Wales (Australia), outdoor summer daytime temperatures can regularly exceed 40 °C, and therefore have significant impacts on the growth of leafy vegetables. Crops under protection are therefore doubly affected, as internal temperatures may exceed 50 °C without adequate ventilation. By the end of the 21st century, atmospheric CO_2 concentrations are projected to rise to between 730 and 1000 ppm. This will lead to average global temperature increases of between 1–3.7 °C (Gray and Brady, 2016). Combined with an increased likelihood of extreme weather events (such as heat waves and drought), protected leafy crops such as rocket are especially vulnerable to losses and or changes in growth rate.

The effects of such growing extremes are presently unknown, and it is likewise unstudied how growth temperature affects regrowth, phytochemical content, or shelf life retention of health-associated compounds. Postharvest work has already demonstrated that these compounds are subject to fluctuation (Bell et al., 2017c; Yahya et al., 2019), but it is unknown to what degree growth temperature and cut influence this process. In light of climate change and global warming effects in future, it is also likely that extremes in temperature will become more common, and therefore it is important to understand how crop growth and quality may be affected.

This study presents phytochemical data relating to the growth of two *D. tenuifolia* and two *E. sativa* rocket cultivars under different growth temperatures. This study is the first to examine the impact of cultivation temperatures on secondary metabolite formation that has consequences for nutrition and flavour qualities of rocket crops. We hypothesised that each species would see increases in GSL concentrations at the higher cultivation temperatures, but that each cultivar would produce differing relative concentrations according to genotype, as has been highlighted in a previous study (Bell et al., 2015). We speculated that the initial concentrations of GSLs postharvest would influence the degree of biosynthesis and retention during the cold storage period, and in turn impact the abundance of GHPs formed.

2. Materials and methods

2.1. Plant material

Two *D. tenuifolia* and two *E. sativa* pre-commercial cultivars were supplied by Elsoms Seeds Ltd. (Spalding, UK). For reasons of commercial sensitivity specific details regarding the genetic origin of these will not be given. *E. sativa* cultivars were designated RS4 and RS8, and *D. tenuifolia* cultivars RW2 and RW3.

Table 1
Numbers of biological replicates under each temperature condition and cut per temperature treatment.

Cultivar	Temperature condition & cut					
	20 °C		30 °C		40 °C	
	1st	2nd	1st	2nd	1st	2nd
RS4	12	12	12	7	16	dns
RS8	11	11	15	8	13	6
RW2	5	3	9	3	dns	dns
RW3	10	7	10	7	2	dns

Abbreviations: RS = salad rocket; RW = wild rocket; dns = did not survive.

2.2. Growing conditions, simulated processing, and shelf life storage sampling

Forty seeds of each cultivar were sown into module trays containing peat-based seedling compost, and germinated under three temperature conditions in Saxcil growth cabinets. The three temperature conditions were as follows: 20 °C (daytime; 15 °C night), 30 °C (daytime; 25 °C night), and 40 °C (daytime; 30 °C night). Lighting conditions were consistent between each chamber and set to a long-day cycle (16 h light, 8 h dark). Light intensity was set to 380 µmol m^{-2} s^{-1}. Humidity was ambient. Healthy seedlings were transplanted into 1 l pots (containing peat-based compost) on an individual basis, upon the development and expansion of two true leaves. Pots were watered daily, as required, to capacity.

Plants were harvested on an individual basis, and were considered of commercial maturity once 10–15 leaves were developed. See Table 1 for numbers of biological replicates harvested for each cultivar under each respective condition. Upon reaching this point, plants were harvested by hand using sterile scissors and left to regrow. It should be noted that not all plants survived the first cut, and that the 40 °C treatment severely impaired growth and survival.

All plants were harvested between 10 a.m. and 12 p.m to minimise the effects of diurnal fluctuations in secondary metabolites (Huseby et al., 2013). The harvested leaves were initially placed in Ziploc bags and then transferred to the laboratory. Upon arrival, plants were hand processed individually by turbulent washing in mildly chlorinated water (30 ppm, sodium hypochlorite; Suslow, 2000) for one minute, followed by gentle rinsing in non-chlorinated water for one minute. Finally leaves were placed in a hand operated salad spinner and dried for another minute. Leaves were divided into equal amounts and designated D0, D4, and D7 according to the beginning of shelf life storage. D0 samples were placed immediately into a -80 °C freezer. D4 and D7 samples were placed in laser perforated bags and closed with an electric heat-sealer, then stored in the dark at 4 °C (Bell et al., 2016). On respective sampling days, each of the bagged leaves were frozen at -80 °C. All sampling took place between 10 a.m. and 12 p.m, as per the initial time of harvest. This entire process was repeated for the second cut of regrown leaves until all plants were either harvested or had died.

2.3. Leaf material preparation, and extraction

Frozen leaf material was lyophilized in batches for three days. Leaves were ground into a fine powder using a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ, USA) and stored in tubes until extraction and analysis.

GSL extraction was performed as per the protocol presented by (Bell et al., 2015) with modifications. Briefly, 40 mg of dried leaf powder was placed into Eppendorf tubes and put into a heat block (80 °C for ten minutes). Afterwards, 1 ml of preheated methanol water (70 % v/v) was added to dried powder, vortexed vigorously, and placed in a water bath

(75 °C) for 20 min. Samples were cooled to halt the extraction and then centrifuged at full speed for five minutes at room temperature (~22 °C), the supernatant collected, and filtered (0.22 µm PVDF Acrodisc syringe filters; VWR, Lutterworth, UK). Crude extracts were stored at -80 °C before dilution (5x) and analysis conducted by LC–MS.

GHPs were extracted according to the protocol published by Ku et al. (2016) with modifications. The extraction duration was optimized for maximum yields of GHPs by comparison of extractions for three hours incubation at 30 °C with immediate dichloromethane (DCM) extraction, and three, nine, and 21 h post incubation with DCM.

50 mg of sample was hydrolysed in 1 ml of d.H_2O for three hours at 30 °C, before subsequent extraction in dichloromethane (DCM) overnight (21 h). The DCM layer was then collected and transferred to glass vials and stored at -80 °C until analysis by GC–MS.

2.4. LC–MS and GC–MS analyses

For LC–MS, samples were analyzed in a random sequence with standards and QC samples. External standards of progoitrin (PRO; 99.07 %, HPLC), glucoraphanin (GRA; 99.86 %, HPLC), glucoerucin (GER; 99.68 %, HPLC), glucobrassicin (GBR; 99.38 %, HPLC), and gluconasturtiin (GNAS; 98.38 %, HPLC) were prepared for quantification of GSL compounds according to the method presented by Jin et al. (2009). GER was used to quantify glucorucolamine (GRM), diglucothiobeinin (DGTB), glucosativin (GSV), and DMB, as no standards are presently available for these compounds. GBR was used to quantify the indole GSLs 4-methoxyglucobrassicin (4MOB) and neoglucobrassicin (NGB; Table 2). All standards were purchased from PhytoPlan (Heidelberg, Germany). Recovery of extracted GSLs was calculated by spiking six random samples with sinigrin upon the addition of pre-heated methanol (Merck, Gillingham, UK). The average recovery of sinigrin was 104.8 %. Limits of detection (LOD) and quantification (LOQ) were established for the method by running serial dilutions of sinigrin (LOD = 5.38 µmol L^{-1}; LOQ = 16.3 µmol L^{-1}).

LC–MS analysis was performed in the negative ion mode on an Agilent 1260 Infinity Series LC system (Agilent, Stockport, UK) equipped with a binary pump, degasser, auto-sampler, column heater and diode array detector; coupled to an Agilent 6120 Series single quadrupole mass spectrometer. Separation of samples was achieved on a Gemini 3 µm C18 110 Å (150 × 4.6 mm) column (with Security Guard column, C18; 4 mm x 3 mm; Phenomenex, Macclesfield, UK). GSLs were separated during a 40 min chromatographic run, with a 5 min post-run sequence. Mobile phases consisted of ammonium formate (0.1 %; A) and acetonitrile (B) with the following gradient timetable: (i) 0 min (A–B, 95:5, v/v); (ii) 0–13 min s (A–B, 95:5, v/v); (iii) 13–22 min s (A–B, 40:60, v/v); (iv) 22 − 30 mins (A–B, 40:60, v/v); 30 − 35 mins (A–B, 95:5, v/v); (v) 35 − 40 mins (A–B, 95:5, v/v). The flow rate was optimized for the system at 0.4 ml min^{-1}, with a column temperature of 30 °C; 20 µl of sample was injected into the system. Quantification was conducted at a wavelength of 229 nm.

MS analysis settings were as follows: Atmospheric pressure electrospray ionization was carried out in negative ion mode (scan range *m/z* 100–1000 Da. Nebulizer pressure was set at 50 psi, gas-drying temperature at 350 °C, and capillary voltage at 2000 V. Compounds were identified using their primary ion mass [M-H]- (Cataldi et al., 2007) and by comparing relative retention times with those of Lelario et a. (2012; Table 2). Data were analyzed using Agilent OpenLAB CDS ChemStation Edition for LC–MS (vA.02.10). GSL concentrations from each time point were averaged; see Table 1 for all *n* per treatment. This approach was also conducted for GHP analysis.

GHPs were identified and analysed according to the method presented by Bell et al. (2017) with the following modification. Extracts were separated on a Zebron ZB-AAA (10 m, 0.25 mm i.d.; Phenomenex) capillary during a seven minute run. GC conditions were as follows: split 1:20 at 250 °C, with a 2.5 µl injection. Helium was used as the carrier gas at a flow rate of 1.5 mL min. The oven program was: 30 °C min from 110 °C to 320 °C, with a one minute hold at 320 °C. Concentrations of all GHPs were calculated as equivalents of SF standard (Sigma).

All concentrations quoted within the text are on a dry weight basis for both GSLs and GHPs.

Table 2
Glucosinolates and glucosinolate hydrolysis products identified in *Eruca sativa* and *Diplotaxis tenuifolia* cultivars.

Glucosinolates					
	Trivial name	Abbreviation	R-group name	Retention time	Identifying *m/z* $[M-H]^-$
1	Glucorucolamine [b]	GRM	4-(cysteine-*S*-yl)butyl	4.7	493
2	Progoitrin [a]	PRO	(2*R*)-2-hydroxybut-3-enyl	5.9	388
3	Glucoraphanin [a]	GRA	4-methylsulfinylbutyl	6.0	436
4	Diglucothiobeinin [b]	DGTB	4-(β-D-glucopyranosyldisulfanyl)	12.7	600
5	Glucosativin [b]	GSV	4-mercaptobutyl	16.4	406
6	Glucoerucin [a]	GER	4-methylthiobutyl	22.7	420
7	–	DMB	Dimeric 4-mercaptobutyl	23.0	405 (811, 731)
8	Glucobrassicin [a]	GBR	Indol-3-ylmethyl	23.5	447
9	Gluconasturtiin [a]	GNAS	2-phenethyl	24.0	422
10	4-methoxyglucobrassicin [c]	4MOB	4-methoxy-3-indolylmethyl	24.2	477
11	Neoglucobrassicin [c]	NGB	1-methoxy-3-indolylmethyl	25.6	477

Glucosinolate hydrolysis products			
	Trivial name	Hydrolysis product of	MS spectra *m/z* (base ion in bold)
1	Sativin [d]	Glucosativin	147, **114**, 87, 72, 60
2	Erucin [d]	Glucoerucin	161, **115**, 72, 61
3	Sulforaphane [a]	Glucoraphanin	177, **160**, 114, 72, 55
4	Bis-(4-isothiocyanatobutyl)-disulfide [d]	DMB	292, 146, **114**, 87, 72, 55

[a] = Quantified using authentic standards.
[b] = Quantified using glucoerucin standard.
[c] = Quantified using glucobrassicin standard.
[d] = Quantified using sulforaphane standard.

2.5. Statistical analysis

ANOVA analyses of all data were performed using XL Stat (Addinsoft, Paris, France). Each respective analysis was conducted with a protected post hoc Tukey's Honest Significance Difference (HSD) test ($P < 0.05$). The Type III Sums of Squares significance values (at 5 %, 1 %, and 0.1 % thresholds) and summary pairwise comparisons tables for GSL and GHP data are available in Supplementary Data File S1.

Principal Component Analysis (PCA) was performed using XL Stat with Pearson correlation analysis (*n*-1), Varimax rotation, and the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy. The KMO value for the analysis was 0.71, indicating a satisfactory level of sampling. The analysis produced four informative Principal Components (PCs) with Eigenvalues >1.0. The cumulative explained variability within these components totalled 78.1 %. After Varimax rotation, PCs 1, 2, and 8 produced the highest degree of explanatory spatial separation.

3. Results and discussion

3.1. The effects of growth temperature on germination and time to first and second cuts

3.1.1. Germination

Germination time was shortest under the 30 °C condition, and *E. sativa* cultivars showed a clear trend for earlier establishment than *D. tenuifolia* (Fig. 1a). RW2 and RW3 had slow germination and growth at both 20 °C and 40 °C, with RW2 not germinating at all under the latter condition. As rocket species have a Mediterranean origin, it is not entirely surprising that germination is optimal at 30 °C, however our data do suggest that *E. sativa* is better adapted to temperature extremes. This could be of particular relevance to growers cultivating rocket under glass or polytunnel where temperatures may regularly exceed 35 °C in the summer. Conversely, growers in cool or temperate regions, cultivating rocket in open field, may find *E. sativa* quicker to establish.

3.1.2. Time to first cut

Harvest age of rocket is highly variable between growth environments (Hall et al., 2015), and as such we selected a physiological growth phase as a benchmark for harvest between each temperature condition (development of 10–15 leaves). At 40 °C all plant growth was severely retarded (Fig. 1b), and no plants were ready for first harvest before 40 days. This suggests that adverse high temperature conditions may have a large impact on the productivity of rocket crops, regardless of species.

Exposure to temperatures >37 °C for prolonged periods of time can be lethal in many plants without acclimatisation. This is due to the inactivation or denaturation of proteins (Schöffl and Panikulangara, 2018). Without adequate time to adjust to heat shock (such as through expression of heat shock proteins) growth is slowed or even halted; ultimately leading to plant death. It is therefore remarkable that the rocket plants (particularly RS8) tested in this experiment were able to tolerate these conditions.

3.1.3. Time to second cut

The differences between temperature conditions for the second harvest were less pronounced (Fig. 1c). At 40 °C however there was no regrowth for RS4, RW2, or RW3. Plants of these cultivars typically senesced a few days after first cut and died. As mentioned previously, given that the 40 °C temperature was extreme (Schöffl and Panikulangara, 2018), *E. sativa* in particular displayed a high tolerance.

Such conditions are not unheard of in protected environments, and may become more common under protected conditions in future due to global warming. It is interesting therefore that RS8 showed little adverse effects to the extreme temperature condition, and regrew within approximately 15 days. While rocket is not bred for temperature tolerance at this time, it does suggest that if climatic conditions become more challenging in future there are cultivars capable of withstanding such high temperature extremes. As will be discussed in subsequent sections, this may also have important implications for biosynthesis of health-related compounds in rocket.

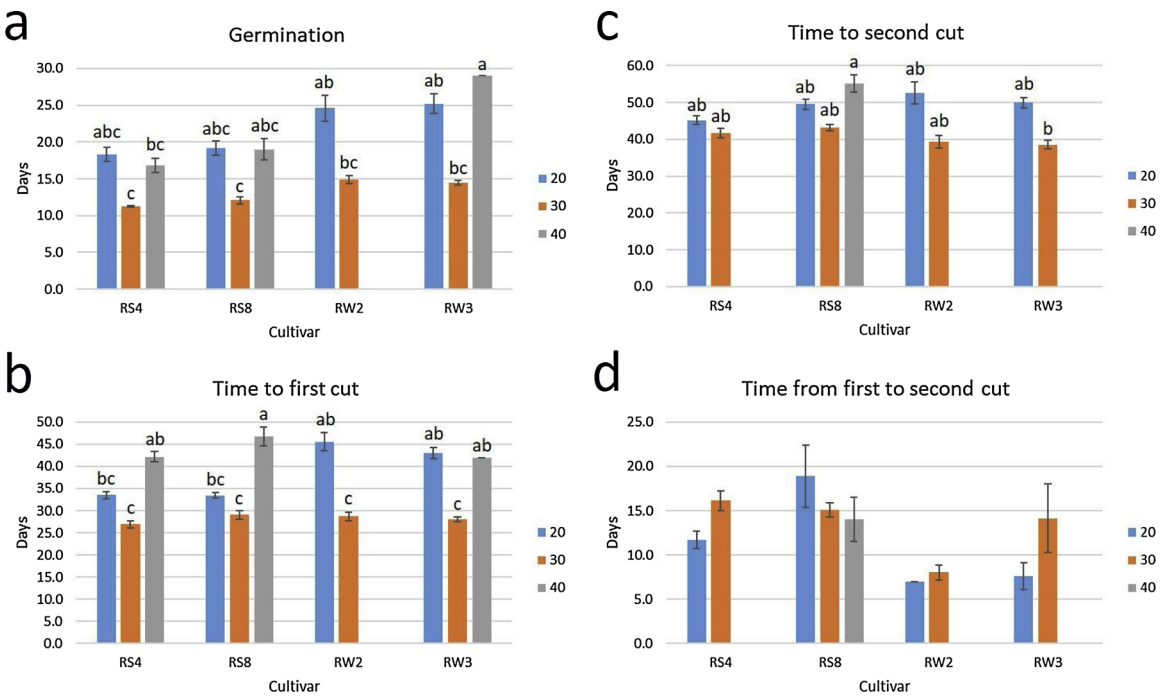

Fig. 1. The number of days taken for *Diplotaxis tenuifolia* (RW) and *Eruca sativa* (RS) cultivars to germinate (a), reach first cut maturity (10 – 15 true leaves; b), reach second cut maturity (10 – 15 regrowth leaves; c), and from first to second cut (d). Error bars represent standard error of the mean. See Table 1 for the numbers of replicates for each cultivar and treatment.

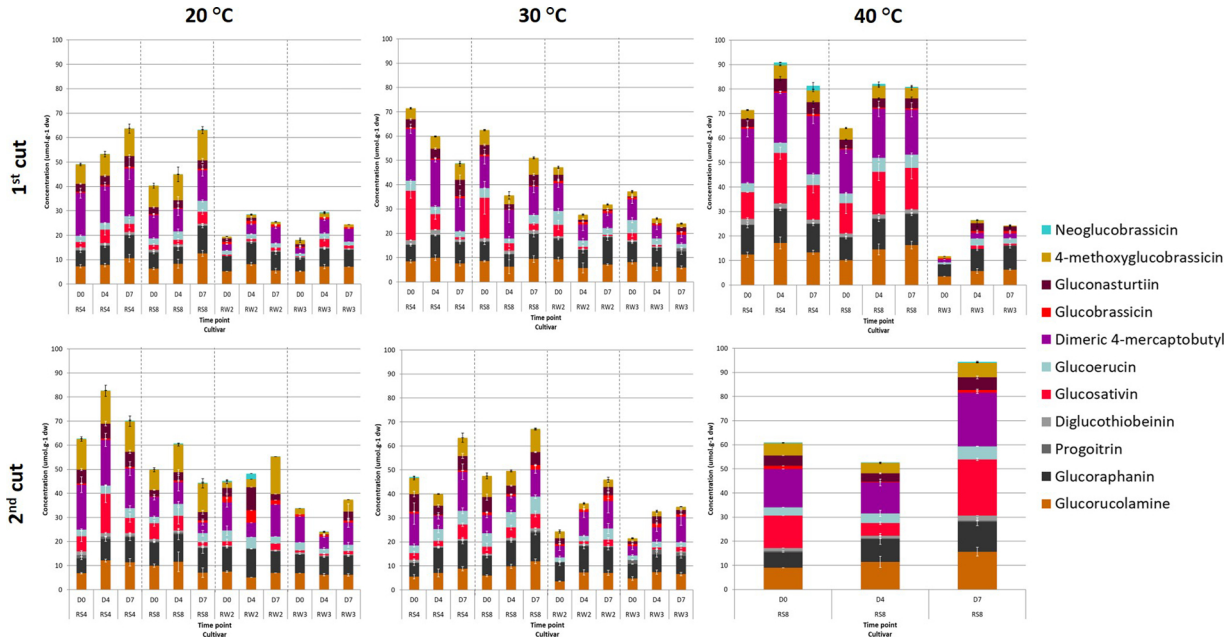

Fig. 2. Glucosinolate concentrations in first and second cut *Diplotaxis tenuifolia* (RW) and *Eruca sativa* (RS) grown at four different temperatures. See inset for glucosinolate colour coding. Abbreviations: D0, start of shelf life; D4, fourth day of shelf life; D7, seventh day and end of commercial shelf life. Error bars represent standard error of the mean for each respective compound. For detailed statistical and Tukey's HSD pairwise comparisons, see Supplementary Data File S1. See Table S1 for the numbers of replicates for each cultivar and treatment. Note that sample RW3 at 40 °C consists of <3 biological replicates due to plant death.

3.1.4. Time from first to second cut

Regrowth of RW2 was extremely fast, taking only seven days to regrow >10 leaves after the first cut at 30 °C (Fig. 1d). These data suggest that *D. tenuifolia* second cuts may be much more productive than *E. sativa* under 20–30 °C conditions. While initial establishment and growth rate is slow in the first cut, this is offset by a much quicker subsequent regrowth rate. Leaves were however much smaller than at first cut, and would likely be much lower yielding (even at high planting densities) than *E. sativa* second cuts.

3.2. The effects of growth temperature on glucosinolate concentrations and hydrolysis product formation during shelf life storage

3.2.1. First cut at 20 °C

There was a clear trend in first cut *E. sativa* cultivars for higher GSL accumulation compared with *D. tenuifolia* (Fig. 2). At D0, ANOVA pairwise comparisons of these samples were non-significant between cultivars, with the exception of 4MOB ($P < 0.0001$; Table 3 & Supplementary Data File S1). RS4 and RS8 both showed a clear trend for increased GSL concentrations over the seven day shelf life period, peaking at the final time point (D7). This is in agreement with observations made by Bell et al. (2017) in a field grown UK *E. sativa* crop. RW2 and RW3 by comparison peaked on D4, but contained almost half the concentrations of RS4 and RS8. These trends were only followed by RS8 and RW2 for hydrolysis product formation, and concentrations were generally low across all time points (Fig. 3). This has implications for consumers, and suggests that health-related benefits are cultivar and time-dependent postharvest. The variability of isothiocyanates and other GHPs in rocket postharvest is documented (Bell and Wagstaff, 2019). The timing of consumption is therefore a critical consideration for determining the efficacy of rocket cultivars against disease and chronic illness, and should be included as a more prominent factor for consideration in clinical investigations.

3.2.2. Second cut at 20 °C

In the second cut at 20 °C the trend between species was reversed: RS4 and RS8 peaked on D4, and RW2 and RW3 peaked on D7 for GSLs (Fig. 2). Again, indolic GSL concentrations were significantly different at D0. RW2 contained significantly higher abundance of GBR than RS4 and RS8 ($P = 0.003$), and conversely, RS4 had higher amounts of 4MOB compared with each of the *D. tenuifolia* cultivars ($12.7 \pm 0.9\ \mu mol\ g^{-1}$; $P < 0.0001$). These differences were repeated in D4 samples (GBR and 4MOB, $P < 0.0001$, Table 3 & Supplementary Data File S1) and suggest a distinct difference in indolic GSL metabolism between the two species; something that has not been previously observed. Indolics are linked with chemopreventative properties, such as promoting cancer cell cycle arrest (Hayes et al., 2008) and selection for improved indolic profiles of rocket could lead to increased health benefits.

Temporal changes in 4MOB were most pronounced in RW2, with a large and significant increase in abundance at D7 ($15.6\ \mu mol\ g^{-1}$) compared to D0 ($2.5 \pm 0.7\ \mu mol\ g^{-1}$) and D4 ($3.3\ \mu mol\ g^{-1}$). Indolic GSLs are involved with abscisic acid (ABA) metabolism and synthesis (Malka and Cheng, 2017), and therefore such large increases over the course of shelf life may be indicative of increased senescence induced by plant hormone activity. This may be of significance when selecting rocket cultivars for improved shelf life traits; especially in second cut *D. tenuifolia*, which is the most common rocket product on global supermarket shelves.

Second cuts of each cultivar produced large average increases in GHP formation (Fig. 3). The patterns of change over shelf life did not match those of GSLs, suggesting that GSL content is not an accurate proxy for the abundance and ratios of GHPs that are formed. Importantly SF, which has been linked with anticarcinogenic effects *in vivo* (Liang et al., 2008) saw large increases in the *E. sativa* cultivars, but not in *D. tenuifolia*; concentrations were significantly higher in RS8 ($2.4 \pm 0.4\ \mu mol\ g^{-1}$; $P < 0.0001$). This was also repeated at D4 (RS8, $2.6 \pm 0.6\ \mu mol\ g^{-1}$; $P < 0.0001$, Table 3 & Supplementary Data File S1) and indicates that second cut *E. sativa* may be better suited for formation of health related SF at lower growth temperatures than *D. tenuifolia*.

3.2.3. First cut at 30 °C

In RS4 the decrease from D0 to D7 (Fig. 2) was predominantly due

Table 3
Results of Analysis of Variance type III sums of squares significance values for four rocket cultivars grown under three environmental temperature conditions, at three postharvest time points.

Factors & interactions	Time point/ cultivar	Glucosinolates							
		Glucoruco-lamine	Glucora-phanin	Progoitrin	Diglucothi-obeinin	Glucosativin	Glucoerucin	Dimeric 4-mercaptobutyl	Glucobras-sicin
Cultivar	D0	***	*	ns	***	***	ns	***	ns
	D4	**	ns	ns	***	***	**	***	***
	D7	***	***	***	***	***	***	***	ns
Growth temperature	D0	ns	ns	ns	ns	ns	*	ns	ns
	D4	ns	ns	ns	ns	ns	ns	ns	***
	D7	ns	ns	***	ns	***	ns	ns	ns
Cut number	D0	**	ns	*	ns	ns	ns	ns	**
	D4	ns	ns	ns	ns	ns	ns	ns	***
	D7	ns	ns	**	ns	ns	ns	ns	ns
Cultivar x temperature	D0	**	ns	ns	ns	ns	ns	ns	*
	D4	ns	ns	**	ns	ns	ns	ns	***
	D7	*	ns	**	**	***	ns	ns	ns
Cultivar x cut number	D0	ns	ns	ns	ns	ns	ns	ns	ns
	D4	ns	ns	ns	ns	ns	ns	ns	***
	D7	ns	ns	***	ns	ns	ns	ns	ns
Cultivar x temperature x cut number	D0	*	ns	*	ns	ns	**	ns	*
	D4	ns	ns	***	ns	ns	ns	ns	***
	D7	ns	ns	***	ns	ns	ns	ns	ns
Temperature x cut number	RS4	*	ns	*	**	***	ns	ns	ns
	RS8	ns	*	ns	ns	ns	**	ns	**
	RW2	ns	ns	ns	ns	ns	ns	ns	ns
	RW3	ns	**	*	ns	ns	ns	ns	ns
Temperature x time point	RS4	ns	ns	ns	ns	***	ns	ns	ns
	RS8	ns	ns	ns	ns	ns	ns	ns	ns
	RW2	ns	ns	*	ns	ns	ns	ns	ns
	RW3	ns	ns	*	ns	ns	ns	ns	ns
Cut number x time point	RS4	ns	ns	ns	ns	**	*	ns	ns
	RS8	ns	ns	ns	ns	ns	ns	ns	ns
	RW2	ns	ns	ns	ns	ns	ns	ns	ns
	RW3	ns	ns	ns	ns	ns	ns	ns	ns
Temperature x cut number x time point	RS4	ns	ns	ns	ns	**	ns	ns	ns
	RS8	ns	ns	ns	ns	*	ns	ns	ns
	RW2	**	ns	ns	**	*	ns	ns	ns
	RW3	ns	*	**	ns	ns	ns	ns	ns

(*continued on next page*)

Table 3 (*continued*)

Factors & interactions	Time point/ cultivar	Glucosinolates					Hydrolysis products			
		Gluconasturtiin	4-methoxy-glucobrassicin	Neoglucobrassicin	Total Glucosinolates	Sativin	Erucin	Sulfora-phane	Bis(4-isothiocyanato-butyl)-disulfide	Total glucosinolate hydrolysis products
Cultivar	D0	***	***	ns	***	***	ns	***	ns	***
	D4	***	***	ns	***	***	***	***	ns	***
	D7	**	***	ns	***	*	**	***	ns	**
Growth temperature	D0	ns	**	ns	ns	ns	ns	ns	ns	*
	D4	ns	**	ns	ns	ns	ns	ns	ns	ns
	D7	ns	***	ns	ns	ns	ns	*	ns	ns
Cut number	D0	ns	ns	*	ns	ns	ns	ns	ns	ns
	D4	ns	ns	ns	ns	***	ns	ns	ns	***
	D7	ns	ns	ns	ns	***	ns	ns	***	***
Cultivar x temperature	D0	ns	**	ns	ns	ns	ns	**	ns	ns
	D4	*	*	ns	ns	ns	ns	**	ns	ns
	D7	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cultivar x cut number	D0	ns	ns	ns	ns	ns	ns	**	ns	*
	D4	ns	ns	ns	ns	ns	ns	***	ns	*
	D7	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cultivar x temperature x cut number	D0	ns	ns	ns	ns	ns	ns	ns	ns	ns
	D4	**	ns	ns	ns	ns	ns	ns	ns	ns
	D7	ns	ns	ns	ns	ns	ns	ns	ns	ns
Temperature x cut number	RS4	ns	ns	ns	**	ns	ns	ns	ns	ns
	RS8	ns	ns	ns	ns	**	ns	**	ns	**
	RW2	ns	***	**	ns	ns	ns	ns	ns	ns
	RW3	ns	ns	ns	ns	ns	ns	ns	ns	ns
Temperature x time point	RS4	ns	ns	ns	ns	ns	ns	ns	ns	ns
	RS8	ns	ns	ns	ns	ns	ns	ns	ns	*
	RW2	ns	***	**	ns	ns	ns	ns	ns	ns
	RW3	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cut number x time point	RS4	ns	ns	ns	ns	ns	ns	ns	ns	ns
	RS8	ns	ns	ns	ns	ns	ns	ns	ns	ns
	RW2	ns	***	**	ns	ns	ns	ns	ns	ns
	RW3	ns	ns	ns	ns	ns	ns	ns	ns	ns
Temperature x cut number x time point	RS4	ns	ns	ns	*	ns	ns	ns	ns	ns
	RS8	ns	ns	ns	ns	ns	ns	ns	ns	ns
	RW2	ns	***	**	ns	ns	ns	**	ns	ns
	RW3	ns	ns	ns	ns	ns	ns	ns	ns	ns

Abbreviations: D0 = day 0 shelf life; D4 = day 4 shelf life; D7 = day 7 shelf life; RS = salad rocket, *Eruca sativa*; RW = wild rocket, *Diplotaxis tenuifolia*.
Significance values: *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$. See Supplementary Data File S1 for detailed P-values and Tukey's Honest Significant Difference pairwise comparisons.

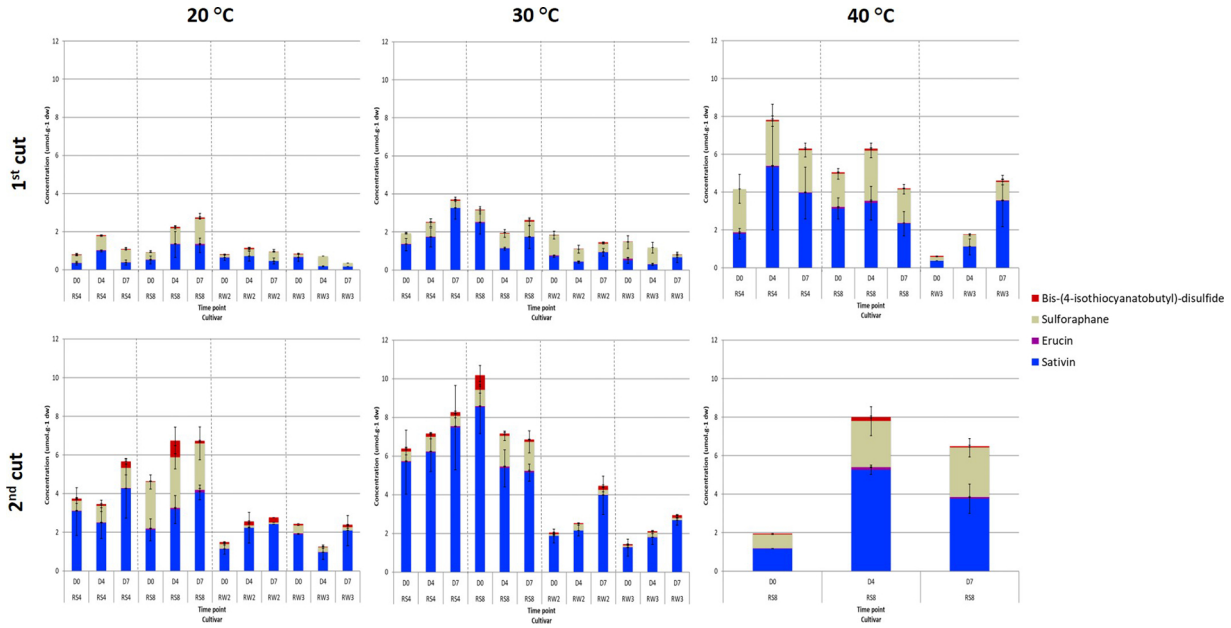

Fig. 3. Glucosinolate hydrolysis product concentrations produced by first and second cut *Diplotaxis tenuifolia* (RW) and *Eruca sativa* (RS) grown at four different temperatures. See inset for hydrolysis product colour coding. Abbreviations: D0, start of shelf life; D4, fourth day of shelf life; D7, seventh day and end of commercial shelf life. Error bars represent standard error of the mean for each respective compound. For detailed statistical and Tukey's HSD pairwise comparisons, see Supplementary Data File S1. See Table S1 for the numbers of replicates for each cultivar and treatment. Note that sample RW3 at 40 °C consists of <3 biological replicates due to plant death.

to a significant reduction in GSV (from 20.4 ± 3.0 μmol g^{-1} to 0.9 ± 0.2 μmol g^{-1}; $P < 0.0001$, Table 3 & Supplementary Data File S1). This trend was not reflected in the GHP profile of RS4 however, where concentrations were highest at D7 (3.7 ± 0.6 μmol g^{-1}). Although no significant differences were found between cultivars or growth temperatures, these trends again suggest that GSL content is not an accurate predictor of GHPs.

3.2.4. Second cut at 30 °C

Second cuts showed no significant differences from the first at 30 °C for GSL content, indicating more consistent biosynthesis. This is a desirable characteristic for growers and processors as it in turn may contribute to improved consistency in taste and flavour between cuts.

In terms of individual GSL differences between cultivars, RS8 again contained significantly higher concentrations of 4MOB (8.9 ± 1.2 μmol g^{-1}) than RW3 at D0 (1.2 ± 0.2 μmol g^{-1}; $P < 0.0001$). On D4 of shelf life, RW3 by contrast contained significantly more PRO than the other cultivars (2.0 ± 0.5 μmol g^{-1}; $P < 0.0001$; Table 3 & Supplementary Data File S1). This compound is known to impart extreme bitterness and is a target for reduction through breeding (Ishida et al., 2014). While concentrations may not be high at the point of harvest, breeding selections should also take into account such possible increases in synthesis postharvest to reduce consumer rejection. PRO is also associated with anti-nutritional properties (Mithen et al., 2000) and efforts should be made to reduce concentrations in rocket cultivars through breeding.

In terms of temporal changes for each cultivar over the shelf life duration, one significant difference was of note: compared to the D0 sample point, D4 and D7 samples of RS4 contained significantly greater concentrations of GRA (5.7 ± 1.1 μmol g^{-1}, 10.6 ± 0.2 μmol g^{-1}, and 11.7 ± 1.3 μmol g^{-1}, respectively; $P < 0.0001$, Table 3 & Supplementary Data File S1). This matches observations made by Bell et al. (2017) where shelf life increases in this GSL were also observed for some cultivars.

Despite negligible changes in GSL content between cuts, GHPs saw large and significant increases compared to the first cut. The exact regulatory mechanisms for GHP formation in rocket are largely unknown, but evidence is mounting that it is not purely a spontaneous process of 1:1 conversion of GSLs. It may be that although GSL concentrations may be lower, myrosinase activity can remain high higher, and/or actively promote the formation of GHPs; such as through the action of ESM1 genes. It is important to understand how this is controlled under abiotic stress conditions, as it will likely influence the nutritional benefits obtained from leaves.

With the exception of RS8, all cultivar concentrations peaked on D7 further supporting previous reports of this (Bell et al., 2017c). At D0, RS8 produced significantly more SAT (8.5 ± 1.4 μmol g^{-1}; $P < 0.0001$) than the *D. tenuifolia* cultivars; and the highest concentrations overall of any tested sample (10.2 ± 2.0 μmol g^{-1}; $P < 0.0001$, Table 3 & Supplementary Data File S1). It is possible that such high concentrations of this compound would greatly increase the pungency of a cultivar, and support the anecdotal observations often made by growers.

3.2.5. First cut at 40 °C

RW3 contained significantly less GRM (3.5 μmol g^{-1}; $P < 0.0001$), GRA (5 μmol g^{-1}; $P = 0.000$), DGTB (not detected; $P < 0.0001$), and DMB (1.3 μmol g^{-1}; $P < 0.0001$) than the *E. sativa* cultivars at D0. In D4 samples, concentrations increased in RW3, however this was significantly lower than RS4 and RS8 for accumulation of GSV (1.3 ± 0.3 μmol g^{-1}; $P < 0.0001$). At D7, concentrations in RW3 declined, with RS4 and RS8 containing significantly more GRM, DGTB, and GSV (all $P < 0.0001$, Table 3 & Supplementary Data File S1).

Relative concentrations of GHPs were higher in RW3, particularly at D7, than the relative amounts of GSLs. The trend for the two *E. sativa* cultivars to contain higher abundances was however similar (Fig. 3). At D4 these contained significantly greater concentrations of SF than both RW2 RW3 ($P < 0.0001$, Table 3 & Supplementary Data File S1), but by D7 there were no significant differences. The retention of SF throughout the shelf life period is an important finding that suggests that potent health-related effects (Sivapalan et al., 2018) may be present in rocket leaves up to a week postharvest, even after the imposition of severe abiotic stress.

3.2.6. Second cut at 40 °C

RS8 was the only cultivar tested that survived and regrew under the 40 °C treatment, and it also contained the highest observed GSL

concentrations of any condition or cut (Fig. 2). While no significant differences between each time point were observed, there is a clear trend for concentrations to increase at D7. This is one of the highest concentrations reported to-date for *E. sativa*, and it is clear that temperature response combined with cut in this cultivar results in extremely high GSL concentrations postharvest. GHPs however were relatively low (Fig. 3) with highest abundance at D4 (8.0 ± 1.1 μmol g^{-1}). This disparity between GSL and GHP abundances may be suggestive of myrosinase impairment or reduced activity.

3.2.7. Cultivar differences between growth temperature treatments

The disparity between rocket species GSL accumulations and GHPs is also evidenced when comparing broadly between growth temperature conditions. Table 3 contains the Type III Sum of Squares analysis results and reveals there are fewer significances between growth temperature and cut treatments for *D. tenuifolia* than *E. sativa* cultivars. This suggests that the former species is much less variable in terms of GSLs and GHPs, however (based on the two cultivars tested) is unable to achieve significant changes in health and flavour-related compounds between growth temperatures. This attribute is important however, and better for (potentially) maintaining uniformity of taste and flavour traits between temperature extremes. If the goal is to make rocket species more nutritionally dense, *E. sativa* possesses a degree of environmental plasticity in response to different growth temperatures that lends itself well to synthesis of GSLs such as GRA and GER. RS4 and RS8 also produced greater concentrations of SF under each condition and cut, therefore making cultivars more efficacious against chronic diseases than the more commonly consumed *D. tenuifolia*.

Considering the factors contributing to differences in concentrations, *E. sativa* is significantly influenced by temperature, with relatively few compounds affected by the respective interactions between temperature, harvest (cut) and sample point. For RW2 indolic GSL concentrations (4MOB and NGB) were most significantly affected by each factor and their interactions. RW2 by contrast had the most variability for GRA and PRO concentrations. GHPs by contrast (regardless of species) were most significantly influenced by the cut number. This indicates that while total GSLs may not be significantly changed after second cut, GHPs are. This may reflect a change in the expression of respective genes and enzymes regulating hydrolysis rather than those involved in GSL biosynthesis *per se*, and may give rise to improvements in nutritional quality.

3.2.8. Effects of growth temperature on postharvest concentrations

Irrespective of cultivar or species, several significant associations between growth temperature and shelf life concentrations of GSL compounds. At D0 GER concentrations were significantly affected by growth temperature ($P = 0.012$), as well as total GHPs ($P = 0.027$). At D4, two significances were observed for the indolic GSLs GBR ($P = 0.001$) and 4MOB ($P = 0.002$). By D7 there were several GSLs and one ITC significantly associated with growth temperature; these were PRO ($P < 0.0001$), GSV ($P = 0.000$), 4MOB ($P = 0.000$), and SF ($P = 0.034$, Table 3 & Supplementary Data File S1). These data are of particular interest for two reasons: the first is that PRO and GSV are thought to contribute significantly to the taste and flavour profile of rocket leaves (Pasini et al., 2011; Raffo et al., 2018). Their relative increases/decreases over the course of shelf life may therefore alter sensory properties, and conceivably consumer preference (Bell et al., 2017b). The second is that SF is associated with health-related benefits, and therefore cultivars could be improved by selecting for plants able to form greater concentrations later into shelf life (*e.g.* RS8; Bell, Yahya, et al., 2017).

As presented in Table 3, there were numerous significant interactions between cultivar, cut, and growth temperature, making exact predictions of postharvest concentrations and profiles difficult. It is clear however that growth temperature influences the potential nutritional and sensory status of cultivars, and goes some way to explain the inconsistencies observed by growers and processors between growing regions and cuts of the same cultivar.

3.3. Principal component analysis

Fig. 4 shows PCs 1 and 2 of the PCA analysis and explain 40.87 % of the observed variation within the data. PC1 separates predominantly for total GSL content, as well as DGBT, GSV and DMB. PC2 by

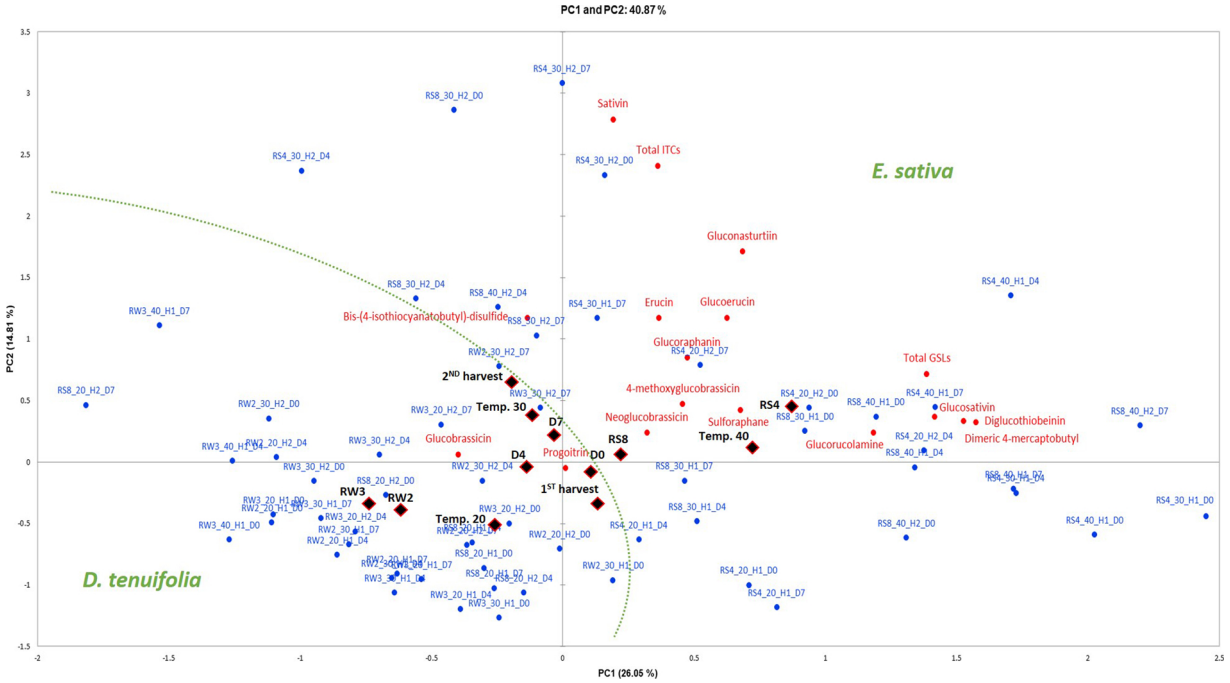

Fig. 4. Principal Component Analysis biplot of glucosinolate and hydrolysis product concentrations or rocket cultivars grown at four different growth temperatures. Components PC1 and PC2 are presented and represent 40.87 % of total variation. Blue data points = sample loadings; red data points = glucosinolate and hydrolysis product scores; black diamonds = cultivar, cut, shelf life time point, and temperature centroids (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

comparison separates strongly for SAT and total GHP formation. This is of note because it indicates that a high GSL concentration does not necessarily correlate with high GHP formation. To give two examples; first cut D0 RS4 plants grown at 30 °C contained relatively reduced concentrations of GHPs compared to the observed GSLs. Conversely, second cut plants of the same cultivar and temperature saw marked increases in GHP formation relative to GSL concentration, which was largely unchanged between cuts at this temperature.

PC1 also separates for species (Fig. 4), as it is clear that the *D. tenuifolia* cultivars tested are generally low accumulators of GSLs, and form relatively few GHPs compared to RS4 and RS8. As a proportion of the overall GSL profile, RW2 and RW3 contained greater concentrations of indolic GSLs (such as GBR) as well as PRO.

As highlighted in previous studies however, concentrations of GSLs/GHPs are not in and of themselves indicators of perceived sensory traits such as pungency; so it may be that the stark differences in the species' profiles may not be reflected in their taste and flavour attributes (Bell et al., 2017a). Other modulating influences such as sugar concentrations may also affect this, so it is therefore important to note that pungency is not indicative of health-related benefits and *vice versa*. A salad rocket may, for example, be very mild tasting but still potentially contain many fold-higher amounts of GHPs which are masked by other compounds.

4. Conclusions

This paper has demonstrated the effects of cultivation temperature and multiple harvests on postharvest GSL and GHP concentrations in rocket species. While it has been anecdotally accepted by growers that pungency increases according to the number of cuts a crop receives, very few previous studies have accounted for this common horticultural practice.

Temperate grown crops (~20 °C average outdoor summer temperatures) are often noted for their less pungent aroma and flavour than those from hotter countries (such as Italy, Portugal, and Morocco). Our data show that total GSL concentrations between growth temperatures are not significantly affected, but that it is the abundance of GHPs produced which differs. It is clear and unsurprising that growth at 40 °C is detrimental to plant development and regrowth, however it is also apparent that there is a significant increase in GSL biosynthesis, and also SF formation postharvest. Our data also highlight that some *E. sativa* cultivars may be better adapted to growth under extreme temperatures, as RS8 showed remarkable tolerance to the 40 °C treatment, and a propensity for increased SF productions under these conditions. More research will be required to determine if this tolerance is indicative of the species more widely when compared with *D. tenuifolia*.

Rocket crop growth under protected conditions can routinely reach or even exceed 40 °C, especially in summer months in countries such as Italy. With such extremes in temperature likely to increase in future due to climate change, it is important to determine the effects on nutritionally dense crops such as rocket. It is likely under such conditions that yields and production will be reduced, but that the nutritional density of crops may actually increase.

The GSL data presented are in agreement with previous studies of other Brassicaceae species (see Bell and Wagstaff, 2017 for a summary) however few other studies have also analysed GHPs in tandem. Our data suggest that it is incorrect to assume that GHP profiles and abundances are affected in a similar fashion to GSLs under different growth temperatures. Fluctuations in GHP abundance and conversion from GSLs is related to both environment and genotype. This is consistent with observations found in *Brassica* vegetables, where the concentrations of hydrolysis products is typically much less than the total concentration of the GSL precursors (Hanschen and Schreiner, 2017). GRA conversion to SF varied from 0.9 % (RW2, second cut, D7, 20 °C) to 25.1 % (RS8, second cut, D4, 40 °C); and GSV/DMB conversion to SAT (Fechner et al., 2018) from 1.7 % (RS4, first cut, D7, 20 °C) to 100 % (RW3, first cut, D7, 40 °C).

The relative changes in the formation of these compounds between growth temperatures indicates that there is an environmental effect upon myrosinase. This may be in terms of total plant content and/or activity, but the differences observed here between genotypes suggests that this is also as a result of genetic variation. The focus of breeding should therefore shift from selecting cultivars with high GSL concentrations, and more towards those that convert them to GHPs most efficiently. This may involve selection for different myrosinase genotypes, but could also feasibly extend to epithiospecifier modifier proteins, such as ESM1, which promote ITC formation (Angelino and Jeffery, 2014).

There are many factors regulating and inhibiting hydrolysis of GSLs other than nascent myrosinase abundance and activity; such as pH, temperature, ascorbic acid concentration, and enzyme co-factor presence/absence. While the kinetics of isolated compounds and myrosinase are well understood, it is still unclear how regulatory mechanisms within plant matrices control GHP formation and abundance. It is important to better understand the postharvest hydrolysis of GSLs since any health-related impacts of consuming Brassicaceae foods (such as rocket) will depend greatly upon pre-harvest environment, rather than postharvest storage conditions alone.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Jake Jasper: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Carol Wagstaff:** Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing. **Luke Bell:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2020.111157.

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