CRANFIELD UNIVERSITY

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TOWARDS A BETTER UNDERSTANDING AND NEW TOOLS FOR SOFT FRUIT QUALITY CONTROL

CRANFIELD HEALTH

Plant Science Laboratory

Ph.D THESIS

2010

CRANFIELD UNIVERSITY

Cranfield Health

Plant Science Laboratory

Ph.D THESIS

Academic Years 2006-2010

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April 2010

This thesis is submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

ABSTRACT

Prospects for the production of new and more tasteful strawberry and blackcurrant fruits may be achieved not only through genetic improvement and release of new varieties but also by adapting current cultivation systems and boosting the development of diagnostics tools for better quality control (QC) by growers and breeders.

The balance between sugar and acid content (S/A ratio) and even the content of certain health-related compounds within the fruit, may act as important indices of consumer acceptability or overall berry quality. The S/A ratio, of fruits from 23 blackcurrant and 19 strawberry cultivars ranged from 1.84-4.39 and 3.9-9.6, respectively. S/A ratios generally increased during blackcurrant ripening (up to 1.3-fold higher for certain cultivars), declined slightly during postharvest storage at different temperatures or even changed when the same cultivar was grown at different locations (up to 30% of variation). Synthesis of health-related compounds (i.e. anthocyanins) occurred even after harvest and was strongly influenced by storage temperature and maturity at harvest. Deficit irrigation (DI) at different fruit developmental stages, was investigated as a potential strategy to improve strawberry fruit quality in a range of cultivars. The S/A ratio and the concentration of health-related compounds (*viz*. individual anthocyanins, antioxidant capacity) were much greater (i.e. 1.4-fold higher antioxidant capacity), for some cultivars, in fruits from DI-treated plants as compared with fully irrigated plants. The taste- and health-related composition of both blackcurrant and strawberries considerably changed from year-to-year demonstrating the influence of agroclimatic conditions on overall fruit quality.

Current standard quality control techniques employed by the soft fruit industry to measure sugars (*viz*. total soluble solids), acids (*viz*. titratable acidity) or total phenolics (*viz*. Folin-Ciocalteu assay), are inappropriate for *in situ* discrimination of fruits based on their biochemical composition. Biosensors may fulfil this niche by providing a rapid and accurate technique for measuring specific analytes that are key indicators of fruit quality. Accordingly, this thesis details the development, optimization and application of new simple, rapid, low cost disposable sensors based on different configurations. Novel glucose oxidase-based, or enzyme-less systems were used to monitor glucose, ascorbic acid (AsA) and a range of compounds with reported antioxidant capacity, respectively, in both blackcurrant and strawberry fruits using a single or a custom-made array of platinised screen-printed electrodes. Excellent linear correlations were

observed when the respective sensors were tested with standard glucose or AsA solutions, in the range of concentrations commonly found in berry fruits ($r^2_{GOx} = 0.97$; $r^2_{AsA} = 0.99$). Glucose and AsA concentrations from freshly-squeezed and diluted berry juice samples from different cultivars and degrees of ripeness were accurately determined in no more than 200 seconds. In addition, potential health-promoting compounds including individual anthocyanins, total phenolics and the antioxidant capacity of the fruits were correctly predicted ($r^2_{AC} > 0.7$) when compared to newly developed standard HPLC or spectrophotometric methods. As a result, the proposed sensors are viable candidates for assisting growers and breeders in determining optimum maturity and eating quality of berries as well as for quality control purposes in the entire soft fruit industry.

The results from this thesis are discussed in relation to potentially improve soft fruit quality by simultaneously improving knowledge of the composition of blackcurrant and strawberry fruits as well as developing new tools for better soft fruit quality control and supporting existing breeding programmes.

ACKNOWLEDGEMENTS

The completion of this thesis was achieved due to the support of numerous people, all of whom contributed in some unique way; without them the work presented herein would not have been possible.

Firstly, I would like to express my most sincere gratitude to Dr. Leon Terry not only for his support, advice and guidance throughout this project but for the numerous opportunities and challenges that he gave me. I would also like to thank Allen Hilton, Balwinder Cook and Rosie Burns for their technical support as well as Charles Marshall for statistical advice. Professor David Cullen and Dr. Jeffrey Newman are also thanked for their advice on some on-going biosensor work. My gratitudes are extended to Dr. Pedro Palencia (Universidad de Huelva, Spain) and Pamela Crespo (Agroscope ACW, Switzerland), to whom I had the opportunity to collaborate on different strawberry trials and share so many hours of encouraging and always motivating discussions.

This studentship was funded by the HDC, and hence I would also like to thank them for the financial support given. This has been without doubt an unforgettable academic and life experience, and as such, would not have been possible without the support and friendship of all Plant Science Laboratory PhD students, Postdocs, etc., as well as many other friends either from Silsoe or Cranfield, to them all: thank you!

Last, but not least, I would like to thank my parents, Idoia and Katarina for always being there and especially for their tireless support and patience in the last, and hardest, stages of this thesis.

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NOTATION

<	less than
>	greater than
≤	less than or equal to
≥	greater than or equal to
%	per cent
=	equals
°C	degree Celsius
β	beta
µA	microamperes
µC	microcoulomb
µl	microlitre
µM	micromolar
µmol	micromoles
1-MCP	1-methylcyclopropene
2-MCE	2-mercaptoethanol
ABA	abscisic acid
AC	antioxidant capacity
ACN	acetonitrile
Ag/AgCl	silver/silver chloride
ANOVA	Analysis of Variance
AOAC	Association of Analytical Communities
AsA	ascorbic acid or ascorbate
AU	arbitrari units
ca.	approximately
Ca	calcium
CaCl2	calcium chloride
CCD	central composite design
CL	citrate lyase

cm	centimetre
Co	Cobalt
CO_2	carbon dioxide
cv./cvs.	cultivar/cultivars
CV	cyclic voltammetry or cyclic voltammograms
cya	cyanidin
cya-3-gluc	cyanidin-3-glucoside
cya-3-rut	cyanidin-3-rutinoside
DAD	diode array detector
DEFRA	Department for Environment Food and Rural Affairs
delp-3-gluc	delphinidin-3-glucoside
delp-3-rut	delphinidin-3-rutinoside
d.f.	degrees of freedom
DI	deficit irrigation
DW	dry weight
E	equilibrium potential
E^0	standard electrode potential
EA	ellagic acid
Eap	Applied potential
EEC	European Economic Community
ELSD	Evaporative Light Scattering Dectector
et al.	and others
EtOH	ethanol
ER	early ripe
F	Faraday constant
FAO	Food and Agriculture Organisation of the United Nations
FC	Foulin-Ciocalteu
FDH	fructose dehydrogenase
FIA	flow injection analysis
FID	flame ionisation detector
FR	fully ripe
FRAP	ferric reducing antioxidant parameter assay
FW	fresh weight
g	gram
GAE	gallic acid equivalents

GC	Gas Chromatography
GOx	glucose oxidase
GSK	GlaxoSmithKline
HCA	hierarchical cluster analysis
HCl	hydrochloric acid
HDC	Horticultural Development Company
HPLC	High Performance Liquid Chromatography
I	current
kg	kilogram
L	litre
LC	Liquid Chromatography
LSD	least significant difference
M	molarity
m	metre
MaB+	meldolas blue
m/d	monosaccharide/disaccharide ratio
MDH	malate dehydrogenase
ME	malic enzyme
MeJa	methyl jasmonate
MeOH	methanol
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
$MnCl_2$	manganese (II) chloride
MP	mobile phase
MS	mass spectrometry
MS/MS	tandem mass spectrometry
mV	milivolts
mv-3-gluc	malvidin-3-glucoside
MW	molecular weight
N	normality
NaCl	sodium chloride
Na_2HPO_4	di-sodium hydrogen orthophosphate

NaH_2PO_4	sodium di-hydrogen orthophosphate
NaOH	sodium hydroxide
NSC	non-structural carbohydrate
nm	nanometre
O_2	oxygen
OR	over-ripe
P	probability
PBS	phosphate buffered solution
PC	principal component
PCA	principal component analysis
PAD	photodiode array detector
pg-3-gluc	pelargonidin-3-glucoside
PVC	polyvinyl chloride
QC	quality control
QE	quercetin equivalents
RID	refractive index detector
rpm	revolutions per minute
S/A	sugar/acid ratio
SCRI	Scottish Crop Research Institute
SLE	Solid liquid extraction
SP	Stationary phase
SPE	Screen printed electrode or solid phase extraction
SPCE	screen printed carbon electrode
S.D.	standard deviation
S.E.	standard error
SWV	square wave voltammetry or square wave voltammograms
TA	total anthocyanins
TP	total phenolics
TSS	total soluble solids
TTA	titrable acidity
UK	United Kingdom
US	United States
USA	United States of America
USDA	United States Department of Agriculture
UV	ultraviolet

Vis	visible
viz.	namely
V	volts
v/v	volume by volume
W	oxidation wave
WHO	World Health Organisation
w/v	weight by volume
w/w	weight by weight

CHAPTER 1

INTRODUCTION

1.0 CHAPTER ONE

Introduction

1.1 Background

Soft fruits are emerging crops with high economic value, not only due to their desired taste but also for their known health-promoting properties. The quality of soft fruit, in terms of taste and consumers acceptance, is fundamentally based on the biochemical composition of the fruit and hence, and not surprisingly, the balance between sugar and acid content within the fruit, referred as sugar/acid ratio, can be used as an important index of consumer acceptability (Perez *et al.*, 1997; Terry *et al.*, 2005), optimum maturity (Perez *et al.*, 1997) and act as a determinant of overall fruit quality and taste (e.g. riper fruit have higher sugar content).

To satisfy current consumer demand together with the requirements of a more competitive market, a better understanding is required on how different cultivation or handling conditions affect the taste- and health-related composition of soft fruits. Blackcurrant and strawberries were the target crops given their importance for the process and fresh soft fruit industry, respectively. Additionally, to guarantee that industry achieves certain quality standards, simple and low-cost alternative QC techniques to the cumbersome and time-consuming nature of titrations are necessary to replace standard QC measurements which are poorly correlated with actual fruit quality.

Biosensors, being traditionally used in medical diagnostics, environmental monitoring and defence, are ideal candidates for discrimination of fruit on the basis of biochemical fruit quality (Terry *et al.*, 2005). Past work at Cranfield University (CU) has successfully demonstrated the novel use of biosensors for quantification of fruit quality attributes (ascorbate, β- D and total D-glucose and sucrose) in tropical fruits (pineapple, mango and pawpaw) (Jawaheer *et al.*, 2003), malate in tomatoes and pome fruit (Arif *et al.*, 2002) and pungency (pyruvate) in onions (Abayomi *et al.* 2006; Abayomi and Terry, 2007).

Rapid determination of sugar concentration (rather than total soluble solids (TSS)), ascorbic and other organic acids, and certain antioxidants in soft fruit would provide a significant improvement over current QC operations. Better feedback vertically through the supply chain as a result of more informative QC could ensure fewer rejections hence reducing unnecessary costs for the soft fruit

industry. Ultimately, biosensors are easily portable devices that may be used by growers and breeders themselves, to assess fruit prior to harvest and hence harvest fruit at the optimum time, when it was at its best.

Consequently, the work proposed in this thesis intended to further understand how different preharvest and postharvest conditions affect the taste- and health-related composition of soft fruits as well as to applied biosensor technology for soft fruit quality control.

1.2 Aims and objectives

1.2.1 Aim

The overall aim of this PhD was to further understand the mechanism underlying the changes in the quality of both blackcurrant and strawberry fruits as a result of different pre- and postharvest conditions. Special emphasis was given to measure both taste- (*viz*. sugars and organic acids) and health-related compounds (*viz*. ascorbic acid, anthocyanins, phenolic acids) as key components governing consumer acceptability. In addition, it was also aimed to develop state-of-the-art and low-cost disposable biosensors and/or a prototype sensor array to accurately measure principal biochemical components (*viz*. sugars, organic acids, antioxidants) intimately related with the taste and potential health-promoting properties of the above-mentioned fruits.

1.2.2 Objectives

- To increase the knowledge of the biochemical composition in strawberry and blackcurrant berries especially focusing on both taste- and health-related compounds (*viz*. sugar and organic acids ratio).
- To assess genotypic differences in quality traits among different UK-grown blackcurrant and strawberry fruits.
- To develop of a prototype multianalyte-sensor device which uses a thick-film screen-printed biosensor array for real-time in-situ objective determination of the principal sugars and organic acid content in soft fruit as a means of improving QC.
- To explore the application of screen-printed technologies for determining health-related compounds increasingly demanded by consumers.
- Provide the UK soft fruit industry with a sustainable competitive advantage.

- Reduce economical losses suffered by suppliers due to processors and retailers returning fruit which is unsatisfactory.
- Allow growers and breeders to improve productivity and reduce losses by enabling them to self-test fruit, enabling harvest time optimisation and, to certain extent, improvements in postharvest management.

1.3 Thesis structure

This thesis is arranged into nine chapters. After a brief background and thesis aims and objectives, *Chapter two* provides a necessary review of the literature, initially covering the importance of the soft fruit industry worldwide and in the UK, and giving background information about the physiology and biochemistry during growing, ripening and postharvest of strawberry and blackcurrant fruits. The efficacy of different treatments applied either before or after fruit harvest on overall berry quality are highlighted. In addition, deficiencies in current analytical techniques commonly used in the soft fruit industry that are poorly correlated with the real fruit quality status are discussed. Subsequently, more modern and reliable approaches such as biosensors, as well as their principles of operation, are described in this chapter as a potential alternative for soft fruit quality control. The biochemical composition of certain soft fruits (i.e. strawberries) is well known whilst for other berries (i.e. blackcurrants) have not yet been studied such in detail. Hence, *Chapters three* and *Chapter four* screen the taste and health-related composition of a wide range of UK-grown strawberry and blackcurrant fruits and describe the impact that different growing conditions or treatments have on the composition of these berries, respectively. In these chapters, besides developing new analytical techniques (*viz.* high performance liquid chromatography (HPLC), spectrophotometry) for the determination of specific analytes (*viz.* sugars, organic acids, anthocyanins, phenolics, ellagic acid, antioxidant capacity of the fruit, etc.), special emphasis was given to the use of chemometric data analysis as an explanatory tool to further understand the role of genotype, maturity at harvest and growing conditions had on the biochemical composition of the berries. Besides, the different blackcurrant and strawberry trials conducted in the above-mentioned chapters (*Chapter 3* and *Chapter 4*) also aided the generation of a wide range of samples for future validation of the prototype biosensors, to better understand the relationship between biosensor response when applied to real samples.

It is noteworthy that the blackcurrant postharvest trial described in chapter four was performed in conjunction with Dr. Gemma A. Chope (CU) yet all other work was conducted solely by The Candidate. The results or methodologies used in these chapters has been published as:

- (**I**) **Giné Bordonaba, J**. & Terry, L.A. (2008) Biochemical profiling and chemometric analysis of 17 UK-grown blackcurrant cultivars. *Journal of Agriculture and Food Chemistry* 56, 7422-7430.
- (**II**) **Giné Bordonaba, J**. & Terry, L.A. (2010) Manipulating the taste related composition of strawberry fruits by deficit irrigation. *Food Chemistry* 122, 1020-1026.
- (**III**) Crespo, P., **Giné Bordonaba, J**., Carlen, C. & Terry, L.A. (2010) Characterisation of major taste and health-related compounds of four strawberry genotypes grown at different Swiss production sites. *Food Chemistry* 122, 16-24.
- (**IV**) **Giné Bordonaba, J**., Chope, G.A. & Terry, L.A. (2010). Maximising blackcurrant anthocyanins: temporal changes during ripening and storage in different genotypes. Journal of Berry Research (in press)

The rise in consumption of soft fruits is, however, not only due to their attractive taste and colour but to their high concentration of natural antioxidants with potential health-related properties. As a result, the analysis of antioxidants in different foodstuffs and beverages has recently become an active area of research which has led to numerous antioxidant-assays being developed. Generally, these spectrophotometric-based assays are costly and not viable to be routinely applied for screening large sample sets. Many antioxidants exhibit inherent electroactivity, and therefore *Chapter five* exploits the application of screen-printed electrodes to measure antioxidant activity and individual antioxidants without the need for added reactive species in both real strawberry and blackcurrant-based juices.

In both strawberry and blackcurrant fruits, glucose is one of the main sugars, and thus it was adopted as an initial marker for sweetness which could readily replace total soluble solids measurements in a more targeted approach of determining strawberry fruit quality. Thus, *Chapter six* reports on the development and optimisation of a glucose oxidase-based amperometric biosensor to determine glucose in strawberry extracts. In this chapter emphasis was given to the experimental design methodology for the optimisation of several experimental variables that directly affect biosensor

performance and to expand the knowledge on the relationship between biosensor response and fruit composition. Results from this chapter have been published as:

- (**V**) **Giné Bordonaba, J**. & Terry, L.A. (2009) Developmentof a glucose biosensor for rapid assessment of strawberry quality: relationship between biosensor response and fruit composition. *Journal of Agriculture and Food Chemistry 57*, 8220-8226.

Following the use of a single screen printed carbon electrode, *Chapter seven* describes the application of a newly developed sensor array to monitor not only glucose but simultaneously other target analytes (viz. glucose, ascorbic and malic acid) in both blackcurrant and strawberry fruits, after different sample pre-treatments.

Chapter eight presents a general discussion which integrates the results from previous chapters, proposes recommendations for future research, and takes into account the implications of the results in terms of the considerations the soft fruit industry. Finally *chapter nine* shows the literature cited.

Following the core chapters of this PhD thesis, several appendices are given. *Appendix A* shows initial work aimed at better understanding the relationship between sugar concentration and TSS values in both blackcurrants and strawberries as well as other horticultural crops. Over the course of this thesis, the scientific community faced a worldwide shortage of acetonitrile which caused on prices to soar drastically. Acetonitrile has been over the past decades the mobile phase of choice for determination of anthocyanins by High Performance Liquid Chromatography, with few alternatives available. Since anthocyanins are among the main health-related compounds and responsible for the attractive colouration in both strawberry and blackcurrant fruits, *Appendix B* reports on the development of an acetonitrile-free mobile phase for HPLC termination of anthocyanins in selected berries. This study was conducted together with the PhD student Pamela Crespo (ACW Agroscope, Switzerland) at CU.

Other appendices include statistical tables (*Appendix C*) and an extended literature review on the health-promoting properties of both *Ribes* and *Rubus* (*Appendices D*) species and Strawberries (*Appendix E*), respectively.

- **(VII) Giné Bordonaba, J**. & Terry, L.A. (2009) *Ribes* and *Rubus* (Chapter 14). In: Health-promoting properties of fruits and vegetables. Ed. Terry, L.A. CABI.
- (**VIII**): **Giné Bordonaba, J**. & Terry, L.A. (2009) Strawberry (Chapter 15). In: Health-promoting properties of fruits and vegetables. Ed. Terry, L.A. CABI.

Appendix F lists the publications and conference presentations, both posters and oral, that have arisen from this PhD.

CHAPTER 2

LITERATURE REVIEW

2.0 CHAPTER TWO

Literature review

2.1 The soft fruit industry in the UK

Fruits account for 47% of the total sales of fruits and fresh vegetables in the UK (£3,796 m) (*Mintel*, 2007). The marginally faster rate of growth experienced by fruits, during the past years, is attributed among others to the increasing sales accounted to higher-value produce such as prepared fruit, soft fruits and tropical fruit (**Table 2.1**). Late in 2004, it was reported that with consumers increasingly focus on health, taste, convenience and value; berries are not only out-performing the soft fruit sector but the whole UK fruit market (FPJ, 2007).

Table 2.1: UK retail sales of fresh fruits by type and value 2002-2006

	2002		2004		2006		% Growth
	£m	%	£m	%	£m	%	
Apples	617	19.5	654	19.1	659	17.4	6.8
Bananas	594	18.8	524	15.3	595	15.7	0.2
Citrus fruit	499	15.8	530	15.5	543	14.3	8.8
Soft fruit	280	8.9	380	11.1	503	13.3	79.6
Grapes	325	10.3	374	10.9	440	11.6	35.4
Stone fruit	278	8.8	309	9.0	340	9.0	22.3
Pears	155	4.9	169	4.9	174	4.6	12.3
Other	410	13	487	14.2	542	14.3	32.2
Total	3,158		3,427		3,796		20.2

Source: Mintel report. Food and Drinks, January 2007.

The soft fruit sector includes strawberries, raspberries, blackcurrants and all other *Ribes* and *Rubus* species grown in the UK. Strawberries, raspberries and blueberries account for most of the

fruit sales in UK (Gray, 2006) while worldwide strawberries, raspberries and currants are the most important crops of the soft fruit industry (Manning, 1993; Gray, 2006).

Worldwide, the production of strawberries has grown steadily during the last 40 years having most of its production in the northern hemisphere (> 95%). The USA is, in official numbers, the leading producing nation, followed by Spain, Turkey and the Russian Federation (**Figure 2.1**). This said, no official statistics are available for the size of the Chinese strawberry industry, even though it is accepted that China is nowadays a direct competitor for most of the major strawberry producing regions with estimated values for the period 2001-2003 of *ca*. 1.5 million tones (Carter *et al.*, 2005). In addition, strawberry production is one of the main parts of the European soft fruit industry, accounting for instance in the UK for a production value of £96m in 2003 (Defra, 2003)

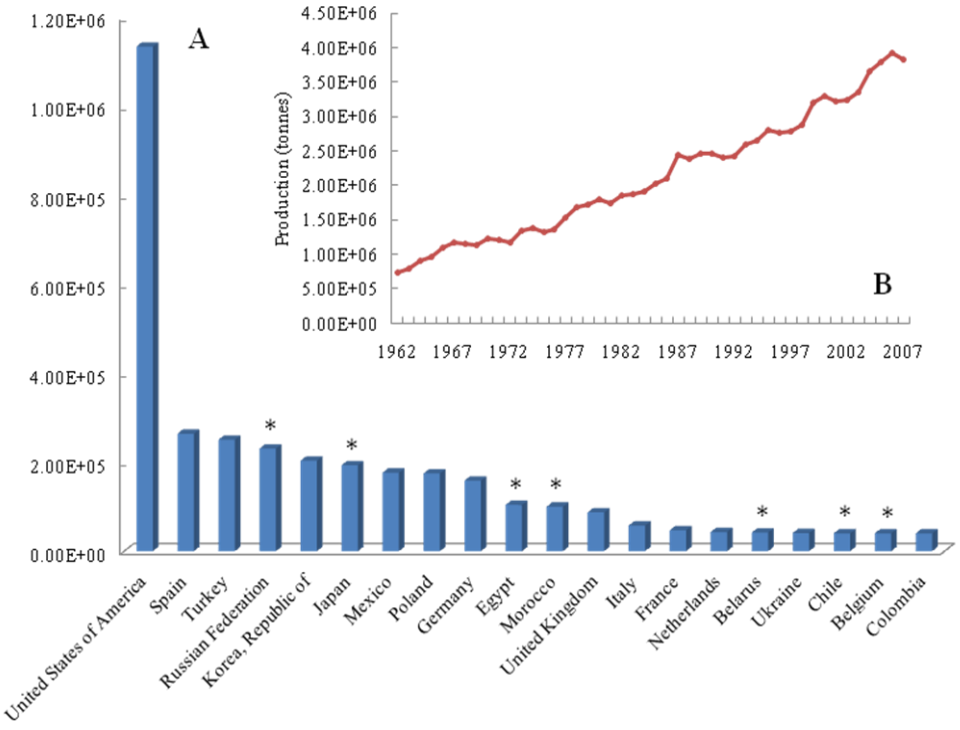


Figure 2.1: (A) Twenty highest strawberry producing countries (tonnes) in 2007. (*) indicate FAO estimates or unoficial figures for production values. (B) Worldwide strawberry production over the last forty-five years (Source: FAOSTAT 2009)

Although strawberry growth is, in general terms, slowing down during recent years in the UK, there are still huge opportunities for UK farmers to produce strawberry and other berries that can satisfy the growing necessities of the current market. Gray (2006) highlighted that some marketing companies in the UK have misled growers into producing high-yielding varieties, which

definitely have cover the market supplied but have devaluated the product through bad taste. The selection of high yielding varieties rather than those with better taste is probably the main reason for the observed decline in strawberry production experienced in UK. Nevertheless, current research focused on the development of new varieties may offer many potential benefits to growers, consumers and the soft fruit market, not just covering increased yield and improved resistance to pest and diseases, but also improved quality, flavor and shelf-life as well as extending the season.

Blackcurrants, on the other hand, represent the major crop of the total world *Ribes* acreage with a relatively steady growth over the past forty years (**Figure 2.2**). Blacurrants are principally processed for juices and also for jams, jellies and liqueurs (such as the French Creme de cassis) and colourings for yogurt and other dairy products (Brennan, 1996). Much of the blackcurrant production in the UK is processed into the proprietary brand name drink `Ribena´ which is characterised by an intense colour, flavour and high antioxidant levels (Brennan, 1996; SCRI, 2007). Worldwide, blackcurrant production averaged about 683,000 tons during the 1990s and increased to above 716,000 tons in 2001 (Hummer and Barney, 2002) (**Table 2.2**). The continuous increase in production until these days has globally lead to low fruit prices (Hummer and Barney, 2002: Brennan, 2006). The Russian Federation, Poland, and Germany lead the world in production of black currants.

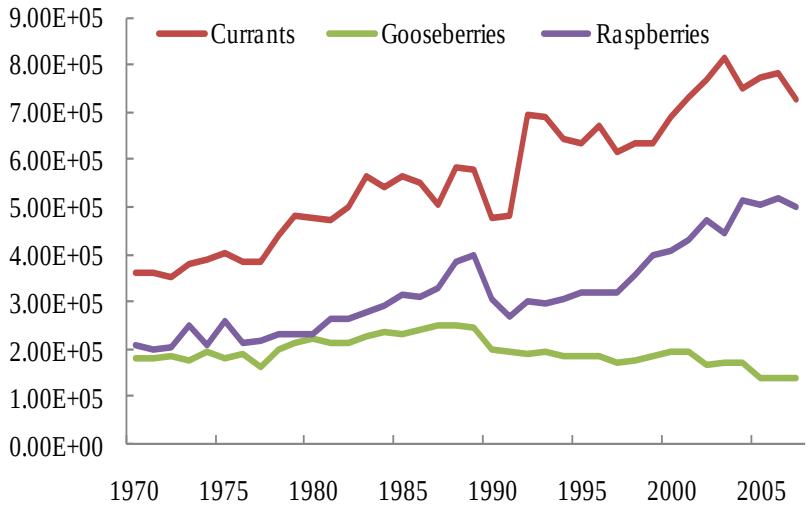


Figure 2.2: Worldwide production (tonnes) of certain *Ribes* and *Rubus* species from 1970 to 2007 (Data: FAOSTAT 2008).

Table 2.2: World production of blackcurrants during 1990, 1995, 2000 and 2001 in metric tons (*from Hummer and Barney, 2002*).

Year	**1990**	**1995**	**2000**	**2001**
Russia	70,000	175,000	208,000	212,000
Poland	130,409	154,591	33,522	180,485
Germany	146,538	169,300	140,000	140,000
Elsewhere	131,810	126,255	118,316	126,213
Total	**478,757**	**625,146**	**499,838**	**658,698**

2.2 Fruit growth and development

2.2.1 Strawberry fruit

The commercial strawberry fruit (*Fragaria ananassa* Duch.) belongs to the Rosoideae order of the Rosaceae family (Mabberely, 1987). It is a perennial plant with rooting runners that usually bears red fruit once it is developed. Hundreds of strawberry cultivars have been grown, however the cultivar Elsanta is the predominant in North Western Europe (Wilson, 1997). The history and evolution of commercial strawberries is described in detail in Appendix E. Strawberry fruit is in fact a "false fruit" being described as a modified receptacle with one-seeded fruits or achenes located on the outer surface (Szczesniak and Smith, 1969; Perkins-Veazie, 1995). Strawberry growth and development is characterised by changes in colour, texture and flavour (Manning, 1993). Four or five different stages of strawberry growth and development are described in the literature (Culperpper *et al.*, 1935; Spay and Morris, 1981; Huber, 1984; Terry *et al.*, 2004) according to the development of non ovarian receptacle tissue. These stages include *small green, large green II, white* and *full red.* Full red stage involves the maximum fresh weight and size (Terry *et al.*, 2004). Due to the above-mentioned characteristics, strawberries have been important as a model for physiological plant studies of fruit growth and development (Perkins-Veazie, 1995). Even though strawberry fruit reaches full red stage within approximately 28 to 30 days after anthesis (period during which a flower is fully opened and functional), several factors including prevailing temperatures (Perkins-Veazie, 1995) and water treatment (Krüger *et al.*, 1999; Terry *et al.*, 2007) can vary this period. The growth of strawberry can be measured by changes in fresh weight, dry

weight and fruit dimensions (Perkins-Veazie, 1995). Initially, strawberry growth was described following a sigmoidal behaviour, characterised by a slow growth rate followed by an exponential phase, and finally a period of declining growth rate (Bollard 1970; Forney and Breen, 1985; Sutte and Darnell, 1987). However, later studies described a double sigmoidal behaviour (**Figure 2.2**) for strawberry growth, characterised by two periods of rapid growth with an intervening slow growth phase (Coombe, 1976; Perkins-Veazie and Huber, 1987; Miura, *et al.,* 1990).

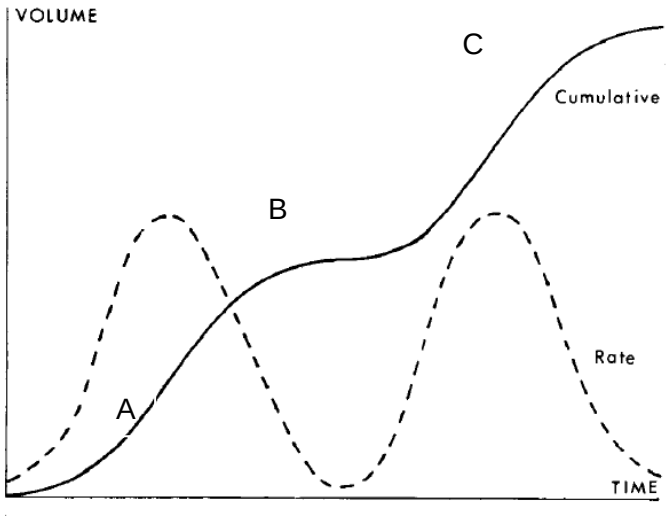


Figure 2.3: Double sigmoidal behaviour of strawberry growth (from Coombe, 1976)

Physiologically, the main factor affecting growth in strawberry has been attributed to stimulation of assimilate transport or mobilization regulated by auxin secretion from achenes (Perkins-Veazie, 1995). Avigdori-Avidov (1986) also suggests that the endogenous control of strawberry growth depends on differences in achene metabolic activity and receptacle plant hormone sensitivity. Therefore, final fruit size and shape are closely correlated with the number and size of fertile achenes on a fruit (Moore *et al.,* 1970; Sistrunk and Morris, 1985; Manning, 1993).

Morphologically, the strawberry cymose is characterised to develop having a terminal primary inflorescence with secondary and tertiary inflorescences attached proximally to this primary bloom (Anttonen *et al.,* 2006) (**Figure 2.4**). Generally, primary fruits have the faster growth rate and achieve larger size on maturity stage than secondary or tertiary fruit (Moore *et al.,* 1970). Webb (1973) suggested that primary fruit had a shorter vascular system and therefore a faster and more efficient assimilate transport which makes them grow and develop faster than secondary or tertiary fruits.

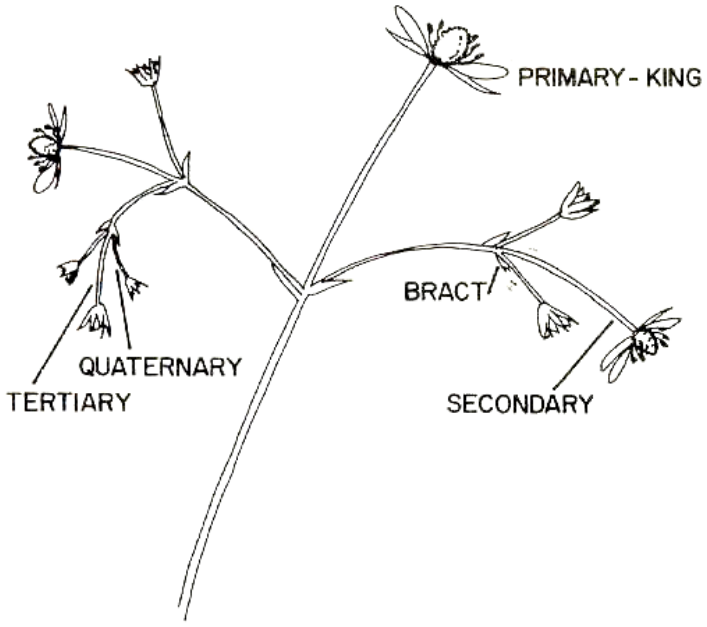


Figure 2.4: Schematic arrangement of strawberry inflorescence showing primary, secondary and tertiary fruits (*from Pritts and Handley, 1998*)

2.2.2 Blackcurrant fruits

Currants are botanically classified in the genera *Ribes* L. which includes more than 150 described species. Initially *Ribes* were included in the family Saxifragaceae however recent classification include them into the family Grossulariaceae (Sinnot, 1985). The Blackcurrant (*Ribes nigrum*) is a species of *Ribes* berry native to central and northern Europe and northern Asia (Brennan, 1996) and it has been cultivated for more than 400 years. Botanically, it is a small shrub growing to 1-2 m tall (Rehder, 1986; Brennan, 1996). Flowers and subsequently the fruit are grouped in racemes (**Figure 2.5**). Each raceme contains four to ten flowers. Oldest flowers are placed close to the stem while youngest flowers are found on the tip. Ripe fruit is up to 1cm or more in diameter and generally a shiny black or purple colour (Hummer and Barney, 2002).

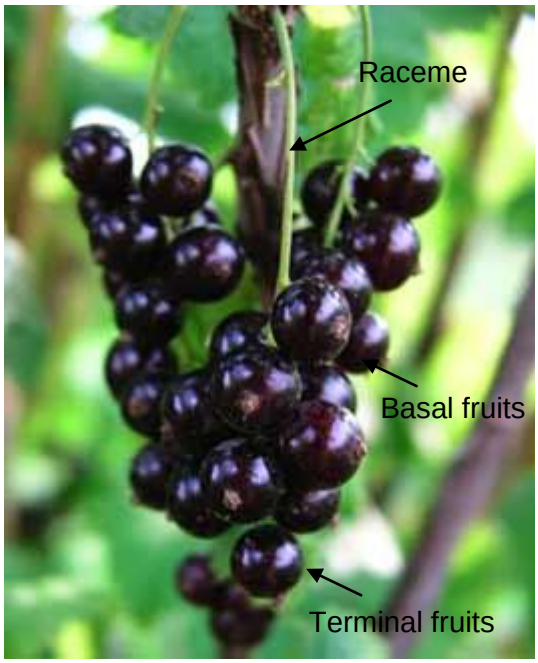


Figure 2.5: Blackcurrant fruits grouped in racemes (*from* Jedwards International Inc., 2005)

For blackcurrant cultivation, cross pollination is essential because most of the cultivars are partially self-sterile (Hummer and Barney, 2002). Basal flowers can bloom up to 20 days earlier than terminal flowers (**Figure 2.5**), being the bloom duration directly related to the temperature at what the plant is exposed (Harmat *et al.*, 1990: Brennan, 1996). Ripening of blackcurrant fruits is much longer than for strawberries, taking place approximately 60 days from flower anthesis. Although little information is available about blackcurrant growth and development it can be assumed that these berries follow a double sigmoidal growth as it occurs with strawberry and other fleshy fruits (Coombe, 1976).

2.3 Fruit composition and biochemistry

2.3.1 Introduction

In addition to being a highly valuable horticultural product (Perkins-Veazie, 1995), strawberries have unique and desirable taste, flavour, and excellent dietary composition that make them one of the most popular fruit (Sturm *et al*, 2003; Kafkas *et al.*, 2006). Some parameters such as colour, texture, odour and the balance between sweetness and acidity/astringency have been identified as being responsible for overall quality of strawberries and other berries (Cordenunsi *et al.*, 2003; Keutgen and Pawelzik, 2008). Flavour was highlighted being the most important attribute giving commercial value to soft fruits. However, it is known that flavour in soft fruits is conditioned

by the balance between sugars and acids expressed in ripe fruits (Montero *et al.*, 1996; Pérez *et al.*, 1997).

Strawberry and blackcurrant colour at their fully ripe stage is fundamentally based in the composition of sugar derivatives of anthocyanidins which in turn is influenced by cellular pH. The characteristic soft melting texture of ripe strawberry is highly valued for consumers, but poses a major problem for growers, handlers and retailers. Changes in texture during ripening are associated with the degradation of the middle lamella of the walls of parenchyma cells with an important increase in pectin solubilisation (Koh and Melton, 2001). Ripe strawberries and blackcurrants contain approximately 90% and 80% of water respectively (**Table 2.3**) and 10% of soluble solids (Hemphill and Martin, 1992).

Table 2.3: Nutritional composition of different fruits per 100g edible portion (*Hummer and Barney, 2002*)

Fruit	**Water (%)**	**Kcalories**	**Protein (g)**	**Carbohydrates (g)**	**Vitamins**				
					A (I.U.)*	B_1 (mg)	B_2 (mg)	Niacin (mg)	C (mg)
Blackcurrant	81.96	63	1.4	15.38	230	0.05	0.05	0.30	181
Redcurrant	83.95	56	1.4	13.8	120	0.04	0.05	0.10	41
Gooseberry	87.87	44	0.88	10.18	290	0.04	0.03	0.30	27.7
Apple	83.93	59	0.19	15.25	90	0.02	0.01	0.08	5.7
Strawberry	91.57	30	0.61	7.02	27	0.02	0.07	0.23	56.7
Orange	82.3	40	1.3	15.5	250	0.10	0.05	0.50	71

*I.U.=International units

2.3.2 Biochemistry of fruit ripening

Maturation, ripening, and senescence are the result of many physiological changes in fruits. Although a strict physiological distinction between fruit ripening and senescence is unclear for many non-climacteric fruits, ripening has been described as a process involving many complex changes including seed maturation, colour changes, abscission from the parent plant, tissue softening, volatile production, ethylene production, changes in respiration rate, tissue permeability and also changes in carbohydrates, organic acids and proteins composition (DeEll *et al.,* 2005).

Therefore, fruit ripening may be considered as a very complex process (Kafkas *et al.,* 2006), including a genetical stage of development and synthesis of novel proteins and mRNAs as well as the production of new pigments and flavour compounds overlapping with senescence (Perkins-Veazie, 1995).

There are important differences in both the rate and pattern of respiration between different fruit types. Fruits are commonly classified as either climacteric or non-climacteric according to their respiration pattern during ripening. Climacteric fruit (such as tomato, avocado, apple, and banana) show a burst of ethylene biosynthesis and an increase in respiration during ripening, whereas non-climacteric fruits (such as strawberry, blackcurrant, grape and citrus) do not. Blackcurrant and strawberry fruits show a gradual increase in respiration with maturation and ripening, and very small increase in ethylene production (Tucker, 1993). In terms of respiration, the major substrates found in fruits are sugars followed by organic acids. The respiratory processes used by fruits are mainly glycolysis, oxidative pentose phosphate pathway (OPP) and the tricarboxilic acid cycle (TCA) (Tucker, 1993).

The pattern of strawberry fruit respiration is a paradigm (Perkins-Veazie, 1995). For many years it has been considered a model for non-climacteric fruits (Knee *et al.,* 1970; Coombe, 1976; Perkins-Veazie, 1995). Strawberry fruit continues to increase in size during ripening, accumulates soluble solids, suffers a decrease in titratable acidity and also shows different changes in pigmentation and softening. Conversely, recent studies carried out by Iannetta *et al.* (2006), using more modern analytical techniques were able to demonstrated that ethylene and carbon dioxide were produced in small amounts during fruit development.

Different changes in flavour, texture, and colour occur as fruit is developed (Perkins-Veazie, 1995).These changes are directly associated with the biochemical changes that occur in the fruit. As reported by several authors (Pérez *et al.,* 1997; Sturm *et al.,* 2003), changes in sugars and organic acids content influence the flavour, taste and texture of strawberry. Soft fruit ripening is influenced by several factors, such as the synthesis and action of hormones responsible for the rate of ripening, the biosynthesis of pigments, the metabolism of sugars acids, and volatile compounds mainly involved in flavour development (Abeles and Takeda, 1990).

Texture in soft fruits is related to the composition of structural polysaccharides. The loss of firmness during ripening of soft fruit is an important factor determining soft fruit quality and postharvest shelf life. Changes in texture during soft fruit ripening are mainly associated to the release of enzymes such as pectins and hemicelluloses (Ponappa *et al.,* 1993). However, expansins, a type of protein associated with the expansion of the cell walls also play an important role during fruit softening (Manning, 1998). In the particular case of strawberries, Abeles and Takeda (1990),

described pectinmethylesterase and cellulose enzymes as the responsible for fruit softening. Marín-Rodríguez *et al.* (2002) reviewed the role of pectate lyases (PEL) in fruit softening and discovered that in certain fruits such as tomato, strawberry, grape, and banana PEL play a significant role in fruit softening.

Until now, few data are available on changes in the biochemical composition of blackcurrant fruit during ripening (Viola *et al.*, 2000; Rubinskiene *et al.*, 2006), if compared to strawberry fruits, and thus this section will be mainly considering strawberry as a model to understand the biochemistry behind the ripening of soft fruit.

2.3.3 Sugars

2.3.3.1 Sugar composition

Sugars in plants are formed as a result of photosynthetic activity. About 80 to 90% of the soluble solids content in ripen soft fruit consist of sugars (Manning, 1993; Perkins-Veazie, 1995; Pérez *et al.*, 1997; Cordenunsi *et al.*, 2003; Keutgen and Pawelzik, 2008). Glucose, fructose and sucrose are the major sugars found in both blackcurrant and strawberry fruits (Pérez *et al.*, 1997; Rubinskiene *et al.*, 2006). In addition to glucose and fructose, the ratios of which differ between blackcurrant and strawberries, other monosaccharides such as xylose are present in trace amounts (Makinen and Söderling, 1980; Perkins-Veazie, 1995; Hakala *et al.*, 2003). Strawberry fruit is characterised for having a glucose:fructose ratio of approximately 1 (Perkins-Veazie, 1995; Sturm *et al.*, 2003; Terry *et al.*, 2007). For blackcurrant berries little information is available about the ratio between different sugars. However, Boccorh *et al,* (1998) reported ratios of fructose/glucose on blackcurrant concentrates differing from 1.2 to 1.6 depending on the year and cultivar (**Table 2.4**). Sucrose is the dominant oligosaccharide found in strawberry (6.0 $mg \cdot g^{-1}$ FW) and most of the berries including blackcurrant (Manning, 1993; Zheng *et al.*, 2010). Generally it was accepted that sucrose concentration in strawberries was relatively low in comparison with other sugars (Kader 1991; Sturm *et al.*, 2003), however recent work by Terry *et al.* (2007) shown that sucrose concentration was comparable to glucose and slightly lower than fructose (cv. Elsanta).

Table 2.4: Non-structural carbohydrates in blackcurrant concentrates *(from* Boccorh *et al.,* 1998)

Season	Post-harvest storage/ geographical origin (n)	Fructose (mg g^{-1})	Glucose (mg g^{-1})	Total sugars (mg g^{-1})	Fructose/glucose ratio
1989	UK Fresh fruit (mean values) (13)	131.69	78.0	209.69	1.69
	SD	4.79	2.59	7.38	0.01
	SE	1.33	0.72	2.05	0.00
	UK Frozen fruit (32)	122.18	75.76	197.94	1.61
	SD	5.69	4.28	9.78	0.04
	SE	1.01	0.76	1.73	0.01
	New Zealand fruit (2)	169.86	112.48	282.34	1.51
	SD	1.17	0.77	1.94	0.00
	SE	0.83	0.55	1.37	0.00

2.3.3.2 Changes during ripening

Soluble solids content tends to increase during soft fruit ripening (Kader, 1991). Montero *et al.* (1996) studied the variation of soluble solids during strawberry ripening and observed that few changes occurred during the first stages. Total soluble solids showed an important increase from day 21 after fruit set until day 28, when maximum values were recorded. In the same work (Montero *et al.*, 1996), a considerable increase in reducing sugars was observed until 35 days after fruit set, showing for both glucose and fructose similar patterns. Glucose and fructose tend to be in similar concentrations in ripe strawberry fruit, being both the main sugars present in ripe strawberry (Roemer, 1989; Montero *et al.*, 1996; Sturm *et al.*, 2003; Terry *et al.*, 2007). The evolution of sucrose content during ripening of strawberries follows a different pattern than that described for glucose and fructose (Montero *et al.*, 1996; Sturm *et al.*, 2003). Highest concentrations of sucrose are found approximately 21 days after fruit set and then decrease considerably. Even though the evolution of sugars and organic acids in blackcurrant berries has not been studied in such detail, recent work has been done understanding the accumulation of ascorbic acid and other constituents such as sugars, during fruit ripening and development (Hancock *et al.*, 2007). The authors suggest that sugars in blackcurrant fruit tend to accumulate following a biphasic pattern with an initial accumulation of sugars, probably resulting from starch breakdown and mobilisation during fruit expansion, followed by a brief plateau and then a second phase of accumulation resulting from the accumulation of sugars from other sources.

2.3.4 Organic acids

2.3.4.1 Organic acid composition

Organic acids are also important flavour components and sugar/acid ratio is an important index for consumer acceptability (Pérez *et al.*, 1997). Acids are not just important for flavour, they are responsible for the regulation of the pH within the berry. Moreover, acids are important components as they affect the formation of off-flavours that can affect the product during processing (Manning, 1993). Citric acid is the main organic acid present in strawberries (8.2 $mg \cdot g^{-1}$ FW) and blackcurrants (44.08 $mg \cdot g^{-1}$ FW) showing considerable differences between genotypes (Sturm *et al.*, 2003; Hummer and Barney, 2002; Skupien and Oszmianski, 2004; Keutgen and Pawelzik, 2008). Other organic acids are present in relatively low concentrations in both strawberry and blackcurrants (Zheng *et al.*, 2010). For instance, ascorbic, citric, fumaric, malic, oxalic, shikimic, succinic and tartaric acid have been identified in strawberry fruit (Sturm *et al.*, 2003; Skupien and Oszmianski, 2004; Terry *et al.*, 2007). Recent work carried out by Terry *et al.*, (2007) on strawberry (cv, Elsanta) show the profile of different organic acids in both primary and secondary fruit of plants submitted to different water treatments (**Table 2.5**).

Table 2.5: Effect of water deficit treatment (mL water day^{-1}) on non-volatile organic acids of strawberry fruit expressed per fresh weight and per dry weight (*modified from Terry et al., 2007).*

Water treatment (mL)	Ascorbate (mg g^{-1})		Citrate (mg g^{-1})		Malate (mg g^{-1})	
	Primary fruit	Secondary fruit	Primary fruit	Secondary fruit	Primary fruit	Secondary fruit
50	0.79^{a}	0.78^{a}	8.81^{a}	9.10^{a}	2.41^{a}	3.04^{b}
100	0.78^{a}	0.76^{a}	9.16^{a}	9.19^{a}	2.24^{a}	2.79^{b}
200	0.71^{a}	0.75^{a}	8.46^{a}	9.22^{a}	2.35^{a}	2.91^{b}

Both blackcurrant and strawberry fruits are important sources of ascorbic acid (AsA) (Antonnen *et al.*, 2006; Walker *et al.*, 2006; Terry *et al.*, 2007), the most abundant water soluble antioxidant present in plants (Smirnoff, 2000). AsA in plants has several biochemical functions, such as, antioxidant, electron transfer, oxalate and tartrate synthesis and cofactor (Smirnoff, 1996). Although

being an important constituent of plants and a major metabolite, the biosynthetic pathway for AsA in plants is not really understood. Two different pathways have been suggested (**Figure 2.6**).

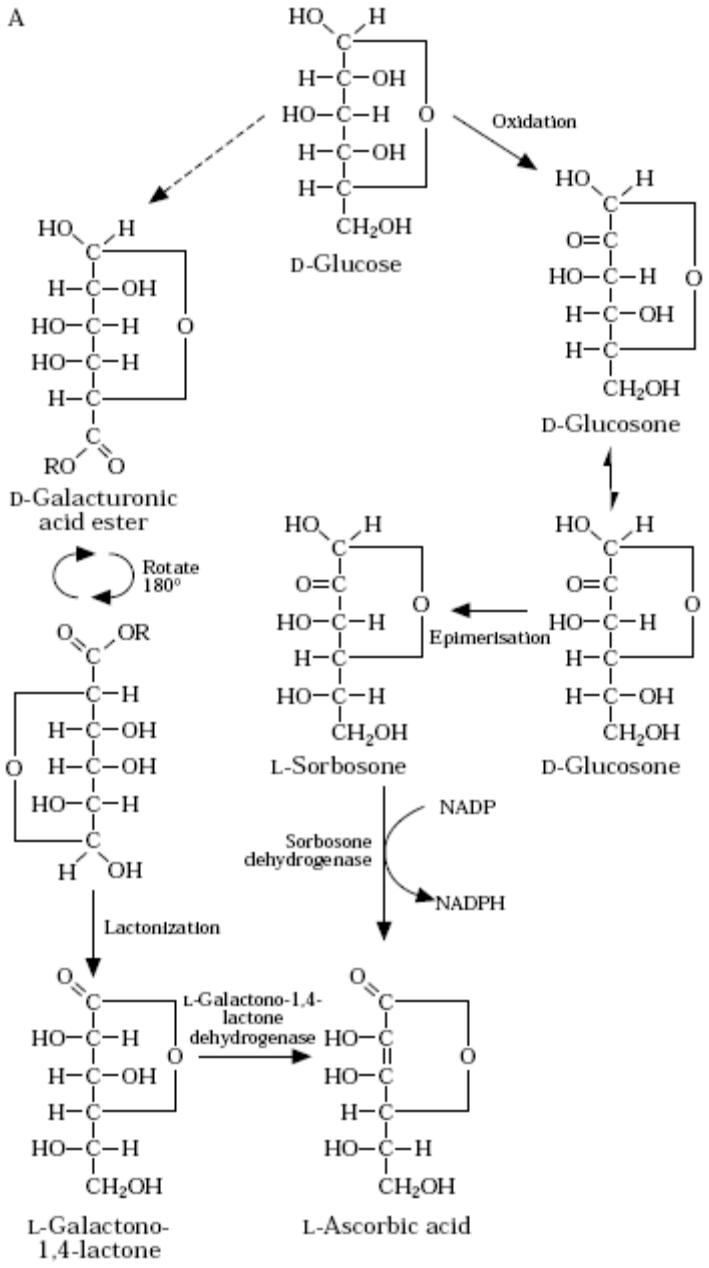


Figure 2.6: Proposed pathways of L-ascorbic acid in plants (*from* Smirnoff, 1996)

Once is ingested, Vitamin C or AsA is an important micronutrient for humans because of its inherent antioxidant properties. As an example, ascorbic acid content in blackcurrant cultivars has been shown to be far higher to those reported for other fruits (**Table 2.6**) (Walker *et al.*, 2006).

Table 2.6: Ascorbic acid content in various fruits (mg $100g^{-1}$ of fruit) (Modified *from* Belitz *et al.*, 2002)

Fruit	Ascorbic acid	Fruit	Ascorbic acid
Apple	3-35	*Blackcurrant*	*177*
Apricot	5-15	Orange	50
Cherry	8-37	Grapefruit	40
Peach	5-29	Lemon	50
Blackberry	17	Pineapple	25
Strawberry	*75*	Banana	7-21
Raspberry	25	Guava	300
Redcurrant	40	Melons	6-32

2.3.4.2 Changes during ripening

Although it is clear the evolution of sugars with maturity, different conclusions have been reported regarding the evolution of organic acids during fruit ripening (Montero *et al.*, 1996; Sturm *et al.*, 2003; Kafkas *et al.*, 2006). Generally, it is accepted that citric acid, being the main organic acid in strawberries, contributes greatly to fruit titrable acidity which has been seen to decline during fruit ripening (Kafkas *et al.*, 2006). On the other hand, acids such as ascorbic, malic, shikimic and fumaric, have been reported to be either in higher or lower concentrations in fully ripe fruits (Sturm *et al.*, 2003; Wang *et al.*, 2003; Rubinskiene *et al.*, 2006).

2.3.5 Phenolic compounds

2.3.5.1 Phenolic composition

Phenolic compounds are produced as secondary metabolites in all plants. They include a wide range of substances and can be defined as compounds containing and aromatic ring with one or more hydroxyl substituents (Antolovich, 2000). These substances are derived from the shikimate pathway and then the phenylpropanoid metabolism (**Figure 2.7**) (Nazck and Sahidi, 2004).

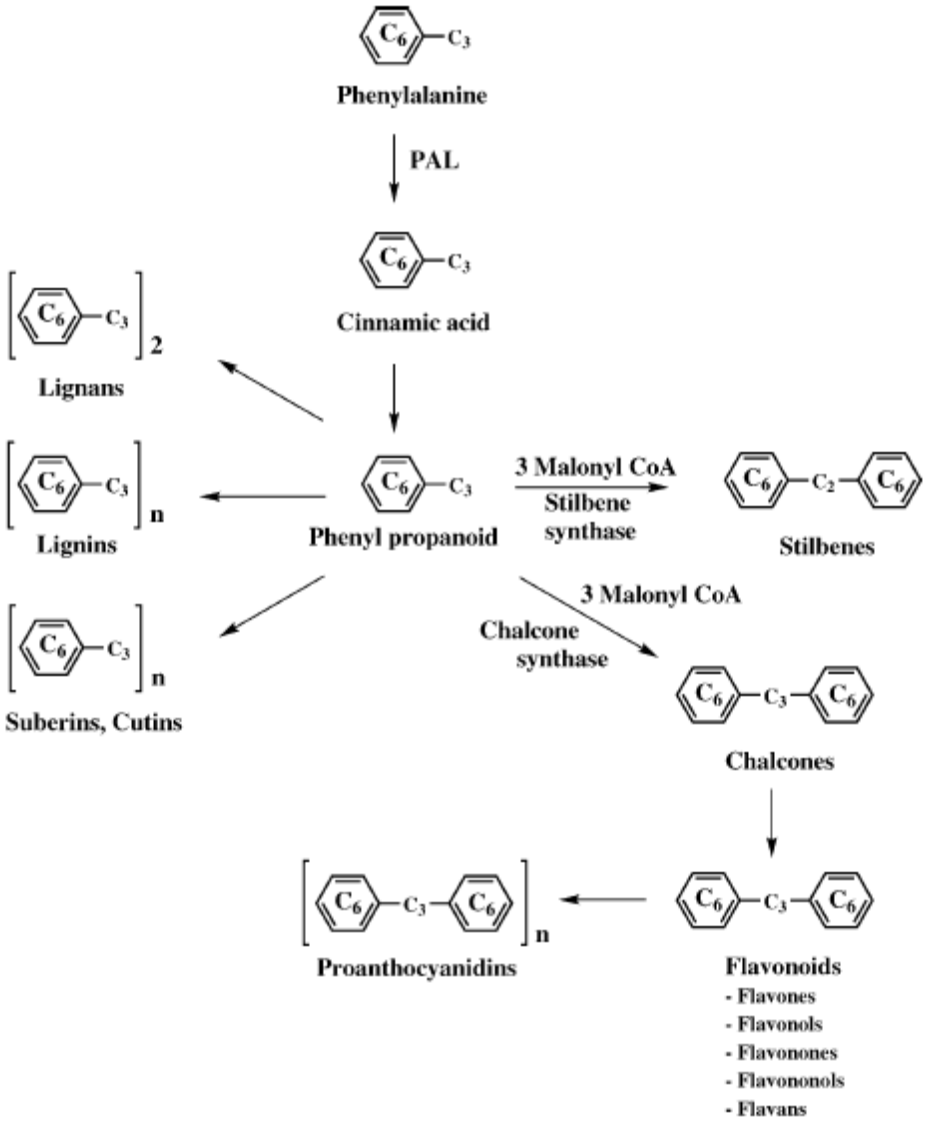


Figure 2.7: Production of different type of phenolics from phenylalanine sources in plants (*from Naczk and Shahidi, 2004*)

Current classification divides phenolic compounds in polyphenols or simple phenols based on the number of phenol subunits present (Robbins, 2003). Plant phenolics include simple phenols containing one phenol subunit, phenolic acids including benzoic and cinnamic acid derivatives, coumarins, flavonoids, stilbenes, hydrolizable and condensed tannins, lignans and lignins (Naczk and Shahidi, 2004). In the plant they have several functions such as to attract seed disseminators or act as antioxidants, scavenging free radicals generated by the plant when this is submitted to any kind of stress (Atkinson *et al.*, 2005). In food they are responsible for the bitterness, astringency, colour, flavour, odour and also for the oxidative stability of the products. Once these compounds are ingested, they act as antioxidants together with ascorbic acid and other food natural substances and hence are considered as bioactive compounds with potential health-promoting properties (Seeram,

2006). A general consensus from health experts worldwide has confirmed that increased dietary intake of antioxidant compounds such as phenolics from fruits and vegetables may protect against the oxidative damage caused by free radicals (McDougall *et al.*, 2005). Moreover, recent epidemiological studies suggest that consumption of products rich in antioxidants such as berry fruits helps towards reducing the incidence of certain cancers and chronic diseases (Konczak and Zhang, 2004; Pantelidis *et al.*, 2006; Seeram *et al.*, 2006). Berries such as blackcurrants and strawberries are important sources of these compounds. The phenolic profile of these berries have been widely studied during recent years (Aaby *et al.*, 2005; Zadernowski *et al.*, 2005; Hernanz *et al.*, 2007; Jordheim *et al.*, 2007; Lopes da Silva *et al.*, 2007; Terry *et al.*, 2007). For instance, ellagic acid, a water soluble phenolic compound, occurs at high concentrations in strawberries and accounted for more than 50% of the total phenolics acids found in this berry (Atkinson *et al.*, 2006). Twelve other phenolics compounds were identified and quantified by Hernanz *et al.* (2007) in five different Spanish cultivars. In this case, the natural occurring pigment (anthocyanin) pelargonidin-3-glucoside accounted for more than 50% of the total phenolic content in the berries studied. In blackcurrants more than 14 different phenolics were identified in Polish berries by Zadernowski *et al.* (2005). In their work, coumaric acid derivatives were the main phenolics accounting for more than 50% of the total phenolic content. Even though, anthocyanins were not quantified in their work, the anthocyanins profile from blackcurrants is also well known and four major anthocyanins have been identified (Delphidin 3-O-glucoside, delphinidin 3-O-rutinoside, cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside) (Häkkinen *et al.*, 1999; Manhita *et al.*, 2004; Rubinskiene *et al.*, 2005; Anttonen *et al.*, 2006). Further details on the health-promoting properties of both blackcurrant and strawberry fruits, as well as their polyphenolic composition, may be found in Appendix D and E, respectively.

2.3.5.2 Changes during ripening

Red colour in strawberry fruit or dark purple colour in blackcurrant develops through the production of anthocyanins during fruit ripening. Recent work carried out on strawberry fruit demonstrated a significant increase of both perlargonidin-3-glucoside and cyanidin-3-glucoside during the different stages of fruit ripening (Kosar *et al.*, 2004; Nunes *et al.*, 2006). Anthocyanins in soft fruit start to appear during the first stage of development, in strawberry they appear during the white stage, when mRNAs from several genes related to phenylpropanoid synthesis and metabolism are up-regulated (Manning 1998). A key step during the biosynthesis of anthocyanins is the synthesis of phenylalanine-ammonia lyase (PAL) (**Figure 2.8**) which in the case of strawberry, raspberry and other berries has been shown to have to main peaks during fruit ripening (Wang *et*

al., 2003). In strawberry fruits, the first peak occurred 5 days approximately after anthesis and the second one at nearly full ripe stage, 27 days after anthesis (Cheng and Breen, 1991).

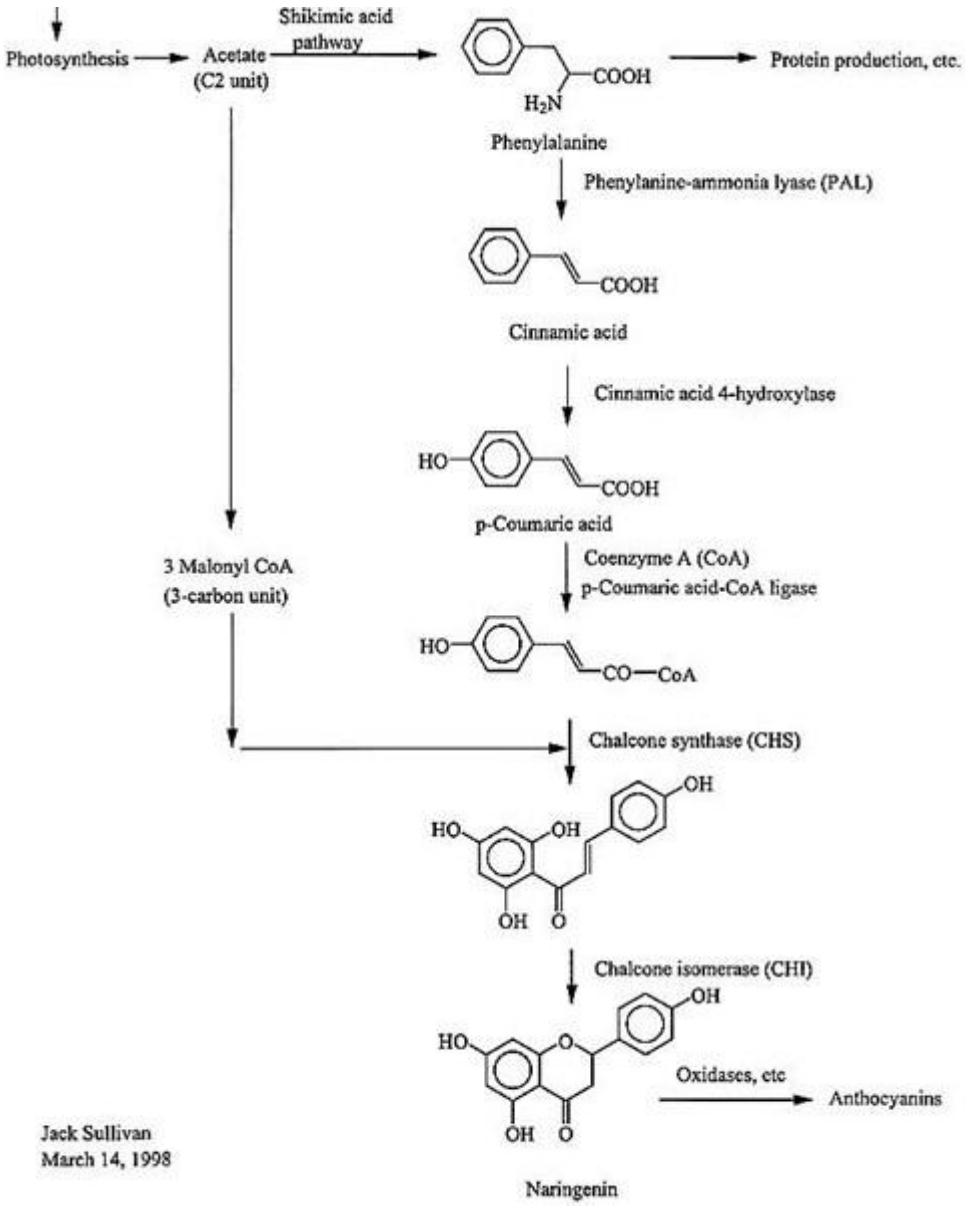


Figure 2.8: Biosynthesis of anthocyanins showing the key step of PAL synthesis (*from Sullivan, 1998*)

Phenolic compounds are normal constituents of fruits which have a direct effect on fruit taste and nutritional value (Schuster and Herrmann, 1985; Tsao and Yang, 2003). As phenolic compounds are related to the astringency of the fruit, and this is generally lost during ripening, it can be assumed that the content in phenolic compounds tend to decline with fruit maturity. Initial studies suggested that total soluble phenols decreased from green strawberries to red ones (Spayd

and Morris, 1981; Terry *et al.*, 2004). This decrease in total phenolics was explained through the shift and increased synthesis of anthocyanins (Perkins-Veazie, 1995). Nevertheless, recent work in strawberry fruit (Nunes *et al.*, 2006) demonstrated that during the first stages of ripening there is a loss of soluble phenolics followed by a plateau and then an increase (16%) during the last stages of maturation.

2.3.6 *Other non-structural components*

Volatile compounds are important substances responsible for the characteristic flavour and aroma of both blackcurrants and strawberries. The characteristic strawberry aroma is known to be composed of almost 200 different volatile compounds including esters, alcohols, carbonyls and sulphur containing compounds (Perez, 1993; Perkins-Veazie, 1995; Perez *et al.*, 1997) (**Table 2.7**). The characteristic aroma of blackcurrant, important for the production of juices and cordials, has been studied in detail during the past 20 years (Bricout *et al.*, 1979; Latrasse *et al.*, 1982; Marriot, 1987 and Ruiz del Castillo, 2002). Mono and sesquiterpens have been highlighted together with diacetyl and butyrates for the particular aroma of fresh blackcurrants (Ruiz del Castillo *et al.*, 2002). Aroma build-up in soft fruits is affected by several external factors such as temperature, day/light variations and humidity (Ayala-Zavala *et al.*, 2004).

Table 2.7*:* Concentration of main odorants in fresh strawberry juice (adapted from Belitz *et al.*, 2002)

Compound	**Concentration (mg kg^{-1})**
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	16.20
(Z)-3-Hexenal	0.33
Methyl butyrate	5.00
Ethyl butyrate	0.41
Isobutiric acid ethylester	0.043
2-/3-Methylbutiric acid ethylester	0.048
Acetic acid	74.50
2,3-Butadione	1.29
Butyric acid	1.83
2-/3-Methylbutiric acid	2.24
(E)-β-Damascenone	< 0.1
(E,E)-2,4-Decadienal	<0.1
Guaiacol	0.8

As described for texture-related, soluble compounds, pigments and polyphenols, aroma related compounds such as volatile esters also change during soft fruit ripening. The biosynthesis of volatiles during soft fruit ripening is complex owing to the large number of compounds for the characteristic aroma of each fruit. More than hundreds of volatile esters have been identified during strawberry (Perez *et al.*, 1997; Ayala-Zavala *et al.*, 2004) or blackcurrant (Bricout *et al.*, 1979; Latrasse *et al.*, 1982; Marriot, 1987; Ruiz del Castillo *et al.*, 2002) ripening. Since unripe strawberry or blackcurrant fruit lack any characteristic flavour it has been assumed that the volatile constituents of ripe fruit are developed during the ripening process.

2.4 Analytical methods used in soft fruit quality control

2.4.1 Introduction to food analysis and quality

Qualitatively, health and pleasure are two main reasons for fruit consumption. However, often the quality of fruit is criticised by the consumers. The concept of fruit quality is an extremely difficult subject and it is influenced by many factors (Abbot, 1998). Quality is not only affected by the biochemical profile of the fruit itself, because in terms of quality the consumers' own perception plays an important role. Therefore fruit quality may be assessed by incorporating consumer criteria into the common analytical methods. Nevertheless, a better knowledge of the exact qualitative and quantitative distribution of the main sugars and organic acids in soft fruit products is of relevant importance to evaluate quality (Pérez *et al.*, 1997). Sugars and organic acids, especially from strawberry fruit, have been investigated during the past decades with different perspectives, such as an index of fruit development and ripening and as components of strawberry flavour and taste. (Pérez *et al.*, 1997; Sturm *et al.*, 2003; Keutgen and Pawelzik, 2008).

Analytically, with few exceptions, food samples can be considered as complex matrices or non-homogenous mixtures of a large range of chemical substances which may include intermediate and side reaction products. The isolation and quantification of the individual target analytes commonly represent a difficult challenge within food analysis techniques. Furthermore, even with powerful and sophisticated modern techniques such as High Performance Liquid Chromatography (HPLC) or Gas Chromatography (GC), rarely is it possible to analyse directly the sample without previous sample preparation and obtain a sensible result (Lichon, 2000). Therefore, before the analysis of the sample, procedures for sample preparation must be developed, evaluated and also reported as an integral part of any analytical method for food analysis.

2.4.2 Determination of sugars and organic acids

2.5.2.1 Introduction

Sugar analysis in food samples can involve many analytical challenges. Sugar molecules have multiple structural forms in water, making it difficult to analyse all of them using the same analytical procedure. Moreover, similar or exact molecular structures can have different solubility or reactivity, making difficult the extraction procedure and thus the analysis (Fournier, 2005). Generally the analysis of sugars uses refractometry, chromatography and enzymatic assays. Different methodologies have been reported for the analysis of organic acids in fruits giving especial interest to the analysis of ascorbic acid due to its known health-related properties. Generally the analysis of organic acids employs titration, spectrophotometry (Arya *et al.*, 1998), amperometry (Arya *et al.*, 2000) and chromatography (Walker *et al.*, 2006). The analysis of organic acids also involves several analytical challenges and most of the current methods may provide unreliable results due to the presence of oxidisable species other than specific organic acids. For example, substances naturally present in fruits such as tannins, sulphydryl compounds, and metals such as copper, iron and cobalt are easily oxidised (Arya *et al.*, 2000) giving overestimates.

2.5.2.2 Analytical techniques

The following sections aim to summarise some of the different analytical techniques available and commonly used for the extraction and quantification of target analytes including sugars and organic acids in soft fruits.

a) Titrimetric analysis

The analysis of organic acids in fruit samples by titration is a standardised technique in the food industry (AOAC, 1990). This analysis involves the measurement of the volume of a solution of a compound of known concentration, the standard, required to react completely with a solution obtained from the sample to be analysed. Even though there are different types of titrations, acid-base titration is the most commonly used being a standard routine method of analysis. Other titrations have also been described and standardised for the analysis of individual organic acids (AOAC, 1990; Kabasakalis *et al.*, 2000). Despite of their simplicity, these techniques encompass important drawbacks. For instance, the AOAC procedure employing titration with 2,6-dichloroindophenol in acidic solution is not applicable to all matrices. Additionally, all titrations results are generally inaccurate and are expressed as a percentage of the predominant organic acid

not providing information about the real organic acid composition of the sample (Pérez *et al.*, 1997). In soft fruits, generally results are expressed as percentage of citric acid, as this is the main organic acid present in this fruit type.

b) Colorimetric quantification

Colorimetric techniques for determination of sugars have been employed in the food industry for years: phenol and sulphuric acid being the most common reagents employed (Dubois *et al.*, 1951; Koch *et al.*, 1951). These techniques were initially developed for the measurement of lactose in dairy products (Barnett and Tawab, 1957), however, they spread to other sectors within the food industry due to the simplicity and availability of the reagents used. Analytical techniques for the spectrophotometric determinations of specific organic acids have also been reported (Arya *et al.*, 1998). However, some of the difficulties of successful application of spectrophotometric methods are that, beyond specified limits, the intensity of absorption is not directly proportional to concentration. Moreover, the well-defined absorption band in the ultraviolet region of the spectrum is subject to interference from many other substances (Raghu *et al.*, 2007). Although colorimetric or spectophotometric determinations are still a valuable tool (Nilsson *et al*, 1997) this technology still has not been sufficiently miniaturised to be portable, is relatively expensive due to the cost of reagents and equipment and also requires training.

c) Refractometry

This technique is the most commonly applied in the food industry due to its simplicity and rapidity. Brix degree (˚Brix) as a measure of total soluble solids (TSS) is widely employed in fresh produce industry as a measure of sweetness of fruits and vegetables (Kopsell and Randle, 1997; Perez *et al.*, 1997). However, the use of ºBrix as a measure of sugars concentration has been found to be poorly correlated to the real sugar content in soft fruit or other fresh products and does not reveal the importance of acid content (Perez *et al.*, 1997; Terry *et al.*, 2005; Chope *et al.*, 2006). Further details on this technique and its limitations can be found in Appendix A.

d) Chromatographic determinations

The analytical techniques used for determination of sugars and acids in fruit samples have improved considerably during the past few decades. Traditional techniques have been replaced by chromatographic techniques both using Gas chromatography (GC) and High Performance liquid chromatography (HPLC). Typically, these methods allow detection of several sugars or organic acids simultaneously offering high selectivity and sensitivity in comparison with enzymatic assays

that are specific for a type of sugar or colorimetric assays which give overestimate results. HPLC is often chosen for sugar determinations and is currently the official method for routine sugar analysis (AOAC, 1995). Working with HPLC, different type of detectors can be used. Refractive Index (RI) is based on the refraction caused when a light beam goes through a specific substance. HPLC-couple to RI detector have extensively been used during the last years for quantification of sugars in fruits (Dolenk and Stampar, 1997; Dolenk-Sturm *et al.*, 1999; Sturm *et al.*, 2003) showing high resolution and precision when low concentration of sugars are present. HPLC coupled to evaporative light scattering (ELS) detection has also been used for the analysis of sugars in fresh products (Chope *et al.*, 2006; Davis *et al.*, 2007; Terry *et al.*, 2007) due to the greater baseline and selectivity in comparison to refractive index (Terry *et al.*, 2005). Other detectors include pulse amperometric detection (PAD) or mass spectrometry (MS), however the use of these detectors has not reach yet the popularity of those mentioned earlier. More recently, liquid chromatography (LC) coupled with electrospray ionization mass spectrometry (ESI-MS) has been reported as being an useful method for analyzing trace sugars in complex matrix (Cheng *et al.*, 2006). Gas chromatography couple to flame ionization detection (FID) and mass spectrometry (MS) have also been used, being GC-MS widely used to provide both qualitative and quantitative analysis due to its capability of molecular identification at high sensitivity (Medeiros *et al.*, 2007).

HPLC for organic acid determination has been also extensively used during recent years (Iversen, 1999; Sturm *et al.*, 2003; Walker *et al.*, 2006; Terry *et al.*, 2007). In this case HPLC techniques are almost exclusively coupled to a DAD and the detection of organic acids is carried out around 250nm.

While chromatographic techniques are very accurate, they are time-consuming and require extensive sample preparation and understanding (Rodriguez-Saona *et al.*, 2001). Furthermore, the application of these techniques to routine quality procedures in the food industry is limited due to the high cost of equipment and maintenance.

e) Enzymatic assays

Different enzymatic assays are used to determine specific enzyme substrates belonging to the compounds frequently present in foods and determining their quality (Shekhovtsova *et al.*, 2006). Enzymatic assays have been widely used in the food industry during the last decades and many commercial kits are currently available and are a prospective technology focus especially to control the content of different vitamins in foods (Shekhovtsova *et al.*, 2006). Enzymatic assays for the determination of ascorbic acid (AsA) are widely used and they are primarily based on the

determination of dehydroascorbic acid (DHAsA) as a product of AsA oxidation by oxygen or hydrogen peroxide in the presence of the enzyme ascorbate oxidase and peroxidase.

Generally, these assays show many advantages for the analysis of specific compounds , such individual organic acids or sugars, however they require single determinations for each compound of interest resulting in time-consuming procedures and high cost analysis when the biochemical profile of a group of compounds want to be determined.

2.4.3 Determination of polyphenolic compounds

It is well established that a diet high in fruits and vegetables is associated with a reduced risk of certain diseases (Seeram *et al.*, 2005) and these beneficial health effects are mainly related to their high levels of certain phytochemicals present in fruits, from which phenolics constitute the greatest proportion. Consequently, several reviews regarding the extraction and quantification of phenolics from fruit samples have been recently published (Antolovich *et al.*, 2000; da Costa *et al.*, 2000; Robbins, 2003; Naczk and Sahidi, 2004; Boudet, 2007). The different assays used for the analysis of phenolics can be classified as either those which measure total phenolic content or those quantifying and individual phenolic or group of phenolics (Naczk and Sahidi, 2004). Due to the high number of polyphenols (>8000) and their different chemical properties (**Figure 2.9**), extraction is a crucial step in the recovery and identification of these compounds from fruits (Waterhouse, 2005). Although several solvents have been reported for the extraction of polyphenols in fruit samples, generally polyphenols are soluble in solvents less polar than water such as methanol, ethanol, acetone, ethyl acetate and their aqueous mixtures.

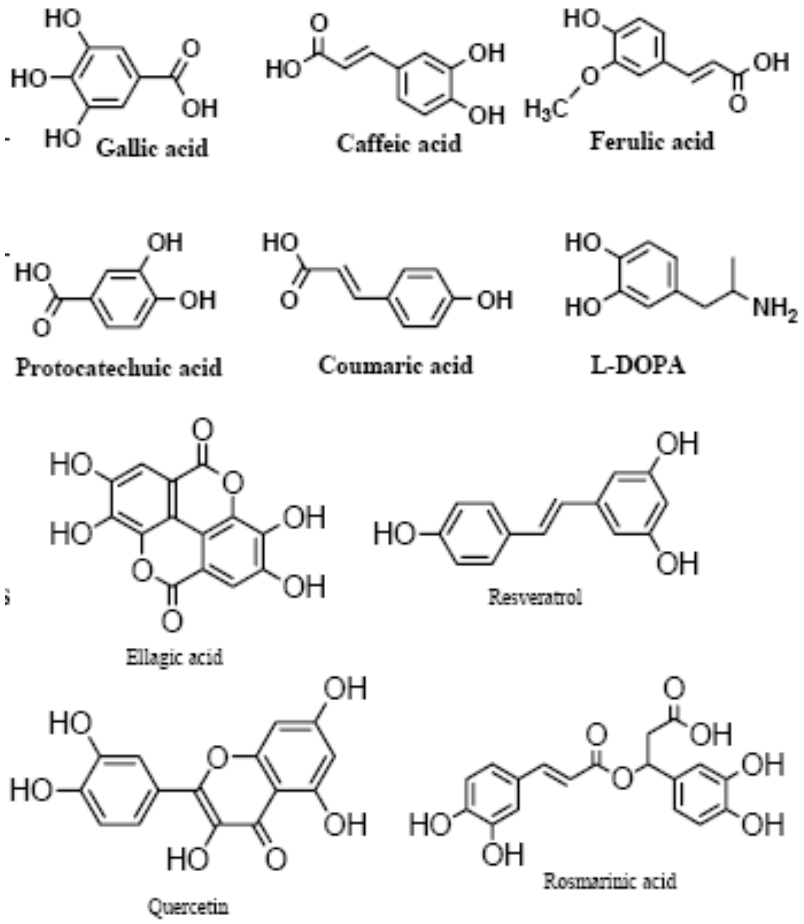


Figure 2.9: Chemical structure of simple phenol, biphenyls, flavavoids and tannins commonly found in plants (from Vattem *et al.*, 2005)

Commonly, polyphenolic compounds are isolated and quantified from fruits either by spectofotometry or chromatography (**Table 2.8**). For instance, the Folin-Ciocalteau method (FCM) (Singleton and Rossi, 1965) is one of the standard spectrophometric procedures in wine analysis as well as in tea analysis (Wiseman *et al.*, 2001). This method is based on the reduction of a phosphowolframate-phosphomolibdate complex by phenolics to blue reaction products (Singleton and Rossi, 1965). Although being a standardised and commonly employed method it has some drawbacks: the most important being interferences caused by either sugars or ascorbic acid when these are present in high concentrations (Atkinson *et al.*, 2005). In addition, the FCM just gives an overall quantification of the polyphenols content in the sample but does not distinguish between different types of compounds. Because polyphenolic compounds display chemical complexities and relatively similar structures, isolation and quantification of individual components has been challenging. However, the use of HPLC, characterised by its high resolution and relative rapidity, have shown a potential alternative for the identification of these compounds. HPLC apparatus

coupled with a diode array detector (DAD) have successfully isolated and quantified the polyphenolic profile of several fruits including strawberries and blackcurrants (**Table 2.8**) being nowadays the most common techniques for identification of these compounds.

Table 2.8: Recent development in the extraction and determination of polyphenolic compounds from fruits

Target polyphenol	Food matrix	Extraction solvent	Type of extraction	Quantification method	Reference
Anthocyanins	Blackcurrants	1g sample in 25ml and partition against 50 ml of solvents of increasing polarities (chloroform, dyethil eter, ethyl acetate)	Solid liquid extraction	Capillary zone electrophoresis	da Costa *et al.*, 1998
Flavonoids and phenolic acids	19 berries	0.5 g sample with 25 ml acidified (1.2M HCl) methanol	Sonicated solid liquid extraction	HPLC-DAD	Häkkinen *et al.*, 1999
Anthocyanins	Blackcurrant nectar	Mixture of water:acetonitrile:formic acid (84:6:10, v/v)	Solid liquid extraction	HPLC-DAD	Iversen, 1999
Anthocyanins	Strawberry	Acidified methanol (0.1% HCl)	Centrifugued solid liquid extraction	HPLC-DAD-ESI-MS	Lopes da Silva *et al.*, 2001
Anthocyanins and other phenolics	Blackcurrants	Acidified Aqueous Sulfur dioxide (pH 3.8 ajusted with acetic acid)	Multiple solid liquid extraction assisted with thermostatic water bath	HPLC-DAD	Cacace and Mazza, 2002
Polyphenolic acids	Strawberry	Acidified aqueous methanol (1.2M HCl)	Sonicated Solid liquid extraction	HPLC-DAD	Sanli *et al.*, 2002
Anthocyanins	Blackcurrants	Acidified aqueous methanol (0.1%TFA, v/v) and partition with ethy acetate/water	Solid liquid extraction adsorption chromatography	HPLC-DAD	Slimestad and Solheim, 2002

Target polyphenol	Food matrix	Extraction solvent	Type of extraction	Quantification method	Reference
Total phenolics	Marionberry and strawberry	3g extract with 40 mL of Acetone: water: acetic acid (70:29.5:0.5, v/v)	Centrifugued solid liquid extraction	FCM	Asami *et al.*, 2003
Phenolic acid profile	Strawberries (flesh and achenes)	70% aqueous acetone (achenes), acetone (flesh)	sonicated solid liquid extraction (3times) + pooled extracts mixed with chloroform (1:1) and centrifuged	FCM, TMA, FRAP, ORAC, HPLC-DAD	Aaby *et al.*, 2005
Phenolic compounds	Strawberry (full fruit)	Acidified aqueous methanol (1.2M HCl) or acidified water (1.2M HCl)	Ultrassound-assisted solid liquid extraction	HPLC-DAD	Herrera and Luque de Castro, 2005
Anthocyanins	Strawberry (full fruit)	Acidified methanol (0.1% HCl)	Multiple solid liquid extraction followed by C-18 SepPak® and ellution using Methanol : 0.1% TFA (95:5, v/v)	HPLC-DAD-MS	Lopes da Silva *et al.*, 2005
Phenolic acid profile	Strawberries (full fruit)	Acidified (0.1% HCl) Methanol or acetone: water (7:3, v/v)	Solid liquid extraction+ centrifugation followed concentration in vacuo and reconstitution with water: acidic methanol (1:1, v/v)	HPLC-DAD-ESI-MS	Seeram *et al.*, 2005
Phenolic acid profile	Small berries	Aqueous 80% methanol	multiple solid liquid extraction (6 times)	GC-MS	Zadernowski *et al.*, 2005
Phenolic acid profile	Blackcurrants	70% acidified aqueous acetone (0.01 M HCl)	Solid liquid extraction	FCM, HPLC-DAD	Anttonen and Karjalainen, 2006

Target polyphenol	Food matrix	Extraction solvent	Type of extraction	Quantification method	Reference
Phenolic acids	Strawberry	Acidified aqueous methanol (1.2M HCl and 80mg Ascorbic acid)	Sonicated (2min) and water-bath solid liquid extraction	HPLC-DAD	Mas *et al.*, 2006
Phenolic acid profile	Strawberries	Acetone (5g in 10 mL)	Sonicated solid liquid extraction followed by centrifugation and reconstitution with 70% acetone	HPLC-DAD	Aaby *et al.*, 2007
Anthocyanins	Strawberries	Acidified methanol (0.1% HCl)			
Flavonols and phenolic acids	Strawberries	Acidified (1.2M HCl and 80mg Ascorbic acid) aqueous methanol 50% (v/v)	Sonicated solid liquid extraction	HPLC-DAD	Hernanz *et al.*, 2007
Anthocyanins	*Ribes* berries (full fruit)	Acidified (0.5% TFA) methanol	multiple solid liquid extraction (6 times)	HPLC-DAD	Jordheim *et al.*, 2007
Phenolic content	Vaccinium berries	Mixture of 5.4M Acetone, 9.9M Methanol and 3.04M formic acid	Solid liquid extraction	ORAC, HPLC-DAD	Kalt *et al.*, 2007
Phenolic compounds	Blackberries	70% Aqueous acetone containing 2% formic acid. acetone (40 °C)	Solid liquid extraction, then the extracts were combined, filtered, and concentrated under vacuum	HPLC-DAD-ESI-MS	Mertz *et al.*, 2007
Individual Anthocyanins and total Phenolics	Strawberry (full fruit)	70% Methanol (v/v) and 0.5%HCl (v/v)	Fast solid liquid extraction	HPLC-DAD FCM	Terry *et al.*, 2007

* DAD: Diode array detector; ESI: Electro-spray ionisation; FCM: Foulin Ciocalteau's method; FRAP: Ferric reducing ability of plasma; GC: Gas Chromatography; HPLC: High Performance liquid chromatography; MS: Mass spectroscopy; ORAC: Oxygen radical absorbance capacity; TFA: Trifuoroacetic acid; TMA: Total monomeric anthocyanins.

2.5 Novel methods for improved soft quality control: Biosensors

2.5.1 Introduction

The development of analytical methods based on low cost, easy to operate, portable instrumentation that is relatively inexpensive is of great importance for the food industry (Tothill, 2001; Terry *et al.*, 2005; Gonzalo-Ruiz *et al.*, 2006). An adequate control of the quality parameters preferably requires real-time, simultaneous and selective quantification of key compounds which establish the quality and taste of most of the soft fruits. In this context, biosensors may have an opportunity to fulfil these needs through measuring target analytes that are directly related with the produce quality (Terry *et al.*, 2005) offering at the same time rapidity and cost-efficiency measurements, without requiring time consuming or expensive sample preparation methods.

2.5.2 Concept of Biosensors

A biosensor can be generally defined as a measuring device that contains a biological element (Buerk, 1993), Even though this definition is very broad many other definitions can be found in the literature. Turner *et al (2000)* described a biosensor as *"A compact analytical device incorporating a biological or biologically-derived sensing element either integrated within or intimately associated with a physicochemical transducer. The usual aim of a biosensor is to produce either a discrete or continuous digital electronic signal that is proportional to a single analyte or a related group of analytes"*

Generally, biosensors are small devices which use the biochemical molecular recognition properties of molecules as the basis for the selective analysis. The major processes involved in a biosensor are analyte recognition, signal transduction and readout (Tothill, 2001). The principle behind the biosensor performance (**Figure 2.10**) can be described as follows: A target analyte in the external medium interacts with the *Molecular Recognition Element (MRE)* of the sensor; this interaction is then detected by a *transducer*. The MRE, also known as bioreceptor, can be an enzyme, cell, microorganism, antibody or nucleic acid. The interaction between the analyte and the MRE can take place in several different ways. The transducer can be electrochemical, optical, piezoelectric, thermometric, magnetic or micromechanical and the function of this is to generate an electrical signal that is proportional to the signal generated by the MRE when it interacts with the

analyte. Therefore, the transducer is a key element in the biosensor performance which confers the sensitivity (Luppa *et al.*, 2001; Kreuzer *et al.*, 2002).

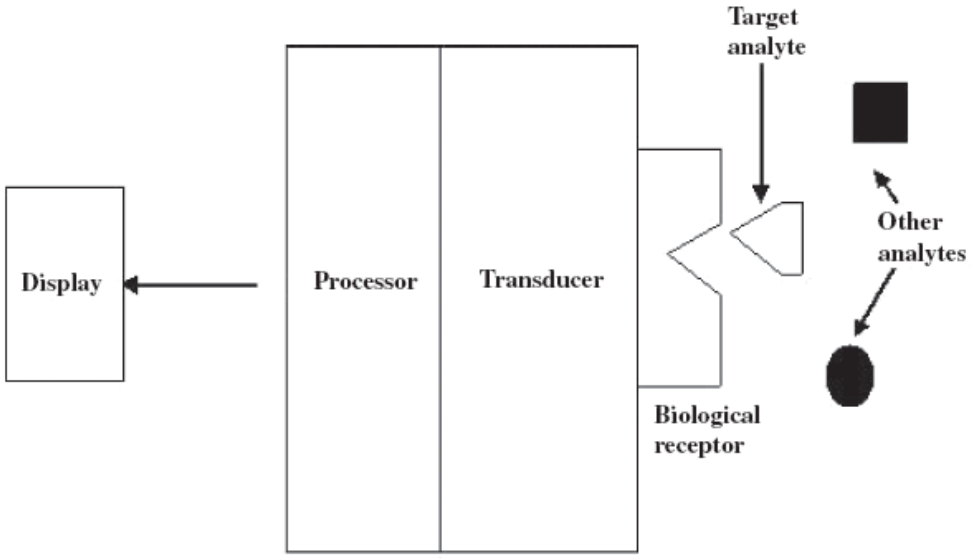


Figure 2.10: Principle of operation of biosensors (*Adapted from Terry and Giné Bordonaba, 2010*)

2.5.3 Types of biosensors and their potential applications in the food industry

Although biosensors can be classified according to either the biological element used or the type of transducer, the following classification aims to characterise the different type of biosensors only on the basis of the transducer.

- ***Electrochemical biosensors***. This type of biosensors has dominated the global market with the majority of biosensors reported based on enzyme catalysis or whole cells (Bardeletti *et al.*, 1991). Electrochemical biosensors are based on the detection of electroactive species that are either formed or produced due to the action of the bioreceptor (e.g., enzymes, antibodies, whole cells) (Terry *et al.*, 2005). Electrochemical biosensors monitor the current at a fixed voltage (amperometry) or the voltage at zero current (potentiometry) (Tothill and Turner, 2003). Amperometric biosensors were the first type of biosensor developed and have been extensively used over the past 35 for the development of glucose biosensors. Their simplicity, ease of production toghether with their relative low costs has made amperometric biosensors the most popular still to date. The signal given by amperometric biosensor is dependent on the rate of mass transfer at the surface of the electrode (Tothill, 2003; *section 2.5.4*). In contrast, potentiometric biosensors operate under conditions of near-zero current and monitor the difference in potential between the working and reference electrode (Tothill and Turner, 2003). The potential difference

between the sensing or working electrode and the reference electrode is proportional to the logarithm of the activity of the analyte in solution (Tapuhi *et al.*, 1996). Potentiometric biosensors have the advantage of operating over a wide range of concentrations. However, the development of these type of biosensors for food quality applications have been limited (Dutta *et al.*, 2001; Rotariu *et al.*, 2002; Kim and Kim, 2003 and Berna and Singh, 2003if compared to amperometric biosensors probably due to the interferences cause by pH changes (Tothill, 2001). Ion-selectice electrodes (ISEs) of which the pH electrode is the most known example, are the most common type of potentiometric biosensors. Conductometric biosensors are based on measuring the time dependence of the change in conductivity as a result of the recognition event between the receptor and its complementary analyte (Eggins, 1996; Tapuhi *et al.*, 1996; Tothill and Turner, 2003). These types of biosensors present some limitations that make them not really appropriate for the food industry. For instance, the signal given by conductometric biosensors reflects the migration of all ions present in the solution, being unselective for specific analytes (Tapuhi *et al.*, 1996).

- ***Optical biosensors***. This type of biosensors are based on a range of principles including the effect of the biological event on light absorption, fluorescence, refractive index (RI), chemiluminescence, surface Plasmon resonance (SPR) or other optical parameters (Tothill and Turner, 2003). These type of biosensors have gain popularity over the past years with many devices being now commercially available (Tothill, 2003; Terry *et al*., 2005). These devices offer important advantages in terms of miniaturisation, low cost, disposability and non susceptibility to electrochemical interferences (Turner and Newman, 1998). In addition these devices provide highly reproducible and high speed measurements (Terry *et al.*, 2005) and in some cases do not require reporter molecules such as enzymes or other labels. For instance, a direct inmunosensor can be design by measuring the change of RI due to the the interaction between the target analyte and the sensing element (Tothill and Turner, 2003). SPR is currently receiving particular attention on the basis of succesfull commercialisation (i.e. BIAcore, Upsala, Sweeden). This technology is based on excitation of the electron plasma (surface Plasmon) of a thin metal layer covering the surface of the wave-guide (Tothill, 2003). Changes in the light incidence angle or the reflectance minima due to changes in RI in the proximity of the thin metalised surface can be recorded (Tothill and Turner, 2003).

- ***Piezoelectric biosensors***. These devices rely on changes in the resonant frequency of wave propagation through a piezoelectric material (Tothill and Turner, 2003). Piezolectric materials, (i.e. quartz crystals) have the property to oscillate when partially or completely immersed into a solution. The surface of the crystal can be modified using a biological element that bind specifically to the analyte of interest. When the crystal is place in an electric field, the crystal is subjected to

mechanical deformations. In this context, if the physicochemical properties of the solution are known a mass change at the crystal surface, for example when the anlayte is bind to the biological recognition element, can be detected by measuring its vibration frequency (Luong and Guilbault, 1991; Turner, 1994).

- ***Thermal biosensors***. These devices are based on the detection of heat generated or consumed during a biological reaction, or in other words, by measuring enthalpy changes during the biological recognition event (Tothill and Turner, 2003). Most reactions produce or absorb heat and could therefore be monitored by sensitive devices including a microcalorimeter with immobilised biological components, pack in a small column and couple to a heat sensing transducer such as thermistors. Although this technique has a broader applicability than other transducer (Mosbach, 1991) the use of sophisticated and costly instrumentation is, most probably, the main limitation for its wider application in the food industry (Luong *et al.*, 1997).

The food industry is very large, but profit margins are relatively low compared to other industrial sectors. Consequently one of the main limitations of this industry is the reduced capacity to invest in novel and considerable expensive analytical methods. As previously stated, all biosensors suffer from a number of limitations. Optical biosensors although being really sensitive show considerable limitations when used in turbid media, and somehow are not acceptable for many food matrixes or require sophisticated sample preparations steps. Thermal biosensors, even though they can be acceptable for most of the major food constituents are of little use where there is little heat exchange, such as when minor constituents (i.e. polyphenols) want to be analysed. Furthermore, these types of devices are difficult to handle (Chaubey and Malhotra, 2002). Amperometric enzyme biosensors may have a limited linear range generally attributed to the low oxygen concentration or deactivation of the enzyme by the hydrogen peroxide generated during the electrochemical enzyme reactions (Luong *et al.*, 1997), however many efforts are currently undertaken to overcome the limitations of this type of sensors. Notwithstanding, biosensor possess a number of advantages such as their simplicity, that favours their use as a portable, and specially their low cost, thereby representing an attractive alternative for quality control within the food industry. Amperometric biosensors have been the most widely used and reported type of biosensors using an electrochemical approach (Tothill and Turner, 2003; Terry *et al.*, 2005), and thus this was the type of biosensor adapted for the following work and will be described in forthcoming chapters (Chapters 6 and 7). Although no biosensor, for food quality applications, has been yet fully commercialised, except of those developed by YSI Inc. (Yellow Springs, USA) and that recently developed by Abayomi and Terry, 2006, numerous studies have been published over the past years highlighting the application of this type of sensors to the food industry (**Table 2.9**).

Table 2.9: Examples of the range of analytes monitored in fruits and derivatives using amperometric biosensors

Analyte	*Food matrix*	*Enzyme used*	*Electrode configuration*	*Reference*
Alcohol	Beverages	Alcohol Dehydrogenase	SPCE modified with Meldola's blue	Sprules *et al.*, 1996
Alcohol	Wines	Alcohol oxidase	SPCE modified with Pt	Patel *et al.*, 2000
Amines	Apricots and cherries	Diamine oxidase and polyamine oxidase	A platinum hydrogen peroxide versus a silver/silver chloride	Esti *et al.*, 1998
Ascorbic acid	Fruit juices	Ascorbate oxidase	YSI 5739 model dissolved oxygen (DO) probes consists of Au(cathode), Ag–AgCl (anode), half-saturated KCl (electrolyte) and a teflon membrane.	Akyilmaz *et al.*, 1999
Ascorbic acid	Tropical fruits	Direct detection	SPCE mediated with rhodium	Jawaheer *et al.*, 2003
Ascorbic acid	Beverages	Direct detection	SPCE modified with electrografted o-aminophenol film	Civit et al., 2008
Carbamate	Fruits and vegetables	Cholinesterase	SPCE modified with CoPC	Nunes, *et al.*, 1999
Fructose	Apple juice	Fructose dehydrogenase	O_2-type Clark using Hexacyanoferrate as a mediator	Xie *et al.*, 1990
Fructose	Citrus	Fructose dehydrogenase	Membrane mimetic gold modified electrode	Kinnear *et al.*, 1997
Glucose, fructose and ethanol	Wine	Glucose oxidase/fructose dehydrogenase/alcohol dehydrogenase	Two hybrid three-channel multibiosensors based on modified graphite surface	Miertus *et al.*, 1998
Glucose and lactate	Tomato juice	Glucose oxidase and lactate oxidase	thin layer flow-through cell equipped with a Pt dual electrode	Palmisano *et al.*, 2000
β-D-Glucose and	Tropical fruits	Glucose oxidase	SPCE mediated with rhodium	Jawaheer *et al.*, 2003

Analyte	*Food matrix*	*Enzyme used*	*Electrode configuration*	*Reference*
Total D-Glucose	Tropical fruits	Mutarotase and glucose oxidase	SPCE mediated with rhodium	Jawaheer *et al.*, 2003
Glucose	Wines	Gluocse oxidase	SPCE mediated with Prusian blue	
Glucose	Wines	HRP and Glucose oxidase	Silicon Microelectrode chips using Pt or MB as a mediators	Alonso Lomillo *et al.*, 2005
L-malic acid	Apple, potato and tomato	Malate dehydrogenase	SPCEs: Three electrode configuration mediated with rhodium	Arif *et al.*, 2001
L-malic acid	Wines	Malate dehydrogenase	SPCE mediated with Meldola's blue	Lupu *et al.*, 2003
Laminarin	Seaweeds	β-1,3-glucanase and glucose oxidase	O2 type electrode for continuous determination	Miyanishi *et al.*, 2003
Polyphenols	Tea	Laccase	O_2-type Clark	Ghindilis *et al.*, 1992
Polyphenols	Beer	Tyrosinase	SPCE: three electrode configuration	Cummings *et al.*, 2001
Polyphenols	Vegetables extract	HRP and DNA	Silica-Titanium electrode	Mello *et al.*, 2003
Polyphenols	Wines	Laccase	SPCE	Gomes *et al.*, 2004
Sucrose	Fruit juices	Sucrose phosporylase, phospoglutaminase and glucose dehydrogenase	Modified Carbon paste electrodes	Maestre *et al.*, 2001
Sucrose	Tropical fruits	Invertase, mutarotase and glucose oxidase	SPCE mediated with rhodium	Jawaheer *et al.*, 2003

Analyte	*Food matrix*	*Enzyme used*	*Electrode configuration*	*Reference*
Pyruvate	Onions	Pyruvate oxidase	SPCE modified with Meldola's blue	Abayomi *et al.*, 2006
Pyruvate	Onions	Pyruvate dehydrogenase	SPCE modified with Meldola's blue	Abayomi *et al.*, 2007

2.5.4 Amperometric Biosensors for Substrate Determination

The use of enzymes associated with amperometric transducers were the first type of biosensors proposed (Clark, 1962). These devices combined the specificity of enzymatic reactions and the analytical sensitivity of electrochemical detection systems. This type of biosensor was chosen for most of the current research and will be described in more detail in forthcoming chapters (Chapter 6 and 7). In this type of biosensor the enzyme is in direct contact with the electrochemical transducer, the enzyme generates or consumes an electroactive species in a stoichiometric relationship with its substrate or target analyte. The amperometric transducer allows the electrochemical reaction, oxidation or reduction, to proceed at the electrode surface giving rise to a current. Therefore, this current can be easily related to the concentration of the substrate.

Currently the most common enzymes used for the construction of amperometric biosensors are oxidases, which is probably associated with their low cost and high versatility. Oxidases catalyse the model reaction shown below.

$$\text{Substrate (S)} + O_2 \xrightarrow{\text{Oxidase enzyme}} \text{Product (P)} + H_2O$$

Oxidases are usually flavoproteins that use O_2 as an electron acceptor to regenerate the reduced enzyme during the reaction (Bardeletti *et al.*, 1991). New systems are developed in which a chemical mediator replaces O_2 as an electron acceptor and allow biosensor preformance at much lower operating potentials, reducing the interferences caused by other electrochemically active species found in many food matrices, such as berries (i.e. phenylpropanoids, AsA). A number of modified electrodes for the regeneration of oxidised enzymes have been developed. These are commonly based on the redox polymer containing *p* and *o* quinine groups adsorbed onto the surface of electrodes (Chaubey and Malhotra, 2002). A growing selection of mediators is being reported for the construction of amperometric biosensors during recent years (**Table 2.10**).

Table 2.10: Example of mediators used for the construction of amperometric biosensors (*modified from Abayomi, 2007*)

Type	Reference
Methylene blue	Kulys *et al.*, 1994a; Munteanu *et al.*, 2003; Arvand *et al.*, 2003
Toluidine	Molina *et al.*, 1999; Munteanu *et al.*, 2003;
Phenazines	Simon and Fabregas, 2004
Prussian blue	O'Halloran ***et al.,*** 2001; Ricci and Palleschi, 2005
Cobalt Phthalocyanine	Crouch *et al.*, 2005
Meldolas blue	Kulys *et al.*, 1994b; Mao and Yamomoto, 2000; Santos *et al.*, 2002; Lupu *et al.*, 2003**;** Munteanu *et al.*, 2003; Vasilescu *et al.*, 2003**;** Santos *et al.*, 2003
Ferrocene	Ghica and Brett, 2005
Ferrocene dicarboxylic acid	Colombari *et al*., 2007
Dichloro-indophenol	Hirano *et al*., 2001
Tetramethylphenylenediamine	Kavanagh *et al*., 2009
Ferricyanide	Ge *et al*., 1998
Benzoquinone	Qian *et al*., 1996

2.5.5 The transducer

2.5.5.1 Screen-Printed Electrodes (SPEs)

Currently, mass production techniques have been applied to the production of electrodes (Icaza and Bilitewski**,** 1993). The substitution of current electrodes for new disposable test strips is a powerful alternative for the food industry where *in situ* measurements are needed. Other methods adapted for the manufacture of biosensors include inkjet printing, air-brush and cavro deposition (Domínguez Renedo *et al.*, 2007). Using these new technologies, these devices are already manufactured on a scale of over millions per month and sold (Terry *et al.*, 2005). Screen-printed electrodes (SPE) are devices that are produced by printing different inks on various types of substrates. They have several advantages such as low cost, ease of use, versatility in configuration (**Figure 2.11**) and a wide range of ways in which

the electrode can be modified (Domínguez Renedo *et al.*, 2007). Potential problems with the mass production of disposable biosensors are reproducibility, sensitivity and linear range that need to be overcome with future research.

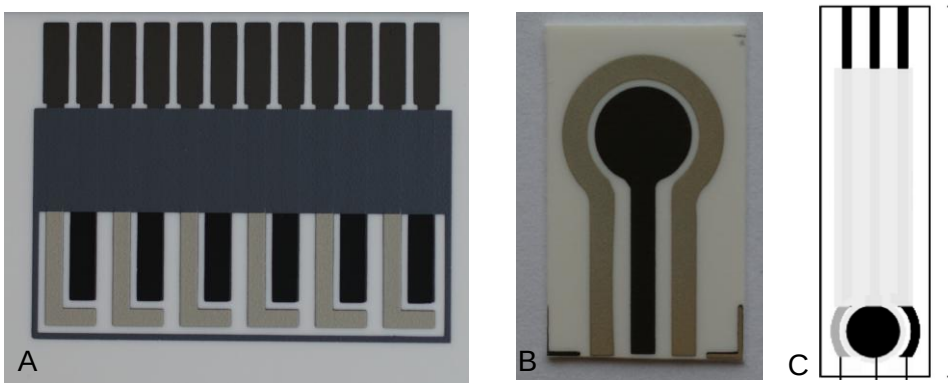


Figure 2.11: Different screen-printed electrode configuration used for the construction of amperometric biosensors. A: SPCE array in two electrode configuration. B: Single SPCE in two electrode configuration. C: Single SPCE in three electrode configuration.

2.5.5.2 Reactions at the electrode

Two types of reactions occur at the electrode. The first one, known as *non-Faradaic* reactions, is related with no charge being transferred. These types of reactions include phenomena such as adsorption and desorption, and also changes in the structure of the electrode-solution interface. In this case, when a potential is applied charge accumulates as a function of the potential across the interface and the composition of the solution analysed. The second type of reactions involves the transfer of a charge across a metal solution interface, resulting in oxidation or reduction. This phenomenon is called a *Faradaic process* and it is governed by the Faraday's law which states that the amount of chemical reaction caused by the current flow at the electrode surface is proportional to the amount of electricity passed. Both Faradaic and non-Faradaic reactions or processes occur when electrode reactions take place (Thevenot *et al.*, 2001).

Chemical reactions associated with charge separation and transfer may be described by the following:

$$O + ne^- \leftrightarrow R \qquad (1)$$

Where n is the number of electrons (e^-) transferred between the oxidant (*O*) and the reductant (*R*). When there is an equilibrium there is no net charge transfer, the chemical reaction can be described by the Nernst equation, which relates the electrode potential to the activity of the compounds participating in the electrode reaction (Riley and Tomlinson, 1987).

$$E = E^0 + RT/F \ln [O]/[R] \qquad (2)$$

Where E = Equilibrium potential; E^0 = Standard electrode potential; R = Gas constant; T = Absolute temperature and F = Faraday constant. The pre-logarithmic factor RT/F is known as the Nerst factor and its value can be found in the literature depending on the temperature.

When a potential is applied, an electrochemical compound can be oxidised or reduced resulting in a generation of current. The current-voltage characteristics of the system depends on several processes such as the transport of reactants in the electrolyte to the surface or the electrode, electrons transfer for the oxidation, reduction of electroactive chemical compounds and removal of the resulting reactants. Subsequently, the resulting electric charge (Q) is described by the following equation:

$$Q = \int_0^t + I\partial t = \frac{nFm}{M} \qquad (3)$$

Where I = current; m = mass of converted chemical compounds; M = molar mass of converted chemical compounds; N = number of electrons transferred for each molecule broken down and t = time.

2.5.5.3 Factors affecting electrode reaction rate and current

Electrode reactions, and thus the signal given by amperometric biosensors, depend on several factors. In general terms, the electron reactions or the current rate is governed by factors such as mass

transfer, electron transfer onto the electrode surface and chemical reactions evolving or preceding the electron transfer (Bard and Faukner, 1980)

At the same time, processes such as mass transfer depend on diffusion, convection or migration. Diffusion can be defined as the movement of ions or molecules due to concentration gradient (Bard and Faukner, 1980). The movement of these molecules occur from regions of high chemical potential to regions where the chemical potential is lower as described by Fick's first law.

$$Q = -D\left[\frac{\partial c}{\partial x}\right] \qquad (4)$$

Where J = coefficient of diffusion; D = coefficient of diffusion and dc/dx = concentration gradient

When convection or migration are omitted or not present, diffusion is the only mechanism to control the flux of reactants. This diffusion then is governed by Fick's second law, which relates to the change in concentration of the solute with time.

$$\frac{\partial c_s}{\partial t} = D\,\frac{\partial^2 c_s}{\partial x^2} \qquad (5)$$

The change in concentration with time at a specific location, x, is described by the differences in flux into and out of an element of width ∂x (Bard and Faukner, 1980).

2.5.5.4 Background signals

For the type of electrodes described in this work, when a potential is applied, non-Faradaic currents decay to a stable background level (**Figure 2.12**) that must be subtracted from gross signals to obtain the real signal given by the analyte measured. High background currents are in all cases undesirable. Working with amperometric biosensors, current leakage, including small potential differences in the electronic instrumentation, dissimilar metal contacts in wire leads of the biosensor, or electrochemically active impurities, such as phenylpropanoids, may all contribute to background signals (Buerk, 1993).

2.5.5.5 Sensitivity and detection limit

When the biosensor response is characterised two different modes are possible: steady-state and dynamic. The steady-state response can be defined as the variation step in current between the baseline or background level signal and the plateau reached after the addition of the analyte to the system (Bardeletti *et al.*, 1991). This step is directly proportional to the concentration of the analyte in the sample. In this context, the slope given by a calibration curve can be considered as the sensor sensitivity which is influenced by mass-transfer limitations and specifically by the membrane permeability and thickness being the sensitivity inversely proportional to the thickness of the membrane.

In addition, the detection limit is defined as the minimum concentration of analyte that can be detected by the biosensor considering an acceptable signal to noise ratio (Bardeletti *et al.*, 1991).

2.5.6 Enzyme kinetics

Generally, an enzyme-based amperometric biosensor either generates or consumes electrochemically active compounds in a stoichiometric with its substrate or target analyte (Bardeletti *et al.*, 1991). Consequently, the signal given by the biosensor is related to the amount of co-reactant consumed or electrochemically active co-product broken down and therefore dependent on the rate of change of reactants to products. Simple enzyme-substrate reactions may be represented by the Michaelis-Menten mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{K_2} P + E \qquad (6)$$

Where E = enzyme; S = substrate; ES = enzyme-substrate complex; P = product and k_1, k_{-1}, k_2 = rate constants. For such a reaction, the velocity (V) is a function of the change in concentration of substrate or product per unit time and can be estimated as follows:

$$V = \frac{dP}{dt} = k_2[ES] \qquad (7)$$

A rate equation which relates the velocity of the reaction to the substrate concentration was proposed by Michaelis and Menten (Voet and Voet, 1995):

$$V = \frac{V_{max}\,[S]}{K_M + [S]} \qquad (8)$$

Where V_{MAX} = maximum velocity and K_M is the Michaelis-Menten constant and is defined as follows

$$K_M = \frac{K_1 + K_2}{K_1} \qquad (9)$$

The equation 7 assumes that the ES complex is in a steady state and thus that after the initial phase, [ES] is constant and also that under saturation conditions, the entire enzyme is converted to ES complex. Thus when the entire enzyme is bound within the ES complex, the rate of formation of products is maximal.

In the type of biosensors considered in this work, at the working electrode, it is accepted that there is a net movement of analyte from the surrounding solution towards the enzyme-sensor system due to the diffusion gradient created by depletion of the analyte at the electrode surface. Besides, at the vicinity of the electrode, the enzyme binds to their specific substrate to yield the products. **Figure 2.12** shows a typical signal given by an enzymatic amperometric biosensor.

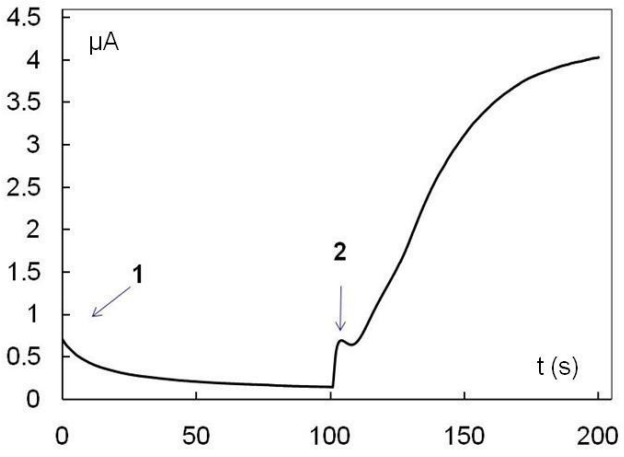


Figure 2.12: Electrocatalytic signal generated from an amperometric GOx-based biosensor. '1' shows non-Faradaic currents decay to a stable background level. '2' depicts the addition of glucose onto the electrode surface

Ideally, since the enzymes utilised for the construction of amperometric enzyme biosensors are specifically gathered to their substrate, the current generated and thus the signal given by the biosensor is originated exclusively by the enzyme-analyte reaction.

2.6 Conclusions

While the major constituents of ripe strawberry fruit are well known, little information is available regarding the biochemical composition of blackcurrants or the biochemical changes during ripening of this berry. Sugars and organic acids, especially from strawberry fruit, have been investigated during the last few decades with different perspectives. Nowadays there is enough evidence that sugar acid ratio can act as an index of fruit development and ripening and as an indicator of fruit quality status in terms of consumer acceptance (Pérez, *et al.*, 1997; Sturm, 2003). However, current routinely techniques applied in the soft fruit industry such as TSS, expressed as ºBrix, are poor indicators of the biochemical status of the fruit in terms of quality. Therefore there is a need for developing new, fast and reliable analytical techniques that can provide the industry and consumers with improved quality standards. There are indications that the soft fruit industry is changing its traditional approach to quality control and therefore, biosensors, which already have had a significant impact in medical diagnostics, offer an attractive alternative to the conventional techniques abovementioned. To date, most of the biosensors developed for fresh produce analysis involve the independent measurement of analytes using individual sensors. However, the information gained from this review in the composition of both strawberry and blackcurrant fruit has the potential to identify target analytes (major sugars and organic acids) that can be incorporated into a multianalyte biosensor to identify optimum soft fruit quality status and therefore provide the UK soft fruit industry with a powerful tool for improvement of current quality control.

CHAPTER 3

PRE-HARVEST MANIPULATION OF STRAWBERRY FRUIT QUALITY:

UNRAVELING THE IMPACTS OF DEFICIT IRRIGATION AND RELATED STRATEGIES

3.0 CHAPTER THREE

Pre-harvest manipulation of strawberry fruit quality: Unraveling the impacts of deficit irrigation and related strategies

3.1 Introduction

Epidemiological studies suggest that consumption of fruit and vegetables contributes towards reducing the risk of certain types of human cancer and cardiovascular diseases (Bazzano *et al.*, 2002; See Appendix C and D). Among fruits, berries are emerging crops with high economic value for which demand and availability have considerably increased over the past few years (FAO, 2007). In the particular case of strawberry fruits, such increase in consumption and demand has been driven, in part, by the highly desired taste of the fruit along with its potential health-promoting properties (Wang *et al.*, 2005; See Appendix E). The characteristic strawberry taste is, however, the result of complex biochemical pathways ending in an appropriate balance between sugars and acids and other taste-related compounds within the fruit. Indeed, sugar/acid ratio has been used as an indicator of strawberry fruit quality (Perez *et al.*, 1997; Terry *et al.*, 2005), degree of ripeness, and even consumer acceptability by several authors (Perez *et al.*, 1997; Keutgen and Pawelzik, 2007). Within the identified potential health-related compounds found in strawberries, vitamin C, anthocyanins, ellagic acid and other polyphenolic type compounds (Meyers *et al.,* 2003; Crespo *et al.*, 2010) are quantitatively the most abundant and hence have received particular attention (**Figure 3.1**). These bioactive compounds are generally secondary metabolites, mainly synthesised by the plant to attract seed disseminators or as a result of exposure to certain biotic or abiotic conditions (Mittler, 2002). Besides, certain bioactives may be over synthesised when the plant is exposed to certain stress conditions (Mittler, 2002).

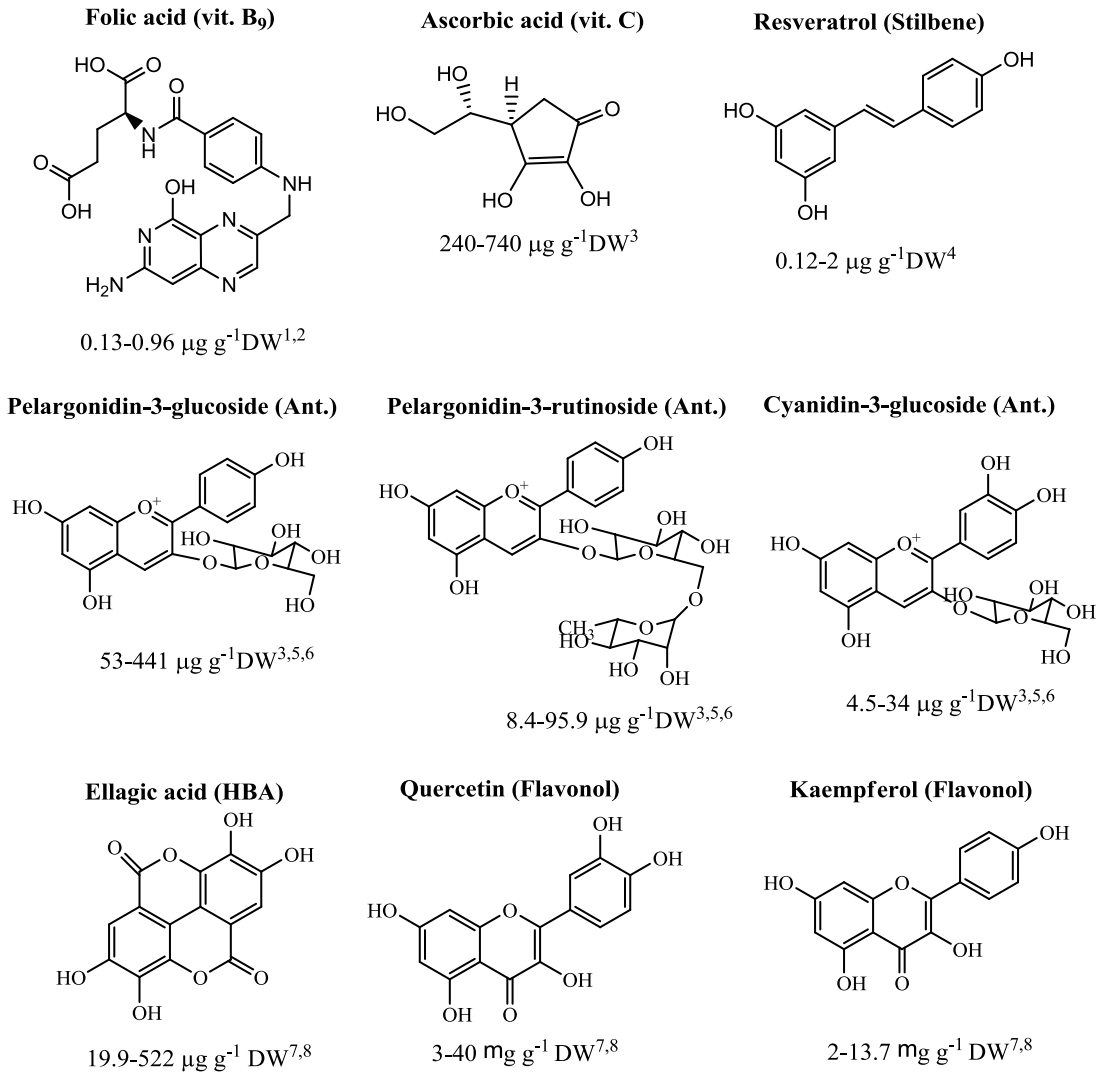


Figure 3.1: Chemical structure and reported concentrations of specific health-related compounds (viz. anthocyanins (Ant.), vitamins (vit.), flavonols, stilbenes and hydroxybenzoic acids (HBA)) in strawberry fruits ([1]Stralsjo *et al.*, 2003; [2]Tulipani *et al.*, 2008; [3]Terry *et al.*, 2007; [4]Wang *et al.*, 2007 ; [5]Wang *et al.*, 2003 ; [6]Kosar *et al.*, 2004 ; [7]Gil *et al.*, 1997 ; [8]Hakkinen and Torronen, 2000).

Over the past years, extensive breeding programmes in the UK and worldwide have aimed to attain fruits of higher quality and greater nutritional value. Nevertheless, strawberry fruit quality is generally a complex trait and hence its control represents a challenge for breeders. Earlier works have demonstrated the existent variability in the quality of fruits from different cultivars and growing locations (Cordenunsi *et al.*, 2003; Tulipani *et al.*, 2008; Crespo *et al.*, 2010) as well as the effect that certain pre-harvest treatments or cultivation practices have on strawberry fruit biochemistry (**Table 3.1**). However, most of the available information focuses on the concentration

of potential health-related compounds rather than taste-related compounds such as sugars and acids. Besides, most of the above-mentioned studies have been mainly conducted with individual cultivars rather than examining the response and variability of a range of genotypes. If considering the diversity of strawberry cultivation systems together with the vast number of cultivars available, elucidating the specific role that different pre-harvest factors may have on the quality of strawberries after harvest remains challenging. Enhancing the concentration of both taste- and health-related compounds in strawberries through different pre-harvest treatments may be a faster approach, if compared to breeding programmes, and may be an key strategy for the production of high-value fruits used not only to satisfy consumer demands but for the prevention and treatment of certain chronic diseases (Liu *et al.,* 2009).

Generally, strawberry plants are irrigated, given the sensitivity of the plant to drought stress during flowering and fruit ripening (Krüger *et al.*, 1999), and hence irrigation becomes one of the main factors that may affect strawberry fruit quality as has been already reported for other horticultural crops (Topcu *et al.*, 2007). Earlier studies conducted on strawberry plants revealed a positive correlation between fruit size, yield and irrigation conditions (Blatt, 1984; Serrano *et al.*, 1992; Krüger *et al.*, 1999; Liu *et al.*, 2007) where reduced water supply to the plant resulted in non-commercially viable sized fruits. Nonetheless, the impacts that deficit irrigation (DI) had on strawberry fruit physiology and biochemistry were recently highlighted to a degree by Terry *et al.* (2007). Indeed, despite berry size being detrimentally affected by DI, monosaccharides and more importantly the sugar/acid ratio or certain health-related components were generally much greater in DI-treated fruits (cv. Elsanta). In the context of climate change where growers are under increasing pressure to demonstrate that their water abstractions for irrigation are reasonable, justified and environmentally sustainable (Fereres and Soriano, 2007; Terry *et al.*, 2007; Leathes *et al.*, 2008) this chapter initially focuses on elucidating the response of different strawberry cultivars to deficit irrigation conditions when applied at different fruit developmental stages (*viz*. flowering, green and white stage). Further attempts to manipulate strawberry fruit quality were done by modifying the supplementation of nutrients to strawberry plants of a wide range of genotypes based on earlier evidence reported in the literature (Hargreaves *et al.*, 2008). Finally, and based on the results from initial experiments and that found by others (Wang, 1999), methyl jasmonate (MeJa), a natural hormone associated with the response of the plant to drought stress, was applied to either fully or deficit irrigated plants of three different strawberry cultivars to further understand the response of strawberry plants to drought stress conditions.

Table 3.1: Effect of different pre-harvest factors/cultivation systems on strawberry fruit quality and nutritional value.

Preharvest factors	Effect on bioactives	Reference
Conventional vs. organic cultivation systems	No effect on total phenolics	Häkkinen and Törrönen, 2000
Growing temperature	Strawberry grown at higher T (ºC) showed higher concentrations of bioactive compounds	Wang and Zheng, 2001
Cultural system (Hill plasticulture vs. matted row)	Hill plasticulture systems resulted in higher content of phenolics, flavonoids and ascorbate	Wang *et al.*, 2002
Soilless vs. conventional	No significant differences in the quality of the fruits	Recamales *et al.*, 2005
Ozone exposure	No significant effect on antioxidant activity or bioactives	Keutgen and Pawelzik, 2007
Salinity stress	Moderate salinity resulted in increase antioxidant activity and bioactives but generally lower sugar concentration	Keutgen and Pawelzik, 2007
Deficit irrigation	Higher content of certain anthocyanins, total phenolics and antioxidant activity	Terry *et al.*, 2007a
Inoculation with *Botrytis cinerea*[1]	No effect on strawberry (cv. Elsanta) bioactives or antioxidant activity	Terry *et al.*, 2007a
Organic and conventional nutrient amendments	No significant differences between treatments on antioxidant activity or sugar concentrations	Hargreaves *et al.*, 2008
Growing location (valley vs. mountain regions)	Lower concentration of both taste- and health-related compounds in plants grown in the mountain region	Crespo *et al.*, 2010

[1]At anthesis of primary flower from the primary truss.

3.2 Plant materials and experimental design

Different experiments were conducted, in either the glasshouse facilities at Cranfield or in collaboration with other institutions/growers, in order to reveal the effect that different pre-harvest conditions had on strawberry fruit quality. The different plant materials, origin of the plants and growing conditions are summarized in **Table 3.2**. Nevertheless, this chapter describes in detail only the results of those trials exclusively conducted by The candidate at Cranfield.

Table 3.2: Summary of strawberry trials and collaborative studies undertaken through the course of this thesis

Experiment	Year	Nº cultivars	Plant supplier/Origin	Growing conditions tested	Collaborative institution
3.1	2007	5	R.W. Walpole Strawberry plants ltd. (Norfolk, UK)	- Deficit (50 mL day^{-1}) or normal irrigation (200 mL day^{-1}) conditions from flowering to fruit harvest.	-
3.2	2007	8	H and H Duncalfe (Cambs., UK)	- Standard commercial growing conditions.	-
3.3	2008	14	Hargreaves Plants (Norfolk, UK)	- Normal irrigation conditions (200 mL day^{-1}) and nutrient supplementation (Half strength or full strength Hoagland's solution) from flowering to fruit harvest	-
3.4	2008	3	Switzerland and Italy	- Standard commercial growing conditions at two different swiss production sites	Agroscope ACW, Switzerland (Pamela Crespo)
3.5	2009	6	R.W. Walpole Strawberry plants ltd. (Norfolk, UK)	- Deficit (50 mL day^{-1}) or normal irrigation (200 mL day^{-1}) conditions from white stage to fruit harvest.	-
3.6	2009	3	Redeva (Dundee, UK)	- Deficit (50 mL day^{-1}) or normal irrigation (200 mL day^{-1}) conditions from green stage to fruit harvest and application of MeJa	-
3.7	2009	3	Local growers (Huelva, Spain)	- Standard commercial soiless growing conditions in different substrates.	Universidad de Huelva, Spain (Dr. Pedro Palencia)

3.2.1 Commercial variety assessments

Eight different strawberry cvs. (*viz*. Christine, Elsanta, Flamenco, Florence, Jubilee, Sonata, Symphony and Pearl) were selected according to individual differences in sugar/acid taste profiles (H. Duncalfe, H&H Duncalfe , *personal communication*). Plants were grown under standard commercial practices and supplied by H&H Duncalfe (Cambs., UK). Samples from this experiment (Exp. 3.2) were used for biosensor development (Chapters 5 and 6) and to assess the variability in the taste- and health-related composition from different commercial genotypes.

3.2.2 DI irrigation trials

Five and six different maiden year cold-stored strawberry cultivars (*viz*. Christine, Elsanta, Flamenco, Florence, Sonata, Symphony) were grown in a glasshouse during 2007 (April to July; Exp.3.1) and 2009 (May to July; Exp. 3.5), respectively, in 1 L capacity pots containing compost. Plants were supplied by R.W. Walpole Strawberry Plants Ltd. (Norfolk, UK). A completely randomised design was adopted for each trial. Water treatments started once the majority of primary fruits from the primary truss were at flower initiation or white stage for Exps. 3.2 and 3.5, respectively. Plants were irrigated with either 50 or 200 ml day^{-1} daily (*ca*. 09:00 h) based on earlier works (Terry, 2007a). In all DI experiments, prior to commencing water treatments, plants were kept at or near field capacity (*ca*. 0.8 m^3 of water per m^3 of soil).

3.2.3 Nutrient fertigation trial

Fourteen different cold-stored maiden-year A+ grade strawberry cultivars (*viz*. Cambridge Favourite, Daisy, Darlisette, Daroyal, Darselect, Elsanta, Florence, Honeoye, Jubidell, Mae, Pandora, Rosie, Sonata and Symphony) were grown during 2008 (Exp.3.3). Plants were supplied by Hargreaves Plants (Norfolk, UK) and grown in similar conditions to that described in section 3.3.2. A completely randomised design was adopted with each of three blocks containing 56 plants (14 cultivars x 4 replicates = 56 x 3 blocks = 168). During the course of the experiment, plants were irrigated with 200 ml day^{-1} (*ca*. 09:00 h). Additionally, all plants received a general N, P, K, Mg nutrient formulation, in the irrigation solution, twice a week but differing in their nutrient strength (half or full strength Hoagland's Solution: Norman, 1996; Terry, 2002). Due to the incidence of misshapen fruits and limited growth for certain cultivars, results are presented exclusively for 12 out of the 14 cultivars initially investigated.

3.2.4 MeJa combined with DI trial

Three different maiden year cold-stored strawberry cultivars (*viz*. '253/29', '279/4' and '279/5') were supplied by Redeva (Dundee, UK) and grown in a glasshouse during 2009 (April to July; Exp. 3.6) in 1 L capacity pots containing commercial standard compost (John Innes nº2) as described earlier. A completely randomised design was adopted considering cultivar, water treatments (50 or 200 ml day^{-1}) and MeJa treatments (none or 0.1 mM) as principal sources of variation. This trial (Exp. 3.6) was conducted to further understand the physiological and biochemical mechanisms behind deficit irrigation conditions. Prior to commencing water treatments plants were kept at or near field capacity (*ca*. 0.8 m^3 of water per m^3 of soil) for approximately three weeks. Water treatments started once the majority of primary fruits from the primary truss were at green stage of development (Anttonnen *et al.,* 2006). Plants were irrigated with either 50 or 200 ml day^{-1} over an eight-week periods daily (*ca*. 09:00 h). Methyl jasmonate (Sigma Aldrich, Dorset, UK) treatment at 0.1 mM + 0.05% Tween-20 (Wang, 1999) was applied as a foliar spray to incipient runoff at 3-days intervals. MeJa treatments started when fruits were at white stage of development (*ca*. 6 days after water treatments started). Similarly, control plants were sprayed with 0.05% Tween-20.

3.3 Materials and methods

3.3.1 Environmental monitoring

Soil moisture content (mV converted to m^3 water per m^3 of growing media) was measured daily (*ca*. 16.00h) by time-domain-reflectometry (TDR) using a Thetaprobe (ThetaKit type TK3, Delta-T devices, Cambs., UK). The water holding characteristics of the soil media were determined as described by Terry *et al.* (2007a). Hourly temperatures within the glasshouse were recorded by means of different Tiny Tag Ultra 2 data loggers (Gemini Data Logger, Sussex, UK), each shielded from solar radiation by a polystyrene cup and placed in each block.

Plants were treated, in all cases, to prevent incidence of spider mites (*Tetranychus* spp.) and powdery mildew (*Podosphaera aphanis*, formerly *Sphaerotheca macularis* (Wallr.: Fr) Jacz f sp. *fragariae* Peries) as described elsewhere (Terry and Joyce, 2000; Terry *et al.*, 2007a). In all experiments, flowers were hand pollinated with a sable paintbrush to minimise the occurrence of misshapen fruit.

3.3.2 Fruit and plant sampling

From each plant, mainly primary and secondary fruits from the primary truss were harvested according to developmental stage. All fruits were harvested at red stage which was considered as optimum ripeness. Days after anthesis (DAA) were monitored for primary and secondary fruits from the primary truss by tagging of flowers at anthesis (Terry and Joyce, 2000). Four fully expanded leaves per plant were excised towards the end of the trial, only for Exp.3.6, and the length and surface of the leaf immediately recorded.

Following objective colour measurements (*section 3.3.3*) berries with and without calyx and leaves weight were measured and recorded. Strawberry secondary fruits without calyxes were cut in half vertically, immediately snap-frozen in liquid nitrogen and stored briefly at -40°C before being freeze-dried in an Edwards Modulyo freeze drier (W. Sussex, UK) for 3 days at 0.015kPa. The same procedure was done for lyophilising leaf samples. Lyophilized samples were then ground in a pestle and mortar, weighed and returned to the freezer until use. All reagents were purchased from Sigma (Dorset, UK) unless otherwise stated.

3.3.3 Colour measurements

After harvest or excision, objective colour of fruits and leaves was measured using a Minolta CR-400 colorimeter and a DP-400 data processor (Minolta Co. Ltd., Japan) with an 8 mm light-path aperture, respectively. The instrument was calibrated with a Minolta standard white tile CR-400 (Y = 93.5, x = 0.3114, y = 0.3190). The mean of three readings at 3 equidistant points around the equatorial axis of the fruits were recorded and the lightness (L*), chroma (colour saturation; C*) and hue angle (H°) automatically calculated (Terry *et al.*, 2007b)

3.3.4 Extraction and quantification of sugars and non-volatile organic acids

Sugars were extracted using 62.5% (v/v) aqueous methanol as described elsewhere (Terry *et al.*, 2007a). Sugar content in strawberry extracts from fruits and leaves was determined using an Agilent 1200 series HPLC binary pump system (Agilent, Berks., UK), equipped with an Agilent refractive index detector (RID) G1362A. Strawberry extracts (20 μL) were diluted (1:10), and injected into a Rezex RCM monosaccharide Ca+ (8%) column of 300 mm x 7.8 mm

diameter (Phenomenex, CA, USA; Part no. 00H-0130-K0) with a Carbo-Ca^{2+} guard column of 4 mm x 3 mm diameter (Phenomenex,; Part no. AJ0-4493). Temperature of the column was set at 80ºC using a G1316A thermostated column compartment. The mobile phase used was HPLC grade water at a flow rate of 0.6 mL min^{-1} (Terry *et al.*, 2007a; Giné Bordonaba and Terry, 2008). Temperature of the optical unit in the detector was set up at 30ºC and temperature of the autosampler at 4ºC using an Agilent cooled autosampler G1330B. The presence and abundance of fructose, glucose and sucrose were automatically calculated by comparing sample peak area to standards (0.025-2.5 mg mL^{-1}) using ChemStation Rev. B.02.01.

Extracts for organic acids determination were prepared as described elsewhere (Terry *et al.*, 2007a). Briefly, freeze-dried strawberry fruit and leaf extracts (50 mg) were dissolved into 3 mL of HPLC grade water. Samples were kept at room temperature (25ºC) for 10 min and then filtered through a 0.2 µm syringe filter. L-ascorbic, citric, and malic acid contents in extracts were detected at 210 nm using the same HPLC system as described above equipped with a Agilent DAD G1315B/G1365B photodiode array with multiple wavelength detector. Extracts (20 µL) were injected into an Alltech Prevail Organic Acid column 250 mm x 4.6 mm diameter, 5 µm particle size (Alltech, CA, USA; Part no. 88645) with an Alltech Prevail Organic Acid guard column of 7.5 mm x 4.6 mm diameter (Alltech; Part no. 96429). The mobile phase was analytical grade degassed 0.2% (w/v) metaphosphoric acid in H_2O. The flow rate of the mobile phase was 1.0 mL min^{-1} under isocratic conditions and the column temperature was set up at 35 ºC. Temperature of the autosampler was set at 4ºC. The presence and quantity of each acid was calculated by comparing the peak area obtained with standards (0.02-2.0 mg mL^{-1}) using ChemStation Rev. B.02.01.

3.3.5 Extraction and quantification of anthocyanins

Individual anthocyanins were extracted using the methodology described in earlier works (Terry *et al.,* 2007a*;* Crespo *et al.*, 2010; Appendix B) by mixing 150 mg of freeze-dried fruit sample with 3 ml of 70% (v/v) methanol and 0.5% (v/v) HCl in HPLC-grade water. The slurry obtained was held at 35 ºC in a water bath with constant shaking for 1.5 h; mixing the samples every 15 min. Finally, the flocculate obtained was filtered through a 0.2 µm Millex-GV syringe driven filter unit (Millipore Corporation, MA) and the clear extract analyzed by HPLC coupled to Diode Array Detector (DAD). The anthocyanin profile of strawberry fruits was determined according to the method described by Crespo *et al.* (2010; Appendix B). Briefly the separation

was performed on an Agilent 1200 series HPLC binary pump system (Agilent, Berks., UK) equipped with an Agilent 1200s DA G1315B/G1365B photodiode array with a multiple wavelength detector. Strawberry diluted (1:5 v:v) extracts were injected (10 μL) into a Zorbax Eclipse XDB-C18 column of 250 mm x 4.6 mm diameter, 5 μm particle size with an XDB-C18 guard column of 12.5 mm x 4.6 mm diameter. The mobile phase consisted of 2% (v/v) acetic acid in HPLC-grade water (A) and 2% (v/v) trifluoroacetic in methanol (B) (Appendix B). Flow rate was set up at 1 mL min^{-1} and the column temperature set at 35 ºC using an Agilent G1316A thermostated column compartment. Temperature of the autosampler was set up at 4ºC using an Agilent G1330B cooled autosampler. Finally, eluted anthocyanins were detected at 520 nm and the presence and quantity of each anthocyanin was calculated by comparing peak area with standards of cya-3-gluc and pg-3-gluc (Extrasynthese, Lyon, France) using Agilent ChemStation Rev. B.02.01.

3.3.6 Extraction and quantification of other health-related compounds

Free ellagic acid was directly quantified from the same extracts as described above (section 3.4.5). Total ellagic acid concentrations were determined after an optimised acid hydrolysis method according to Vrhovsek *et al.* (2006) with some modifications. Briefly, 300 mg of strawberry freeze-dried extract were dissolved in 5 or 4 mL of 70% (v/v) aqueous methanol and 1 or 2 mL of 32 % HCl (v/v), respectively, making a final extract volume of 6 mL. Extraction was performed in a water bath at 90ºC during different time intervals (0, 60, 90, 120 and 180 min). The flocculate obtained after acid hydrolysis was filtered through a 0.2 μm Millex-GV syringe driven filter unit and the clear extract analyzed. Both free and total ellagic acid (EA) concentrations in fruits were determined using the same HPLC conditions as described in section 3.4.5. Eluted EA concentrations were detected at 254 nm and quantification was performed by comparing peak area with ellagic acid standard (Sigma Aldrich), retention time and literature data (Koponen *et al.,* 2007) using Agilent ChemStation Rev. B.02.01. Stock solutions of ellagic acid standard (1.0 mg mL^{-1}) were prepared daily before analysis by dissolving it in two part of dimethyl sulfoxide (DMSO) followed by two parts of methanol (Koponen *et al.,* 2007). Working standard solutions were then prepared by dissolving initial stock solution in methanol.

3.3.7 Antioxidant capacity measurements

Antioxidant capacity from strawberry fruits and leaves was measured using the FRAP assay as described in earlier works (Benzie and Strain, 1996; Terry *et al.*, 2007) with some modifications. A 50 µL aliquot of diluted sample extract (1:9; v/v) or Fe^{2+} ($FeSO_4$. $7H_20$) standards (0 – 5.0 mM) was added to 3.6 mL of freshly prepared FRAP working solution (*viz*. 5 mL of 10 mM TPTZ (2,4,6-tripyridyl-2-triazine) in 40 mM HCl + 5 mL of 10 mM $FeCl_3$ in 50 mL of 300 mM acetate buffer). The reaction mixture was incubated at 37°C for 10 min and absorbance measured spectrophotometrically at 593 nm using a Camspec M501 UV/Vis spectrophotometer (Camspec Ltd., Cambs., UK). Antioxidant capacity was expressed as the concentration of antioxidants having a ferric reducing ability (mmols Fe^{2+} g^{-1} DW).

3.3.8 Estimation of strawberry taste parameters

Sweetness index (SI) for the different cultivars analysed was calculated as previously described (Keutgen and Pawelzik, 2007a). Briefly, the contribution of each major sugar found in strawberry fruits was calculated considering that fructose and sucrose are 2.3 and 1.35 times sweeter than glucose, respectively. Accordingly, SI = 1.0 [Glucose] + 1.35 [Sucrose] + 2.3 [fructose].

3.3.9 Statistical and chemometric data analysis

All statistical analysis were carried out using Genstat for Windows, Version 10 (VSN International Ltd., Herts., UK). Data for each experiment, and whenever possible between experiments, were subjected to analysis of variance (ANOVA) tests based on a completely randomised design within blocks. Least significant difference values (LSD; P = 0.05) were calculated for mean separation using critical values of t for two-tailed tests. Correlations between experimental variables were made using Spearman's Rank Correlations and, if required, presented as Spearman's Correlation Coefficient (r) and P value based on a two-tailed test. Unless otherwise stated, significant differences were $P < 0.05$. Where no significant differences between factors were found, results are presented, if required, as mean values ± standard deviation of the means. Chemometric data analysis was performed using the same software as described above. Concentration values for taste-related compounds were used as an input analytical data for principal component analysis (PCA). Dimensions of the corresponding data matrix were > 500 samples, corresponding to the triplicate values of each sample and different

sets of variables. Data was autoscaled in order to provide similar weights for all the variables as described elsewhere (Bereton, 2007).

3.4 Results and discussion

3.4.1 Soil water status and water use efficiency of DI treated plants

Soil water content values of the growing media differed between treatments but also between cultivars when submitted to DI conditions, indicating the existence of genotypic differences in the response of strawberry plants to drought stress, as well as differences in water usage between different cultivars. In all DI experiments (Exp. 3.1, 3.5 and 3.6), values for non-DI-treated plants were consistent between cultivars, ranging from 0.73 to 0.90 m^3 of water per m^3 of growing media (**Figure 3.2**). Similar water contents were observed by Terry *et al.* (2007a) when assessing water DI in cv. Elsanta fruits grown under comparable conditions. When DI was applied from flower initiation, greater differences between water content of soils from water stress plants and plants kept at or near field capacity were observed for cv. Elsanta during the whole duration of exp. 3.1 (**Figure 3.2**). Elsanta plants grown under drought stress conditions used more water (up to 20% more) from the growing medium during the first days after commencing water treatments than the rest of cultivars. Water usage of DI-treated plants for cvs. Sonata and Symphony were similar during Exp. 3.1, as was also observed for cvs. Florence and Christine. Nevertheless, drought stress applied at later developmental stages (i.e. white stage; Exp. 3.5) resulted in greater differences between irrigation regimes in plants from cvs. Christine, Florence and Symphony. The observed higher water uptake for certain cultivars may be the result of either the increase in root growth or root hydraulic conductivity or even greater water usage as compared to other cvs. (Savić *et al.*, 2008). Yuan *et al.* (2004) already reported that water use efficiency was increased in plants (cv. Sachinkoka) that received less water. As reported by others (Krüger *et al.*, 1999; Terry *et al.*, 2007a), differences in water uptake between plants from different experiments (Exps. 3.1 and 3.5) highlighted that the sensitivity of the plant to drought stress, during flowering and fruit ripening, was strongly influenced by the genotype and the timing of when drought conditions started. In all experiments, water content in the growing media of DI-treated plants declined following similar water-soil dynamics to that previously described (Liu *et al.*, 2007; Terry *et al.*, 2007a; Savic *et al.*, 2008).

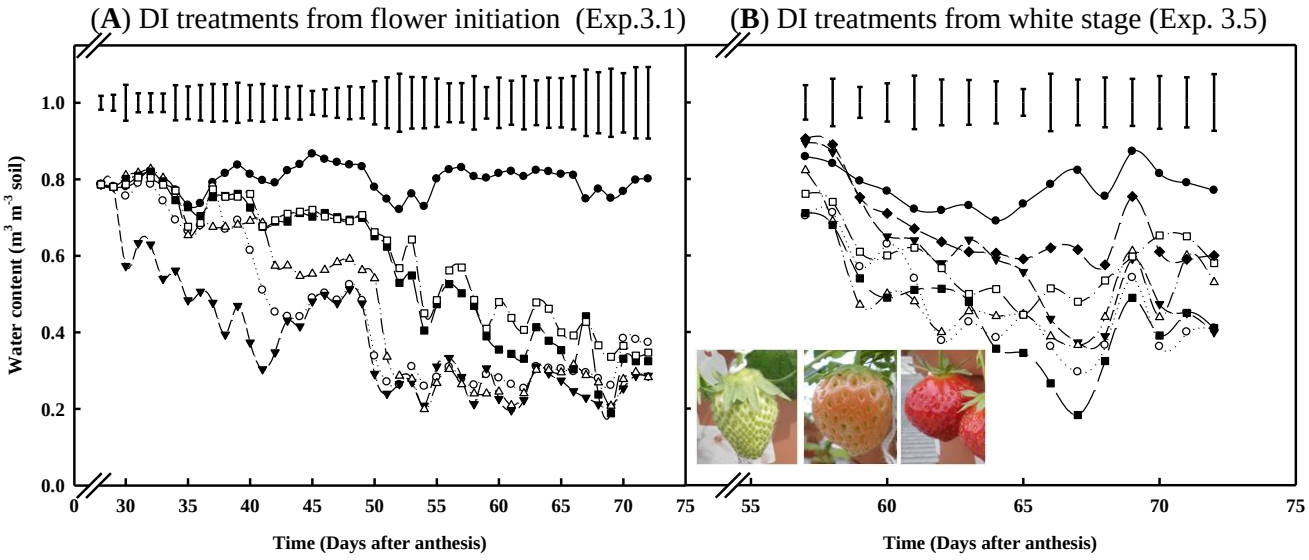


Figure 3.2: Water volume of the growing media of five different strawberry cultivar (Symphony (-○-), Elsanta (-▼-), Sonata (-Δ-), Florence (-■-), Christine (-□-) and Flamenco (-♦-)) grown under deficit irrigation (50 mL day^{-1}) conditions. Water volume of growing media from fully irrigated (-●- 200 mL day-^{1}) plants was similar between cultivars and values are presented as the mean per day of the five (Exp. 3.1; A) or six (Exp.3.5; B) cultivars . Error bars indicate LSD ($P < 0.05$) value for the daily interaction cultivar*water treatment. LSD ($P < 0.05$) values for the overall interaction days*cultivar*water treatment were 0.182 (A) and 0.146 (B).

To better understand the response of strawberry plants to drought stress conditions, methyl jasmonate (MeJa), a natural hormone associated with the response of the plant to drought and other biotic/abiotic stresses (Wang *et al.*, 1999), was applied in either fully or deficit irrigated plants from three different strawberry cultivars. Plants from different selections (*viz*. 253/29, 279/4 and 279/5) responded differently to drought stress conditions as well as to pre-harvest foliar application of MeJa. Whereas the pre-harvest application of this stress-related hormone did not have an effect on the amount of water extracted from the growing media in plants from cv. 253/29, higher or lower water abstractions were observed in MeJa-treated plants from cultivars 279/4 and 279/5, respectively (**Figure 3.3**).

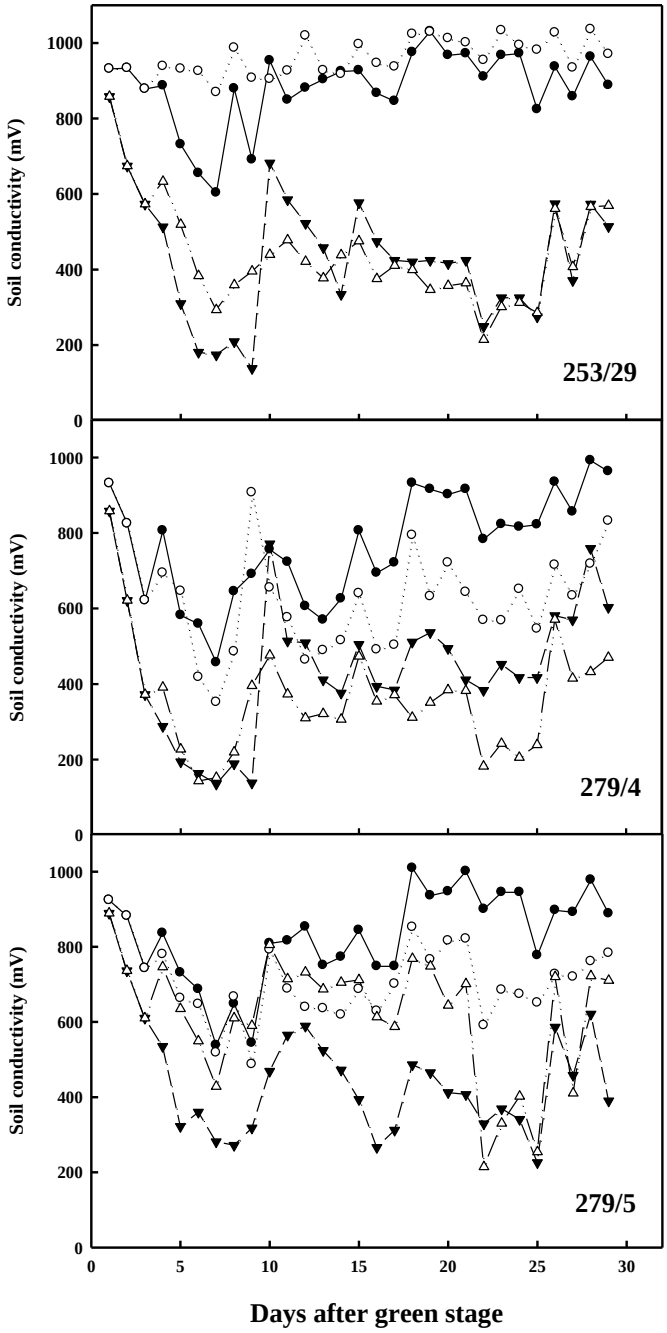


Figure 3.3: Soil conductivity (mV) of the growing medium of three different strawberry cultivars (viz. 253/29, 279/4, 279/5) grown under deficit irrigation (50 mL day^{-1}) or normal irrigation conditions (200 mL day^{-1}) and treated or not with 0.1 mM MeJa . (●) Control; (○) Control + MeJa; (▼) Deficit and (Δ) Deficit + MeJa. Values represent mean conductivities for (n = 6) plants per cultivar and treatment. LSD ($P < 0.05$) value for the overall interaction days*cultivar*water treatment was 307.

The mechanisms by which MeJa may act on the response of the plant to wounding or drought stress conditions are vast and still remain unclear for most cultivated species (Wang *et al.*, 1999). Earlier works conducted on model plants or other horticultural crops showed the effect that MeJa had on root development (Staswick *et al.*, 1992; Maksymiec and Krupa, 2007). For instance, primary root growth was inhibited by 50% when seedlings of *Arabidopsis thaliana* were grown on medium containing MeJa (Staswick *et al.*, 1992). Visual inspection of root development for each plant, at the end of the trial, indicated the positive correlation between the capacity of the plant to extract water and root development but supported only partially the associations between MeJa and root development found by others (Staswick *et al.*, 1992; Maksymiec and Krupa, 2007). In detail, no differences were observed in visual root development of MeJA-treated or control plants from cv. 253/29, however, MeJA applied to DI-treated plants from cultivar 279/5 resulted in reduced root growth and hence accounting, at least in part, for the observed higher soil conductivities recorded for these plants. In contrast, greater root development was observed in MeJA treated plants of cv. 279/4 regardless of the water treatment applied (**Figure 3.4**).

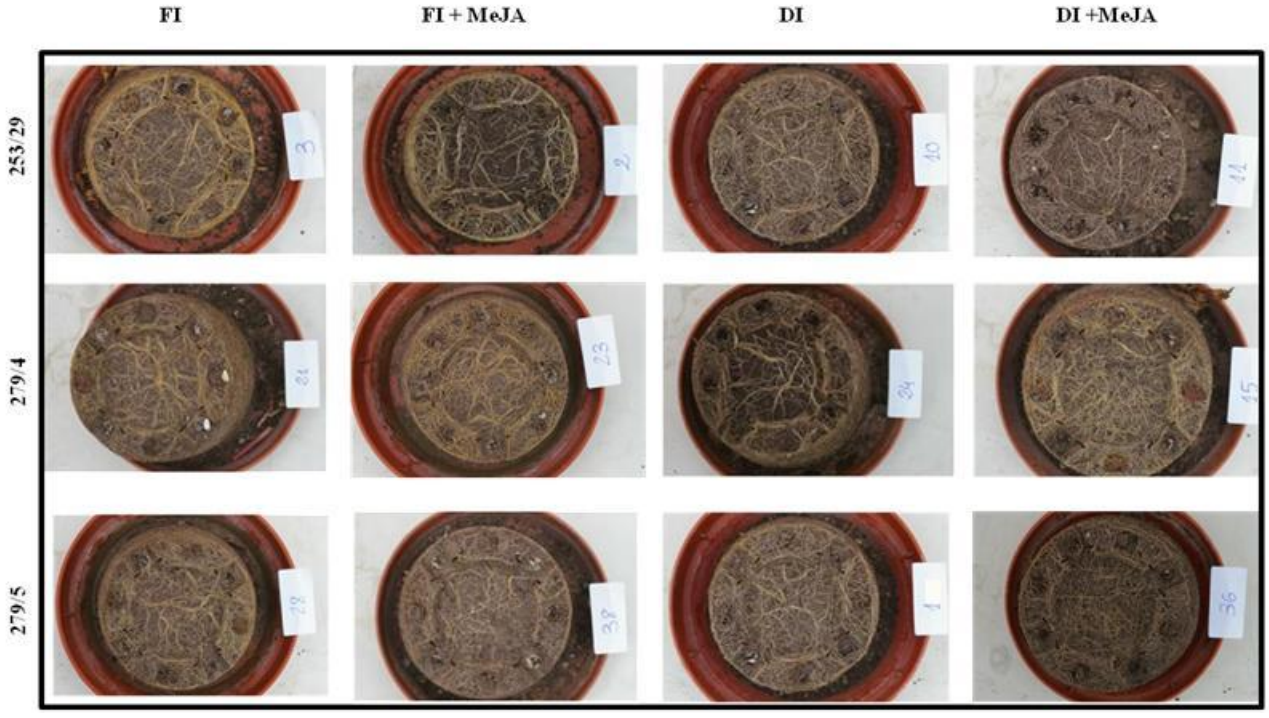


Figure 3.4: Visual investigation of root development from strawberry plants from different cultivars (viz. 253/29, 279/4, 279/5) grown under deficit irrigation (DI: 50 mL day^{-1}) or normal irrigation conditions (FI:200 mL day^{-1}) and treated or not with 0.1 mM MeJa (Exp.3.6).

Differences between this and early studies may be the result of different species being investigated as well as differences in the way and dosage of MeJa. Besides, growing the plants in 1L capacity pots may have restricted normal root development as well as up-regulated the synthesis of abscisic acid (ABA) and other stress-hormones as reported by others (Giannina *et al.*, 1997), hence affecting the response of the plants to exogenous application of MeJa.

3 .4.2 Effect of DI and related strategies on fruit physiology

In all the experiments, the response to water stress on fruit physiology was dependent on genotype. Strawberry fruit size is governed by the interaction between several inherent factors, including blossom position, the number of developed achenes, fruit competition, and leaf number (Janick and Eggert, 1968). Berry weight from secondary strawberry fruits was significantly reduced (approximately 30% lower) by DI in cvs. Symphony, Elsanta and Sonata when DI conditions started at flowering stage (Exp. 3.1; **Table 3.3**). Nevertheless, both cvs. Christine and Florence showed similar weights for both water-stressed and non water-stressed plants, regardless on the time of application of DI conditions (**Table 3.3**). Concomitant to this and in those cultivars where berry size was reduced, dry matter as a proportion of fresh weight was considerably higher in fruits from water-stressed plants as compared to plants kept at or near field capacity. Previous studies also showed that fruits from strawberry plants that received full irrigation had higher water content and greater berry fresh weight as compared to plants grown under reduced irrigation (Blatt, 1984; Serrano *et al.,* 1992; Krüger *et al.,* 1999; Kirnak *et al.*, 2001; Kirnak *et al.*, 2003; Liu *et al.,* 2007; Terry *et al.,* 2007a). In Exp. 3.1, fruits from cv. Elsanta showed the greatest increase (1.24-fold higher) in dry matter content as a proportion of fresh weight in response to reduced water supply. These findings were in agreement with Terry *et al.* (2007a), since strawberry fruits (cv. Elsanta) from DI-treated plants also showed one quarter higher dry matter as compared to non-water stressed plants. Similarly, Kirnak *et al.* (2003) reported higher soluble dry matter content for cvs. Oso Grande and Camarosa fruits subjected to DI in trials conducted in the field. Differences in fruit physiology among cultivars may be accounted by differences in ABA or other hormones in plants exposed to drought stress. For instance, the plant hormone ABA regulates various physiological reactions in plants and its role in the response to drought stress is known for many horticultural crops (Seki *et al.,* 2007). In

strawberry plants (cv. Elsanta), grown under comparable DI conditions, ABA was substantially but not significantly greater than that of fully irrigated plants (Terry *et al.*, 2007a).

Table 3.3: Effect of water deficit irrigation (50 mL day^{-1}) or full irrigation (200 mL day^{-1}) on weight characteristics of secondary strawberry fruits from the primary trusses of six different cultivars. Water treatments started when the majority of secondary fruits from each cultivar were at flower initiation or white stage for Exps. 3.1 (2007) and 3.5 (2009), respectively.

	W (g)				Dry matter (g 100g^{-1} FW)			
Time of DI application	Flowering		White stage		Flowering		White stage	
Cv/ Irrigation (mL day^{-1})	200	50	200	50	200	50	200	50
Christine	6.34	5.90	10.20	9.08	12.55	13.95	13.21	13.13
Florence	7.66	7.98	10.37	8.92	12.07	12.00	12.02	13.91
Symphony	10.20	7.36	9.40	7.70	9.57	11.26	12.38	12.72
Sonata	11.97	7.24	11.54	8.27	11.52	14.29	13.43	15.29
Flamenco[a]	-	-	8.14	7.98	-	-	13.92	15.20
Elsanta	9.63	7.24	9.77	7.10	10.98	14.75	14.11	14.30
LSD ($P < 0.05$)	1.301		1.396		1.121		1.378	

[a]Cultivar Flamenco was only introduced in experiment 3.5 and hence results are only available when DI was applied at white development stage

Plant responses to either water or salt stress have been reported to have much in common (Munns, 2002). For instance, salinity reduces the capacity of plants to take up water which may result in reduce growth rate and metabolic changes similar to that observed in plants grown under water stress. In this context, strawberry plants grown under high salinity conditions resulted in both higher (Awang and Atherton, 1995) and/or lower (Keutgen and Pawelzik, 2008b) dry matter content. The greater dry matter content observed in the results from experiments 3.1 and 3.5 (**Table 3.3**) suggests a concentration effect by either limitation of water uptake and/or enhanced import of solutes into the fruit. Strawberry growth follows a double sigmoidal curve with two rapid growth periods generally attributed to water accumulation into the fruit (Coombe, 1976). Hence, the most pronounced effect of Exp. 3.1 on fruit weight and dry matter content may be related to limiting fruit water uptake during both water accumulation

periods instead of only the last one as occurred for plants submitted to DI from white stage (Exp.3.5). In Exps. 3.1 and 3.5, dry matter content from other parts of the plants were not investigated and therefore, it is difficult to state whether or not the increased dry matter may be the result of a trade off in resource allocation within the plant (e.g. vegetative and root growth versus fruit growth or trade off between leaves and fruits). This said, the dilution effect observed in plants that received more water was limited to certain cultivars since fruits from cv. Christine and/or Florence showed similar dry matter content between DI-treated and non-water-stressed plants (**Table 3.3**). Dry matter content from leaves was, however, investigated in samples from Exp. 3.6. Whereas no differences were observed in dry matter content of leaves from different cultivars or submitted to different irrigation regimes, foliar application of MeJa resulted in lower dry matter content (35.8 mg DW $100g^{-1}$ FW) as compared to control plants (36.5 mg DW $100g^{-1}$ FW). Nevertheless, there was a significant interaction between MeJa, irrigation treatments and cultivar which indicated that different cultivars responded in a different manner to the combination of treatments applied (**Table 3.4**). In contrast to the results presented earlier (Table 3.4) and that reported by others (Blatt, 1984; Serrano *et al.*, 1992; Krüger *et al.*, 1999; Kirnak *et al.*, 2001; Kirnak *et al.*, 2003; Liu *et al.*, 2007; Terry *et al.*, 2007a) no significant differences were observed for dry matter content of fruits from plants (cvs. 279/4, 279/5 and 253/29) submitted or not to DI conditions (Exp. 3.6). Differences between experiments may be due, in part, to different cultivars being investigated as well as the application of drought stress conditions at later fruit development stages than in Exp. 3.1. Besides, plants from Exps. 3.1 and 3.5 were June-bearers cultivars whereas samples from Exp. 3.6 were neutral day varieties which generally give smaller fruits.

In Exp. 3.6, plant vegetative growth in fully irrigated plants from cvs. 279/4 and 279/5, but not in cv.253/29, was detrimentally affected by DI conditions, since lower runner biomass was recorded in plants that received less water. Similar results were observed by others when investigating the effects of salinity on strawberry production (Awang and Atherton, 1995; Keutgen and Pawelzik, 2008b). Other indicators of vegetative growth including foliar density and leaf length were not affected by any of the treatments applied (data not shown). Yuan *et al.* (2004) also reported that strawberry plants that received more water had greater runner biomass as compared to plants receiving lesser amounts. Pre-harvest application of MeJa was observed to improve plant vegetative growth regardless of the water treatment (**Table 3.4**), and hence confirmed, at least in part, the findings by Wang (1999) in which foliar application of MeJa

render strawberry plants to withstand better drought stress conditions. Greater runner biomass, or plant biomass above the ground level, is indeed a desired attribute in strawberry nurseries where propagation rather than fruit quality is the main target, and consequently, the application of low concentrations of MeJa should be further investigated.

Table 3.4: Runners biomass (g plant^{-1}) and weight characteristics of fruits and leaves from three strawberry cultivars grown under deficit irrigation (50 mL day^{-1}) or full irrigation (200 mL day^{-1}) and treated with 0 or 0.1 mM foliar application of MeJa. Water treatments started when the majority of secondary fruits from each cultivar were at green stage.

	Runners weight (g)				Dry matter (%) leaves				Dry matter (%) fruits			
Irrigation	50		200		50		200		50		200	
Cv/ MeJa (mM)	0.1	None	0.1	None	0.1	None	0.1	None	0.1	None	0.1	None
253/29	18.8	17.3	18.6	4.2	34.2	34.8	34.9	39.3	11.7	11.28	14.11	13.84
279/4	24.7	16.2	55.9	31.4	36.2	37.2	35.0	36.9	15.8	14.37	14.62	14.98
279/5	13.3	6.1	35.9	34.8	35.1	37.6	36.6	36.2	14.43	13.15	11.88	13.97
LSD ($p<0.05$)	26.33				2.41				7.56			

Despite differences in fruit weight and dry matter content of fruits from plants grown under different irrigation conditions, manipulating the strength of the nutrient solution resulted in no significant differences in either fruit size or dry matter content from fruits in a range of cultivars (**Figure 3.5**). Others have already showed that high nutrient concentrations during flower formation of strawberries (cvs. Elsanta or Korona) favoured final fruit size (Terry, 2002; Opstad and Sonsteby, 2008). In contrast, Andriolo *et al.* (2009), reported that not only fruit size, but plant yield and growth were reduced by increasing the concentration of a general NPK nutrient solution. Nevertheless, in the former study, nutrient solutions were provided daily and during the whole growing period instead of in three day intervals as described herein or during specific developing periods as described by Opstad and Sonsteby (2008). Since nutrient solutions are an important source for both Na^+ and K^+ it may be reasonable to speculate that the results obtained by Andriolo *et al.* (2009) would rather reflect the effect that moderate salinity stress has on strawberry fruit quality as earlier pointed out by others (Keutgen and Pawelzik, 2008). Minor effects of other nutrient applications (i.e. potassium, K) on yield performance of strawberry plants have been demonstrated by others in the past (Albregts *et al.*, 1996; Miner *et al.*, 1997). In experiment 3.3, however, nutrient concentration differing in up to 2-fold in specific nutrient concentrations (N, P, K, Ca and Mg) were not likely to be sufficient to reveal any positive or negative effect on fruit physiology (**Figure 3.5**).

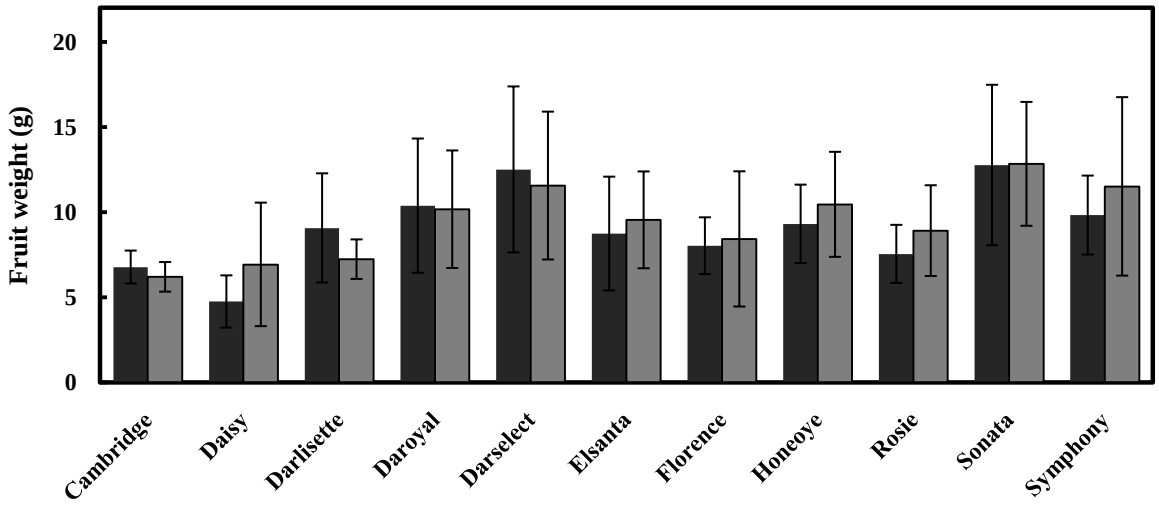


Figure 3.5: Fruit weight of secondary fruits from the primary truss of twelve strawberry cultivars grown under normal irrigation conditions (200 mL day^{-1}) and receiving half (■) or full (■) strength modified nutrient Hoagland's solution every three days interval.

Anthesis occurred significantly ($P < 0.05$) later in water stressed plants for cvs. Elsanta, Florence and Christine and was generally reduced for the other cultivars investigated. In a similar manner, fruit maturation was also slower for some water-stressed cvs. (*viz*. Elsanta and Christine) taking 33 days after anthesis for plants treated with 50 mL of water per day in comparison to 31 days for plants receiving 200 mL per day (**Figure 3.6**). Time from anthesis to harvest for the other cvs. investigated was not affected by either DI (**Figure 3.6**) or the strength of the nutrient solution (**Figure 3.7**) . Terry *et al.* (2007a) also found that fruit maturation was slower (not significantly) in Elsanta fruit grown under drought stress conditions. Indeed, it has been shown that for certain crops, drought stress may result in considerable increase of the time to anthesis and to physiological maturity (Geerts *et al.*, 2008). In these lines, DI could be adopted as an strategy to extend fruit availability matching with periods of greater consumer demand.

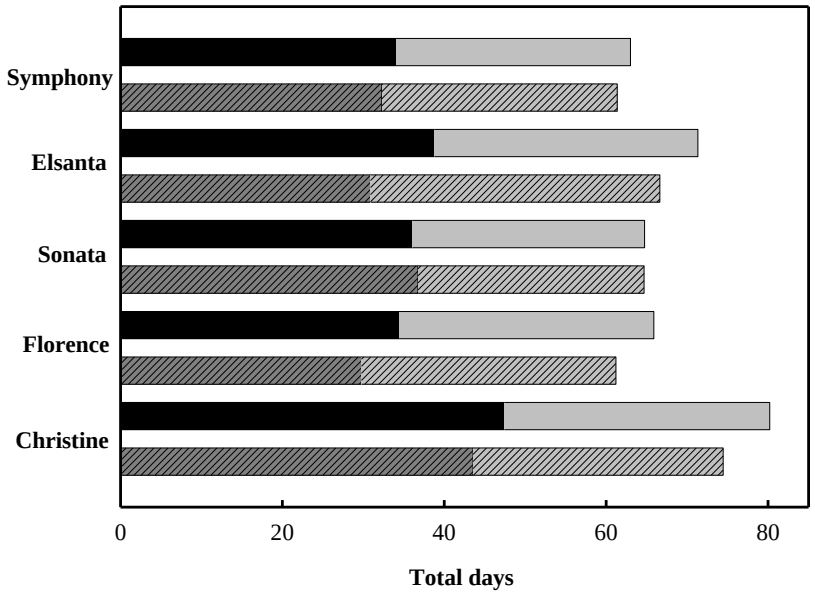


Figure 3.6: Days of anthesis (▬) and post anthesis (PA; ▬) of five different cultivars grown under normal irrigation (NI; striped bars, 200 mL day^{-1}) or deficit irrigation (DI; solid bars, 50 mL day^{-1}). LSD value for days of anthesis = 2.6; LSD value for days post anthesis = 2.9.

Little information is available which describes the effect of nutrient supplementation on flower and fruit development (Opstad and Sonsteby, 2008). High nitrogen application was shown to hasten flower bud development (Yoshida *et al.*, 1992) and even advance in a moderate manner the ripening period of strawberry fruits (Vang-Petersen, 1998). In Exp. 3.3, different cultivars had variable lengths of the ripening period (**Figure 3.7**), but differences in the

concentrations of nutrients supplied to the plant did not either slow down or speed up the ripening period (**Figure 3.7**). Shorter ripening of the fruits, as determined by the numbers of days from anthesis to fruit harvest, were encountered in fruits from cvs. Daroyal and Honeoye. Differences between the results from Exp. 3.3 and proceeding literature may be that N and other nutrient concentrations (i.e. 8.88 g N Kg^{-1}) in the growing media were too high to allow any differences between the treatments applied.

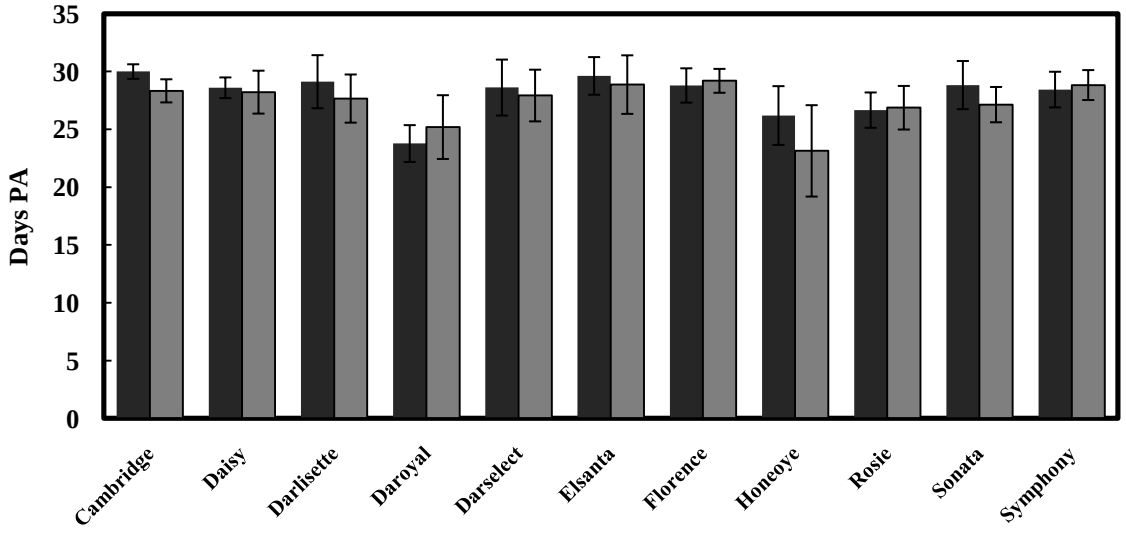


Figure 3.7: Days of post anthesis until harvest (PA) from twelve strawberry cultivars grown under normal irrigation conditions (200 mL day^{-1}) and receiving half (■) or full (■) nutrient strength modified Hoagland's solution every three days interval (Exp. 3.3). Error bars indicate standard deviations (n = 168).

3.4.3 Effects of DI and related strategies on fruit colour

Colour of strawberries is without doubt one of the main attributes which governs consumer perception and therefore acceptability (Francis, 1995; Garzón andWrolstad, 2002; Hernanz *et al.*, 2008). Objective colour of each fruit was measured, in each experiment, when fruit was adjudged to be at optimum ripeness (when fully red) and significant differences were encountered between the different cultivars investigated for each experiment (**Figure 3.8**; **Table 3.5**). Similarly, significant differences in objective colour have been reported by others when

studying different cultivars (Sacks and Shaw, 1994; Hernanz *et al.,* 2008; Capocasa *et al.,* 2008; Crespo *et al.,* 2010) or between fruits from plants grown under different conditions (Hernanz *et al.,* 2008). Generally, though, no significant differences were observed for colour (L*, C*, H°) parameters between each secondary fruits within a cultivar. Nonetheless, even if all fruit were picked at the full red stage, significant differences were encountered for C* values between fruits from Elsanta regardless of the water application regime. Accordingly, previous work carried out by Terry *et al.* (2007a) also found significant differences in the objective colour of strawberry cv. Elsanta fruits when harvested at optimum maturity. Generally, DI had a considerable effect on objective fruit colour, especially when drought stress was initiated at flowering developmental stage. In this particular case (Exp. 3.1), plants receiving 50 mL of water per day showed lower C* values as compared to non-water-stressed plants (**Table 3.5**). These differences were especially highlighted for cvs. Elsanta, Sonata and Florence ($P < 0.05$). The effect of DI on L* and H^o values was, similarly, dependent on the cultivar and time of application of DI conditions. Some cultivars (*viz.* Elsanta and Symphony) showed higher values for lightness (L*) and lower redness (higher H^o) for plants receiving 50 mL per day as compared to non-water-stressed plants. The remaining cultivars tended to show lower L* and H° values for DI-treated plants (**Table 3.5**). Likewise, Terry *et al.* (2007a) also found higher Hue angles in cv. Elsanta fruits treated with 50 ml day^{-1} as compared to plants receiving either 100 or 200 ml day^{-1}. Strawberry fruit colour is due, in part, to anthocyanins pigment concentrations which account for the attractive red/orange colour of the fruits. It will be expected, therefore, that if lower red coloration (higher H^o) is reported for water-stressed plants, lower anthocyanins will also be encountered in the fruit. However, in the study carried out by Terry *et al.* (2007a), and as will be presented later, DI resulted in lower redness but higher concentration of pelargonidin-3-glucoside and derivatives of this anthocyanin on a FW basis. In view of that, the authors suggested that the lower redness in smaller fruits was most probably an artefact of the objective colorimeter measurement since shorter distances between achenes will exist in smaller fruits resulting in lower recorded redness when measured with an 8mm aperture. These findings were further confirmed in experiments 3.5 and 3.6, since lower reductions in berry size, as a result of later application of drought stress conditions, were accompanied by lower differences in H^o values between DI and FI plants (**Table 3.5**).

Colour characteristics of fruits, from 12 different cultivars, were also investigated in plants that received a standard nutrient formulation but differing in their strength. In this

particular case, and despite significant differences between cultivars, no significant differences were encountered between fruits from different treatments (**Figure 3.8**).

Table 3.5: Objective colour characteristics (L*,C*, Hº) from different strawberry cultivars grown under full (FI; 200 mL day^{-1}) or deficit irrigation conditions (50 mL day^{-1}). Deficit irrigation conditions started at flower initiation or when the majority of primary fruits from the primary truss where at white development stage for Exps. 3.1 and 3.5, respectively.

	L*				**C***				**H°**			
Time of DI application	Flowering		White stage		Flowering		White stage		Flowering		White stage	
Cultivar/Irrigation (mL day^{-1})	**DI**	**FI**	**DI**	**FI**	**DI**	**FI**	**DI**	**FI**	**DI**	**FI**	**DI**	**FI**
Christine	44.85	46.87	47.71	43.97	51.92	52.78	56.24	55.55	42.6	43.09	36.79	35.76
Sonata	44.73	46.08	45.87	44.06	51.69	53.84	55.49	53.39	43.42	42.98	35.26	33.72
Elsanta	44.91	44.12	48.98	47.82	47.49	49.56	57.06	56.00	46.68	41.84	37.66	37.44
Symphony	41.76	39.89	43.55	42.55	46.21	46.65	52.55	52.08	43.2	38.15	33.20	31.5
Florence	36.49	40.77	42.37	42.7	38.06	44.27	50.28	52.38	36.22	37.12	32.07	31.31
Flamenco[a]	-	-	46.47	49.41	-	-	56.14	57.89	-	-	36.51	39.38
LSD (*P < 0.05*)	1.857		2.718		2.486		2.953		2.31		2.234	

[a]Cultivar Flamenco was only introduced in experiment 3.5 and hence results are only available when DI was applied at white development stage.

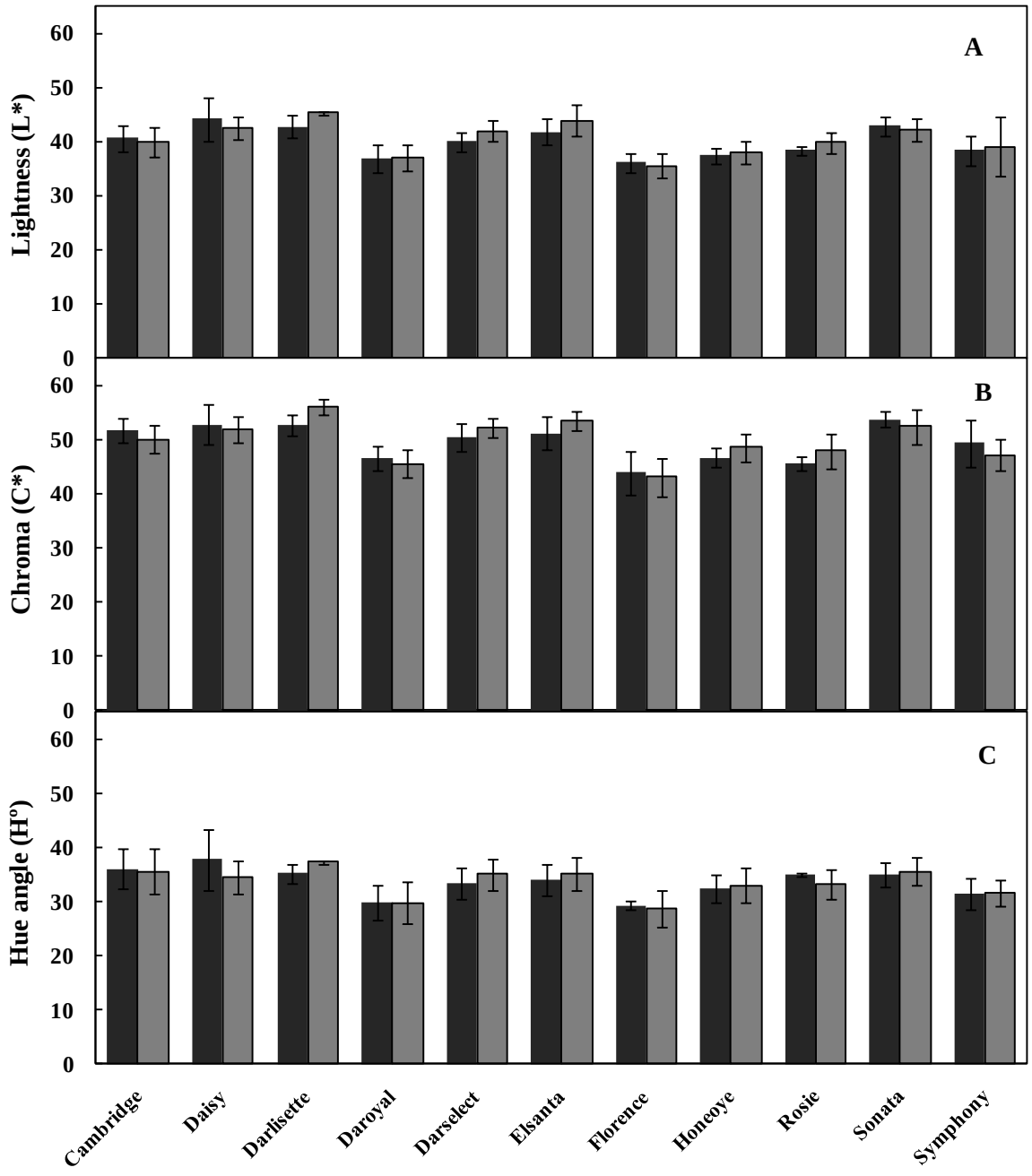


Figure 3.8: Objective colour characteristics (L*(A),C*(B), Hº(C)) from twelve strawberry cultivars grown under normal irrigation conditions (200 mL day^{-1}) and receiving half (■) or full nutrient strength (■) modified Hoagland's solution every three days intervals. Error bars indicate standard deviations.

3.4.4 Effect of DI and related strategies on the taste-related composition of strawberries

Little information is available which describes the effect of DI or other pre-harvest strategies on the taste-related attributes (*viz*. sugars and acids) of strawberries (Terry *et al.*, 2007a). In contrast, ample data is available on the effect of either cultivation practices or other stress conditions (*viz*. ozone exposure, salinity) on overall strawberry fruit quality and especially regarding the effect on specific bioactive compounds (Wang *et al.*, 2002; Davik *et al.*, 2006; Keutgen and Pawelzik, 2007a and 2007b; Hargreaves *et al.*, 2008; Keutgen and Pawelzik, 2008b; Hargreaves *et al.*, 2009). In Exp. 3.1, both on a DW and FW basis, fruits from cvs. Florence and Sonata had higher sugar content for non-water-stressed plants (70.75 and 68.79 mg g^{-1} FW, respectively; **Table 3.6**) as compared to other cultivars. DI conditions applied at flower initiation stage resulted in fruits from cvs. Elsanta and Sonata containing greater sugar content (82.34 and 81.57 mg g^{-1} FW, respectively; **Table 3.6**). Fructose content in cvs. Elsanta and Sonata plants as well as glucose content in Elsanta were the only sugars significantly greater in DI-treated plants as compared to plants kept at or near field capacity (**Table 3.6**). Application of DI conditions at later development stages resulted in little or non-significant changes in sugar concentrations of fruits from the same cultivars (**Table 3.6**). Previous studies (Terry *et al.*, 2007a) also highlighted that although sucrose was not affected by DI, monossacharides (glucose and fructose) were significantly higher in DI plants. The authors concluded that lower concentrations of sugars in fruits that received more water was most probably due to a dilution effect. Similarly, Crespo *et al.* (2010) demonstrated that monosaccharide concentrations but not disaccharides in strawberry fruits may be manipulated through different preharvest strategies. Despite total soluble solids not always being well correlated with sugar content in strawberry fruits (Perez *et al.*, 1997; Terry *et al.*, 2005; Appendix A), in the study by Awang *et al.* (1995), higher soluble solids in cv. Rapella grown under salinity stress was associated with restricted vegetative growth and shift of photoassimilates to fruits. In all cases the results from the experiments presented herein may support such findings. Although no evidence exists for strawberry fruits, it is well documented that plants grown under water stress undergo a process of osmotic adjustment (Mahajan and Tuteja, 2005). Thereby, greater sugar content in DI-treated plants may be an attempt by the plant to reduce osmotic potential by the accumulation of solutes. Differences in the osmotic potential between sucrose and glucose or fructose may partially explain the differential effect of DI for each major sugar.

Table 3.6: Effect of water deficit irrigation (DI; 50 mL day^{-1}) or full irrigation (FI; 200 mL day^{-1}) on sugar concentrations of different strawberry cultivars. Deficit irrigation conditions started at flower initiation or when the majority of primary fruits from the primary truss where at white development stage for Exps. 3.1 (n = 120) and 3.5 (n = 190), respectively.

		mg g^{-1} DW						mg g^{-1} FW					
	Cultivar	**Sucrose**		**Fructose**		**Glucose**		**Sucrose**		**Fructose**		**Glucose**	
		DI	FI	DI	FI	DI	FI	DI	FI	DI	FI	DI	FI
Flower initiation	Sonata	252.1	290.5	143.2	139.4	149.9	168.7	39.0	33.5	20.7	15.9	21.8	19.3
	Florence	202.0	243.4	140.9	155.9	144.4	165.3	27.3	30.7	19.7	19.4	20.1	20.6
	Elsanta	191.4	223.2	183.2	158.0	187.6	168.8	27.9	24.0	26.8	16.7	27.5	17.9
	Symphony	187.1	172.8	145.4	153.7	149.7	170.8	23.3	16.7	17.6	14.6	18.3	16.2
	Christine	188.3	161.2	158.1	147.6	168.1	149.5	22.9	19.6	19.3	18.2	20.4	18.3
	LSD (P < 0.05)	49.25		21.15		24.17		7.20		3.78		4.26	
White stage	Sonata	241.5	264.5	192.7	185.8	180.6	171.9	37.6	35.4	29.7	24.9	27.8	23.1
	Florence	217.1	232.4	201.0	196.7	181.3	182.5	30.9	27.7	28	23	25.3	21.3
	Elsanta	155.8	146.6	225.0	242.1	214.1	222.5	23.2	20.4	32.8	34.6	31.3	31.8
	Symphony	173.9	176.9	214.5	210.5	194.4	194.0	22.6	22.5	27.6	26	25.3	24
	Christine	162.7	148.6	215.6	237.2	195.1	215.4	21.6	19.1	28.3	30.7	25.7	27.9
	Flamenco	244.9	276.6	195.8	187.2	180.5	175.7	35.9	37.5	28.5	25.8	26.3	24.2
	LSD (P < 0.05)	43.16		17.46		24.17		7.20		3.78		3.83	

Sweetness of strawberry fruits is an important factor which can characterise the acceptance of the fruits by consumers (Keutgen and Pawelzik, 2008b). If considering that fructose is *ca*. 1.8 times sweeter than sucrose, and the sweetness of glucose is 60% that of sucrose, DI resulted in generally sweeter berries as determined by the sweetness index (**Figure 3.9**) and regardless of the time of initiation of DI conditions. Fruits from Elsanta plants subjected to drought stress from flower intiation stage showed as much as one third higher sweetness as compared to plants kept at or near field capacity. A significant increase in the sweetness index of Sonata was also observed for DI-treated plants in both experiments 3.1 and 3.5. Nevertheless, these results should be further corroborated by sensory evaluation.

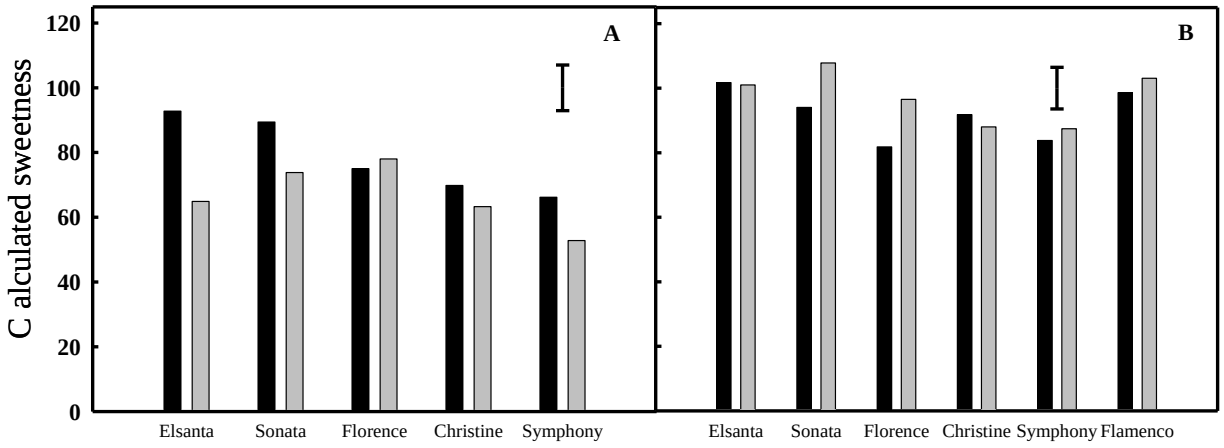


Figure 3.9: Effect of water deficit irrigation (▬50 mL day^{-1}) or full irrigation (▬ FI; 200 mL day^{-1}) on calculated sweetness of different strawberry cultivars. Deficit irrigation conditions started at flower initiation (Exp. 3.1 (n = 120); A) or when the majority of primary fruits from the primary truss where at white development stage (Exp. 3.5 (n= 190); B). Error bar indicates LSD value ($P < 0.05$).

In an attempt to further understand changes in sugar metabolism as a result of drought stress, sugar concentrations in both fruits and leaves were investigated in Exp. 3.6 (**Table 3.7**). Generally DI resulted in substantially but not significantly higher glucose and fructose concentrations as compared to fruits from plants kept at or near field capacity. It is well known that both sugars and organic acids originate from photosynthetic assimilates. Strawberries accumulate sugars during the ripening process by translocation from leaves to fruits (Villareal *et*

al., 2010), thereby higher sugar concentrations are found in fully ripe fruits (Basson *et al.*, 2010). No other studies thus far have investigated sugar and organic acid concentrations in both fruits and leaves and therefore the results presented herein may assist in better understanding the metabolism of strawberry photoassimilates. Monosaccharide and disaccharide concentrations were nearly five times greater in fruits than in leaves (**Table 3.7**). Generally, significant differences were encountered in glucose and fructose but not sucrose concentrations of leaves from different cultivars as well as a result to different irrigation strategies. Interestingly, sucrose which was the only sugar not affected, by either deficit irrigation or MeJa application, is the main sugar loaded into and translocated through the phloem from leaves to fruits (Slewinski and Braun, 2010). Sucrose accumulation in strawberry and other plants (Basson *et al.*, 2010) is subjected to carbohydrate sink strength. Sink strength may be defined as the rate at which photosynthate is used by the sink cell in accumulative, biosynthetic and respirative processes (Doehlert, 1993). In this context, Basson *et al.* (2010) recently pointed out that complex biochemical pathways within the plant use sucrose degradation products, diverting them into different carbon pools including sugars and organic acids. Given that no differences were encountered for sucrose concentrations between the different treatments applied, it may be reasonable to speculate that DI and related strategies do not directly affect sucrose accumulation but rather resulted in differences in channeling sucrose degradation products to different carbon pools (i.e. monossacharides (**Table 3.7**) and/or organic acids (**Table 3.10**).

No significant differences were found in sugar content of leaves between MeJa treated plants and the respective controls. Nevertheless, the interaction of DI with this natural hormone resulted in significant differences for glucose and fructose concentrations. In DI-treated plants, the application of MeJa resulted in greater sugar content whereas none or little effect was observed in fully irrigated plants. Both DI and MeJa application are known to have an effect on stomata opening, with MeJa reducing transpiration and aiding the plant to withstand better water stress conditions (Wang, 1999). In contrast, any of the treatments applied (i.e. DI, MeJa or DI + MeJa) had by itself a significant effect on fruit sugar concentration. A significant interaction between treatments was however observed for glucose concentration from fruits (**Table 3.7**). Limitation of transpiration by inhibiting stomata opening may slow down sugar metabolism in the plant and hence causing a reduced transport from leaves to fruits, favouring the accumulation of sugars into the leave tissue as observed herein.

Table 3.7: Sugar concentration (mg g^{-1} DW) of strawberry fruits and leaves from three different cultivars when submitted to full (200 mL day^{-1}) or deficit irrigation conditions (50 mL day^{-1}) and after none or 0.1 mM application of MeJa. Deficit irrigation conditions started when the majority of primary fruits from the primary truss where at green development stage and foliar application of MeJa started ca. 3 days after DI treatments were initiated and was applied at three days intervals.

		Leaves						**Fruits**					
		Fructose		**Glucose**		**Sucrose**		**Fructose**		**Glucose**		**Sucrose**	
Cv	**Irrigation**	0	0.1	0	0.1	0	0.1	0	0.1	0	0.1	0	0.1
253/29	50	35.5	42.3	41.3	48.7	59.3	64.3	189.5	191.2	183.6	190.2	213.5	262.4
	200	36.9	33.0	51.9	41.2	65.1	48.5	178.9	191.3	174.4	185.3	264.3	221.4
279/4	50	45.1	55.9	51.8	65.2	82.4	75.9	209.2	201.3	213.9	194.8	210.7	193.1
	200	32.4	36.1	41.4	45.1	66.8	75.6	204.2	211.4	206.7	210.2	210.0	201.9
279/5	50	51.4	69.0	53.4	70.3	84.7	87.6	248.0	226.4	181.5	228.8	249.8	232.7
	200	52.8	50.3	56.3	56.4	82.3	87.2	216.5	209.4	218.9	204.4	284.3	251.6
LSD ($P < 0.05$)		15.05		15.00		20.45		38.27		30.68		51.22	

In experiment 3.4, sucrose concentration in the 12 genotypes investigated ranged from 108.3 to 377.7 mg g^{-1} DW and was higher when plants were supplied with a full strength nutrient solution (255.5 mg g^{-1} DW) as compared to half strength (230.0 mg g^{-1} DW). In contrast and despite a high variability between cultivars, full strength modified Hoagland's solution resulted only in higher glucose concentration in cultivars Darlisette and Symphony and greater fructose in cvs. Rosie and Symphony. Fruits from cv. Mae responded differently than the rest of cultivars to the treatments applied since higher nutrient concentrations resulted in fruits with lower glucose and fructose concentrations (**Table 3.8**). These findings were in agreement with Nestby (1998) who reported that greater N application rate increased the sugar content of strawberries. Hargreaves *et al.* (2008) reported no changes in sugar content of fruits after different nutrient amendments. The monossacharide/dissacharide (M/D) ratio was significantly affected by the interaction cultivar and treatment, but for most cultivars, high nutrient concentrations resulted in greater M/D ratios.

Table 3.8: Sugar concentration (mg g^{-1} DW), monosaccharide/disaccharide (M/D) ratio and calculated sweetness of strawberry fruits from twelve cultivars grown under normal irrigation conditions (*ca*. 200 mL day^{-1}) but with different nutrient strength formulations (HS: Half strength modified Hoagland's solution; FS: Full strength modified Hoagland's solution).

	Sucrose (mg g^{-1} DW)		Glucose (mg g^{-1} DW)		Fructose (mg g^{-1} DW)		M/D		Sweetness	
Cultivar	**HS**	**FS**	**HS**	**FS**	**HS**	**FS**	**HS**	**FS**	**HS**	**FS**
Cambridge	307.5	273.5	141	121.7	152.2	131.8	0.97	1.06	63.55	55.75
Daisy	375.7	377.7	138.7	155.9	144.3	161.0	0.76	0.84	68.98	72.89
Darlisette	268.9	323.1	86.8	152.0	131.5	160.2	0.79	0.97	53.13	67.07
Daroyal	114.3	201.6	114.5	120.4	113.1	125.3	2.01	1.34	36.4	47.43
Darselect	200.9	204.3	116.5	86.9	122.3	90.0	1.19	0.97	46.65	40.05
Elsanta	250.9	226.2	175.3	172.6	192.6	188.1	1.69	1.60	66.43	63.07
Florence	297.1	355.3	164.2	156.7	176.5	165.4	1.15	0.91	67.81	71.41
Honeoye	106.5	221.5	134.2	127.4	165.7	136.5	3.33	1.26	45.21	51.63
Mae	128.1	157.1	220.3	173.0	241.1	184.7	3.60	2.29	64.6	55.64
Rosie	108.3	130.4	80.5	100.4	88.1	129.1	1.56	2.94	29.77	39.73
Sonata	354.7	348.9	151.4	156.1	156.2	162.8	0.87	0.90	69.54	70.3
Symphony	246.5	246.2	119.3	164.2	129.6	181.2	1.02	1.40	52.54	63.47
LSD (P<0.05)	86.05		43.92		40.11		0.9329		11.983	

Taste in strawberry fruits is, however, not just influenced by sugars. Acids and volatile compounds are also important contributors to strawberry taste and flavour (Cordenunsi *et al.*, 2002) and hence were investigated in Exp. 3.1, 3.5 and 3.6. In all experiments, and as earlier reported by others (Perez *et al.*, 1992; Keutgen and Pawelzik, 2007; Terry *et al.*, 2007a), three major organic acids were found within the cultivars studied: citric, malic and ascorbic acid. In all experiments, citric acid was the major acid found in the different cultivars investigated herein, accounting for approximately 1% of the total fresh weight and was in agreement with that found in the literature (Terry *et al.*, 2007a; Keutgen and Pawelzik, 2008). Malic and ascorbic acid were also identified in all cultivars in lower concentrations, up to 4.49 and 0.78 mg g^{-1} FW, respectively. Drought stress also affected acid composition (**Table 3.9**). On a DW basis, plants kept at or near field capacity tended to have greater acid content as compared to those exposed to drought stress. Christine and Florence were the only cultivars where DI resulted in higher acid content. However, if considering the results on a FW basis, DI resulted in greater acid content for Symphony and Florence whilst it did not significantly affect acid content for the rest of cultivars. In an earlier study, DI resulted in lower ascorbic, citric and malic acid content on a DW basis for cv. Elsanta plants (Terry *et al.*, 2007a); however it is clear that, in Exp.3.1, DI had a genotype-dependent effect on acid metabolism. As indicated by differences in fruit physiology and sugar metabolism, it may be plausible to speculate that DI resulted in different respiratory metabolism between cultivars and hence different utilisation of respiratory substrates such as organic acids. Strawberry fruits are an important source of ascorbic acid (AsA), since this vitamin in combination with other phytochemicals (*viz.* anthocyanins, phenolic acids, etc.) found in strawberry have been reported to be responsible for the numerous health benefits associated with these berries. In this context, strawberries were recently ranked as one of the most important sources of cellular antioxidant activity in the North American diet (Wolfe *et al.*,2008). In the present study, concentration of AsA ranged from 0.42 (DI treated cv. Elsanta) to 0.73 (DI treated cv. Florence) mg g^{-1} FW and was dependent on the water regime and cultivar. It is noteworthy that only AsA rather than vitamin C was measured in the present work. Values were in agreement with that previously reported (Perez *et al.*, 1992; Davik *et al.*, 2006; Terry *et al.*, 2007a). DI resulted in significantly lower and higher concentration of AsA only for cultivars Elsanta and Florence, respectively (**Table 3.9**). DI, applied from flower initiation stage, had the greatest effect in cultivar Florence which showed 1.3-fold higher AsA concentration in fruits from water stressed plants, and hence perhaps resulting in more healthful berries. This said,

Keutgen and Pawelzik (2007b) observed that the content of ascorbic acid was reduced in fruits from cvs. Elsanta and Korona plants subjected to moderate salt stress.

Table 3.9: Effect of water deficit irrigation (DI: 50 mL day^{-1}) or full irrigation (FI: 200 mL day^{-1}) on main non-volatile organic acids of secondary strawberry fruits from the primary trusses of five different cultivars. Cultivars are arranged in descending order of total acid concentrations for fully irrigated plants on DW basis.

Cultivar	mg g^{-1} DW								mg g^{-1} FW							
	Citric		Malic		Ascorbic		Total Acids		Citric		Malic		Ascorbic		Total Acids	
	DI	FI	DI	FI	DI	FI	DI	FI	DI	FI	DI	FI	DI	FI	DI	FI
Symphony	90.47	99.12	25.24	35.14	4.86	5.68	120.57	139.92	11.06	9.45	3.10	3.35	0.59	0.54	14.75	13.34
Sonata	62.10	83.56	29.46	38.95	3.50	4.13	95.06	126.68	8.59	9.67	4.13	4.49	0.47	0.47	13.19	14.63
Florence	82.43	80.34	21.41	23.37	5.33	4.31	109.17	108.02	11.42	10.09	2.91	2.97	0.73	0.54	15.06	13.61
Christine	80.39	66.18	29.09	21.45	5.09	5.10	114.58	92.75	9.63	7.87	3.46	2.55	0.60	0.61	13.70	11.04
Elsanta	64.72	63.60	16.41	22.22	3.07	5.22	84.20	91.04	9.22	6.88	2.36	2.43	0.42	0.57	11.99	9.88
LSD (P < 0.05)	17.012		7.114		1.258		20.302		2.116		0.876		0.143		2.814	

When investigating organic concentrations from leaves, ascorbic acid (AsA) was significantly different among genotypes, irrigation regimes and its interaction. Specifically, AsA was greater in leaves from plants subjected to reduced irrigation (1.528 mg g^{-1} DW) as compared to plants kept at or near field capacity (1.149 mg g^{-1} DW) which may be related with the mechanisms of the plants to fight reactive oxygen species generated as a result of stress conditions (i.e. deficit irrigation) (Wang, 1999). The greatest increase in AsA, *ca*. 2-fold greater, as a result of limited irrigation was observed in leaves from cv. 279/5. Ascorbic acid from plants is oxidized in antioxidative reactions where a system for the regeneration of this compound is vital for maintaining the antioxidative system and hence survival of the plant. Dehydroascorbate (DHA) reductase (DHAR) is an enzyme found in strawberry and other plants which catalyses the re-reduction of DHA to ascorbate (Amako and Ushimaru, 2009). It is know that under certain abiotic stress conditions the transcription levels and activity of DHAR are increased thereby resulting in higher ascorbic acid concentrations in plants submitted to stress conditions (Amako and Ushimaru, 2009). Similarly to the changes observed in ascorbic acid content of leaves, malic acid on both FW and DW basis was affected by the interaction between water treatments and genotypes. Except for cv. 279/5, where malic acid was lower in leaves from DI-treated plants, irrigation resulted in greater amounts of malic acid in leaves from cvs. 279/4 and 253/29. Despite not being statistically significant, MeJa treatments resulted in substantially greater amounts of AsA and malic acid depending on the cultivar (**Table 3.10**). In contrast, leaves of drought-stressed strawberry plants had lower AsA content if compared to leaves from control-treated leaves (Byers and Perry, 1992; Wang 1999), this said different cultivars and experimental conditions were tested in the earlier works as compared to those presented herein. A study conducted with *Arabidopsis thaliana,* showed that cell cultures treated with MeJa had 64% more AsA than untreated cells (Wolucka *et al.,* 2005). In experiment 3.6, citric acid was the only acid affected by the interaction between cultivar, water treatments and MeJa application (**Table 3.10**).

Whereas citric acid is the major organic acid in strawberry fruits, with concentrations up to 3.5-fold higher than malic acid, leaf tissue contained lower concentrations of citric if compared to malic. These findings suggested that citric acid may be the preferred organic acid transported to the phloem and accumulated in fruit tissues, whereas other acids may be mainly stored in the leaves and transported to a lesser extent.

In fruits, AsA was the only acid not affected by the conditions imposed in this study rather than differences between genotypes. In agreement, earlier experiments (Exp. 3.1) found

AsA to be either higher, lower or not affected in plants submitted to drought stress depending on the cultivar. Besides, high variability in the AsA content among different strawberry cultivars have been described earlier (Tulipani *et al.,* 2008; Crespo *et al.,* 2010). AsA concentration in cultivar 253/29 (17.1 mg g^{-1} DW) was 2.3- or 1.5-fold higher than that found in fruits from cvs. 279/4 (7.3 mg g^{-1} DW) and 279/5 (11.7 mg g^{-1} DW), respectively (Exp. 3.6). The mechanisms regulating ascorbic acid content in fruits result from a balance between synthesis and degradation. Additionally, ascorbic acid content results from *in situ* synthesis and long distance transport from the leaves via the phloem (Barata-Soares *et al.,* 2004). The *in situ* synthesis occurs in strawberries through D-galacturonic acid, a component of cell wall pectins (Agius *et al.* 2003). Glucose is also used for ascorbic acid biosynthesis as described by Wheeler *et al.* (1998) and the synthesis of ascorbic acid from glucose seems to be genetically regulated. It is possible, as pointed out earlier, that DI and related strategies affected AsA synthesis in leaves tissues and hence limiting the amount of this acid transported to fruits. Nevertheless, further research should study whether the same treatments have an effect on *in situ* synthesis or further degradation of this vitamin in the fruit.

In contrast, citric acid from fruits of the different cultivars analysed was quite even (56 mg g^{-1} DW) but significantly affected by deficit irrigation ($P = 0.015$) or MeJa treatments ($P = 0.035$). Plants subjected to drought stress resulted in either higher or lower citric acid content depending on the cultivar which corroborates the genotype-specific response shown above, as well as earlier evidence reported by others (Carbone *et al.,* 2009; Crespo *et al.* 2010; Giné Bordonaba and Terry, 2010). Preharvest foliar application of MeJa resulted in fruits with greater citric acid content (60.2 mg g^{-1} DW) as compared to fruits from non-treated plants (51.1 mg g^{-1} DW). The concentration of malic acid was affected by differences in genotypes as well as the interactions between irrigation and cultivar or interactions between the different pre-harvest treatments applied. Specifically, in non-DI treated plants MeJa applications resulted in lower malic acid content of fruits, whereas no changes were observed when plants were fully irrigated (**Table 3.10**).

Table 3.10: Organic acid concentration (mg g^{-1} DW) of strawberry fruits and leaves from three different cultivars when submitted to full (200 mL day^{-1}) or deficit irrigation conditions (50 mL day^{-1}) and after none ([a]) or 0.1 mM ([b]) application of MeJa. Deficit irrigation conditions started when the majority of primary fruits from the primary truss where at green development stage and foliar application of MeJa started ca. 3 days after DI conditions were initiated and was applied at three days intervals.

		Leaves						**Fruits**					
		Ascorbic		**Citric**		**Malic**		**Ascorbic**		**Citric**		**Malic**	
Cv	**Irrigation**	**0[a]**	**0.1[b]**	**0**	**0.1**	**0**	**0.1**	**0**	**0.1**	**0**	**0.1**	**0**	**0.1**
253/29	50	1.74	1.54	11.3	10.5	27.21	25.46	15.60	25.50	62.40	82.40	8.26	14.13
	200	1.34	1.11	8.43	8	24.63	15.8	16.60	10.50	56.60	44.70	13.12	10.51
279/4	50	1.11	1.39	10.95	7.64	28.01	32.9	5.40	5.00	42.70	50.10	6.12	6.62
	200	0.96	1.04	9.01	10.79	26.28	20.13	8.50	10.50	54.90	60.90	13.90	8.53
279/5	50	2.83	1.98	12.23	16.01	16.64	19.5	12.50	15.00	59.40	68.10	16.90	13
	200	1.18	1.26	16.45	12.37	28.92	27.07	5.50	13.90	34.80	54.90	13.57	11.62
LSD (P < 0.05)		0.783		5.036		14.003		10.273		19.072		5.186	

The balance between sugars and acids in strawberries and other berries are important indicators of fruit taste (Terry *et al.,* 2005 and 2007a; Giné Bordonaba and Terry, 2008), additionally the ratio can be used as an indicator of fruit ripeness (Pérez *et al.,* 1997) or even as an index of consumer acceptability (Keutgen and Pawelzik, 2007a). It is worth noting than as different sugars have a different apparent sweetness (Keutgen and Pawelzik, 2007a), each acid may have a different impact on perceived acidity (i.e. different pKa and influence on pH), therefore the information given by the sugar acid ratio should be treated with some caution. Recently, Terry *et al.* (2007a) showed that sugar/ acid ratios were significantly greater in fruits from DI-treated plants as compared to those from non-water-stressed plants. The results from experiment 3.1 showed that although sugar/acid ratios for DI-treated were substantially higher in cvs. Symphony, Elsanta and Sonata, significant differences between treatments ($P < 0.05$) were only encountered in fruits from cv. Sonata (**Figure 3.10**). In the same experiment, water DI did not have a significant effect on the sugar / acid ratio of either Christine nor Florence, and comparable values were observed between both irrigation regimes. In Exp. 3.6, DI resulted in either higher or lower sugar/acid ratios only for cultivars 279/4 and 279/5, respectively. Generally, foliar applications of MeJa did not have a substantial effect on the sugar/acid ratio of any of the cultivars investigated, except for cv. 279/5 where MeJa applied to fully irrigated plants resulted in lower values as compared to non-MeJa treated plants. Cordenunsi *et al.* (2002) reported that good quality strawberry fruits were those with a ratio higher than 5.3. However, such information must be taken with some prudence as values will directly depend on the nature of the measurements. In experiment 3.1, plants kept at or near field capacity for all the cultivars except Symphony had sugar acid ratio higher than 5 (i.e. Elsanta and Christine with values of 5.9). In addition, the quality of strawberries was significantly and substantially higher in Sonata and Elsanta with values of 6.2 and 6.9, respectively. Fruits from plants in experiment 3.6 had in average 1.8-fold greater sugar acid ratios which were due to the lower acid concentrations found in these fruits.

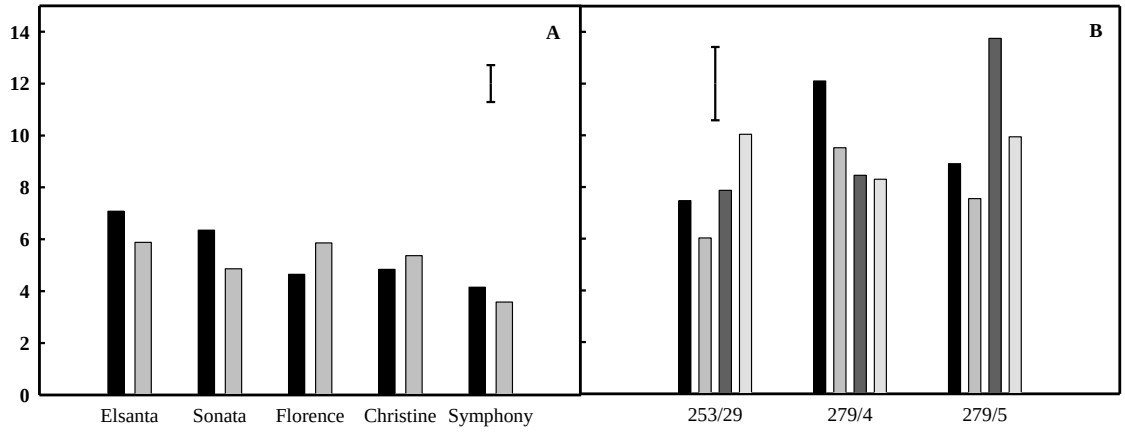


Figure 3.10: (A) Effect of water deficit irrigation (▬50 mL day^{-1}) or full irrigation (▬ FI; 200 mL day^{-1}) on the sugar acid ratio of different strawberry cultivars. (B) Sugar acid ratio of strawberry fruits from plants grown under (■) full irrigation, (■) deficit irrigation, (■) full irrigation + 0.1 mM of MeJa and (■) deficit irrigation + 0.1 mM of MeJa. Deficit irrigation conditions started at flower initiation (A) or when the majority of primary fruits from the primary truss where at green development stage (B). Error bar indicates LSD value ($P < 0.05$).

3.4.5 Year-to-year variation in the sugar composition of strawberry fruits

It is well documented that variation in agroclimatic conditions results in fruits with different biochemical composition (Carbone *et al.*, 2009; Crespo *et al.*, 2010). Indeed, Haynes and Goh (1987) found that fruit quality varied more year to year than due to treatment differences, which make it difficult to elucidate the effect that any pre-harvest treatment may have on strawberry quality, especially if assessed in multiple or different years. Accordingly, and even though plants were purchased from the same supplier, grown under equal conditions and planted in the same week or close enough, significant differences were found in the sugar composition, especially for sucrose values (up to 1.6-fold), of strawberry fruits from cv. Elsanta grown at or near field capacity (**Figure 3.11**).

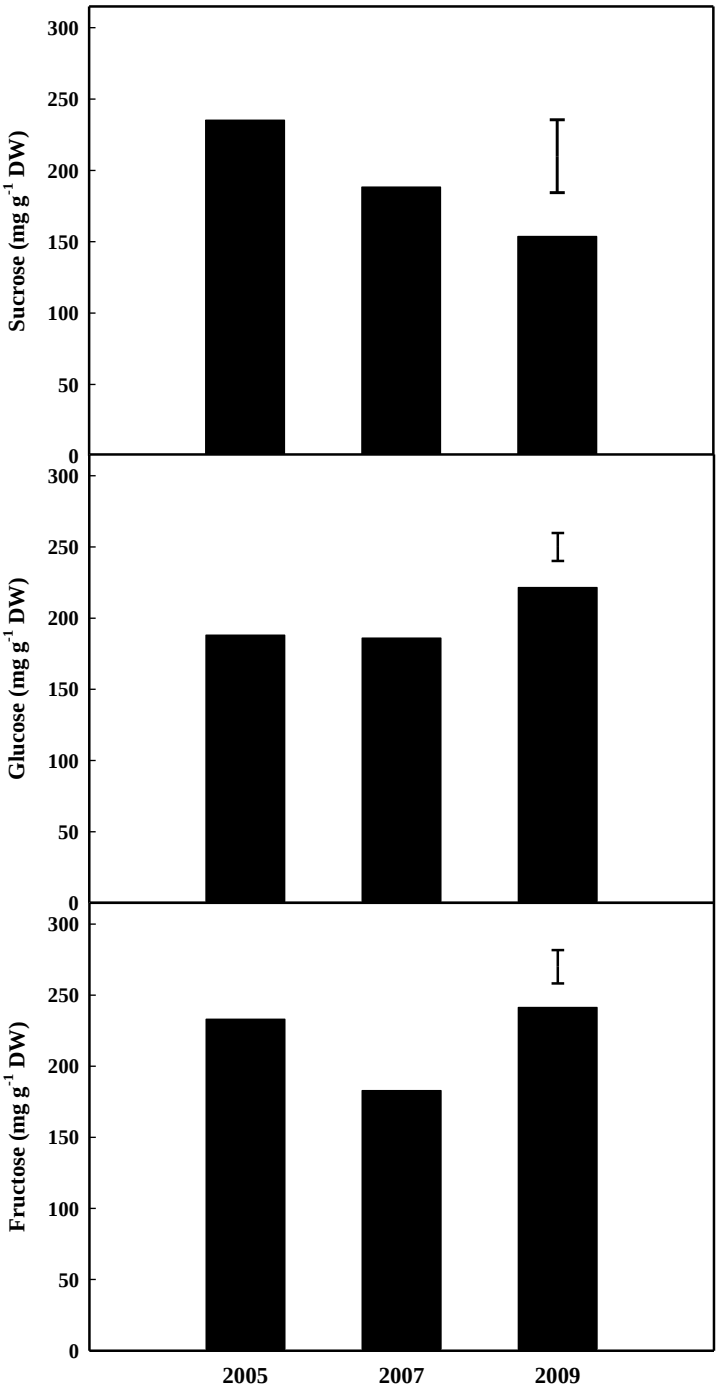


Figure 3.11: Year-to-year variation in the sugar composition of strawberry fruits (cv. Elsanta) from plants kept at or near field capacity. Error bar indicates LSD value ($P < 0.05$). 2005 data from Terry *et al.* (2007a).

Even though all experiments were conducted inside glasshouse and under similar conditions, variations in external temperatures and outside weather conditions resulted in different average temperatures and light exposure for each trial. Average temperatures within the glasshouse, during the plant growing period, for years 2005, 2007 and 2009 were 23, 21 and 23ºC, respectively. Higher monosaccharide and lower sucrose concentrations in fruits grown during 2009 may hence be the result of greater temperatures and also days with greater exposure to sunshine (data not available) which may favour plant respiration, photoassimilates production and degradation of sucrose into glucose and fructose. Accordingly, several authors have reported that strawberry fruits grown with greater exposure to sunshine had greater sugar content, as well as, a lower sugar to organic acid ratio compared to fruit that were sampled following periods of predominately overcast weather conditions (Daugaard, 2001; Hargreaves *et al.*, 2008; Crespo *et al.*, 2010). To further elucidate the role that climatic conditions have on strawberry fruit quality more information on weather conditions and measurements of photosynthetic activity would be needed.

3.4.6 Effects of DI and other preharvest strategies on strawberry bioactives

Anthocyanins together with ascorbic and ellagic acid are quantitatively the most important antioxidant compounds present in strawberry fruits (Appendix E; Crespo *et al.*, 2010). It is therefore not surprising that the analysis of these compounds in strawberry and other berry fruits have received particular attention over the past years. As shown in earlier sections, ascorbic acid concentrations strongly varied when plants were ground under different conditions, although such variations depended on the genotype. Four different anthocyanins, namely cyanidin-3-glucoside (cya-3-gluc), pelargonidin-3-glucoside (pg-3-gluc) and two pelargonidin-derivatives (pg-deriv), were identified in the present study according to their retention time, UV spectra and comparison with standards (Appendix B; Crespo *et al.*, 2010) (**Figure 3.12**) and using a newly developed and optimised method (Appendix B). Anthocyanin concentrations reported in this work strongly depended on the cultivar and treatment applied.

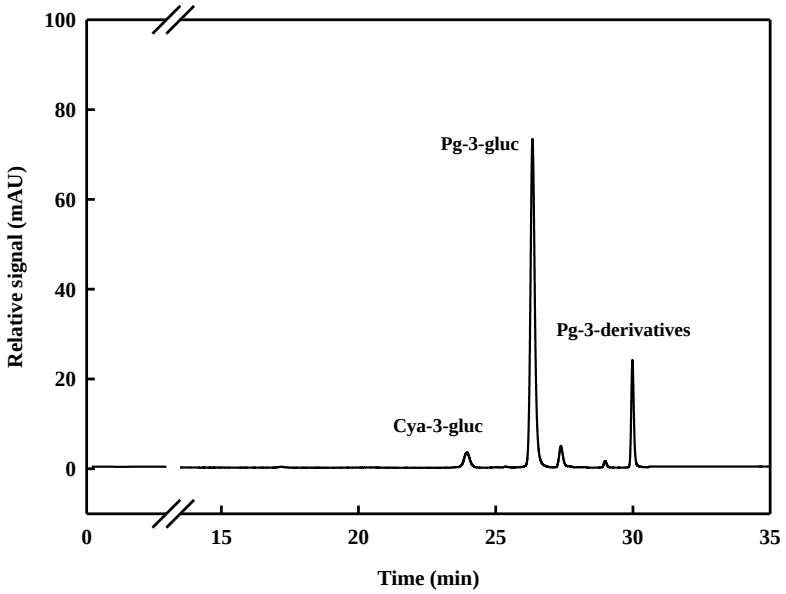


Figure 3.12: HPLC anthocyanin profile of strawberry extracts. HPLC conditions for the determination of anthocyanins. The mobile phase consisted of 2% (v/v) acetic acid in HPLC-grade water (A) and 2% (v/v) trifluoroacetic in methanol (B) with a flow rate of 1 mL min^{-1}. The gradient conditions were 0-10min, 2-20% B; 10-20min, 20-25% B; 20-25min, 25-35% B; 25-35min, 35-75% B.

In Exp. 3.6, fruits from cultivar 253/29 were characterised by the presence of these four pigments whereas fruits from cultivars 279/4 and 279/5 lacked the pg-derivative 2, as earlier observed by Terry *et al.* (2007) in fruits from cv. Elsanta. Significant differences between cultivars were encountered for each individual anthocyanin and hence corroborating the strong influence of genetic background in determining anthocyanin content of strawberry fruits (Carbone *et al.*, 2009; Crespo *et al.*, 2009). Anthocyanin concentrations for strawberry fruits differ markedly in the literature available (Lopez da Silva *et al.,* 2007; Hernanz *et al.*, 2007; Terry *et al.,* 2007). Nevertheless, total anthocyanin contents found in these experiments were lower than the contents reported in five strawberry cultivars by Lopes da Silva *et al.* (2007), but slightly higher than the values reported by Hernanz *et al.* (2007). In addition, values were comparable to those from Terry *et al.,* (2007) and Crespo *et al.* (2010) which used a similar methodology (i.e. extraction and quantification). Despite the fact that different genotypes were used in each study, the higher values obtained by Lopes da Silva *et al.* (2007) may be partially explained by different extraction procedures used as the extraction was repeated several times

until complete removal of the colour was achieved. In contrast, in the present study, and as earlier reported by Hernanz *et al.* (2007), only one extraction step was performed even though total removal of the colour from the extracts was still achieved. Furthermore, pelargonidin derivatives such as pg-3-rutinoside, pg-3-malonylglucoside and pg-3-acetylglucoside have been identified in strawberries by other authors (Aaby *et al.,* 2007; Lopes da Silva *et al.,* 2007; Yoshida *et al.,* 2002; Hernanz *et al.,* 2007). Indeed, the elution order of the pelargonidin derivatives and their occurrence in strawberry cultivars reported by others, strongly suggest pg derivative 1 to be pg-3-rutinoside. However, to further confirm this assumption the identification of the substituting sugar by mass spectra analysis would be necessary.

In Experiment 3.6, fruits from cultivar 279/5 had *ca*. 2-fold greater pg-3-gluc concentrations than that of fruits from cultivars 279/4 and 253/29 (**Table 3.11**). Deficit irrigation applied at green-stage of fruit development had no or only a minimal effect on the anthocyanin concentrations of the different cultivars analysed. In contrast, earlier works (Terry *et al.,* 2007) found anthocyanin concentrations to be greater in fruits from plants (cv. Elsanta) that received less water. In the above-mentioned study DI was applied at flower initiation and hence it may be feasible to speculate that despite anthocyanin accumulation occur during later developmental stages (Carbone *et al.,* 2009) the enhanced synthesis of this pigments as a result of drought stress may be up-regulated at much earlier development stages (i.e. from flower initiation up to green-stage). In addition, there was a significant genotype and irrigation interaction, revealing the differential effect that water DI may have on strawberry anthocyanins as also observed for other metabolites herein and elsewhere (Terry *et al.,* 2007; Carbone *et al.,* 2009; Giné Bordonaba and Terry, 2010; Crespo *et al.,* 2010).

Methyl jasmonate applied preharvest has been earlier reported to enhance anthocyanin concentrations in berries (Wang *et al.,* 2008; Perez *et al.,* 1997) and other fruits (Kondo *et al.,* 2001; Rudell and Mattheis, 2008). In the present study, anthocyanin concentration of the fruits was also positively influenced by MeJa applications, and such effects were seen to be genotype-specific (**Table 3.11**). The similarities between MeJA and abscisic acid (ABA) (**Figure 3.13**) in their physiological effects including the response of plants to certain stress conditions are well documented (Melan *et al*., 1993; Kondo and Fukuda, 2001). Despite the lack of significance, earlier works (Terry *et al.,* 2007) showed endogenous ABA concentrations to be greater in DI-treated fruits as compared to fruits from plants that received full irrigation.

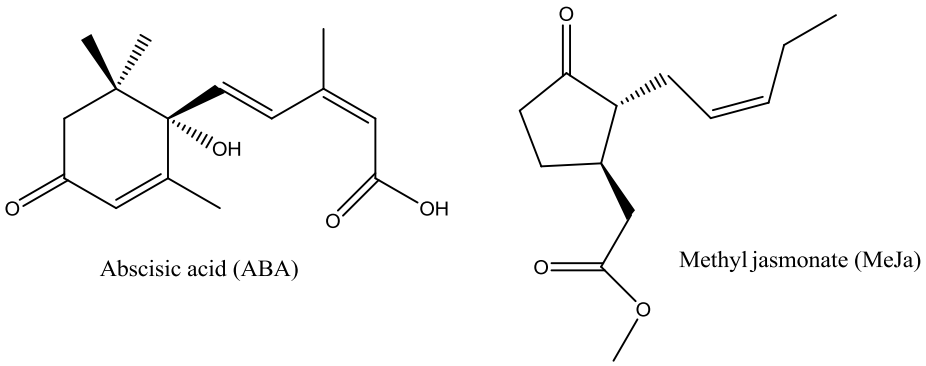


Figure 3.13: Chemical structure of strawberry hormones, abscisic acid (left) and methyl jasmonate (right).

Higher ABA concentrations were accompanied by greater anthocyanin concentrations, mainly pg-3-gluc, in the fruits (Terry *et al.,* 2007a). As mentioned earlier, in Exp. 3.6, DI was applied at later fruit development stages, when endogenous MeJa levels in the fruit are low (Gansser *et al.,* 1997; Mukkun and Singh, 2009), resulting in no enhanced synthesis of anthocyanins. However, exogenous MeJa applications resulted in higher anthocyanin concentrations in the fruit depending on the cultivar. Generally, when a plant hormone influences fruit physiology when applied exogenously, it is accepted that the same effect might be expected from the endogenous hormone (Kondo and Fukuda, 2001). Discrepancies between the results presented herein and preceding studies (Terry *et al.,* 2007) strongly suggest that the boost in anthocyanin synthesis as a result of pre-harvest conditions is modulated, at least in part, by the levels of natural hormones found in the fruit (i.e. ABA and MeJa), therefore highlighting the importance of these hormones on secondary metabolism probably by increasing PAL activity as recently pointed out by Wang *et al.* (2009). Accordingly, Wang *et al.* (2009) speculated that MeJA applied postharvest delayed fruit ripening of Chinese red bayberries, thereby reducing decay incidence. In the present study, given that other biochemical markers of fruit ripening (*viz*. sugars and acid) where not significantly affected by MeJa treatments, it is then difficult to corroborate the role that MeJa may have on delaying fruit ripening, at least on strawberry fruits, rather than having just a marked effect on pigment metabolism.

Table 3.11: Concentration of individual anthocyanins in strawberry fruits from three different cultivars subjected to deficit (50 mL day^{-1}) or full irrigation (200 mL day^{-1}) conditions and after 0 or 0.1 mM foliar application of methyl jasmonate at three days intervals during the growing period (Exp.3.6).

		Cya-3-gluc		Pg-3-gluc		Pg-deriv. 1		Pg-deriv. 2	
Cv	Irrigation	0	0.1	0	0.1	0	0.1	0	0.1
253/29	50	44.2	33.5	1559.2	880.2	148.3	89.8	377.7	227.2
	200	41.4	33.3	776	874	68.1	94.8	166.6	237.2
279/4	50	20.1	30.8	764	587	47.5	42.6	0	0
	200	23.2	27.9	1252	1054	59.7	58.8	0	0
279/5	50	26	48.3	1174	3198	72.4	132.3	0	0
	200	22.9	31.5	890	2782	57.6	123.6	0	0
LSD (P<0.05)		10.22		712.53		30.99		82.83	

Total antioxidant capacity of fruits and leaves depends on the presence of oxygen radical scavengers such as phenolic compounds and ascorbic ubiquitously present in different plant tissues. Generally though, antioxidant capacity was found to be better correlated with ascorbic acid rather than anthocyanin concentrations as was also found by other studies (Crespo *et al.*, 2010). Antioxidant capacity, measured by the standardised FRAP assay, was greater in DI-treated fruits (Terry *et al.*, 2007) or in fruits grown in a valley as compared to those grown in a mountain region (Crespo *et al.*, 2010). The effect that other preharvest strategies have on the antioxidant capacity of the fruits is also well documented (Hargreaves *et al.*, 2008; Keutgen and Pawelzik, 2008; **Table 3.1**).

In Exp.3.6, antioxidant capacity was measured not in fruits but in leaves, and results clearly revealed that leaves from DI-treated plants had greater antioxidant capacity as compared to leaves from plants kept at or near field capacity. Foliar application of MeJa enhanced antioxidant capacity of strawberry leaves but only when plants were subjected to drought conditions (**Figure 3.14**).

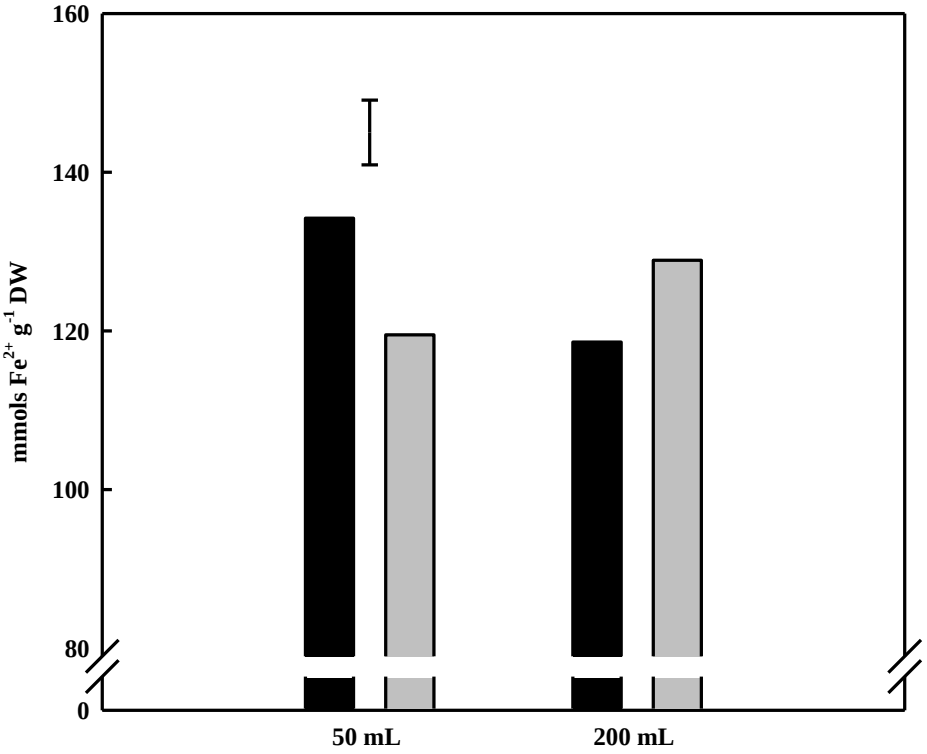


Figure 3.14: Antioxidant capacity, measured by the FRAP assay, on strawberry leaves, when plants were submitted to full (200 mL day^{-1}) or deficit irrigation conditions (50 mL day^{-1}) and after none (■) or 0.1 mM foliar application of MeJa (■). Deficit irrigation conditions started when the majority of primary fruits from the primary truss where at green development stage and foliar application of MeJa started ca. 3 days after DI were initiated and was applied at three day intervals. Error bar indicates LSD value ($P < 0.05$).

The effect that other preharvest conditions had on strawberry fruit bioactives was also investigated through different trials in collaboration with other institutions. Soilless cultivation systems, in all its variations (*viz*. supported or suspended, open or closed, with or without substrate) are considered as potential alternatives whereby soil disinfestations are not an issue and when water and nutrients are used in a more sustainable manner (Hernanz *et al.*, 2007; Hernanz *et al.*, 2008). In Europe, the land area dedicated for the soilless cultivation of strawberries is expected to rise considerably over the next years as it is already happening in one of the main strawberry producing areas such as Huelva, Spain. Studies throughout the last

decade have compared productivity and yield from plants grown under soilless cultivations and those grown in a more traditional manner (Tagliavini *et al.*, 2005) and it was concluded not to be significantly different. Similarly, Recamales *et al.* (2007), compared the fruit quality between soilless (open and close systems) and traditional cultivated strawberry plants and found that soilless production resulted in not significant but lower amount of soluble sugars, organic acids and mineral content. Besides technological variations among soilless systems, the amount and nature of substrates that can be employed is vast (*viz*. peat, sand, gravel, polyurethane foam, expanded polystyrene, geotextiles, perlite, rockwool, vermiculite, coconut fibre, grape debris, rice husks, solid urban waste, etc). To date no studies have compared the health-related composition of fruits grown under different substrates. In the present experiment (Exp. 3.7), the nature of soilless substrate significantly affected the concentration of these compounds, specifically the main strawberry anthocyanin, pg-3-gluc (**Figure 3.15**) and to a lesser extent the concentration of other bioactives such as ellagic acid and anthocyanidins (data not shown).

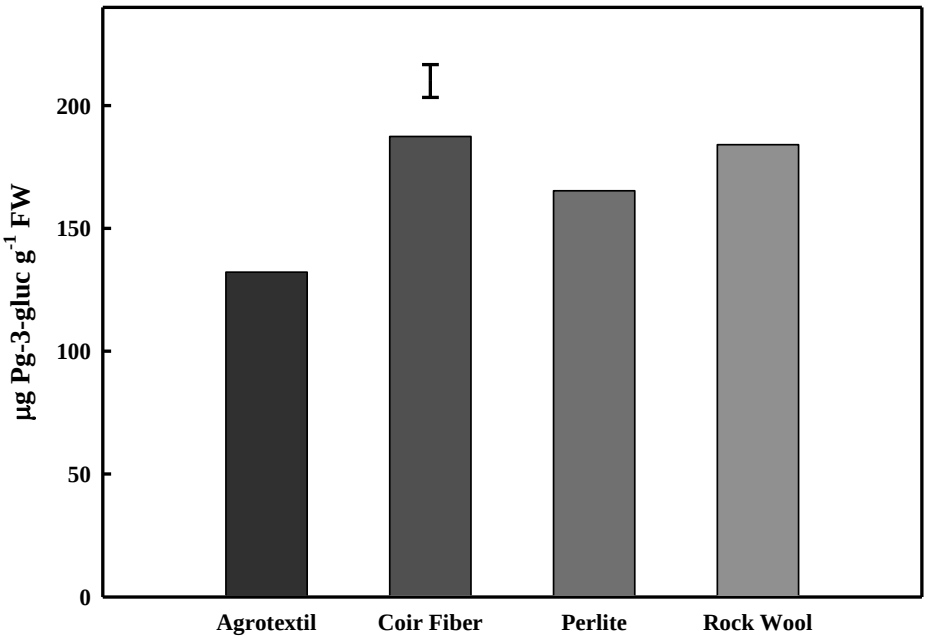


Figure 3.15: Concentrations of the main strawberry anthocyanin (pg-3-gluc) from strawberry fruits from plants grown using different soilless substrates (viz. agrotextil, coir fibre, perlite and rock wool).

3.4.7 Variability in the taste-related composition of fruits from different European markets

As shown in Table 3.2, and in addition to the experiments described in earlier sections, several trials were conducted with other research groups, investigating the composition of strawberry fruits from different European countries. Concentrations for all taste-related compounds not only significantly differed between cultivars but also between different origins (*viz*. Spain, Switzerland and United Kingdom). Generally, though, results for all analytes were in the range of those found by others (Perez, *et al.*, 1997; Terry *et al.*, 2007; Keutgen and Pawelzik, 2008) and clearly illustrated the vast variability in the composition of strawberries (**Figure 3.16**), as can be also observed when comparing the ample literature available.

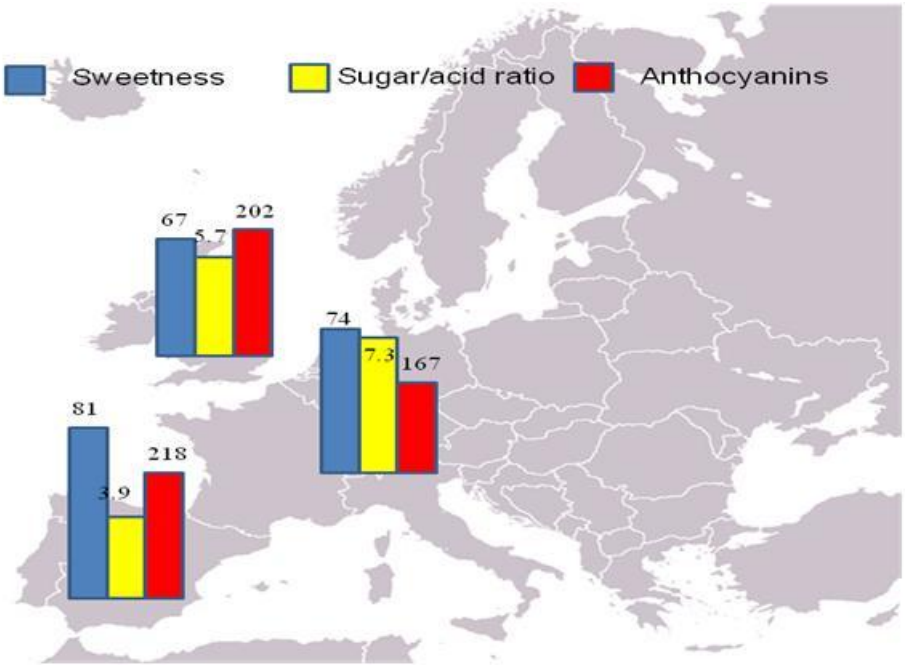


Figure 3.16: Average concentrations of different taste and health-related parameters of strawberry fruits from different European countries (*viz*. Spain, Switzerland and United Kingdom). Values for each country are means for fruits from different cultivars, locations as well as grown under different conditions.

Such variability may be the result of several factors including different cultivars being investigated (Tulipani *et al.*, 2008), the conditions under which the plants are grown under (Terry *et al.*, 2007; Exp. 3.1, 3.3, 3.5 and 3.6) as well as different agroclimatic conditions between different growing locations (Crespo *et al.*, 2010). In the present section, a chemometric approach

was therefore undertaken to further study the extent to which of the above-mentioned factors affected the taste-related composition of strawberry fruits. Indeed, chemometric interpretation of analytical data is increasingly applied to food analysis (García-Villar *et al.*, 2007; Plessi *et al.*, 2007) and may present several advantages, as compared to traditional statistical approaches, when large data sets need to be analysed or when the influence of multiple factors needs to be studied. For instance, principal component analysis (PCA) has been used for the differentiation and classification of food products according to geographical origin or for the chemotaxonomic approach to botanical classification (García-Villar *et al.*, 2007; Plessi *et al.*, 2007). The chemometric interpretation of the biochemical profile of seventeen UK-grown blackcurrant cvs. was also assessed to test the possible heterogeneity between different genotypes (Chapter 4; section 4.5.1.4; Giné Bordonaba and Terry, 2008).

Considering the taste-related composition of a collection of strawberry samples ($n > 600$) as analytical data, PCA was able to discriminate mainly between origins but also between genotypes and conditions by which the plants were grown (**Figure 3.17**). It is worth noting that the methods used for adquiring the biochemical data were for all samples the same and hence the chemometric analysis and comparison was justified. Three Principal Components (PCs) were required to capture over 75% of the variance between the samples investigated. PC1 explained 41% of the total variation and was mainly related, according to factor loadings values, to glucose, total sugars and the sugar/acid ratio. PC2 explained 22% of the variation captured between samples and was mainly related to sucrose, calculated sweetness and the monosaccharide to disaccharide ratio. PC3 captured a marginal 10 % of the variation between samples, and the inclusion of additional PCs failed to improve clustering. Consequently, the interpretation of clustering between samples was mainly characterized by the information provided by PC1 and PC2 (**Figure 3.17**), in which the different growing locations were especially highlighted.

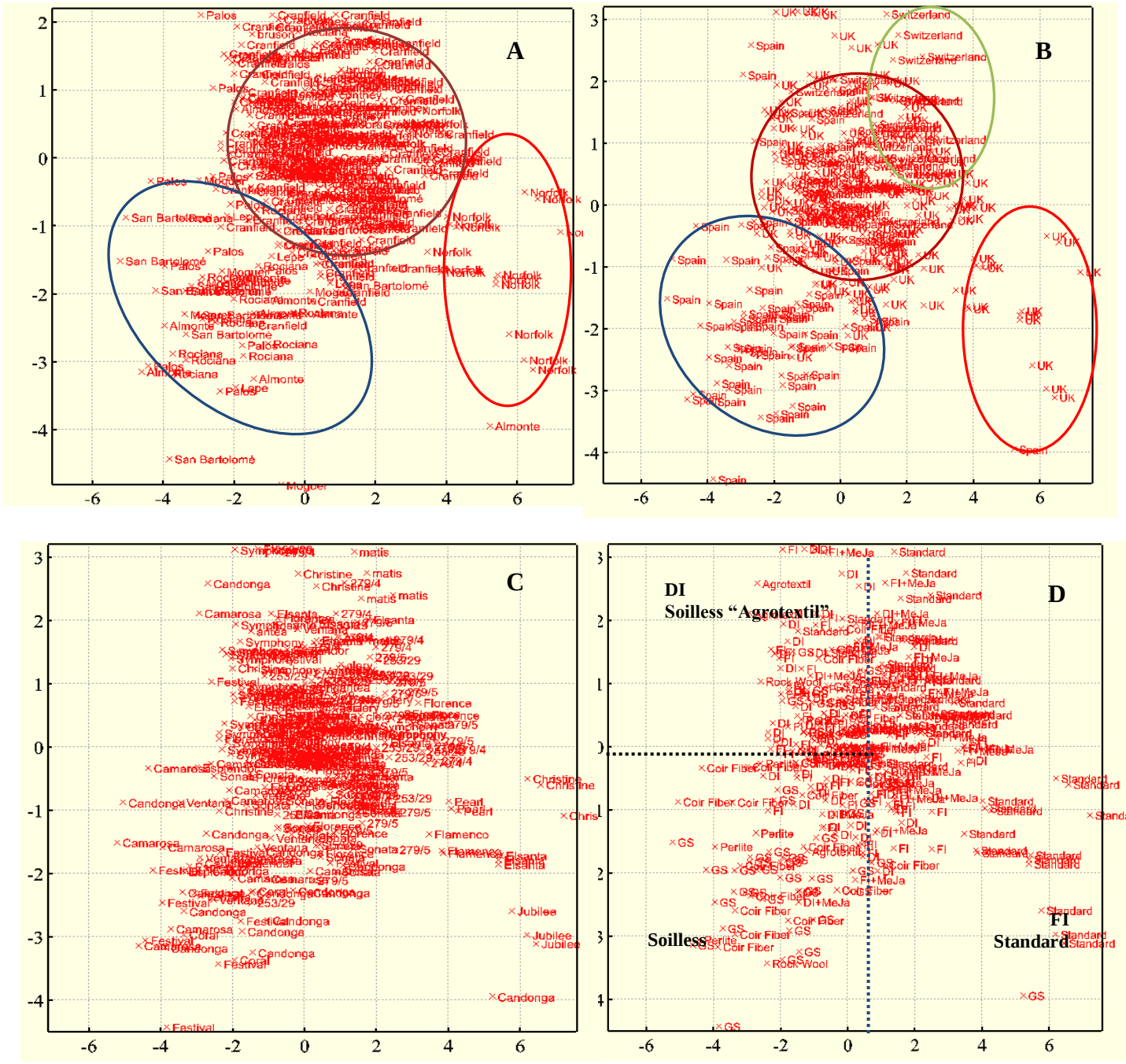


Figure 3.17: Principal component analysis (PCA) characterisation of strawberry fruits from different cultivars (C), locations (A and B) and grown using different cultivation techniques (D) using taste-related compounds as analytical data. Y axis = PC1 (44%) and X axis = PC2 (22%).

Grouping along PC1 revealed differences in sugar composition as well as the sugar acid ratio between strawberry samples commercially grown in UK and Spain to those grown in

Switzerland (A and B) (**Figure 3.17**). In addition, it was noticed that there vast variability in the glucose and sugar acid ratio from samples originated from Spain, which indeed was attributed not to genotypic differences (C) but due to the nature of the different soilless substrates where the plants were grown (D). On the other hand, grouping along PC2 clearly distinguished between commercially grown samples from UK and Spain, mainly due to their differences in Sweetness (**Figure 3.17**) as well as the monosaccharide ratio, indicating that even though total sugar concentrations were relatively similar, significant differences between those fruits existed for their relative sugar proportions.

3.5 Conclusions and future research needs

Results from this chapter have shown the existing variability in the composition of strawberry fruits from different cultivars, growing locations as well as due to different pre-harvest strategies. Indeed, earlier evidence already demonstrated the role that certain pre-harvest conditions had on strawberry fruit quality, although most of the information available referred to the health-related composition of the fruits rather than compounds directly related to taste, and hence consumer acceptance. In the present chapter it was verified that DI on strawberry plants can reduce berry size and yield of certain cultivars but more importantly can have a marked effect on the concentration of certain compounds directly related to fruit quality (*viz*. sugar and acids, sweetness and antioxidants). Similarly, manipulating the concentration of nutrients supplied to the plant resulted in considerable changes in sugar concentrations within the fruit. Nevertheless, the response of strawberry plants to drought conditions clearly depended on the development stage of the plant as well as the genotype investigated, since little or no differences were found for certain cultivars when plants were grown under full or deficit irrigation conditions or when drought conditions were initiated at white development stage. In this context, future work should assess the response and variability of a range of cultivars when DI conditions are applied at different development stages so that year-to-year variation can be excluded

Results suggested that greater sugar concentrations, in DI treated plants, are due to changes in respiratory metabolism and trade-off in resource allocation rather than a dilution effect as hypothesised in earlier works. For instance, evidence was shown that DI did not directly affect sucrose accumulation in leaf tissues but rather resulted in differences in channelling

sucrose degradation products to different carbon pools such as monosaccharides and organic acids.

The levels of antioxidant compounds in fruits and other plant tissues were also greater in DI as compared to plants kept at or near field capacity, which may be the result of *in situ* synthesis of antioxidants thereby limiting the amount of ROS generated by the plant under stress conditions. Nonetheless, anthocyanin concentrations were not significantly affected by DI treatments but were enhanced after treatments with MeJa. However, if taking together the results presented herein and earlier studies (Terry *et al.*, 2007a), and considering the physiological similarities between ABA and MeJA, the observed changes in anthocyanin concentrations may be modulated by the levels of these natural hormones in the fruit. Therefore it is noticeable the importance of these hormones on secondary plant metabolism probably by increasing PAL activity. Future studies should assess changes in endogenous ABA and MeJa and relate these changes with primary and secondary strawberry plant metabolism.

The results and methodologies detailed in this chapter have been published as:

Giné Bordonaba, **J**. and Terry, L.A. (2010). Manipulating the taste-related composition of strawberry fruits (*Fragaria* x *ananassa*) from different cultivars using deficit irrigation. Food Chemistry (in press).

Crespo, P., **Giné Bordonaba, J**., Carlen, C. & Terry, L.A. (2010) Characterisation of major taste and health-related compounds of four strawberry genotypes grown at different Swiss production sites. *Food Chemistry* 122, 16-24.

CHAPTER 4

TASTE- AND HEALTH-RELATED COMPOSITION OF BLACKCURRANT BERRIES:

VARIATIONS AMONG GENOTYPES, GROWING LOCATIONS, MATURITIES AT HARVEST AND DURING STORAGE

4.0 CHAPTER FOUR

Taste- and health-related composition of blackcurrant berries: variations among genotypes, growing locations, maturities at harvest and during storage

4.1 Introduction

Blackcurrant (*Ribes nigrum* L.) is a species of *Ribes* berries native to central and northern Europe and northern Asia (Brennan, 2005). Nowadays, most of the production of blackcurrant berries is for use in processed products, including juices and cordials (Brennan *et al.,* 2003; Zheng *et al.,* 2009). Nevertheless, fresh blackcurrant berries are recognised as one of the richest sources of antioxidants, notably anthocyanins, hydroxycinnamic acids (Koeppen and Hermann, 1977), and ascorbic acid (Walker *et al.,* 2006). Experts worldwide have stated that an increased dietary intake in antioxidant compounds from fruits and vegetables may protect against the oxidative damage caused by free radicals (McDougall *et al.,* 2005; WHO, 2006). Recent studies suggest that consumption of products rich in certain phytochemicals (*viz*. phenylpropanoids) (Duthie, 2007; Pantelidis *et al.,* 2006; Seeram *et al.,* 2008), such as berry fruits, help towards reducing the incidence of certain cancers and chronic diseases (WHO, 2006; Seeram *et al.,* 2008*; See Appendix D*).

If compared to other berry types, scarce information is available in the literature which describes the taste-related composition (*viz*. sugars and acids) of blackcurrant fruits, which is probably due to their limited commercial importance on the fresh market. Nevertheless, sugar and acid concentrations within the fruit as well as the corresponding sugar/acid ratio are among the primary quality factors of fruits and fruit juices (Terry *et al.,* 2005). Berries with pleasant taste characteristics often have high contents of sugars and relatively low contents of acids (Ruiz del Castillo *et al.,* 2002), although scarce data exist regarding the sugar and acid content for blackcurrant berries (Boccorh *et al.,* 1998). In contrast, quite a few methods are reported for the extraction and quantification of specific quality-related target analytes in blackcurrant fruit (Ruiz del Castillo and Dobson, 2002; Zadernowski *et al.,* 2005; Brennan *et al.,* 1997), with the majority of studies focused on quantification of anthocyanins and other phenylpropanoids from processed fruit or blackcurrant-based beverages. Only limited

information is available which refers to the genotypic variability of sensory properties of blackcurrant juices (Brennan *et al.*, 2003) or fresh berries (Ruiz del Castillo and Dobson, 2002). Nevertheless, recent research demonstrated that processing (Hollands *et al.*, 2008) or postharvest storage (Harb *et al.*, 2008) of blackcurrant fruits dramatically reduced the content and bioavailability of taste-and health-related compounds when compared to fresh fruits.

Given this situation, and considering the variety of cultivars available, it is perhaps surprising that more research has not been conducted to characterize the biochemical profile of different genotypes, especially for fresh fruits, or to assess changes in blackcurrant berries during the later stages of ripening or storage. Accordingly, the present chapter describes the results from several experiments conducted on blackcurrant berries harvested during the summers of 2006 and 2007.

The aims of the present trials were (I) to optimize different standard extraction and analytical methods for quantification and comparison of a wide range of health- and taste-related compounds in berries from a range of UK-grown blackcurrant genotypes, (II) to assess the variation in the composition of blackcurrants during the latest stages of ripening or due to different growing locations, (III) to better understand the biochemical changes that occur during postharvest storage of blackcurrant fruit and hence optimise storage conditions, and (IV) to assess the variation in the composition of blackcurrant fruits using chemometric data analysis.

4.2 Plant materials and experimental design

4.2.1 Effect of genotype on blackcurrant fruit composition

Seventeen different blackcurrant cvs. (**Table 4.1**) from 6 year-old bushes were hand-harvested at optimum ripeness (when fully black) in summer 2006 and used for this experiment (Exp.4.1). Bushes were grown under standard field conditions and were supplied by the Scottish Crop Research Institute (SCRI; Invergowrie, UK; 56°27′ N, 3° 3′ W). Samples were transported at 4ºC by road to Cranfield University overnight.

4.2.2 Effect of maturity at harvest, genotype and growing location

Two experiments (Exp. 4.2 and Exp. 4.3) were conducted on blackcurrant berries from different cultivars, hand-harvested at different edible maturity stages and grown at two different locations within UK during summer 2007. Fruits used for experiment two (Exp.4.2) were supplied by GlaxoSmithKline, grown at Norfolk, UK (52º39’ N, 0º 54’ E) and consisted

of berries from three different blackcurrant cvs. (**Table 4.1**) harvested at three different maturity stages (early ripe (ER), fully ripe (FR) and overripe (OR)) based on standard commercial evaluation, and hence samples from the different cultivars were harvested on different dates reflecting the time course variability of each cultivar. Berries for the third experiment (Exp.4.3), were supplied by the Scottish Crop Research Institute (SCRI) grown at Invergowrie, UK and consisted of fruits from eleven different cultivars and selections (**Table 4.1**) harvested at both ER and FR stage. All fruits (*ca*. 2 kg per cultivar and maturity stage) were originated from bushes of similar age and grown under commercial standard practices.

Table 4.10: Percentage information of a range of UK-grown blackcurrant cultivars used for each experiment.

Cultivar/Genotype	Parentage (♀ x ♂)	Experiments
Ben Tirran	Ben Lomond x N29/17	4.1, 4.2
Ben Alder	Ben Lomond x Ben More	4.1, 4.3
Ben Dorain	Ben Alder x C2/1/62	4.1, 4.2, 4.3
Ben Starav	Ben Alder x C2/10/72	4.1
Ben Klibreck	S13/17/3 x (Ben More x 74020-16)	4.1
Ben Lomond	(Consort x Magnus) x (Brodtorp x Janslunda)	4.1, 4.3
Ben Hope	Westra x (233/36 x EM21/15)	4.4
Ben Gairn	Ben Alder x Golubka	4.2
Ben Vane	(S240/18 x S243/7) x (Westra x S243/7)	4.3
Baldwin	Ben Sarek x Ben Lomond	4.3
Hedda	Ojebyn x Melalahti	4.3
871-5	AB3/7 x C6/12/36	4.1, 4.3
9199-4	S18-1-2 x B1834-120	4.1
9198-1	S18-15-1 open pollinated	4.1, 4.3
9141-6	Ben Alder x S18-15-1	4.1
9137-2	Ben Avon x Ben Hope	4.1
9111-14	Ben Alder x B1610-68	4.1
S26-5-3	C2/4/51 x Ben Alder	4.1
9328-45	(Ben Alder x Ben Loyal) x S10-2-27/28	4.1, 4.3
9148-9	Ben Dorain x S18-23-5	4.1
9311-82	S36-2-8 x B1834-120	4.1
91129-1	Ben Dorain x B1836-120	4.1, 4.3
9154-3	Ben Alder x S18-15-1	4.3

4.2.3 Effect of postharvest storage temperature on blackcurrant fruit composition

Blackcurrants cv. Ben Hope were supplied by GlaxoSmithKline Plc and used for this trial (Exp. 4.4). The fruit were grown on the same farm in Norfolk, UK (52º39' N, 0º 54' E) using conventional methods (Rob A. Saunders, GSK, *pers. comm.*), and harvested by hand at two different commercial maturities; early ripe (ER; 9th July) or fully ripe (FR; 23rd July) in 2007.

In order to investigate the effect of temperature during postharvest storage of blackcurrant berries, a temperature gradient was set up, based on a solid aluminium block (86.0 cm x 20.2 cm x 10.0 cm) with 5 rows of 20 precision-drilled holes of 2.5 cm diameter and 7 cm depth, with sufficient metal between each hole (1 cm) to ensure adequate heat and / or cold transfer along the block (Beylaggoun, 1999) (**Figure 4.1**). To avoid any heat leakage, the block was totally insulated with expanded polystyrene (7.5 cm thickness). Two small reservoirs, with water tight cover plates at each end of the aluminium block were linked to hot water circulating equipment (water bath) at one end and to coolant circulating equipment at the other. The temperature gradient was measured using a digital thermometer (Jenway, Essex, UK) and was set at 20ºC at one end of the block and 1ºC at the other end, resulting in increments of 1ºC. Blackcurrant berries (*ca*. 12 g) were placed in glass vials within each hole. Where possible, fruit were left as racemes, with individual berries added to make up the total mass. The experiment was a completely randomized design, with the structure consisting of different maturity stages, postharvest storage temperature and storage durations. Temperature intervals (*viz*. 1-4, 5-8, 9-12, 12-16, 17-20 ºC) rather than individual temperature points were considered in order to increase the number of replicates per treatment (n = 4) and further assess the interactions of storage temperature with other factors. Organic acids, anthocyanins and non-structural carbohydrates were measured at four time points (day 0; n=10, days 1, 3, and 7; n=20), whereas respiration rate was recorded at days 0, 1, 3, 5 and 7. Control measurements (day 0) were made on freshly received fruit, where respiration rate was measured in berries held at 1.5 h for 5ºC (approximately the temperature of samples after reception and initial sample preparation steps).

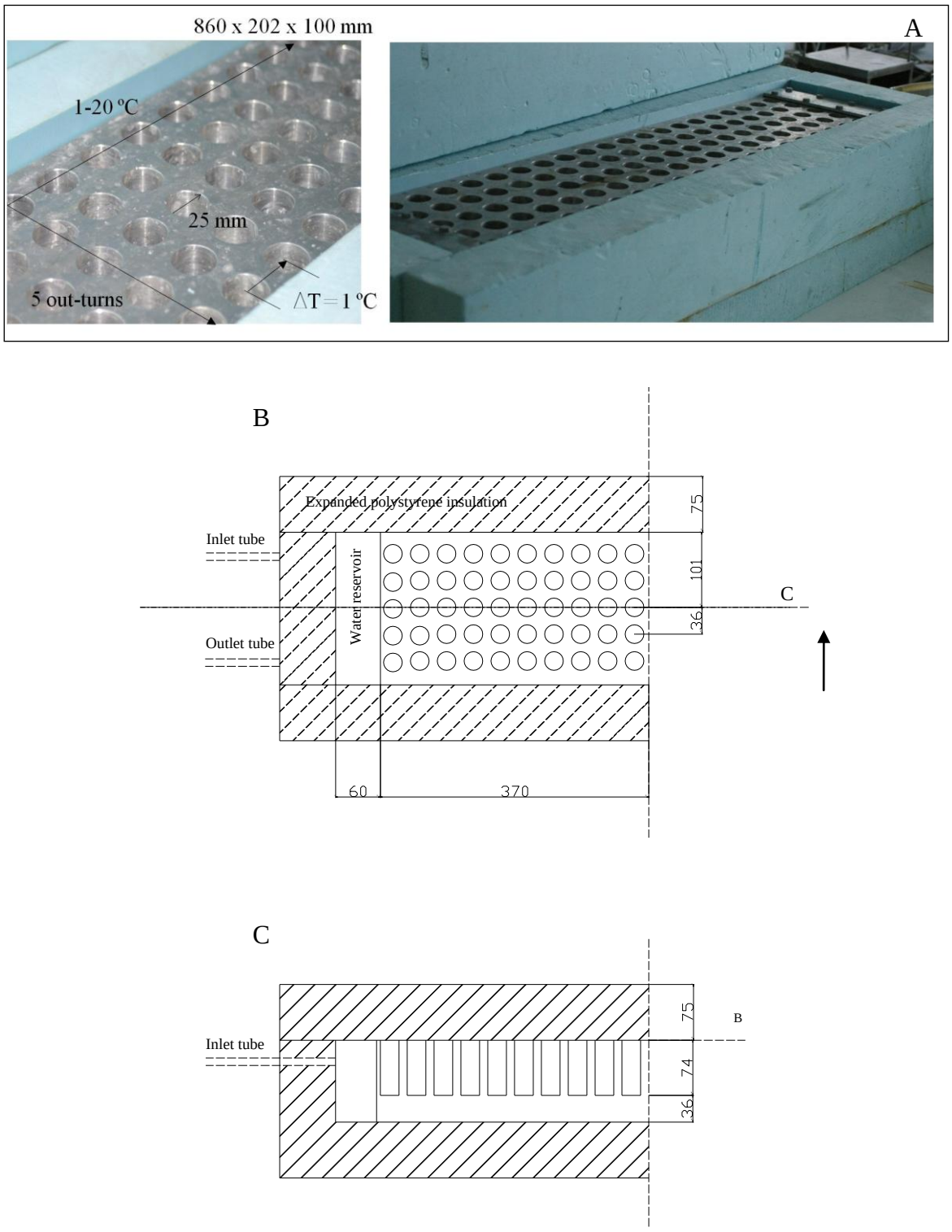


Figure 4.1: Image (A) and schematic diagram of the temperature block; plan view (B) and longitudinal section (C). Numbers (B, C) indicate the dimensions (mm).

4.3 Materials and methods

4.3.1 Fruit sampling

Immediately after harvest fruits were stored on ice and transported by road to Cranfield University overnight or within the same day. After reception total soluble solids (TSS), tritrable acidity (TTA) and pH were measured. Briefly, 150 g of sample were allowed to defrost at room temperature for 1h (Ruiz del Castillo and Dobson, 2002) and were homogenised. The pH of the samples was determined using a Jenway 3020 pH meter (Jenway, Essex, UK) whereas TTA measurements were based on that previously reported (AOAC, 1984)

The rest of the samples were immediately snap-frozen in liquid nitrogen and stored at -40ºC. From each cultivar and maturity a sub sample (150 g) was lyophilized using a Christ ALPHA-RVC freeze drier with cooling-trap ALPHA 1- 4 (Christ, Lower Saxony, Germany) for 5 days at 0.015 kPa. Samples were subsequently weighed, ground in a pestle and mortar to a fine powder, divided into separate vials, and returned to the freezer (-40ºC) prior to analysis. All reagents were purchased from Sigma (Dorset, UK) and assays carried out in triplicate unless otherwise stated.

4.3.2 Extraction and quantification of sugars and non-volatile organic acids

Sugars (*viz*. glucose, fructose and sucrose) and organic acids (viz. ascorbic, citric and malic acid) were extracted according to methods described in Chapter 3 (section 3.3.4). Sugar and organic acid concentrations in blackcurrant extracts were determined using an Agilent 1200 series HPLC binary pump system (Agilent, Berks, UK), equipped with an Agilent refractive index detector (RID) G1362A and a DA G1315B/G1365B photodiode array with multiple wavelength detector, respectively (Chapter 3; section 3.3.4).

4.3.3 Extraction and quantification of blackcurrant anthocyanins

Individual anthocyanins were extracted by mixing freeze-dried sample (150 mg) with 3 ml of different solvent combinations including (methanol: HCl: water (70: 0.5: 29.5; v/v/v); methanol: HCl: water (50: 0.5: 29.5; v/v/v); ethanol: HCl: water (70: 0.5: 29.5; v/v/v); ethanol: HCl: water (50: 0.5: 29.5; v/v/v) and HCl: water (99.5: 0.5; v/v). After choosing the optimum

extraction solvent the anthocyanin profile was determined as described in Chapter 3 (section 3.3.5) with some modifications to achieve superior peak separation and resolution. The HPLC system comprised an Agilent 1200 series HPLC system (Agilent, Berks., UK), equipped with an Agilent 1200s DA G1315B/G1365B photodiode array with multiple wavelength detector. Blackcurrant extracts (20 µl) were injected into a Zorbax Eclipse XDB-C18 column of 250 mm x 4.6 mm diameter, 5 µm particle size with an XDB-C18 guard column of 12.5 mm x 4.6 mm diameter (Agilent) The mobile phase consisted of degassed 2.5% (v/v) acetonitrile and 5% (v/v) formic acid in HPLC-grade water (A) and acetonitrile (B) following a linear gradient program of increasing polarity in several steps (from 0 to 13% B in 10 min, 18.7 min-18% B, 22 min-65% B and 27 min-65% B). The flow rate was 1 ml min^{-1} and the column temperature set at 40ºC (Agilent 1200s, G1316A, Berks., UK). Anthocyanins were detected at 520 nm and the presence and abundance of each anthocyanin was calculated by comparing the peak area with external standards (delphinidin-3-glucoside (delp-3-gluc), cyanidin-3-glucoside (cya-3-gluc) and cyanidin-3-rutinoside (cya-3-rut) (Extrasynthèse, Genay, France) using Agilent ChemStation Rev. B.02.01. Due to the lack of standard for delphinidin-3-glucoside, the concentration of this compound was calculated from the calibration curve of delphinidin-3-glucoside.

Over the course of this thesis, the scientific community faced a worldwide shortage of acetonitrile which resulted on prices to soar drastically. Acetonitrile has been, over the past decades, the mobile phase of choice for determination of anthocyanins by HPLC, with few alternatives being available. Accordingly, during the last year of these experiments, efforts were made through the development of an acetonitrile-free mobile phase for HPLC determination of anthocyanins in selected berries (Appendix B)

4.3.4 Extraction and quantification of total phenolics, total flavonoids and antioxidant capacity

Total phenolic concentrations and antioxidant concentrations were measured from freeze-dried material as described earlier (Chapter 3; section 3.3.7; Terry *et al.*, 2007a). Briefly, total phenolic concentrations were measured by means of the Folin-Ciocalteu Method (FCM), based on the reduction of a phosphowolframate-phosphomolibdate complex by phenolics to

blue reaction products, whereas antioxidant capacity from berries was based on the FRAP assay (Terry *et al.*, 2007a; Chapter 3; section 3.3.7).

For the extraction of total flavonoids, two different solvent combinations were studied based on earlier works (Davis and Terry, unpublished). Briefly, 150 mg of lyophilized sample was dissolved into 3 ml of either 70% (v/v) methanol and 0.5% (v/v) HCl in HPLC-grade water or ethanol: water (80:20; v/v). Samples were extracted in a HAAKE SWB 20 water-bath at 70°C (Thermo Scientific, Germany) for 2h, mixing every 20 min. The solution was filtered using a syringe filter of 0.2 μm and the clear filtrate analyzed. And aliquot (100 μl of sample or quercetin standard) were mixed with 3 ml of NaOH (4 g NaOH in 100 ml ethanol:water (50:50; v/v)). Samples were then left to develop for 10 min prior to measuring the absorbance at 420nm. Total flavonoid content was estimated from a standard curve of quercetin and results were expressed as mg quercetin equivalents (QE) g^{-1} dry weight (DW). Seven point calibration was linear to a concentration of 5 mg mL^{-1} ($R^2 > 0.993$) and reproducible results were obtained for all samples.

4.3.5 Statistical and chemometric data analysis

All statistical analyses were carried out using Genstat for Windows Version 12 (VSN International Ltd., Herts., UK). Least significant difference values (LSD; $P = 0.05$) were calculated for mean separation using critical values of t for two-tailed tests for each individual experiment. Comparison between experiments (Exp. 4.2 and 4.3) was only conducted taking into account two maturity stages and common cultivars grown in both locations. Tests for correlations between mean values for analyte concentrations were made using Spearman's Rank Correlation. Correlations are presented with the Spearman's Correlation Coefficient (r) and P value based on a two-tailed test. Chemometric data analysis, including principal component analysis and hierarchical cluster analysis, was performed using the same software described above; the triplicate per sample values were used as an input for chemometric analysis (PCA) (Chapter 3; section 3.3.9).

4.4 Results and discussion

4.4.1 Effect of genotype on blackcurrant fruit composition

4.4.1.1 Sugars and non-volatile organic acids.

For the extraction of both sugars and non-volatile organic acids, the methods described herein were successfully adapted and slightly modified from previous reported methods applied to other fresh produce types (Wang *et al.,* 1996; Davis *et al.,* 2007; Terry *et al.,* 2007a). Although little information is available describing the sugar and acid composition of fresh blackcurrant berries, concentrations were in accordance with those reported in the literature for blackcurrant derivative products (Boccorh *et al.,* 1998)

In all experiments, the content of sugars was significantly influenced by genotype (**Table 4.2;** Exp. 4.1), on both a FW and DW basis. However, sugar concentrations were correlated with water content ($r^2 = 0.63$) and therefore some of the sugar variation between cvs. was most probably caused by a 'dilution effect'. Sucrose, glucose and fructose were identified in all cultivars and concentrations ranged between 3.38-36.89 mg g^{-1} FW, 36.86-82.78 mg g^{-1} FW and 43.43-85.73 mg g^{-1} FW, respectively. The proportion of each sugar also varied with genotype. Glucose and fructose were the dominant sugars found in all cultivars making up *ca*. 40% and 49%, respectively, of total sugars. Sucrose, on the other hand, made up *ca*. 10% of the total sugar concentration but was more variable within genotypes. Fructose/glucose ratios ranged from 0.91 (cv. Ben Lomond) to 1.53 (cv. S26-5-3) and therefore were in agreement with those reported by Boccorh *et al.* (1998) for blackcurrant concentrates. Concomitant to this, significant differences in the fructose/glucose ratio were observed (Boccorh *et al.,* 1998) when different origins and harvest year were investigated. Some cvs., e.g. 871-5, Ben Alder and Ben Lomond had a total sugar concentration that was 1.25-fold higher than the mean of the 17 cultivars tested (**Table 4.2**; Exp. 4.1).

Table 4.2: Sugar concentrations in 17 UK-grown blackcurrant cultivars on a fresh weight (FW) and dry weight (DW) basis. Cultivars were arranged in descending order according to total sugar content. Data presented corresponds to Exp. 4.1.

Genotype	Sucrose	Glucose	Fructose	Total Sugars	S/A[A]	m/d[B]
	mg g^{-1} FW (DW)					
871-5	17.64 (48.23)	82.78 (226.35)	79.50 (217.51)	179.92 (492.10)	1.89	3.41
Ben Alder	3.38 (9.59)	78.02 (221.09)	85.37 (241.53)	166.78 (472.61)	2.61	48.58
Ben Lomond	16.38 (43.78)	74.85 (200.05)	68.52 (183.14)	159.75 (426.97)	2.50	8.71
Ben Dorain	15.38 (42.40)	60.36 (166.58)	73.48 (202.77)	149.22 (411.79)	1.99	29.55
9199-4	10.93 (29.49)	57.24 (154.40)	72.06 (194.35)	140.24 (378.24)	1.84	7.82
Ben Starav	36.88 (127.72)	45.53 (156.75)	57.35 (198.63)	139.76 (483.10)	2.57	11.83
9198-1	31.54 (90.48)	45.96 (131.84)	61.53 (176.50)	139.04 (398.82)	2.96	8.76
9141-6	19.26 (53.54)	49.92 (138.79)	60.10 (167.09)	129.28 (359.42)	1.90	17.33
9137-2	14.04 (47.71)	45.96 (156.19)	57.98 (197.04)	117.99 (400.94)	2.47	5.72
Ben Tirran	3.83 (11.99)	51.75 (162.02)	61.37 (192.11)	116.95 (366.12)	2.22	5.24
9111-14	17.86 (64.01)	39.52 (141.66)	54.03 (193.68)	111.40 (399.36)	2.27	11.3
S26-5-3	9.06 (30.92)	40.38 (137.88)	61.89 (211.30)	111.33 (380.11)	2.54	7.41
Ben Klibreck	17.72 (67.05)	43.19 (163.39)	50.10 (189.53)	111.01 (419.97)	3.20	2.78
9328-45	8.34 (28.58)	44.83 (153.67)	53.45 (183.22)	106.62 (365.47)	4.39	9.21
9148-9	11.86 (42.48)	36.86 (132.07)	55.76 (199.79)	104.48 (374.30)	2.18	22.82
9311-82	5.46 (17.03)	43.62 (135.99)	50.81 (158.39)	99.89 (311.41)	2.85	5.27
91129-1	3.57 (12.46)	38.09 (132.76)	43.43 (151.38)	85.09 (296.60)	2.92	11.83
Mean	14.30 (45.15)	51.68 (159.50)	61.58 (191.67)	127.19 (396.32)	2.51	12.73
LSD (P < 0.05)	1.040 (3.328)	1.025 (3.103)	1.633 (5.393)	2.842 (9.216)	0.082	1.956

[A] Total sugars / Total organic acids; [B] monosaccharide/disaccharide ratio (m/d) = (Glucose +Fructose) / Sucrose

Non-volatile organic acids on both a FW and DW basis were also influenced by genotype (**Table 4.3**; Exp. 4.1). Five different organic acids were identified in all cvs. and significant genotypic differences were observed (**Table 4.3**). In all genotypes, citric acid was the predominant organic acid and accounted for ≥73% (FW basis), of the total organic acid content in blackcurrant fruits. Comparable results, in terms of proportion of individual organic acids, were obtained by (Boccorh *et al.*, 1998) when blackcurrant concentrates were analyzed for citric, ascorbic and malic acids. Concentration on a FW basis of oxalic, tartaric, malic, ascorbic and citric acids varied from 0.23-0.47 mg g^{-1}, 0.46-1.12 mg g^{-1}, 0.70-6.91 mg g^{-1}, 1.92-5.42 mg g^{-1} and 28.84-59.79 mg g^{-1}, respectively. Results from this study confirmed that blackcurrant fruits are known to be a good source of ascorbic acid (AsA) (Rubinskiene *et al.*, 2006; Walker *et al.*, 2006) and, for the first time, minor organic acids, including oxalic and tartaric acid, have been reported in fresh blackcurrant berries. Concentration of AsA ranged from 1.92 to 5.42 mg g^{-1} FW and therefore was far higher as compared to other common berry fruits where AsA content is generally >1 mg g^{-1} FW (Iversen, 1999; Walker *et al.*, 2006). Significant differences for AsA concentrations were also found across cvs. on both a FW and DW basis (**Table 4.3**). Although AsA was not significantly correlated to total organic acid content, results suggest a strong relationship between both parameters for certain cvs.

Table 4.3: Concentration of main organic acids in 17 UK-grown blackcurrant cultivars on a fresh weight (FW) and dry weight (DW) basis. Cultivars were arranged in descending order according to total organic acid content. Data presented corresponds to Exp. 4.1.

Genotype	Oxalic	Tartaric	Malic	Ascorbic	Citric	Total
			mg g^{-1} FW (DW)			
9198-1	0.42 (1.22)	0.82 (2.34)	6.90 (19.81)	5.41 (15.53)	59.79 (171.50)	73.35 (210.4)
Ben Alder	0.47 (1.33)	0.76 (2.16)	4.05 (11.47)	2.53 (7.18)	56.05 (158.83)	63.86 (180.96)
Ben Dorain	0.45 (1.25)	0.91 (2.50)	5.04 (13.91)	3.31 (9.15)	49.98 (137.93)	59.69 (164.74)
Ben Tirran	0.37 (1.17)	0.70 (2.19)	3.74 (11.70)	3.10 (9.72)	50.61 (158.45)	58.53 (183.23)
9148-9	0.30 (1.08)	0.53 (1.91)	2.59 (9.29)	2.75 (9.84)	50.74 (181.79)	56.92 (203.92)
9199-4	0.38 (1.02)	1.12 (3.03)	6.59 (17.77)	3.76 (10.14)	42.74 (115.28)	54.59 (147.23)
Ben Lomond	0.31 (0.82)	0.69 (1.84)	3.34 (8.94)	3.96 (10.60)	45.69 (122.13)	54.00 (144.34)
9311-82	0.30 (0.95)	0.95 (2.97)	6.37 (19.86)	3.51 (10.94)	41.37 (128.97)	52.51 (163.70)
9141-6	0.37 (1.04)	0.91 (2.53)	1.75 (4.86)	3.30 (9.19)	46.02 (127.95)	52.36 (145.55)
9111-14	0.35 (1.26)	0.46 (1.66)	1.33 (4.76)	2.38 (8.55)	45.78 (164.10)	50.30 (180.33)
S26-5-3	0.32 (1.11)	0.65 (2.23)	0.99 (3.38)	3.49 (11.90)	43.68 (149.13)	49.13 (167.74)
9137-2	0.36 (1.22)	0.73 (2.49)	3.35 (11.37)	4.00 (13.60)	38.05 (129.31)	46.49 (158.00)
Ben Starav	0.29 (1.02)	0.60 (2.80)	1.30 (4.52)	2.26 (7.82)	39.09 (135.38)	43.55 (150.83)
871-5	0.30 (0.82)	0.77 (2.09)	0.82 (2.23)	2.26 (6.19)	36.84 (100.75)	40.99 (112.09)
91129-1	0.29 (1.04)	1.12 (3.89)	5.03 (17.53)	3.79 (13.23)	28.84 (100.53)	39.08 (136.21)
Ben Klibreck	0.24 (0.90)	0.54 (2.06)	0.70 (2.65)	2.05 (7.74)	35.46 (134.16)	38.99 (147.52)
9328-45	0.23 (0.78)	0.61 (2.076)	4.35 (14.93)	1.92 (6.59)	29.45 (100.95)	36.56 (125.32)
Mean	0.34 (1.06)	0.75 (2.36)	3.42 (10.53)	3.16 (9.88)	43.54 (136.60)	51.23 (160.12)
LSD (P < 0.05)	0.029 (0.096)	0.108 (0.335)	0.377 (1.275)	0.145 (0.483)	0.814 (2.628)	1.018 (3.346)

Sugar and acid contents are important indicators of fruit flavor (Perez *et al.,* 1997; Terry *et al.,* 2005). Moreover, sugar acid ratio is often used as an indicator of fruit ripeness (Perez *et al.,* 1997). TSS and to a lesser extent TTA are commonly used during routine quality control in the blackcurrant industry as a measure of sugar and acid content, respectively. In the present study TSS and TTA were measured and compared to results obtained by HPLC. In agreement with that previously reported for other fresh produce type (Terry *et al.,* 2005; Chope *et al., 2006*) poor correlations were found between TSS and total sugar concentration (r^2 =0.53) or between sugar: acid ratio measured as TSS/TTA with the ratio obtained from HPLC analysis (r^2 = 0.54). Sugar: acid ratios reported in the present work (**Table 4.2**) were slightly higher than those previously reported (Boccorh *et al.,* 1998). However, these authors did not quantify sucrose content and therefore total sugar content may have been underestimated.

4.4.1.2 Individual anthocyanins

Different solvent combinations appear in the literature describing the extraction of individual anthocyanins from blackcurrant berries. Some authors have refer to the use of methanol as the most efficient solvent for the extraction of these compounds in blackcurrant samples (Koeppen and Herrmann, 1977; Wang *et al.,* 1996; Tsao and Yang, 2003; Seeram *et al.,* 2008), but others instead, have shown that acetone aqueous extracts were more efficient extracting these compounds from blackcurrant and other berries (Antonnen and Karjalainen, 2006). In the method described herein, different methanol (MeOH), ethanol (EtOH) and water- based solvent combinations were tested (**Figure 4.2**). Results showed that, within the solvents tested, 70% (v/v) methanol and 0.5% HCl (v/v) in HPLC-grade water was the best solvent mixture for the extraction of these target analytes from freeze-dried blackcurrant samples. Methanol solubilizes the anthocyanins mainly present in the skin of the berries (Koeppen and Herrmann, 1977) while the HCl fraction helps maintain these compounds in their stable flavylium cation form (Naczk and Shahidi,2004; Aaby *et al.,* 2005; Antonnen and Karjalainen, 2006). The choice of extraction solvent was in agreement with that previously described (Lapornik *et al.,* 2005), since methanol extracts from blackcurrants had approximately 2 and 1.5-fold higher values of anthocyanins as compared to water and ethanol based solvents, respectively.

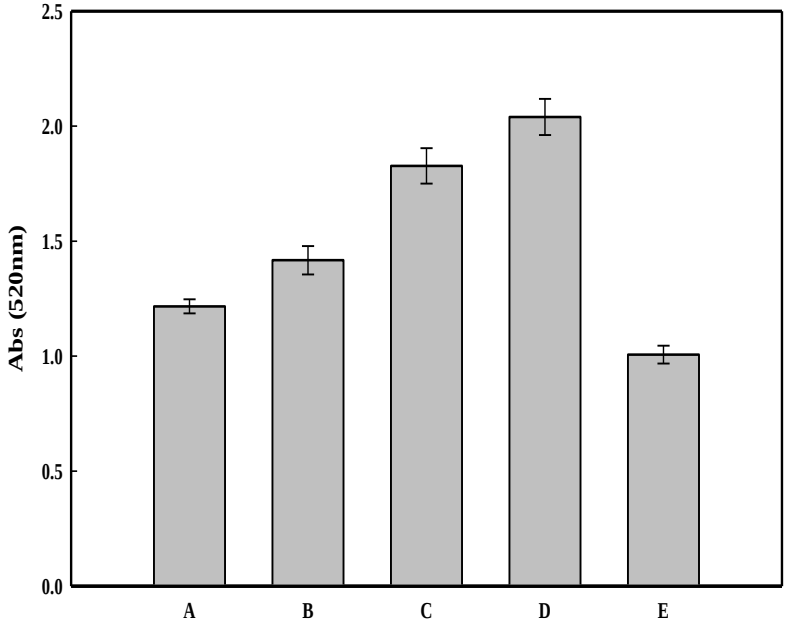


Figure 4.2: Effect of different solvents combinations (v/v) on the extraction of total anthocyanins at 20ºC for 1.5h, from freeze-dried blackcurrant (cv. Ben Dorain) samples. (A) EtOH:H2O:HCl (50:49.5:0.5); (B) EtOH:H2O:HCl (70:29.5:0.5); (C) MeOH:H2O:HCl (50:49.5:0.5); (D) MeOH:H2O:HCl (70:29.5:0.5); (E) H2O:HCl (99.5:0.5). Error bars represent standard error for n = 3.

For the identification and quantification of anthocyanins in blackcurrant extracts, a method based on that previously published (Manhita *et al.,* 2006*)* was adapted and optimized for each experiment. Generally, final runs below 30 min were required to elute all the anthocyanins present in the samples investigated. The anthocyanins profile of blackcurrants is well known (Appendix B). Four major anthocyanins (viz. delp-3-gluc, delp-3-rut, cya-3-gluc and cya-3-rut) were first reported by Chandler and Harper in 1958. Since then, numerous studies have identified these compounds in different blackcurrants or blackcurrant-based products (Koeppen and Herrman, 1977; Antonnen and Karjalainen, 2006; See Appendix B for further details). In agreement with previous work (Manhita *et al.,* 2006; Rubinskiene *et al.,* 2006), the aforementioned major anthocyanins accounted for over 80% of the total anthocyanin concentration and, were identified and quantified, according to their retention time, UV-Vis spectra and comparison with standards. Other minor anthocyanins were also observed in some of the cultivars analysed. The content of individual anthocyanins among the seventeen genotypes studied (Exp. 4.1) varied considerably (**Figure 4.3**). The relative

concentration of cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside and delphinidin 3-rutinoside was 3.1-7.9%, 35.4- 47.0%, 7.6-12.5% and 36.9-50.9%, respectively, and were comparable to that previously reported (Iversen, 1999; Landbo and Meyer, 2004; Antonnen and Karjalainen, 2006). Consequently, the rutinoside forms of anthocyanins were predominant in all the cases. In contrast, Rubinskiene *et al.*(2006) also reported similar relative concentrations of these pigments in blackcurrant fruit; however, the authors concluded that cyanidin was the major anthocyanin in all the UK-grown cultivars examined. Conversely, the results presented herein show that although having similar concentrations, in most of the cvs. delphinidin was the main pigment identified. This dichotomy could be explained because during the last few years blackcurrant breeding programmes have tended to select cultivars with high delphinidin/cyanidin ratio due to the higher stability of delphinidin (Brennan, 2005). The overall quantification of anthocyanins in blackcurrant samples, expressed as the sum of individual anthocyanins, (1.99-0.83 mg g^{-1} FW) was slightly lower than that previously reported (Antonnen and Karjalainen, 2006). Although different harvest year, cultivar, and locations are known to be a source of variation, most of the differences encountered are probably due to the extraction procedure as the authors combined the extracts from several exhaustive extractions using 70% aqueous acetone containing 0.01M HCl.

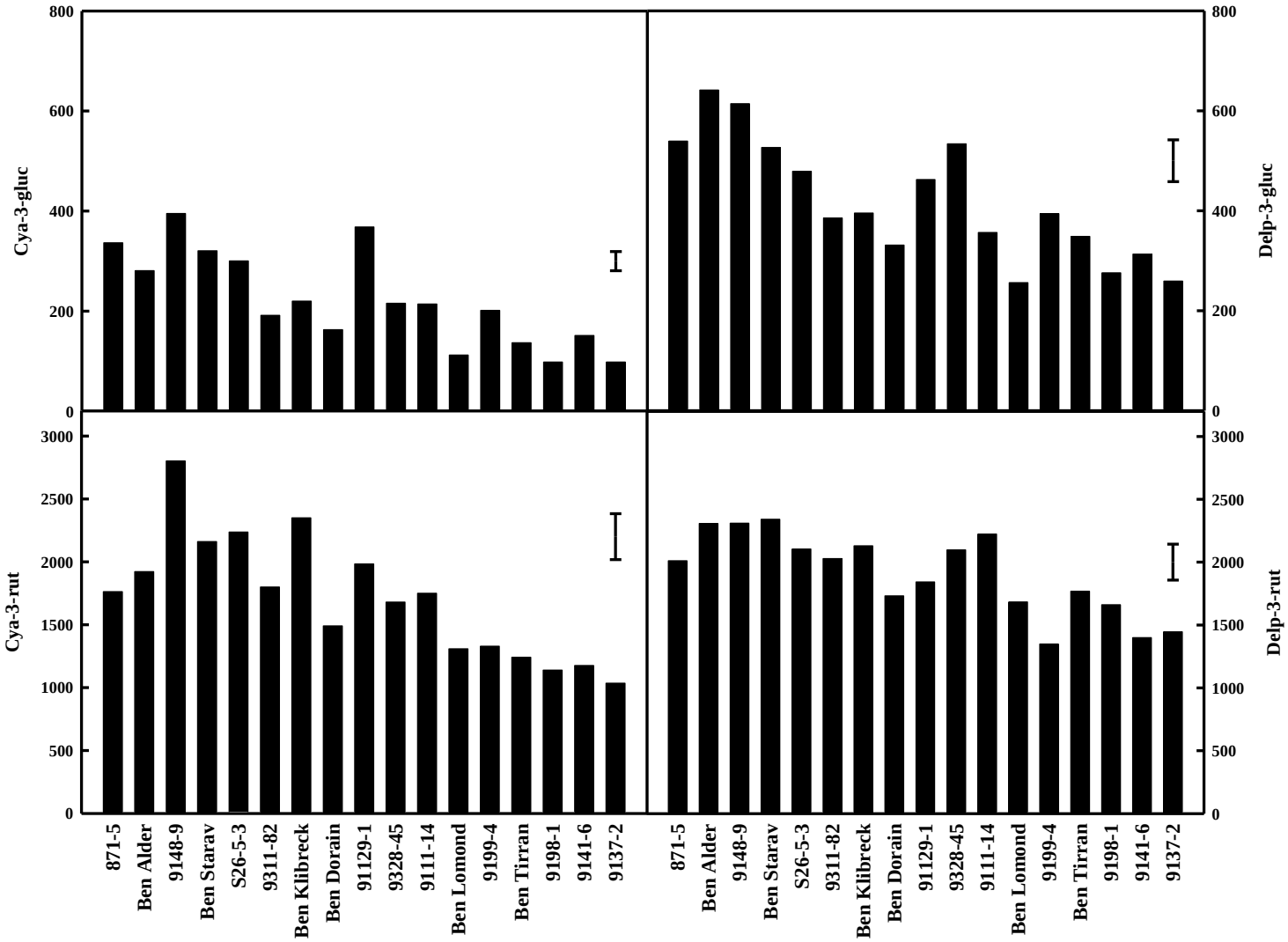


Figure 4.3: Anthocyanin concentrations (µg g^{-1} DW) of 17 UK-grown blackcurrant cultivars (Exp. 4.1).

4.4.1.3 Total phenolics concentrations

The content of TP from plant materials has been studied in great detail using several methods (Cacace and Mazza, 2002; Moyer *et al.,* 2002; Stratil *et al.,* 2006; Terry *et al.,* 2007a), most of them relying on spectrophotometric techniques, such as the herein described Folin-Ciocalteu (FC) method. In the present study, previously described spectrophotometric techniques (FC) were adapted (Singleton and Rossi, 1965) and results obtained were in the range of those previously reported (Cacace and Mazza, 2002). However, the exact assessment and comparison of results obtained is difficult due to the heterogeneity between experimental conditions applied and differences in chemical properties of oxidizable substrates within the methods found in the literature (Singleton *et al.,* 1999; da Costa *et al.,* 2000; Atkinson *et al.,* 2005; Stratil *et al.,* 2006). In these lines, the advantages and main drawbacks of the Folin-Ciocalteu method for the determination of total phenolics are described in detail in following chapters (Chapter 5; section 5.3.3). As for other analytes studied, TP concentrations were significantly affected by the genotype (**Figure 4.4**).

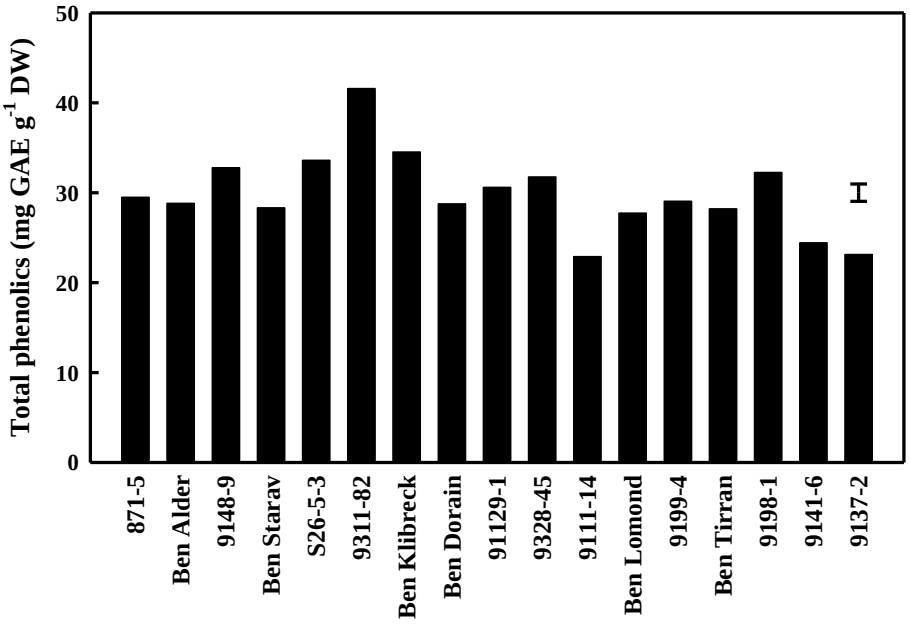


Figure 4.4: Total phenolic concentrations (mg gallic acid equivalents (GAE) g^{-1} DW) in 17 UK-grown blackcurrant cultivars.

Despite anthocyanins being one of the main groups of polyphenols present in blackcurrant (Koeppen and Herrmann, 1977; Jordheim *et al.*, 2007) no correlation was found between TP and total anthocyanin values (**Figure 4.5**). In this context, total phenolic concentrations, determined by the FCM, may have been detrimentally affected by the presence of high concentrations of ascorbic acid and saccharides, which are both relatively high in blackcurrant berries (Singleton *et al.,* 1999; Atkinson *et al.*, 2005; Jordheim *et al.,* 2007). Accordingly, anthocyanins represented only ca. 20% of the total phenolic concentrations determined in this work (**Figure. 4.5**)

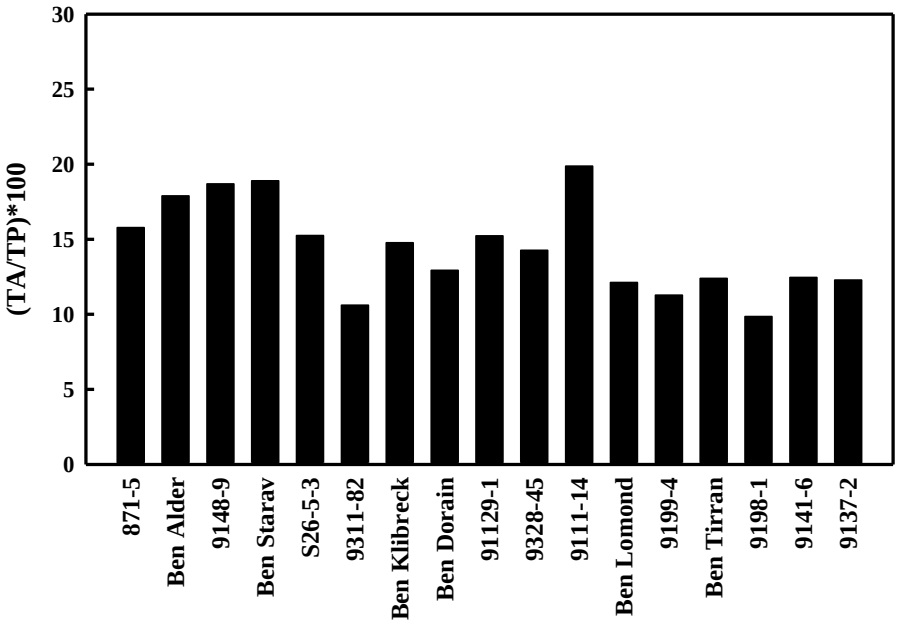


Figure 4.5: Percentage of total anthocyanins, expressed as the sum of individual anthocyanins, in comparison to total phenolic concentrations.

4.4.1.4 Principal component analysis (PCA).

Considering 14 variables as analytical data (complete biochemical profile excluding correlated variables), PCA was able to discriminate within cvs. Generally, all replicates from each sample were clustered together and separated from other genotypes, confirming the repeatability of the methods used in this study. Three Principal Components (PCs) were required to capture almost 73% of the variance. PC1 explained 32% of the total variation and was mainly related, according to factor

loadings values, to TP. Glucose, fructose and especially each delphinidin anthocyanin were also important variables to define PC1. PC2 explained 27% of the variation captured within cvs. and was related to ascorbic acid, and both cyanidin 3-glucoside and 3-rutinoside. PC3 captured 15.1% of the total variation between cvs. and was mainly related to citric and oxalic acid, fructose and again TP. The inclusion of additional PCs failed to improve clustering between cvs. Consequently, the interpretation of clustering between cultivars was mainly characterized by the information provided by PC1 and PC2 (**Figure 4.6-A**), in which the genotypic differences between cultivars were emphasized. The study of the distribution of cultivars, bearing in mind the parentage information, did not shown relevant patterns. However, most of the related cultivars were grouped within the two main central clusters, while non-related genotypes were clustered individually in the surroundings of the space plot (**Figure 4.6-A**). Initial exploration of the clustering revealed that the content of minor compounds such as individual anthocyanins, TP and ascorbic acid were relevant variables to characterize and classify different cultivars. Accordingly, several authors have referred to use of minor constituents as providing a versatile and a valuable tool for the characterization and, therefore, quality assurance of certain food products (García-Villar *et al.*, 2007; Kalithraka *et al.*, 2007; Plessi *et al.*, 2007). For instance, minor polyphenols (García-Villar *et al.*, 2007) or biogenic amines (Kalithraka *et al.*, 2007) have been used to satisfactorily characterize different wine types while the phenolic profile has suitably classified different quince jams (Plessi *et al.*, 2007).

When considering health-related compounds/parameters only (7 variables; *viz*. individual anthocyanins, ascorbic acid, TP and TA) as analytical data for the chemometric analysis (**Figure 4.6-B**), the clustering between cultivars was slightly different to that obtained when all analytical data was considered (**Figure 4.6-A**). However, the variation captured by these variables (health-related compounds) was significantly improved. Two main PCs were required to capture 80% of the variance within genotypes. PC1 explained most of the variance observed (60.2%) and was closely related to individual anthocyanins. PC2 accounted for 19.4% of total variation and was related to TP and AsA. The inclusion of a third PC did not improve significantly the clustering between cvs. (PC3 = 7.5% of the total variation). As described for general PCA, the interpretation of the results was mainly characterized by the information given by PC1 and PC2 (**Figure 4.6-B**), in which the differences in AsA and anthocyanins between cvs. were specially emphasized since four different clusters were clearly identified. Bearing in mind all the analytical information, individuals from cluster 1 were characterized for having high anthocyanins and low ascorbic acid content. Generally significant

differences in both sugar and acid content were found between the genotypes of this cluster. Cluster 2 grouped genotypes containing low anthocyanin and high AsA content. While sugar concentration for individuals of group 2 was generally similar to the mean value between the different cultivars studied, significant differences were found in their acid content. Genotypes from group 3 showed significant differences in both anthocyanins and sugar content. Similar concentration of AsA and generally mean acid content was found for all the genotypes grouped in cluster 3. Finally, genotypes from cluster 4 showed low sugar and AsA content in comparison with other clusters. Similar anthocyanin concentrations were found for the genotypes grouped in cluster 4.

Current blackcurrant breeding programmes are focused on selection of genotypes with either high anthocyanin (Brennan, 2005) and/or ascorbic acid content (Walker *et al.*, 2006). Therefore, considering the results from this study it is assumed that if both anthocyanins and AsA content are considered individually, blackcurrant breeding programmes may tend to increase the existent variability between cultivars. For instance, the selection of new cultivars with high AsA content may lead to a significant reduction in anthocyanins concentration.

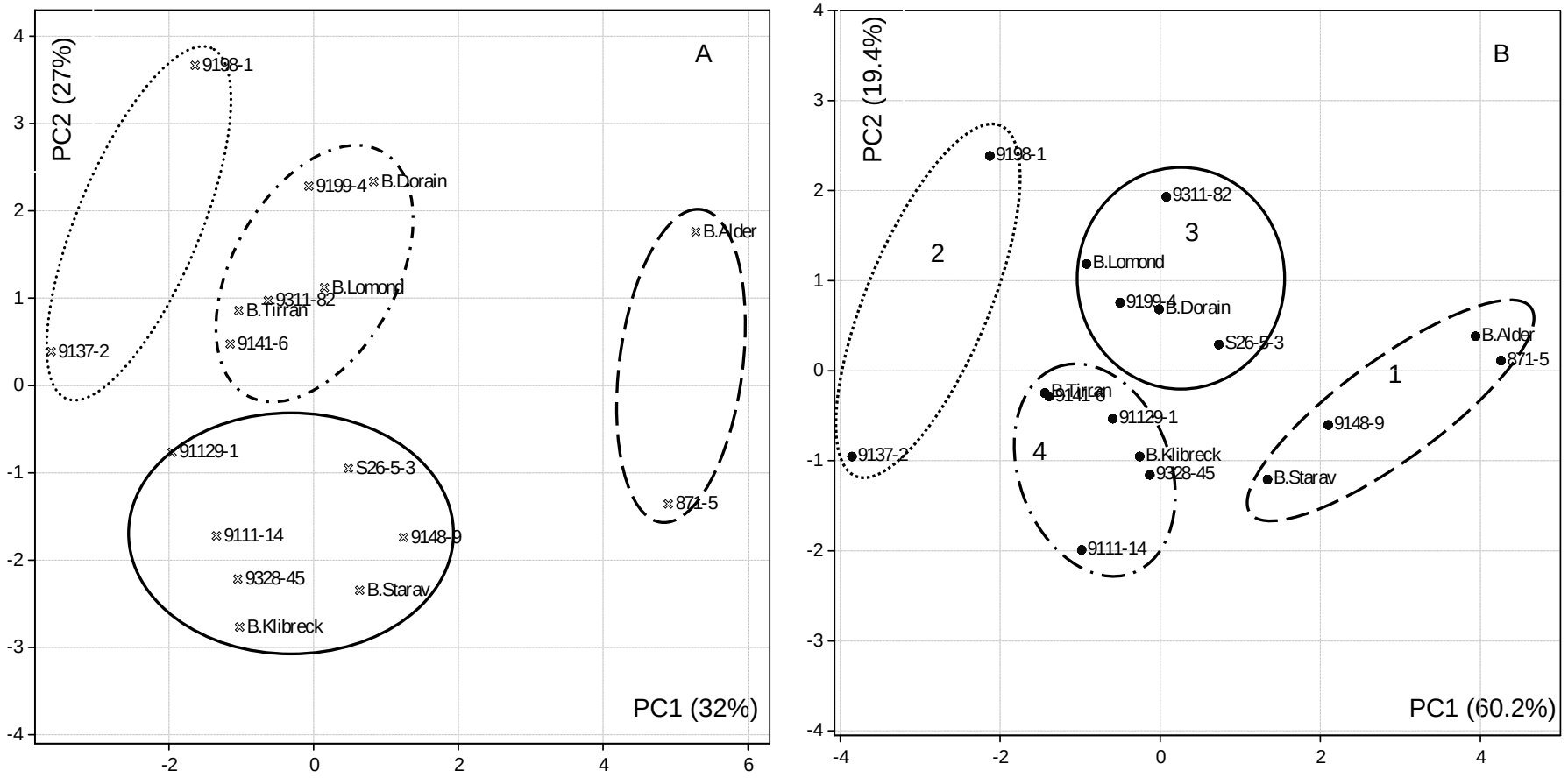


Figure 4.6: PCA characterization (PC1 vs. PC2) of 17 UK-grown blackcurrant cultivars using (A) the biochemical profile (14 variables) as analytical data and (B) health-related compounds (7 variables) as analytical data.

4.4.1.6 Hierarchical cluster analysis (HCA).

Like PCA, cluster analysis is an unsupervised data analysis method (Bereton, 2007), meaning that prior knowledge of the sample is not required. Such methods allow the clustering of the samples according to intrinsic variance between them but without being biased by desired outcomes. As compared to PCA, HCA allows interpretation of the results in a fairly intuitive graphical way. Cluster analysis was hence used as an additional exploratory tool to assess the heterogeneity between different blackcurrant genotypes and to relate the results obtained with parentage information (**Table 4.1**). Generally, HCA showed two clear clusters, with 70% of similarity, of 2 and 15 genotypes (**Figure 4.7-A)** referred to as groups 1A and 2A, respectively. Inspection of the groups showed that individuals from group 1A, called Ben Alder and 871-5, although having different parentage were reported as those containing higher concentrations of polyphenolic compounds, mainly cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside and delphinidin 3-rutinoside, and also non-structural carbohydrates. In terms of parentage, no clear relationships were observed between parentage information and biochemical profile when all the analytical data was considered. For instance, cultivars derived from a cross with Ben Alder as a parent, did not show substantial similarities (75%) in comparison with other genotypes. When considering only health-related compounds, results showed also two clear clusters of 2 and 15 genotypes respectively (**Figure 4.7-B**). In this case, the similarity between the two main clusters (1B and 2B) was lower than 0.6, and clustering of genotypes was slightly different than the HCA which considered all analytical data (14 variables) (**Figure 4.7-A**). Conversely to that described for cluster analysis of all analytical data, the use of health-related compounds was more suitable to classify cultivars according to parentage. Within cluster 2B, two clear subclusters were identified (2B' and 2B''). Most of the individuals from subcluster 2B'' (**Figure 4.7-B)** were intimately related by the genotype (Ben Lomond descendents) and showed the highest similarities (>0.95). Despite this, cvs. 9199-4 and 9311-82 although being directly related by genotype were not related to Ben Lomond, but grouped within the same cluster. On the other hand, cultivars from subcluster 2B' were intimately related to cv. Ben Alder (mainly first generation descendents) and showed also high similarities (<0.9). This said, cv. Ben Alder was clustered, using either all analytical data or health-related compounds, completely separately from its descendents. Although significant differences may exist between the parents and descendents in blackcurrant breeding programmes, it appears that a key set of biochemical traits are inherited that allow parentage to be closely grouped.

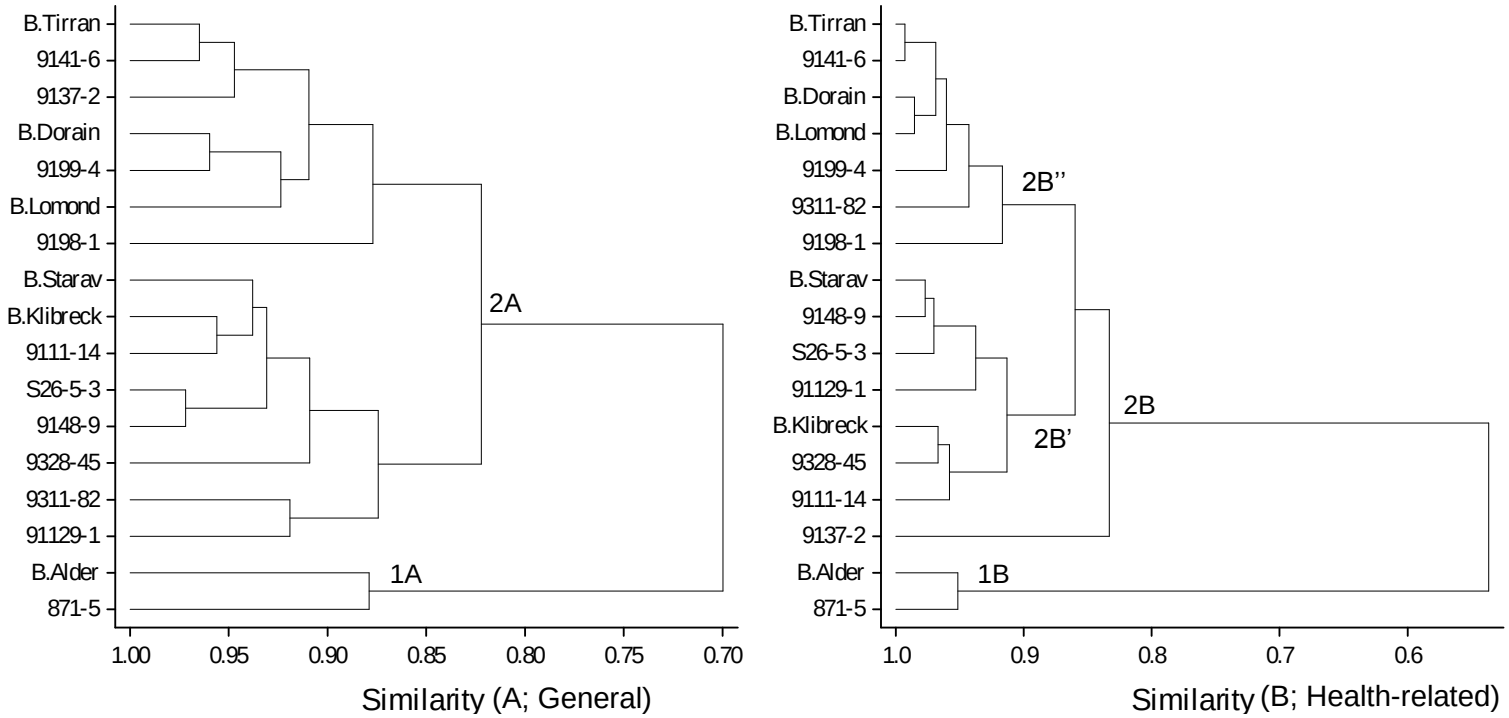


Figure 4.7: Hierarchical Cluster analysis of 17 UK-grown blackcurrant cultivars based on group average cluster analysis of (**A**) the biochemical profile (14 variables) as analytical data and (**B**) health-related compounds (7 variables) as analytical data.

4.4.2 Effect of maturity at harvest on blackcurrant fruit composition

4.4.2.1 Sugar and non-volatile organic acids

Primary metabolite concentrations in blackcurrant fruits were subject to considerable changes during ripening (Exp. 4.2 and Exp. 4.3) and agreed well with changes that occur in berries, tomato and other horticultural crops (Wang 2003; Rubisnkiene *et al.*, 2005; Gautier *et al.*, 2008; Wang et al, 2009). No other studies, thus far, have quantified changes in a range of both primary and secondary metabolites during blackcurrant ripening. On a DW basis, fructose, glucose and sucrose content in blackcurrant varied significantly among the fruits harvested at different moments but not between the different cultivars studied (**Table 4.4**). Differences between cvs., for Exps. 4.2 and 4.3, were accentuated when considering the results on a FW basis as was also noticed in earlier experiments (section 4.5.1.1; Exp. 4.1).

Table 4.4: Sugar concentration, on a fresh weight (FW) and dry weight (DW) basis, in three UK-grown blackcurrant cultivars harvested at three different maturities (Exp. 4.2).

Genotype	Maturity	Fructose	Glucose	Sucrose	Total Sugars	S/A[A]	m/d[B]
				mg g^{-1} FW (DW)			
Ben Dorain	ER	31.1 (171.8)	22.8 (126.2)	8.5 (47.2)	62.4 (345.2)	1.41	6.3
	FR	44.2 (198.9)	34.0 (138.9)	9.7 (43.5)	87.8 (395.5)	1.78	8.1
	OR	38.3 (183.0)	27.9 (118.6)	7.8 (37.1)	74.1 (353.6)	1.31	8.57
Ben Gairn	ER	36.5 (180.9)	28.1 (153.0)	20.8 (103.1)	85.4 (422.9)	2.44	3.17
	FR	40.2 (182.7)	31.9 (145.2)	14.2 (64.4)	86.2 (392.3)	2.07	5.09
	OR	53.1 (203.9)	40.6 (153.9)	10.6 (48.3)	104.3 (376.7)	2.59	23.38
Ben Tirran	ER	28.9 (160.4)	21.3 (133.5)	14.1 (78.7)	64.4 (357.7)	1.26	4.67
	FR	34.7 (198.4)	26.9 (155.8)	4.1 (23.6)	65.8 (375.9)	1.58	14.96
	OR	44.0 (215.5)	32.4 (158.9)	7.6 (37.2)	84.1 (411.6)	1.72	10.06
LSD ($P < 0.05$)		2.36 (18.03)	2.32 (17.37)	2.50 (26.26)	4.53 (47.56)	0.350	4.846

[A] Total sugars / Total organic acids; [B] monosaccharide/disaccharide ratio (m/d) = (Glucose +Fructose) / Sucrose
ER = Early ripe; FR = Ripe and OR = Overripe berries

In both Exps. 4.2 and 4.3, fructose was the dominant sugar (159.57-213.31 mg g^{-1} DW) in all cultivars followed by glucose (113.78-169.16 mg g^{-1} DW) and sucrose (21.89-105.74 mg g^{-1} DW). Generally, total sugar content and particularly glucose and fructose, tended to significantly increase as fruit ripened while sucrose content tended to decline during ripening. Greater concentration of reducing sugars in ripe fruits is common for most cultivated species, and it may be related to starch degradation as well as hydrolysis of sucrose into glucose and fructose (Ho, 1988). Invertase (P-fructosidase; EC 3.2.1.26) levels in the vacuoles, the enzyme responsible for catalyzing the conversion of sucrose to its monosaccharide constituents, clearly defines the final concentration of these sugars into the fruit (Davies *et al.*, 1996). In Exp. 4.4 (section 4.5.3.1), berries harvested at the FR stage contained 1.5-fold the fructose, 1.3-fold the glucose and 0.75-fold the sucrose concentration than those harvested at the ER stage. Similar results were observed in Exp. 4.2 (**Table 4.4**) but not in Exp. 4.3 (**Figure 4.8**), where even with similar trends, differences in sugar concentrations between the two maturities investigated were, despite being again significant, not so exaggerated. In red raspberries, fructose and glucose were also present at higher concentrations in FR berries (100% maturity) than at earlier stages (Wang, Chen, and Wang, 2009). In this context, the lower sucrose content observed in OR berries may be the result of the hydrolysis of this disaccharide into the corresponding reducing sugars during the ripening process by up-regulation of invertase levels within the fruit. In Exp. 4.2, total sugars concentrations, on a FW but not on a DW basis, were greatest in OR berries (**Table 4.4**) which clearly revealed that the observed higher concentrations found in fresh over-ripe berries were associated to reduced water content as compared to ER or FR. Indeed, dry matter as a proportion of fresh weight was greater in over-ripe berries as compared to ripe or early ripe fruits, and significantly differed between cultivars with values ranging from 17.5 to 26.2 g DW 100 g^{-1} FW. The differences in dry matter content between maturities were most pronounced in cv. Ben Gairn, where OR fruits had almost 31% higher dry mater than ER fruits. Greater dry matter content in OR berries may be associated with increase water loss, which occurs in over-ripe berries due to a considerable increase in respiratory metabolism (Ho, 1988).

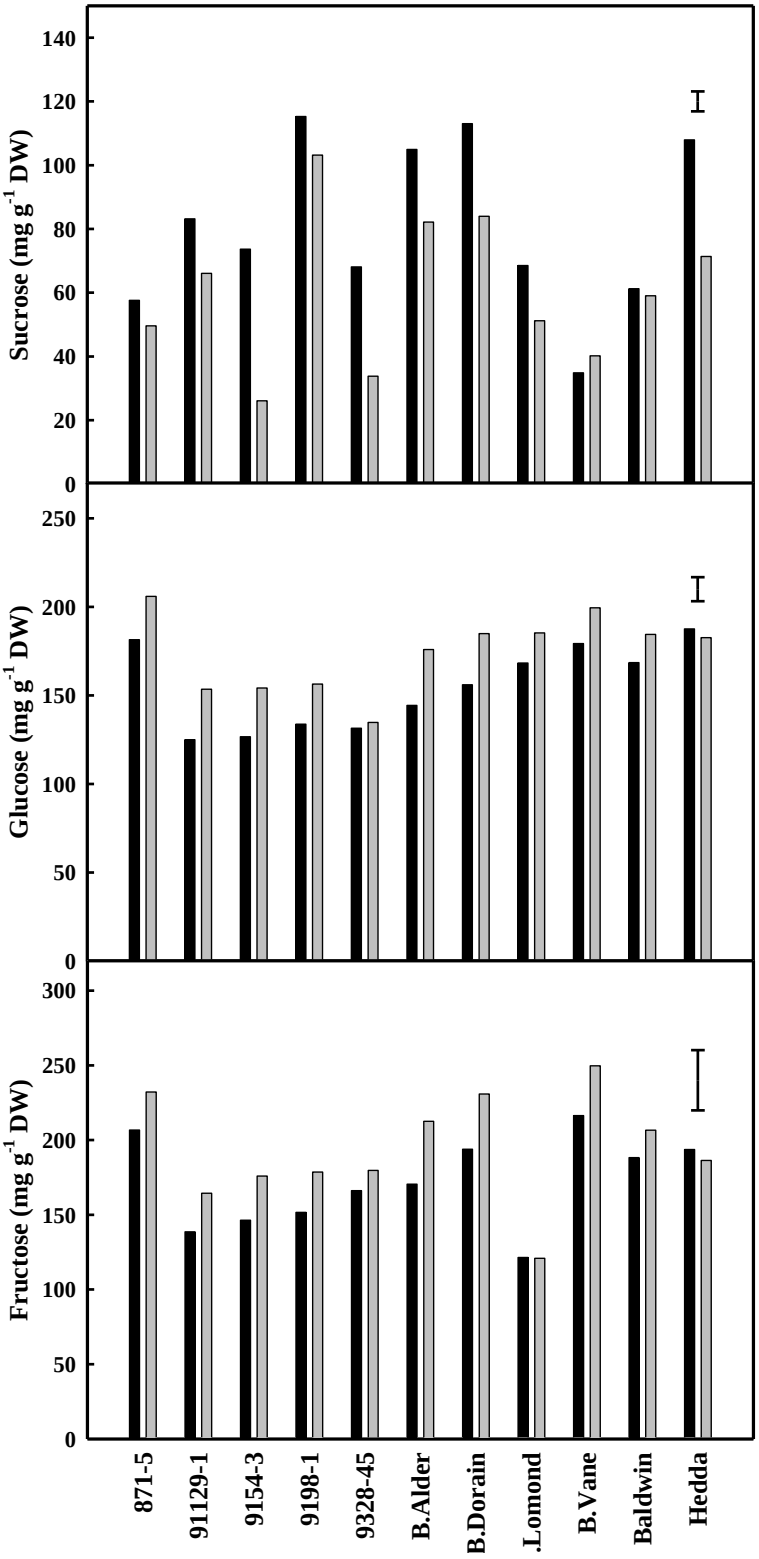


Figure 4.8: Sugar concentration (mg g^{-1} DW) in early ripe (ER; ■) or fully ripe (FR; ■) berries from eleven UK-grown blackcurrant cultivars (Exp. 4.3).

In Exps. 4.2, 4.3 and 4.4, citric acid accounted for almost 75 % of total organic acids quantified in blackcurrant cultivars and hence confirmed the organic acid profile of blackcurrant berries presented in section 4.5.1.1, as well as those reported by others (Zheng *et al.,* 2009). In Exp.4.2, significant differences were encountered for all organic acid, on a FW basis, between different cvs. and degrees of maturity, but not when results were considered on a DW basis. Nevertheless, changes in organic concentrations during fruit ripening were cultivar dependent as also observed in Exps. 4.3 and 4.4. Citric acid concentrations, in Exp. 4.2, increased from ER to FR but then decreased in OR fruits reaching values similar to those found in ER fruits (**Table 4.5**). In Exp.4.3, citric acid remained fairly unchanged among the different ripening stages investigated for all cvs. except for cv. 9154-3, where 1.2-fold concentrations were found in FR as compared to ER fruits (**Figure 4.9**). Both ascorbic and malic acids were present in greater concentrations in ER than FR berries (**Table 4.5**; **Figure 4.9**), except in cvs. Ben Gairn (Exp. 4.2), 9198-1 and 9328-45 (Exp. 4.3), where greater ascorbic acid concentrations were encountered in berries with advanced maturity. In tomatoes, greater ascorbate content is found in deep red fruits as compared to less ripe fruits (Jiménez *et al.,* 2002; Gautier *et al.,* 2008). In berries, however, the temporal changes in ascorbic acid content have been reported to depend on several physiological factors and growth conditions during berry ripening of different cultivars (Viola *et al.,* 2000; Walker *et al.,* 2006). AsA synthesis was generally most intense in ER berries and hence results were in agreement with Rubinskiene *et al.* (2005) since higher ascorbic acid content was observed in blackcurrant berries which were not fully ripe. In the former study, the authors related changes in ascorbic acid concentration to ascorbinoxidase levels which tend to be low in unripe fruits (Rubin, 1970). Previous studies (Rubinskiene *et al.,* 2005; Maksimenko, 1999), also described that in early fruiting cultivars (i.e. Ben Gairn), ascorbic acid content suddenly decreased as berry colour intensified. In the present study, AsA content in early cultivars such as Ben Gairn was observed to decline between ER and R stages followed by a considerable increase up to OR stages.

Similar to the results observed herein, malic acid concentrations were present in greater concentrations in red raspberries prior to reaching the fully ripe stage (Wang, 2003) and decreased during ripening of seabuckthorn berries (Raffo *et al.,* 2004). In contrast to strawberry fruits, were a dramatic malate loss does not occur during ripening, results from this study reveal a clear decrease in malic acid concentrations and hence highlighted the metabolic active role of this acid in the ripening process of blackcurrants (Sweetman *et al.,* 2009). During initial ripening steps, blackcurrant berries may accumulate malate mainly through sugar metabolism and translocation from leaves to fruit. In ripe fruits, and as reported for grapes (Sweetman *et al.,* 2009), malate may be released from fruit vacuoles,

becoming available for catabolism through different pathways including the TCA cycle and respiration, gluconeogenesis, aminoacid interconversions, and the production of complex secondary compounds such as anthocyanins and flavonols (Famiani *et al.*, 2000).

Table 4.5: Organic acid concentrations, on a fresh weight and dry weight basis, of three UK-grown blackcurrant cultivars harvested at three different maturities (Exp. 4.2).

Genotype	Maturity	Ascorbic	Citric	Malic	Total acids
			mg g^{-1} FW (DW)		
Ben Dorain	ER	1.9 (10.8)	32.5 (179.8)	9.2 (51.0)	43.7 (245.1)
	FR	1.9 (8.5)	38.1 (171.6)	8.4 (38.0)	48.4 (221.8)
	OR	2.0 (9.6)	46.4 (221.7)	7.4 (35.3)	55.8 (271.1)
Ben Gairn	ER	1.5 (7.4)	29.4 (145.7)	3.5 (17.2)	34.4 (174.0)
	FR	1.3 (6.1)	36.9 (167.9)	2.4 (11.1)	40.7 (190.3)
	OR	2.6 (10.0)	36.0 (138.3)	0.7 (2.7)	39.4 (146.2)
Ben Tirran	ER	2.8 (15.6)	40.6 (225.6)	7.3 (40.4)	50.7 (285.3)
	FR	1.8 (10.3)	34.9 (199.2)	4.3 (24.5)	41.0 (238.1)
	OR	2.6 (12.7)	41.6 (203.5)	4.1 (20.2)	48.3 (240.2)
LSD ($P < 0.005$)		0.25 (1.07)	4.16 (20.21)	0.71 (3.65)	4.69(24.13)

ER = Early ripe; FR = Ripe and OR = Overripe berries

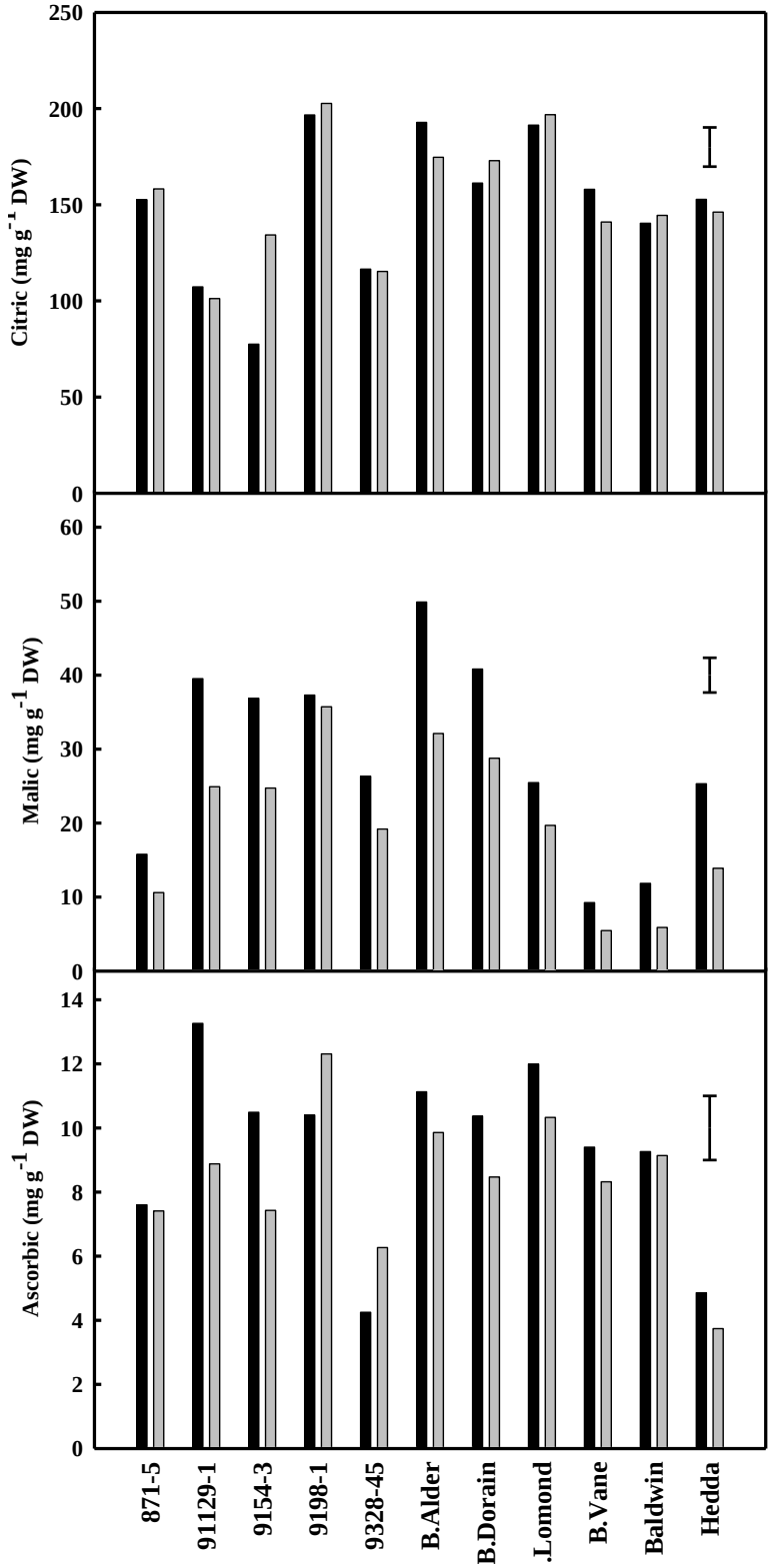


Figure 4.9: Organic acid concentrations (mg g^{-1} DW) in early ripe (ER; ■) or fully ripe (FR; ■) berries from eleven UK-grown blackcurrant cultivars (Exp. 4.3).

Titratable acidity declined from early to fully ripe fruits which was probably due to the reductions in malic rather than citric as reported by others (Gautier *et al.*, 2008). The influence of both acids in TSS measurements are described in Appendix A. Interestingly, titratable acidity is generally expressed as the amount (%) of the major acid, in this case, citric acid even though it does not reflect the stability of this acid during ripening as observed in Exp. 4.3 (**Figure 4.9**). TTA acidity values were not well correlated with either citric or total organic acid concentrations for fruits from Exp. 4.2 (**Figure 4.10**)

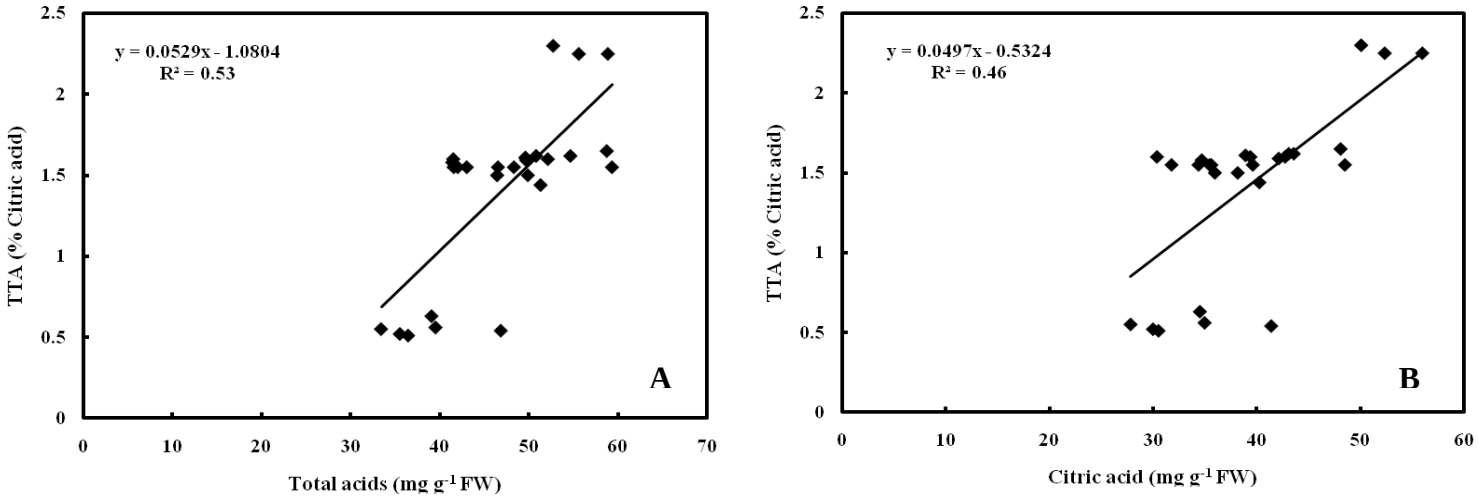


Figure 4.10: Correlations between TTA, expressed as % Citric acid, and total (A) or citric acid (B) concentrations in blackcurrant berries harvested at different maturities (Exp. 4.2).

4.4.2.4 Individual anthocyanins.

As shown for Exp. 4.1, four major anthocyanins were identified and quantified according to their retention time, UV spectra and comparison with standards. In Exp. 4.2, however, other minor anthocyanins were observed in all the cvs. analysed. For instance, the chromatographic profile of berries from cv. Ben Tirran clearly revealed eight different peaks as compared to six peaks observed in the other cultiars investigated (**Figure 4.11**) and hence confirmed that minor anthocyanins are present in blackcurrant berries depending on the cultivar (Iversen, 1999; Landbo and Meyer, 2004; Antonnen and Karjalainen, 2006; Section 4.5.2.1).

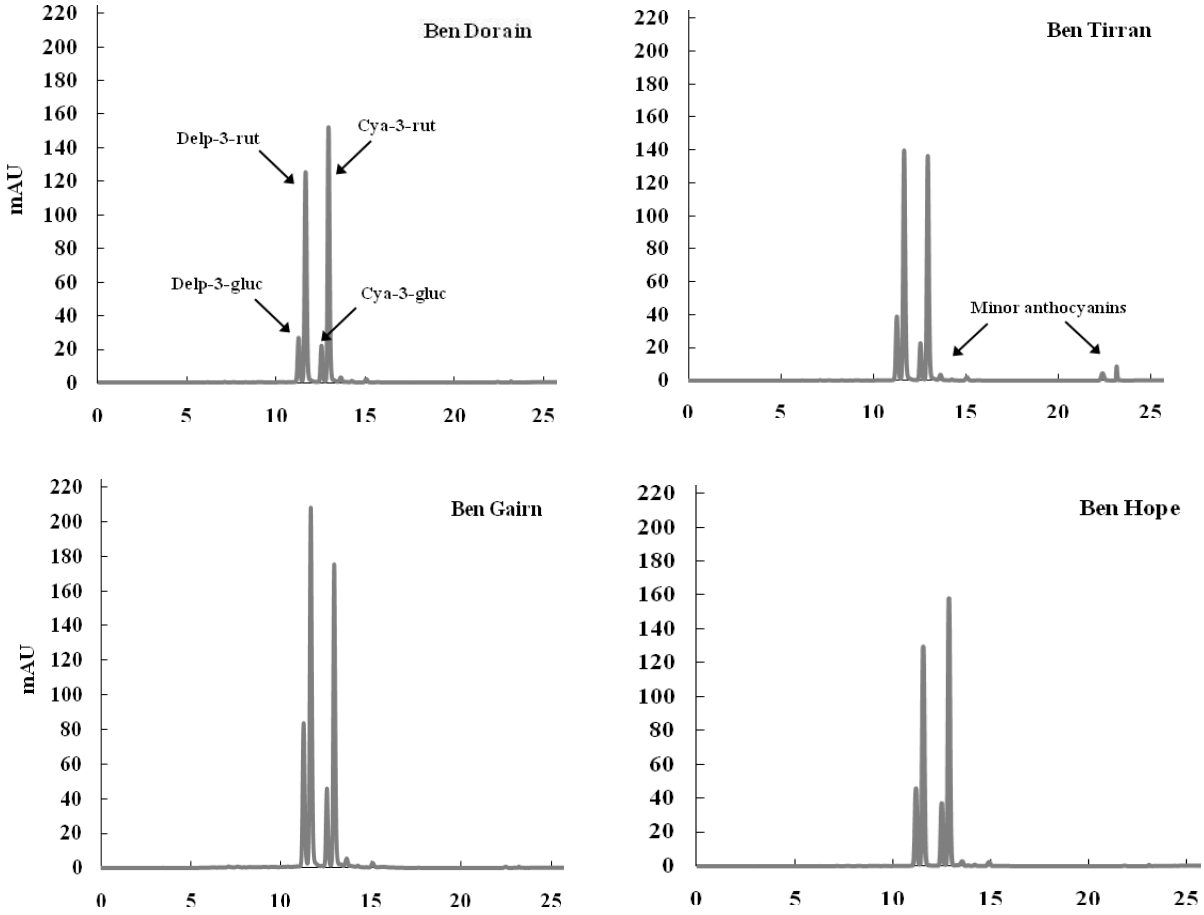


Figure 4.11: Chromatographic profile of anthocyanins found in fully ripe blackcurrant berries from different cultivars (Exps. 4.2 and 4.4) (*viz*. cya-3-gluc (peak 3), cya-3-rut (peak 4), delp-3-gluc (peak 1), delp-3-rut (peak 2)).

In the present study (Exp. 4.2), berry darkening during ripening was related to an increase in anthocyanin concentrations and accompanied by a decline in organic acids and an increase in sugar concentration as reported for other berries (Robbins *et al.,* 1989; Kalt *et al.,* 1999; Perkins-Veazie *et al.,* 1999). On a FW basis (**Fig. 4.12**), individual anthocyanins concentrations significantly differed between cultivars and degrees of maturity. Individual anthocyanin concentration increased gradually as fruit ripened for all the cultivars investigated which was in agreement with earlier works on blackcurrants (Rubinskiene *et al.,* 2006) and other berry fruits (Carbone *et al.,* 2009). This said, similar amounts were recorded for FR or OR berries from cv. Ben Dorain. Mimicking the results obtain in Exp. 4.2 (**Figure 4.12**), total anthocyanin concentration was generally higher in FR (> 3500 µg g^{-1} FW) compared to ER (< 1500 µg g^{-1} FW) blackcurrants (cv. Ben Hope), with nearly double the

concentrations of cyanidin and delphinidin rutinosides in OR than in berries harvested at earlier stages (Exp.4.4; section 4.5.3.2).

In Exp. 4.3, the relative concentration of each individual anthocyanin remained fairly constant regardless of fruit development stage, except for cv. Ben Gairn where OR fruits had three times as much anthocyanin glucosides than the other cultivars investigated (**Figure 4.12**). In contrast, in berries from cv. Ben Hope (Exp. 4.4; section 4.5.3.2), the proportions of cyanidin and delphinidin glucosides were higher in ER (ca. 8% and 12% respectively) than in FR blackcurrants (6% and 9%), whereas the proportions of the respective rutinosides were greater in FR (49% and 35% respectively) than in ER fruit (51% and 31%). In agreement with that reported for other berry fruits (Carbone *et al.,* 2009; Crespo *et al.,* 2010), these results suggest that anthocyanin profile is mainly genetically inherited rather than being affected by external environmental factors or different maturities.

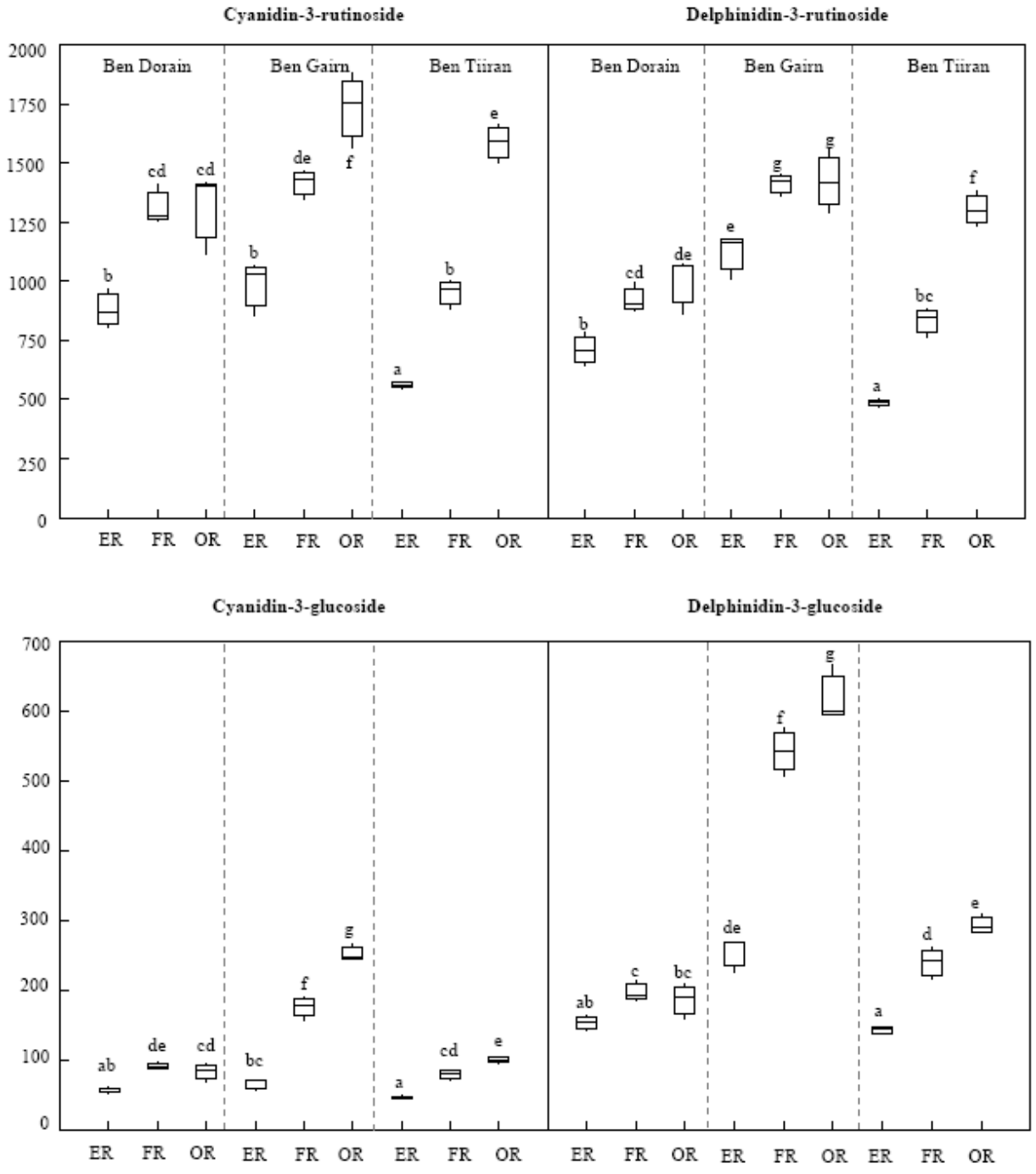


Figure 4.12: Accumulation patterns of individual anthocyanins (µg g^{-1} FW) through different development stages (viz. early ripe (ER), fully ripe (FR) and over ripe (OR)) in different blackcurrant cultivars. Letters indicate significant differences among mean values ($P < 0.05$).

Differences in anthocyanin accumulation pattern between different cultivars may be related to timing differences for each cultivar and hence influenced by agroclimatic conditions during the ripening period. Indeed, soil and water status, light incidence and temperature are known to affect anthocyanin concentrations in several berries (Terry *et al.*, 2007; Wang *et al.*, 2009). In the present study, no clear associations were observed when considering metereological data (*viz.* rain incidence, sunshine and minimum and maximum temperatures; METOFFICE, 2010) with anthocyanin accumulation patterns for the different cultivars investigated (**Figure 4.13**). This said, higher anthocyanin concentrations in fruits from cvs. Ben Gairn and Ben Dorain were obtained after periods with higher rain fall (**Figure 4.13**).

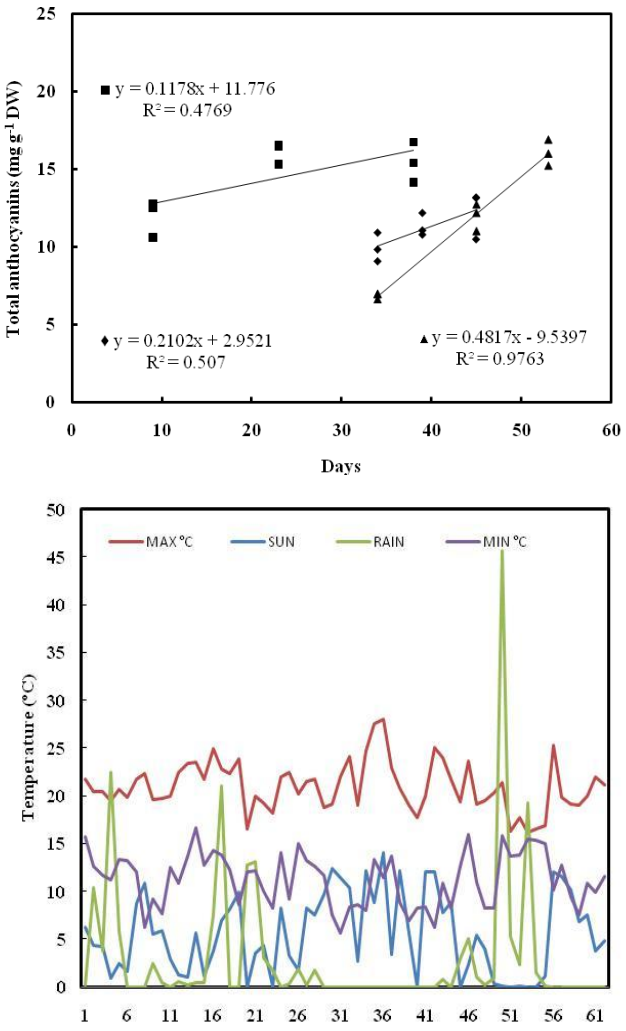


Figure 4.13: Relationship between total anthocyanin accumulation in fruits of different blackcurrant cultivars (Ben Gairn (■), Ben Tirran (▲) and Ben Dorain (●)) harvested at different maturities (Exp. 4.2) and possible relationship with metereological data (— max. temperature; — minimum temperature; — sunshine; — rain; METOFFICE2010).

4.4.2.5 Total phenolics, total flavonols and antioxidant capacity

On a DW basis, no significant differences were observed for total phenolic concentrations between fruits from different cultivars or harvested at different maturities. However, different patterns were observed for the evolution of total phenolics between cultivars. For instance, in fruits from cvs. Ben Gairn and Ben Tirran, total phenolic content decreased as fruit ripened whilst for Ben Tirran a significant decrease was observed between ER and FR stages but followed by a peak in total phenolic concentrations from OR fruits (**Table 4.6**). There is evidence that polyphenol biosynthesis in different berries is intimately associated with the development stages of the fruit. For instance, Jaakola *et al.* (2002) demonstrated that the expression of flavonoid biosynthetic genes in bilberry (*Vaccinium myrtillus*) was coordinated with the accumulation of anthocyanins and other phenylpropanoids in developing fruits. In certain berry species, the flavonoid biosynthetic pathway showed two differentiated enzymatic peaks at early and late ripening stages. If the same applies to blackcurrant fruits, the noticeable increase in anthocyanins, accompanied by a slight decrease in total phenolic concentrations, strongly suggest that the second peak corresponds to anthocyanin biosynthesis in detriment of other phenolic compounds (**Table 4.6**). Kalt *et al.* (2003) also reported an increase of anthocyanin content and a decrease of total phenolics during blueberry ripening. In the former study the authors suggested that in the later stages of fruit development there is a shift in the pool of total phenolics towards anthocyanin synthesis, resulting in an overall decline in the content of other phenolic components. The results from Exp. 4.2 can be interpreted accordingly. Nonetheless, it may be also possible that the reduction in TP values during ripening is related to AsA concentrations (**Table 4.5**; **Figure 4.9**), since this compound is a common interferent in the FC method (Chapter 5; section 5.3.3).

Total flavonoid concentrations were also investigated using an optimized unpublished method by Davis and Terry. Two different solvent combinations were used for the extraction of these group of phenolic compounds. A methanol based extraction solvent was more effective at extracting the flavonoids present in blackcurrant samples as compared to ethanol:water (ES2) (**Table 4.6**). Results were in agreement with that presented in earlier sections, since acidified aqueous methanol was best for extracting the anthocyanin pigments from blackcurrants. Anthocyanins represent most of the flavonoid pool present in blackcurrant fruits (Antonnen and Karjalainen, 2006) and hence is not surprising that the same results were obtained in both cases. Flavonoid content was significantly higher in OR fruits as compared to FR or ER, and again, correlated well with the sum of individual anthocyanins ($r > 0.72$; $P < 0.01$; data not shown). On a DW basis, antioxidant capacity was different between cultivars but not

between different fruits harvested at different developmental stages. Nevertheless, significant differences were found for the interaction between cultivar and maturity, which clearly indicated that accumulation patterns for antioxidant capacity during ripening was strongly influenced by the genotype. For instance, antioxidant capacity increased during the ripening of fruits from cv. Ben Tirran but was greater in ER fruits from cvs. Ben Gairn and Ben Dorain. Results agreed well with that found for other berries (Wang and Lin, 2000; Carbone *et al.,* 2009; Wang *et al.,* 2009), since greater antioxidant capacities are generally found in ER fruits. This may be due to the abundant procyanidin, ascorbate or other phenolic acid concentrations found in berries prior of reaching optimum maturity (Wang *et al.,* 2009).

Table 4.6: Total phenolic (mg GAE g^{-1} DW), antioxidant capacity (mg Fe2+ g-1 DW) and total flavonoids (mg QE g^{-1} DW) of three UK-grown blackcurrant cultivars harvested at three different maturities.

Cultivar	Maturity	Total Phenolics	Antioxidant Capacity	Total Flavonols (1)[A]	Total Flavonols (2)[B]
	ER	31.3	982	10.47	11.58
Ben Dorain	FR	29.21	837	11.75	11.31
	OR	25.54	962	13.59	13.03
	ER	31.74	1164	16.6	15.59
Ben Gairn	FR	31.19	1083	21.33	17.6
	OR	26.29	1105	25.4	19.38
	ER	29.4	928	7.8	8.69
Ben Tirran	FR	22.15	1169	12.03	10.92
	OR	33.33	1257	17.1	15.76
Lsd (P<0.05)		**3.997**	**140.7**	**1.217**	**1.455**

[A] Extraction solvent (ES) = 70: 29.5:0.5 (methanol:water: HCl; v/v/v); [B] ES = 80:20 (ethanol:water; v/v)
ER = Early ripe; FR = Ripe and OR = Overripe berries

4.4.2.6 Principal component analysis

The study of all variables simultaneously was necessary to explore the temporal changes in the quality and health related attributes of different blackcurrant cultivars harvested at different maturities. Considering all analytical data except correlated variables, PCA was able to discriminate between

cultivars and different degrees of maturity. Three Principal Components (PCs) were required to capture > 83% of the biochemical variance. PC 1 explained 57.58% of the variation and was mainly related, according to factor loadings, to ascorbic acid, each individual anthocyanin, fructose and glucose. PC2 explained 17.25% of the total variation and was mainly related to sucrose, titratable acidity and other minor anthocyanins. PC 3 was responsible for capturing 11.38% of the variation. It is noteworthy that anthocyanins and ascorbic acid were again key components to assess the genotypic variability between different genotypes (Exp. 4.1; section 4.5.1.4). The addition of further PCs failed to improve the clustering between cvs and degree of maturity. Consequently, the interpretation of clustering between cvs. and degrees of maturity was mainly characterised by the information given by PC1 and PC2 (**Figure 4.14**). PCA revealed that the largest contribution to the variation between samples was the degree of maturity, since different ripening stages were clearly discriminated along PC1. Additionally, the different cultivars examined were clustered along PC2

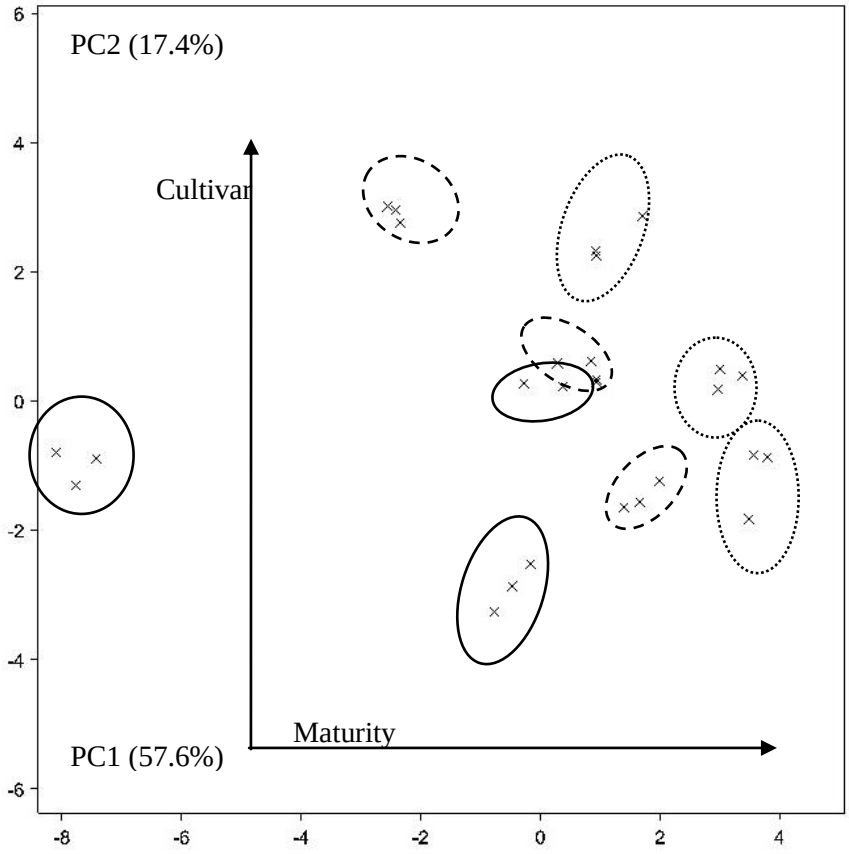


Figure 4.14: PCA characterization (PC1 vs. PC2) of three UK-grown blackcurrant cultivars harvested at three different maturities (viz. early ripe (·····), fully ripe (– – –) and over-ripe (——)) using the biochemical profile as analytical data.

4.4.3 Effect of growing location and year on the taste-related composition of blackcurrants

Differences among berries grown at different growing locations (i.e. Norfolk vs. Scotland) may be related to differences in agroclimatic conditions. Similarly, year-to-year variation in the composition of blackcurrant fruits from the same cultivars and grown at identical locations is undoubtedly due to differences in weather conditions. In Exp.4.2 and 4.3, berries from the same cultivar (cv. Ben Dorain) were sourced from different locations within UK. Although little is known about the relation between different locations on the composition of fruits (Crespo *et al.*, 2010), the effect of weather conditions is fairly well documented for several horticultural crops. Recently, Zheng *et al.* (2009) studied the variation in the composition of blackcurrant fruits grown at two different latitudes within Finland. Latitude, and accordingly, weather conditions affected significantly the composition of the fruits although it was difficult to draw any direct relationship between fruit composition and weather conditions during the growing period. Nevertheless, such effects were observed to be genotype-dependent. In the present study, samples from cv. Ben Dorain showed greater sugar concentrations, sugar to acid and M/D ratios as well as lower malic acid concentrations when bushes where grown at higher latitudes (i.e. Scotland) (**Table 4.7**). Other variables including age of the bushes, soil type and the known differences in weather conditions between Norfolk and Scotland may have obviously led to these results. Citric acid was the only component not affected by the location where the plants were grown with average concentrations of 172 mg g^{-1} DW.

Table 4.7: Taste-related composition of UK-grown blackcurrant berries (cv. Ben Dorain) harvest during 2007 at two different locations

Compound (mg g^{-1} DW)	Dundee	Norfolk
Sucrose	84.0[a]	43.5[b]
Glucose	184.9[a]	153.0[b]
Fructose	230.8[a]	198.9[b]
Citric	172.9[a]	171.6[a]
Malic	28.8[a]	38.0[b]
Ascorbic	8.5[a]	8.5[a]
Total sugars	500.3[a]	395.4[b]
Total acids	210.1[a]	218.1[a]
M/D	4.9[a]	8.1[b]
S/A	2.3[a]	1.8[b]

Significant differences were also observed between berries from the same cultivars, grown at the same location but harvested in two successive years (**Figure 4.15**). Greater temperatures during June (17.0ºC) and July of 2006 (20.3ºC) if compared to those from 2007 (15.5 and 16.2ºC for June and July, respectively) (METOFFICE, 2010) may have contributed to the lower sucrose values and greater glucose and fructose in fruits (**Figure 4.15**). In agreement, glucose and fructose were observed to slightly increase in strawberry fruits grown at higher temperatures (Chapter 3; section 3.4.5), as was also observed by Utsunomiya (1992) in purple passion fruit. Citric and malic acid were lower and greater, respectively, in fruits harvest during high temperature periods, as was also observed in peach (Lobit *et al.*, 2003) or grapes (Lakso and Kliewer, 1975).

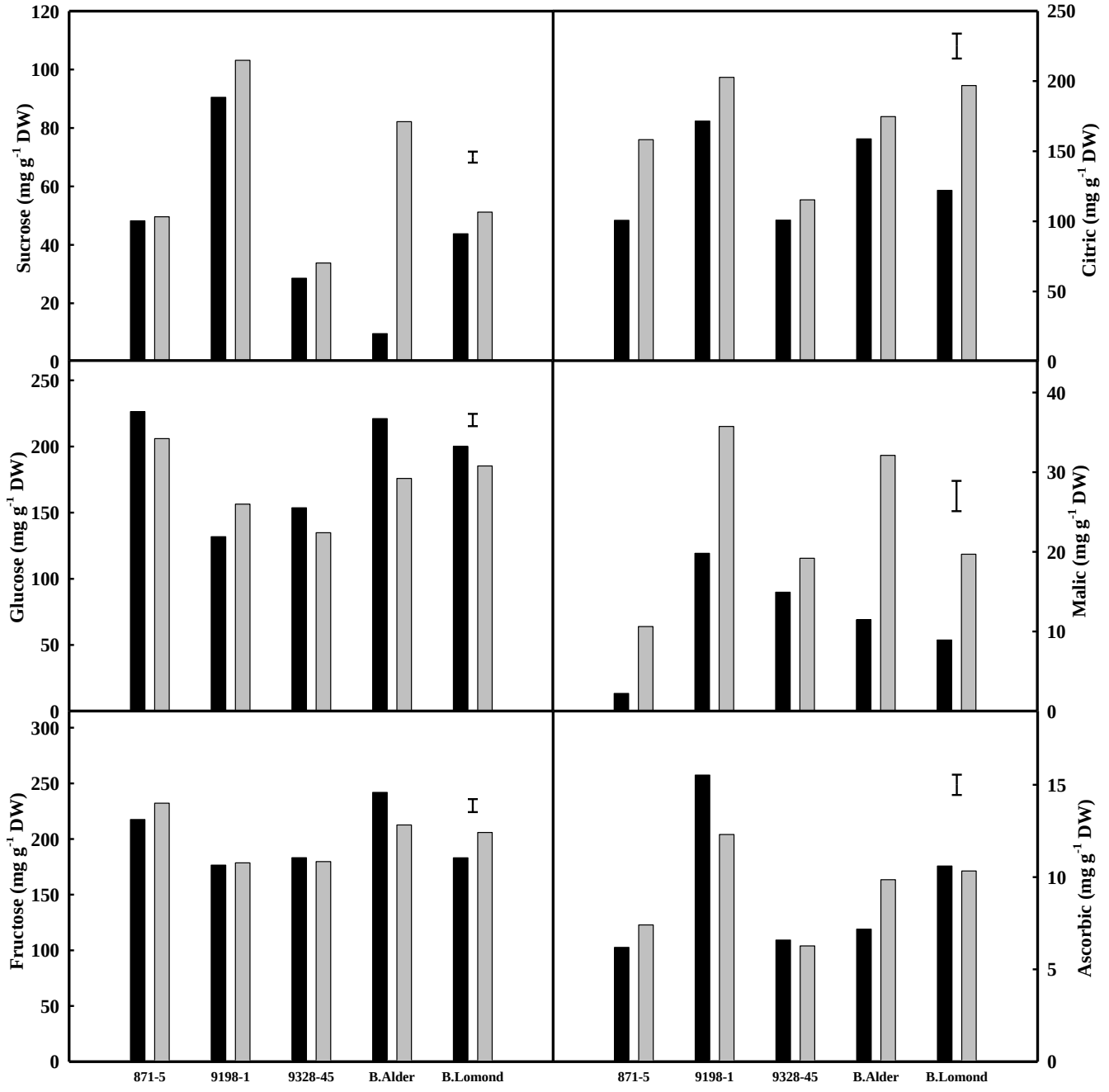


Figure 4.15: Variation in sugar and acid concentration (mg g^{-1} DW) of blackcurrant berries from different cultivars harvested in 2006 (■) and 2007 (■).

4.4.4 Effect of postharvest storage conditions on blackcurrant fruit quality

4.5.3.1 Sugars and non-volatile organic acids.

Since carbohydrates are a respiratory substrate, it was expected that changes in the respiration rate of blackcurrant berries during postharvest storage would affect sugar metabolism and hence final sugar concentrations. Temperature is a crucial variable influencing the respiration rate of many fruits during postharvest storage and berries are no exception (Nunes *et al.*, 2004). Results from this study showed that respiration rate increased with increasing storage temperature (**Figure 4.16**).

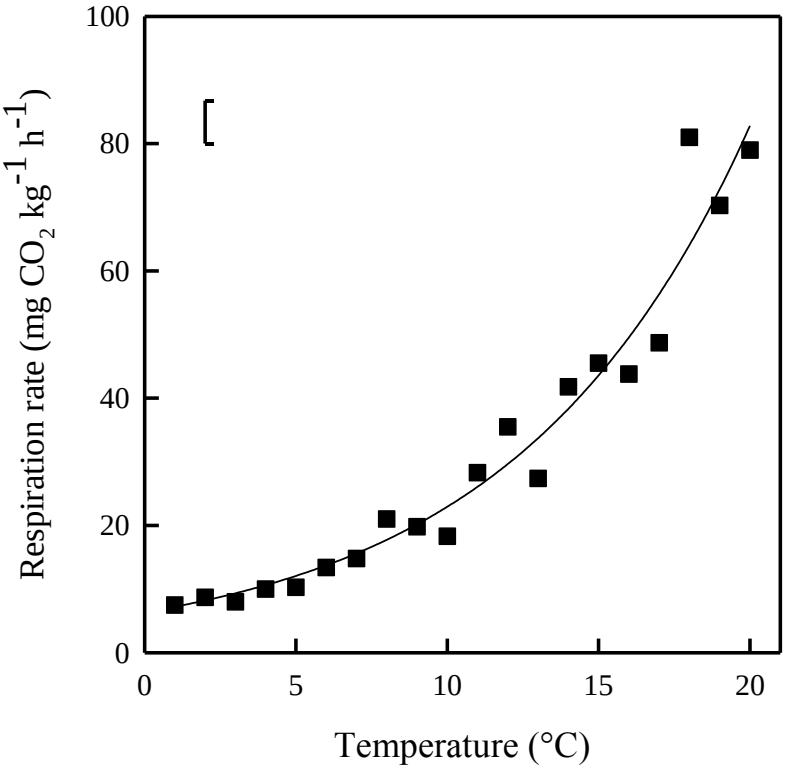


Figure 4.16: Respiration rate of blackcurrant berries stored at different temperatures (values are means of 5 days and two maturity stages). LSD bar (P = 0.05) is shown. The equation for the exponential curve is f=a*exp(b*x) where a=6.3501 and b=0.1284.

Thus, the observed decrease in the major blackcurrant sugars not only occurred during storage but with increasing temperatures (**Table 4.8**) was most probably caused by the hydrolysis of sucrose into glucose and fructose and the corresponding utilization of these reducing sugars as respiratory substrates; however, it is also likely that these simple sugars were used in anabolic processes. Similar

findings were reported concerning changes in sugars during postharvest storage of strawberries (Pelayo *et al.*, 2003). Lower glucose concentrations may be a desirable attribute for the production of blackcurrant beverages since this sugar is the preferred substrate for the Maillard reactions that occur during conventional juice production (Boccorh *et al.*, 1998) and hence storage of FR harvested berries at lower temperatures which limit the rise in glucose concentration during storage may be preferred. There was no difference in respiration rate of ER and FR berries (data not shown), however, sugar metabolism was affected by maturity at harvest (**Table 4.8**). These results indicate that mechanisms other than respiratory metabolism may contribute to the observed postharvest sugar changes. Sugars in their activated form (i.e. UDP-glucose) are common substrates for glucosyltransferase (GT) enzymes (e.g. flavonoid GTs) involved in biosynthesis of anthocyanins. Glycosylation of anthocyanidins is an important step for increasing the hydrophobicity and stability of these molecules. The observed lower sugar concentrations accompanied by an increase in anthocyanins (**Figure 4.17**) during storage may imply a rise in flavonoid GT enzyme activity (Halwirth *et al.*, 2006) or even suggest that the carbon required for the *de novo* synthesis of anthocyanins could be sourced from organic acids or sugars. There is a finite amount of carbon in harvested fresh produce, so there must be a trade-off in carbon allocation between various pathways, for instance, sugars may enter into the Shikimate pathway (Herrmann and Weaver, 1999), resulting in the production of flavonoids (i.e. glycosylated anthocyanins) via phenylalanine, or may be used for respiration via glycolysis.

Table 4.8: Sugar concentration of blackcurrant berries (cv. Ben Hope) harvested at early ripe or fully ripe maturity stage and stored for 7 days at different temperatures.

		Early ripe					Fully ripe				
		Storage temperature (ºC)									
Compound	**Day**	**20-17**	**16-13**	**12-9**	**8-5**	**4-1**	**20-17**	**16-13**	**12-9**	**8-5**	**4-1**
Fructose $LSD_{0.05}$ = 8.994 (P = 0.042)	0*	-	-	-	32.00	-	-	-	-	48.31	-
	1	32.85	34.11	33.70	32.53	29.84	50.66	55.54	53.00	50.33	50.02
	3	35.22	25.43	23.48	32.71	34.96	67.98	59.12	54.71	48.47	41.75
	7	32.26	32.96	35.06	37.22	37.82	55.83	52.82	48.34	46.88	45.13
Glucose $LSD_{0.05}$ = 4.632 (P = 0.009)	0*	-	-	-	21.98	-	-	-	-	30.30	-
	1	25.24	24.82	24.69	23.73	20.91	32.02	34.79	32.79	28.77	31.37
	3	26.11	24.48	22.37	23.28	25.09	42.57	37.05	34.20	29.05	25.98
	7	22.92	22.64	22.65	24.39	23.88	35.10	32.34	28.69	23.97	23.68
Sucrose $LSD_{0.05}$ = 3.377 (P = 0.232)	0*	-	-	-	23.61		-	-	-	18.29	-
	1	22.77	21.28	22.68	20.87	15.02	17.19	15.93	14.06	13.70	12.12
	3	20.42	19.35	18.60	18.07	16.31	16.72	14.32	12.14	11.51	6.87
	7	16.12	13.25	16.02	13.54	12.20	10.88	10.70	7.59	6.41	5.49
Total sugars $LSD_{0.05}$ = 14.295 (P = 0.011)	0*	-	-	-	77.62	-	-	-	-	96.87	-
	1	80.86	80.21	81.07	77.14	34.76	99.87	106.26	99.85	92.81	93.51
	3	81.75	69.26	64.45	74.06	76.35	127.26	110.49	101.05	89.03	74.60
	7	71.30	68.86	73.73	75.14	73.90	101.81	95.86	84.62	77.26	74.30

*Values at day 0 correspond to anthocyanin concentration measured in blackcurrant berries kept at 5 ± 1 ºC before the start of the storage trial.

Despite differences in the actual concentrations reported for ER or FR berries, malic acid declined during storage in both maturity stages, which has also been extensively reported for other fruits (Wang, 2003; Alique *et al.*, 2005). Increased respiratory stress at greater storage temperatures may account for most of the malic acid losses reported in this study, highlighting the importance of malic acid as a possible respiratory substrate in blackcurrant postharvest metabolism.

In general, the AsA concentration of stored blackcurrants harvested at FR stage tended to decline during the duration of the trial with a *ca*. 40 % decrease observed by day 7 as compared with the initial content of 2.49 mg g^{-1} FW, however, there was little change in ER berries (**Table 4.9**). In addition to the changes in sugar and acid content which may be directly related to respiration, the nutritional quality of blackcurrant berries was also significantly affected by storage temperature and time. Rubinskiene *et al.* (2006) reported that AsA content steadily decreased with maturity, but was relatively similar between the brown and black ripe stages in nine Lithuanian-grown blackcurrant cultivars. In the present study AsA concentration was similar between both maturity stages but was only detrimentally affected by storage period in FR-harvested berries (**Table 4.9**). Similarly, AsA content of blackcurrants (Viola *et al.*, 2000), redcurrants (Roelofs *et al.*, 1993), raspberries (Kalt *et al.*, 1999), blackberries (Antunes *et al.*, 2003) and strawberries (Cordenunsi *et al.*, 2003) has been shown to decrease with storage time. This said, none of the above mentioned studies have looked at a series of defined temperature bands as the ones described herein. For blackberries, the decrease in AsA was shown to be greater in berries stored at 20ºC (50%) as compared to 2ºC (25%) (Antunes *et al.*, 2003). However, in the present study, storage temperature did not have a detrimental effect on AsA concentration and hence contradicts previous works in which AsA in currants was reported to decrease with increasing storage temperature (Häkkinen *et al.*, 2000). Discrepancies between previous studies and the results presented herein may be due to the use of different cultivars or other variables between storage conditions such as light or relative humidity. In addition, it may be feasible to speculate that the storage conditions described herein may have, to certain extent, limited the oxidation of AsA to dehydroascorbic acid. However, this study has examined a range of temperatures in detail, rather than being limited to just one or two storage regimes, and as such, has been able to detect different trends occurring between 1 and 20 ºC.

Table 4.9: Organic acid concentration of blackcurrant berries (cv. Ben Hope) harvested at early ripe or fully ripe maturity stage and stored for 7 days at different temperatures.

		Early ripe					Fully ripe				
		Storage temperature (ºC)									
Compound	**Day**	**20-17**	**16-13**	**12-9**	**8-5**	**4-1**	**20-17**	**16-13**	**12-9**	**8-5**	**4-1**
Ascorbic $LSD_{0.05}$ **= 0.480 (p = 0.029)**	0*	-	-	-	2.57	-	-	-	-	2.49	-
	1	2.85	2.64	2.83	2.94	2.60	2.03	1.99	2.29	2.46	2.22
	3	2.80	2.95	2.69	2.77	2.39	2.50	2.07	2.05	1.85	1.71
	7	2.30	2.51	2.97	2.33	2.31	1.90	1.91	1.22	1.25	1.46
Citric $LSD_{0.05}$ **= 6.341 (p = 0.048)**	0*	-	-	-	39.03	-	-	-	-	40.23	-
	1	39.10	38.39	37.32	38.28	38.41	36.93	37.51	39.87	38.12	40.81
	3	38.57	39.26	37.85	40.91	35.59	46.45	42.56	37.92	31.53	30.23
	7	36.39	41.48	43.50	41.20	42.03	40.55	38.71	37.40	34.50	42.27
Malic $LSD_{0.05}$ **= 1.097 (p = 0.277)**	0*	-	-	-	7.20	-	-	-	-	4.52	-
	1	7.04	6.27	6.21	6.12	5.35	4.19	4.32	3.47	4.02	3.97
	3	6.64	6.71	5.70	5.35	3.77	3.51	2.99	2.48	2.03	2.09
	7	4.43	4.96	3.86	3.30	3.52	3.74	2.72	2.30	1.98	1.90
Total acids $LSD_{0.05}$ **= 7.280 (p = 0.090)**	0*	-	-	-	48.77	-	-	-	-	47.24	-
	1	49.01	47.32	46.37	47.41	46.37	43.15	43.80	45.67	44.54	47.03
	3	48.05	48.93	46.24	49.06	41.73	52.41	47.67	42.41	35.39	33.94
	7	43.11	49.00	50.34	46.82	47.79	46.21	43.35	40.92	37.78	45.65

*Values at day 0 correspond to anthocyanin concentration measured in blackcurrant berries kept at 5 ± 1 ºC before the start of the storage trial.

4.4.3.2 Individual anthocyanins

There were significant main effects and interactions between maturity stage, storage time and temperature on the concentrations of total anthocyanins, individual anthocyanins and proportions of anthocyanins (**Figure 4.17**). Increasing evidence suggests that anthocyanins, as natural antioxidants, exert a wide range of health-promoting properties (*viz*. anti-carcinogenic, anti-inflammatory, vaso-protective and anti-obesity) when tested *in vitro* or *in vivo* (Prior, 2004). The plasma concentration relative to the dose received was greater for rutinosides than glucosides in both rabbits and humans (Nielsen *et al.*, 2003), indicating that they are more bioavailable. This suggests that although total anthocyanins were present in lower concentrations in ER blackcurrants, as was also observed by Wang *et al.* (2009) in raspberries and by Shin *et al.* (2008) in strawberries, proportionally they contained greater amounts of the more bioavailable rutinosides, which could compensate for the lower overall concentrations.

In ER fruit, there was no significant effect of time on the proportions of delp-3-rut, however, the proportion of cya-3-rut increased, and delp-3-gluc decreased, between days 3 and 7. Overall, the concentrations of all anthocyanins increased with time in ER berries (**Figure 4.17**). In FR fruit, the proportion of cya-3-gluc peaked in the middle of storage at day 3, whereas the proportions of cya-3-rut and delp-3-gluc reached a minimum on day 3, and that of delp-3-rut on day 7. Similarly, the actual concentration of cya-3-gluc reached a peak on day 3, while cya-3-rut and delp-3-rut and delp-3-gluc fell to a minimum on day 3. Total anthocyanin concentration also reached a minimum on day 3.

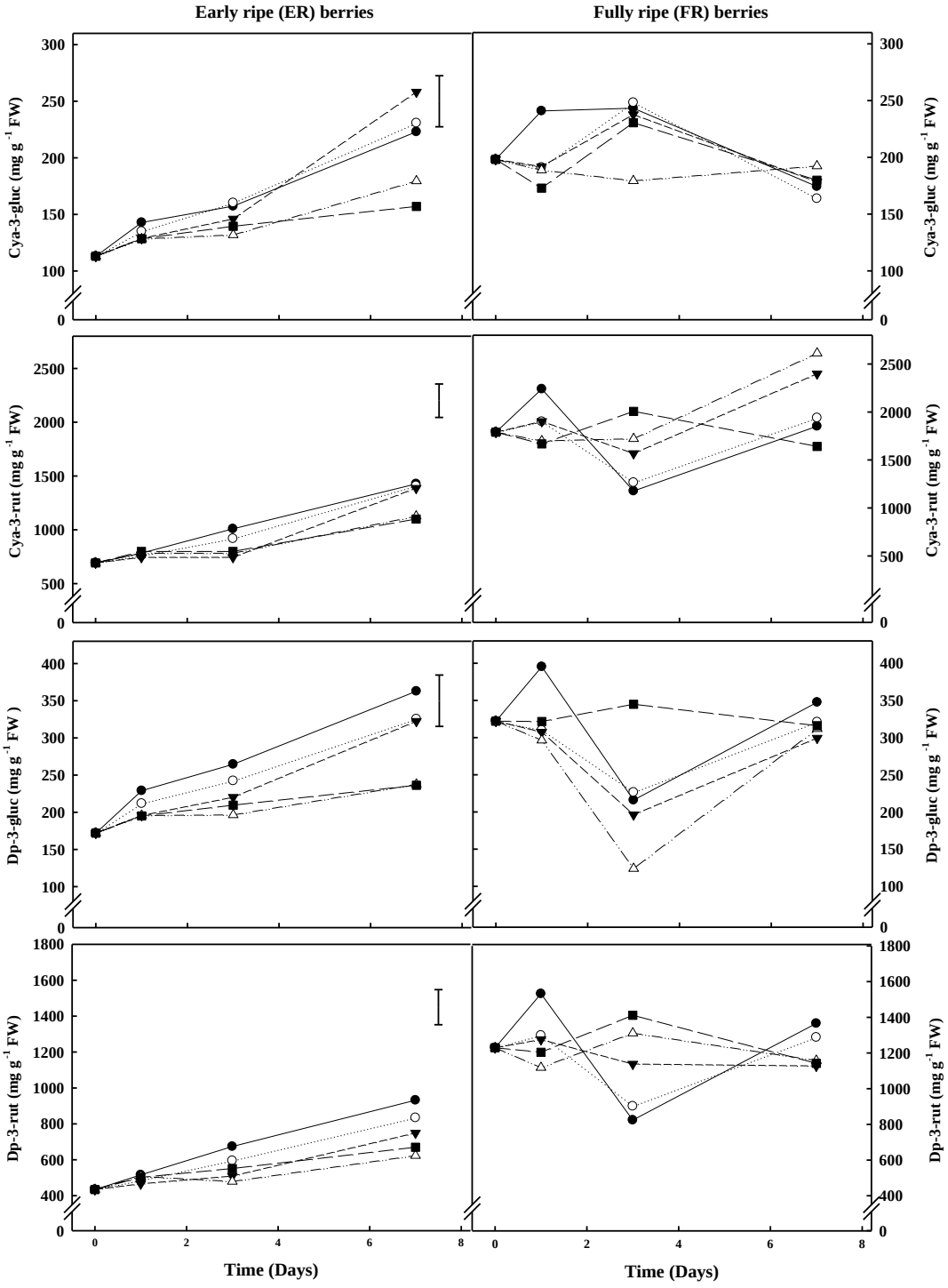


Figure 4.17: Anthocyanins (A: cya-3-gluc; B: cya-3-rut; C: delp-3-gluc; D: delp-3-rut) in blackcurrant berries harvested at early ripe or fully ripe maturity stage and stored for 7 days at (1-4 ºC (●), 5-8 ºC (○), 9-12 ºC (▼), 13-16 ºC (Δ) and 17-20 ºC (■)). LSD bar (p = 0.05) is shown for the interaction of time*temperature*maturity at harvest for each individual anthocyanin.

Total anthocyanins were generally present in higher concentrations in FR berries stored at higher temperatures. The concentration of cya-3-gluc was greater at temperatures above 12°C than below in both ER and FR berries. There was no effect of temperature on the concentration of cya-3-rut or delp-3-gluc in ER berries, but in FR berries, both were greatest at temperatures between 5 and 12°C, and the concentration of delp-3-rut in FR berries was not affected by temperature. The proportion of delp-3-gluc tended to increase with increasing temperature in FR berries, while the proportion of cya-3-gluc was also greater at higher temperatures, but reached a maximum at intermediate storage temperatures (between 9 and 12°C). The proportion of cya-3-rut was higher in berries of both maturities stored at lower temperatures.Anthocyanin synthesis was observed in both ER and FR berries during storage; this said, the anthocyanin concentrations in ER berries never reached levels equal to those in berries harvested at FR stage, which has also been the case for highbush blueberries (Kalt *et al*., 2003). Synthesis of anthocyanins in stored fruit can occur in the darkness, but light can enhance the process (Austin *et al*., 1960) with both UV-A, and to a lesser extent UV-B, inducing the overexpression of anthocyanin biosynthetic genes. The limited synthesis of anthocyanins observed herein as compared to earlier works (Robbins *et al.*, 1989; Wang, 2003) may therefore be the result of light as the limiting factor as berries were stored in darkness. Total anthocyanin content of berries is affected by temperature and storage period. Anthocyanins decreased in strawberries stored at 0ºC and 5ºC, but gradually increased during storage at 10ºC (Ayala-Zavala *et al.*, 2004). In contrast, anthocyanins in raspberries increased by 70% during storage at 0ºC for 24 days (Robbins *et al.*, 1989), but decreased during storage at 10ºC for 7 or 10 days (Chanjirakul *et al.*, 2006). In the present study, total anthocyanins significantly increased with increasing storage temperature. This may be due to the increase in metabolic activity which occurs at high temperatures which in turn leads to greater oxidative stress (i.e. generation of radical species) and hence a greater requirement for antioxidants within the fruit.

4.5 Conclusion and directions for future work

The work presented in this chapter represents an attempt to explore the genotypic variation in the major taste- and health-related compounds from a wide range of UK-grown fresh blackcurrant berries. Wide variations in the biochemical profile of the target analytes examined were observed and thus, it is clear that the differences in blackcurrant cultivars play an important role in determining fruit

composition. Increasing evidence demonstrates the negative correlation between intake of naturally rich products in antioxidants, such as blackcurrants, and the incidence of certain cancers and chronic diseases. Nevertheless, both taste- and health-related compounds drastically change during berry ripening, with differences in the maturity of harvested berries representing the main source of variation in the composition of fruits. The results presented herein may assist in the selection of cultivars as well as in the preharvest conditions (i.e. optimum maturity, temperature during the growing period) leading to fruits with enhanced taste and potential health-related properties. Within the relatively short window of commercial maturity, there were large variations in the concentrations of blackcurrant health-promoting compounds. The application of chemometric techniques was demonstrated to be a suitable tool to compare and assess the variability between the biochemical profiles of different blackcurrant genotypes and maturities at harvest and therefore could be employed as a complementary tool to standard genomics in breeding programmes. Indeed, PCA and HCA revealed that the determination of health-related compounds alone was more suitable to discriminate between different genotypes than using other major components such as citric acid and non-structural carbohydrates. Differences in cultivars having high AsA or anthocyanin concentrations were especially emphasized by PCA. Taken as a whole, the results from initial experiments (Exp. 4.1) may help to better inform breeding programmes in the selection of appropriate cultivars based on their biochemical profile.

Furthermore, the temporal variations in blackcurrant taste- and health-related compounds during storage at different temperatures was strongly influenced by the maturity of the berries at harvest. When berries were harvested at the ER stage, they never reached the same taste-related composition or nutritional quality of those harvested at the FR stage regardless of storage conditions and time. No other studies thus far have studied such in detail the effect that storage temperatures, ranging from 1 to 20ºC on blackcurrant composition, and hence, results from the postharvest trial may provide suitable information on the storage conditions required to maximize the quality of blackcurrant berries specially addressing individual requirements for each of the different blackcurrant-based products available in the market.

Future work should repeat the experiments presented here across successive growing seasons to assess the interaction of climactic conditions, genotype and maturity stage to further identify potential quality markers in blackcurrants that could easily be taken into consideration by breeding programmes.

The results and methodologies detailed in this chapter have been published as:

Giné Bordonaba, J. and Terry, L.A. (2008). Biochemical profiling and chemometric analysis of seventeen UK-grown blackcurrant cultivars. *Journal of Agricultural and Food Chemistry*, 56, 7422-7430.

Giné Bordonaba, J., Chope G.A. and Terry, L.A (2010). Maximising blackcurrant anthocyanins: temporal changes during ripening and storage in different genotypes. *Journal of Berry Research* (in press).

CHAPTER 5

ELECTROCHEMICAL BEHAVIOUR OF BLACKCURRANT AND STRAWBERRY FRUIT JUICES ON DISPOSABLE SCREEN-PRINTED CARBON ELECTRODES:

TOWARDS A RAPID SENSOR FOR ANTIOXIDANT CAPACITY AND INDIVIDUAL ANTIOXIDANTS

5.0 CHAPTER FIVE

Electrochemical behavior of blackcurrant and strawberry fruit juices on disposable screen-printed carbon electrodes: Towards a rapid sensor for antioxidant capacity and individual antioxidants

5.1 Introduction

The vast majority of biochemical reactions which ensure life run with the production of free radicals, which in turn lead to oxidative stress and associated damage to the living organism. Complex biochemical pathways within the human body are responsible for fighting oxidative stress by ensuring an appropriate balance between prooxidants (i.e. free radicals) and antioxidants. Epidemiological data have strongly suggested an inverse correlation between the intake of fruits and vegetables (FAV), naturally rich in antioxidants, and the incidence of certain diseases (*viz.* cancer, coronary, diabetes) (Ames *et al.*, 1995; Litescu and Radu, 2000; Blomhoff, 2005). From the earlier-mentioned studies, it is unquestionable that dietary antioxidants play a role in maintaining an optimum oxidative balance within the body, and hence, it is not surprising that the analysis of antioxidants or antioxidant capacity (AC) in different foodstuffs and beverages has become an expanding area of research (Prior *et al.*, 2005).

Both blackcurrant and strawberry fruits are among the richest sources of antioxidants (see chapters 3 and 4), especially anthocyanins (Aaby *et al.*, 2007; Terry *et al.*, 2007; Chapter 3; section 3.3.5; Chapter 4; section 4.4.2.4) and other phenolic compounds as well as ascorbic acid (Viola *et al.*, 2000; Walker *et al.*, 2006; Tulipani *et al.*, 2008). Recently, Wolfe *et al.* (2008) and Haleem *et al.* (2008) reported that strawberry fruits were amongst the top sources of antioxidants from FAV in the American and Scottish populations, respectively. Nevertheless, comparison of AC between different food sources still remains challenging due to the diversity of AC assays found in the literature (Prior *et al.*, 2005). Antioxidant capacity assays rely on two different reaction mechanisms differing on whether hydrogen atom transfer (HAT) or single electron transfer (SET) are responsible to deactivate radicals (Prior *et al.*, 2005). HAT-based assays monitor competitive reaction kinetics and these would include common assays such as oxygen radical scavenging capacity (ORAC) and the total radical-trapping

antioxidant parameter assay (TRAP). In contrast, SET-based assays involve one redox reaction with the antioxidants and included standardised methods in the food arena such as Trolox equivalent antioxidant capacity assay (TEAC), ferric ion reducing antioxidant parameter assay (FRAP) and total phenolics assay by Folin-Ciocalteu (Prior *et al.*, 2005; Blasco *et al.*, 2007). As an example, the Folin-Ciocalteu (FC) assay, since its development in the sixties (Singleton and Rossi, 1965), has been a widely used assay in which an oxidant, the Folin-Ciocalteu reagent, extracts an electron from the antioxidant (i.e. polyphenol) causing the colour change of the reagent. The degree of colour change monitored at 765 nm is proportional to the concentration of the antioxidant. Despite being recognised as a total phenolics assay, this method instead measures the sample reducing capacity (Blasco *et al.*, 2007). Like the FC assay, most of the available methods are based on spectrophotometric techniques which are relatively costly and unfeasible to be routinely applied for screening large sample sets as well as suffering important interferences when working with colourful or turbid samples (Huang *et al.*, 2005)

Many antioxidants exhibit inherent electroactivity, acting as reductants in solutions (Kilmartin *et al.*, 2001). Therefore, employing electrochemical methods could be a viable approach for evaluating the overall reducing power of antioxidant compounds within a fresh produce matrix without the need for added reactive species (Blasco *et al.*, 2007). In fact, over the past years electrochemical techniques have been used, mainly as HPLC detection systems (Aaby *et al.*, 2004), flow injection measurements (Mannino *et al.*, 1998) and, to a lesser extent, for direct determination of antioxidants at inner electrodes (Blasco *et al.*, 2004; Piljac-Zegarac *et al.*, 2008). No studies, thus far, have studied the potential application of simple screen-printed carbon electrodes aiming to develop sensors for direct determination of antioxidants in berries or berry-derived products. Accordingly, the aims of the present chapter were first to study the electrochemical behaviour of natural antioxidants and soft fruit juices on screen-printed carbon electrodes and secondly to assess the relationship between the electrochemical signals and the composition of the fruit juices with particular emphasis to their antioxidant capacity.

5.2 Materials and methods

5.2.1 Reagents

Anthocyanin standards (*viz.* cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-glucoside, malvidin-3-glucoside and pelargonidin-3-glucoside) were purchased from Extrasynthèse (Genay, France). All other reagents and standards, including quercetin and myrecitin, were HPLC-

analytical grade and purchased from Sigma (Dorset, UK). Solvents were prepared immediately before analysis, unless otherwise stated.

5.2.2 Plant materials and sample preparation

Blackcurrant and strawberry samples used in this study corresponded to samples used for experiments 4.2 and 3.2, respectively. Briefly, blackcurrant samples from three different cultivars and harvested at different maturities were chosen due to known differences in their antioxidant profile (Chapter 4; section 4.2.2). Similarly, strawberry samples from six different cultivars were grown under commercial practices and supplied by H.H. Duncalfe (Chapter 3; sections 3.2). A detailed study on the composition of the strawberry samples described herein can be found in the following chapter (Chapter 6). Frozen berries (50 g) were diluted in phosphate buffer (500 mL), homogenised with a domestic blender for 1 min and the solution obtained was filtered through a Whatman filter (No.2) prior to analysis. HPLC and antioxidant capacity analysis, except for the different antioxidant fractions (section 5.2.4.1), were performed on freeze-dried samples as described earlier (Chapter 3; section 3.3.5).

5.2.3 Apparatus and electrochemical measurements

All electrochemical measurements were performed using a Palmsens potentiostat (Palm Instruments BV, The Netherlands) and results processed using Ivium Palmsens PC software (Palm Instruments BV, The Netherlands). Screen-printed electrodes (SPE) (Gwent Electronic Materials Ltd. GEM, Gwent, UK) were used for all measurements (**Figure 5.1**). The electrodes were screen-printed in a two electrode configuration comprising a generic carbon working electrode (28 mm^2) and a combined Ag/AgCl reference/counter electrodes onto a PVC substrate.

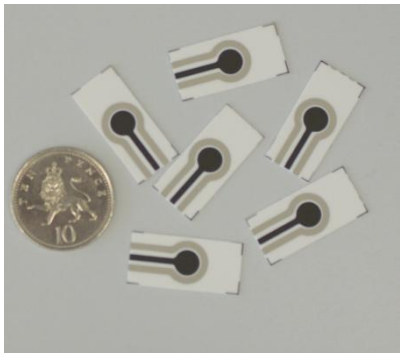


Figure 5.1: Two electrode configuration screen-printed carbon electrodes.

Sensor measurements were taken on standards or juice samples after adjusting the pH to 4 or 7 which corresponded to moderated acid pH, as commonly encountered in fruit juices, or neutral pH, respectively. The pH of the buffer/electrolyte solutions was determined using a JENWAY 3020 pH meter (Jenway, Essex, UK). A 50 µl aliquot of sample or standard was deposited onto the surface of the electrode in order to complete the electrochemical cell. Immediately after sample deposition, square wave voltammograms (SWV) were recorded by establishing the appropriate scan rate (50 mV s^{-1}) and scanning from 0 to 1.2 V. All experiments were carried out at room temperature (~22 ºC).

5.2.4 Characterisation of berry antioxidants

5.2.4.1 Separation of anthocyanin and non-anthocyanin polyphenolic fractions.

The separation of anthocyanin and non-anthocyanin fractions was performed on Waters C18 solid phase extraction (SPE) vacuum cartridges as described by Wrolstad *et al.* (2004), with some modifications. Briefly, cartridges were preconditioned by sequentially passing 10 mL of ethyl acetate, 10 mL of acidified aqueous methanol (MeOH:H_2O; 70:30 v/v) and 10 mL of 0.01 N aqueous HCl. Subsequently, 3 mL of blackcurrant juice (fully ripe berries from cv. Ben Gairn; 20 g fruit and 180 g Millipore H_2O) and strawberry juice (cv. Elsanta; 40 g fruit in 260 g Millipore H_2O) sample were loaded into each cartridge. Cartridges were washed with 6 mL of 0.01 N aqueous HCl to remove sugars, organic acids and other water soluble compounds (fraction 1 (F1)). Following removal of water soluble compounds, cartridges were dried by continuously applying a pump generated vacuum for 5 min. Non-anthocyanin polyphenolic compounds were eluted from the cartridge by using 20 mL of ethyl acetate (fraction 2 (F2)). Finally, absorbed anthocyanins (fraction 3 (F3)) were eluted from the cartridges with 6 mL of 70:29.5:0.5 (v/v/v) MeOH:H_2O:HCl (**Figure 5.2**). All fractions were separately collected and 3 mL of each sample were placed in amber glass vials. Solvents were removed overnight in an Edwards Modulyo freeze-drier and resuspended in 0.1 M PBS for FRAP and sensor measurements.

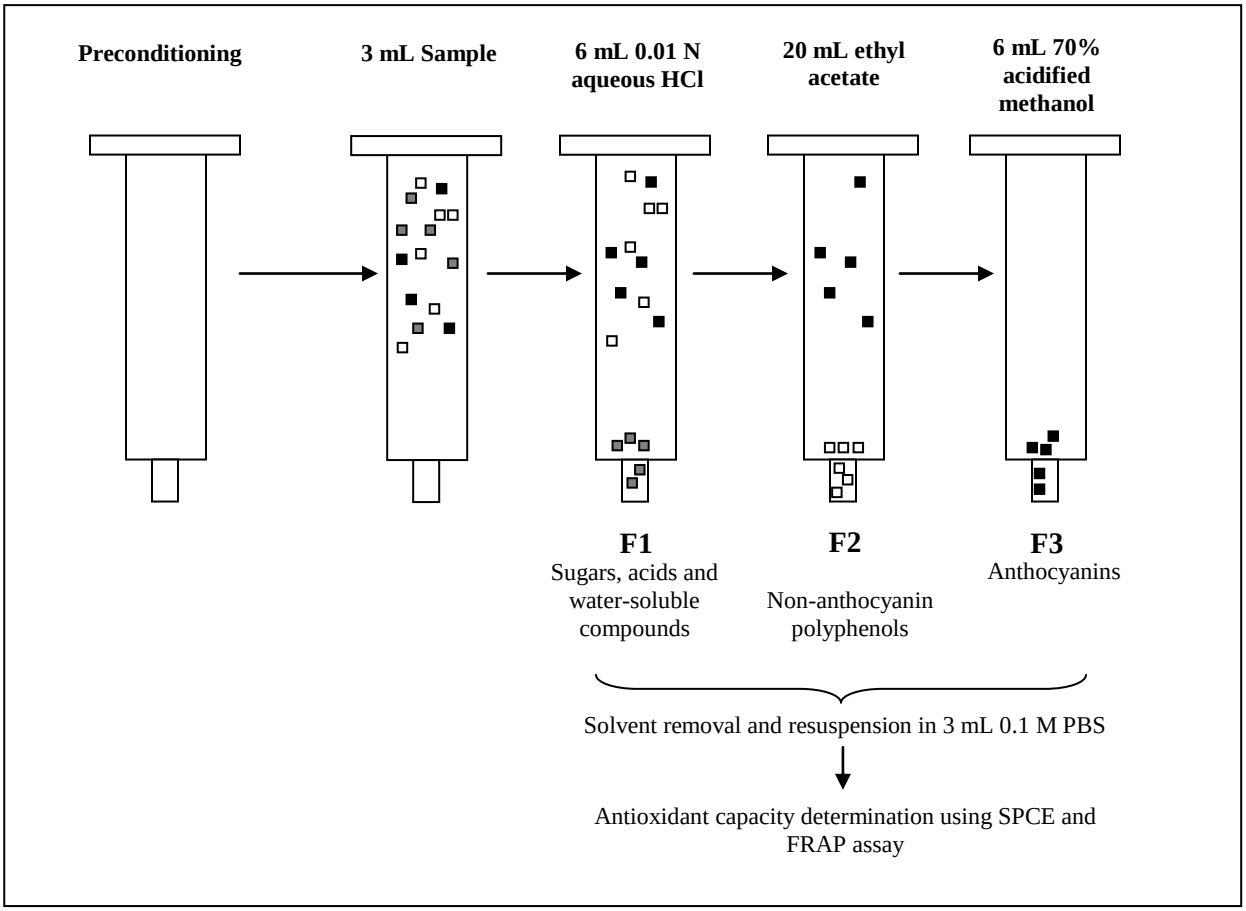


Figure 5.2: Schematic representation of the procedure used for the extraction of anthocyanins and non-anthocyanin polyphenolic compounds from blackcurrant and strawberry fruits (■ sugars, acids and water-soluble compounds, □ non-anthocyanin polyphenols and ■ anthocyanins)

5.2.4.2 HPLC measurements

The HPLC system comprised an Agilent 1200 series HPLC system (Agilent, Berks., UK), equipped with an Agilent 1200s DA G1315B/G1365B photodiode array with multiple wavelength detector. Individual anthocyanins were extracted by mixing freeze-dried sample (150 mg) with 3 ml of HPLC grade methanol: HCl: water (70: 0.5: 29.5; v/v/v) and the anthocyanin profile was determined as described elsewhere (Chapter 4; section 4.4.3) by means of HPLC-DAD (520 nm). Similarly, ascorbic acid concentrations in strawberry and blackcurrant fruits were measured after a simple aqueous extraction of the freeze-dried material (Chapter 3; section 3.3.4) and analysed by HPLC-DAD (210 nm).

5.2.4.3 Determination of total phenolics and antioxidant capacity.

Total phenolic concentrations and antioxidant capacity of the fruits, and different polyphenolic fractions, were quantified from freeze-dried material as described earlier (Chapter 3; Terry *et al.*, 2007). Briefly, total phenolic concentrations were measured by means of the Folin-Ciocalteu method (FCM) and antioxidant capacity measured by the FRAP assay (Chapter 3; Terry *et al.*, 2007). Both AC assays are based on the same electron transfer principle; the FCM is based on the oxidation of phenolics and other antioxidants by a molybdotungstane-based reagent yielding a coloured product with λ_{max} at 765 nm whereas the FRAP assay measures the reduction of a ferric 2,4,6-tripyridyl-s-triazine (TPTZ) to a coloured product with λ_{max} at 593 nm.

5.2.5 Data analysis

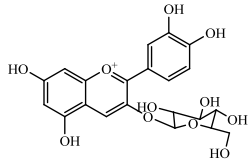
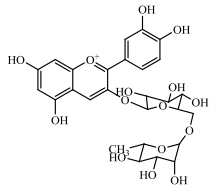
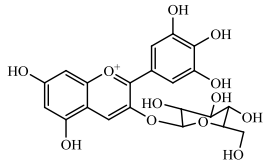
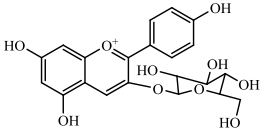
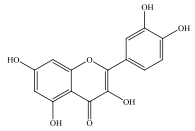
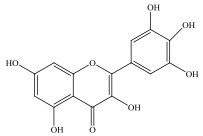
All statistical analyses were carried out using Genstat for Windows Version 12 (VSN International Ltd., Herts., UK). Data presented corresponds to average of minimum triplicate measurements. Tests for correlations between mean values were made using Spearman's rank correlation. Correlations are presented with the Spearman's correlation coefficient (r) based on a two-tailed test only when $P < 0.01$. Regression analysis was performed in order to explain the possible relationship between the cumulative sensor responses (Q) at different formal potentials (300, 500 and 1000 mV) and antioxidant capacity (FRAP), total phenolics (TP), ascorbic acid and anthocyanin content in different berry juices.

5.3 Results and discussion

5.3.1 Electrochemistry of standard antioxidants at screen-printed carbon electrodes

Due to its reducing properties, which indeed are directly related to their antioxidant capacities, different berry antioxidants (*viz.* cyanidin-3-rutinoside, delphinidin-3-glucoside, pelargonidin-3-glucoside, myricetin and quercetin; **Table 5.1**) were analysed by means of square wave voltammetry (SWV) at screen printed electrodes. Screen-printed technology is mainly used for the production of disposable sensors. This type of sensors is of particular interest in the analysis of antioxidants because during the oxidation process of these compounds (i.e. phenolics) a polymeric film is created leading to the inactivation of the electrode (Romani *et al.*, 2000).

Table 5.1: Voltammetric behavior, at screen printed carbon electrodes, and chemical structure of berry antioxidants studied at different pH conditions.

Compound	Phenolic class	Chemical structure	pH studied	Peaks	Oxidation potential (mV)
Cyanidin-3-glucoside	Anthocyanin		4	I	300
			7	I	258
			4	II	820
			7	II	763
Cyanidin-3-rutinoside	Anthocyanin		4	I	296
			7	I	-
			4	II	818
			7	II	-
Delphinidin-3-glucoside	Anthocyanin		4	I	275
			7	I	249
			4	II	800
			7	II	747
Pelargonidin-3-glucoside	Anthocyanin		4	I	318
			7	I	299
			4	II	828
			7	II	790
Quercetin	Flavonol		4	I	207
			7	I	-
Myricetin	Flavonol		4	I	168
			7	I	-

- Analysis for these compounds was not performed at pH 7

All the compounds measured acted as powerful antioxidants and were easily oxidised at screen-printed carbon electrodes. Even though different electrochemical techniques and electrode configurations were used, the electrochemical behaviour of the different compounds analysed agreed with those found in the literature (Blasco *et al.*, 2004; Janeiro *et al.*, 2007; Pijac-Zegarac *et al.*, 2008; Piljac-Zegarac *et al.*, 2009; Aguirre *et al.*, 2010). Cyclic voltammetry (CV) has been, so far, the method of choice when analysing antioxidants in wines (Kilmartin *et al.*, 2002), fruit juices (Piljac-Zegarac *et al.*, 2007) and even plant extracts (Chevion *et al.*, 1999). In the present study, SWV was chosen rather than CV. Besides sharing most of the advantages described for CV (Piljac-Zegarac *et al.*, 2008), SWV allows easy interpretation of the results, rejects background current 'noise' and can have very low detection limits. In addition, SWV enables, while performing the scan, to see whether an oxidation reaction is reversible or not, since the current is sampled in both positive and negative pulses. Accordingly, oxidation and reduction peaks can be obtained in the same experiment (Janeiro *et al.*, 2007).

The square wave voltammogram of pelargonidin-3-glucoside showed two peaks, the first one (P1), reversible, at 318 and the second one (P2), irreversible, at 828 mV (**Table 5.1**). Similar results were obtained for cyanidin-3-rutinoside (**Figure 5.3**) which at pH 4 presented two peaks at 296 and 820 mV. No significant changes were observed in P1 of either cyanidin-3-glucoside or 3-rutinoside, as was also highlighted by Janeiro and Oliveira-Brett (2007), indicating that differences in the sugar moiety attached to the anthocyanidins (**Table 5.1**) did not change the first oxidation peak (P1) (**Table 5.1**). Both oxidation peaks from the cyanidin molecule were shifted towards lower potentials in the case of delphinidin-3-glucoside (pH 4). In this context, the first oxidation peak for each anthocyanin corresponded to the oxidation of the OH groups in the beta ring of the molecule whereas the peak at greater potentials was most probably related to the 5,7-dihydroxyl moiety of the A ring (Janeiro and Oliveira Brett, 2007; Aguirre *et al.*, 2010). Besides anthocyanins, both quercetin and myricetin were also investigated. Their voltammetric profile differed significantly from that observed for anthocyanin molecules, where the first oxidation peak was observed for both compounds at lower potentials (150-200 mV) (**Table 5.1**). In contrast, at a glassy carbon electrode, quercetin led to two oxidation peaks (Filipiak, 2001). Lower oxidation potentials for these compounds, but in particular for quercetin, was also reported by others (Born *et al.*, 1996; Filipiak, 2001) when investigating different natural antioxidants using cyclic voltammetry. It is well accepted, that adjacent hydroxy groups, as in the catechol (1,2-dihydroxy; cyanidin) and pyrogallol (1,2,3-trihydroxy; delphinidin), stabilise the phenoxy radical leading to a lower oxidation potential of the compound and hence accounting for the potential

shift observed herein and elsewhere (Filipiak, 2001; Aaby *et al.*, 2004). Lower oxidation potential has been referred as high antioxidant power (Mannino *et al.*, 1998) and consequently, based on their antioxidant power, the results pointed out the following sequence: myricetin > quercetin > delphinidin > cyanidin > pelargonidin.

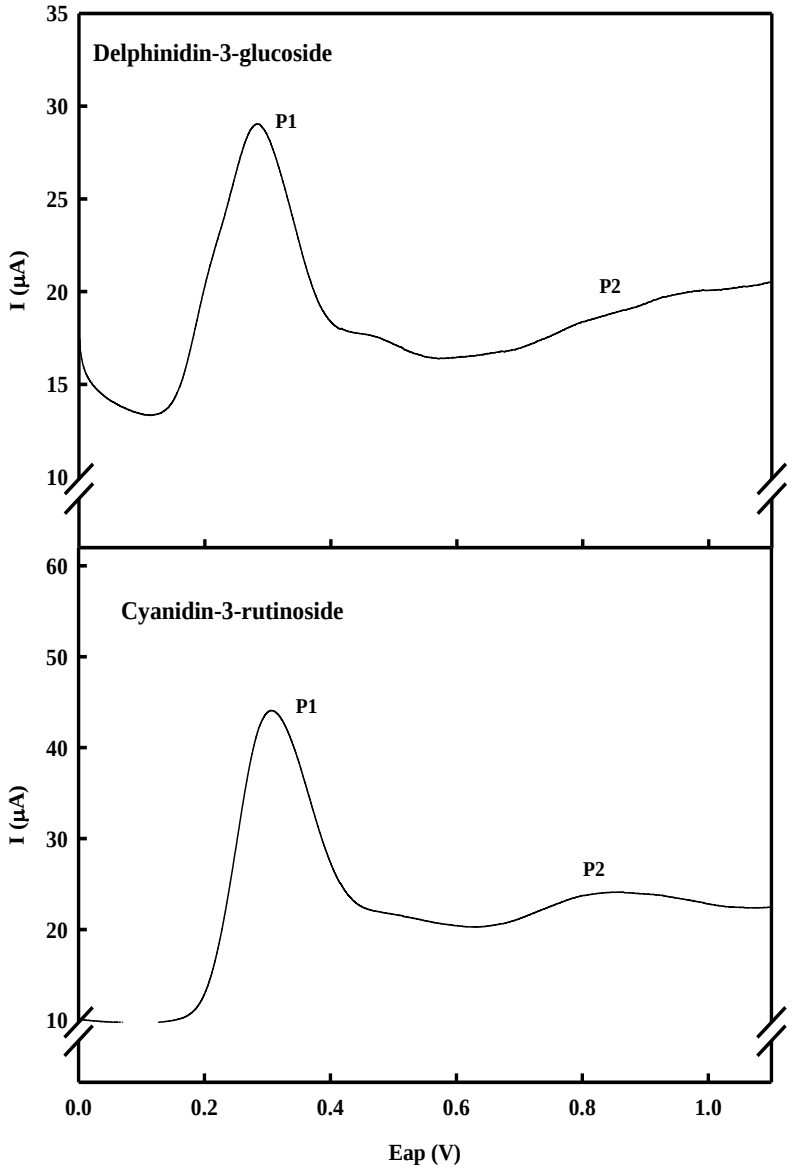


Figure 5.3: Square wave voltammograms of delphinidin-3-glucoside (A) and cyanidin-3-rutinoside (B) diluted in 0.1 mM PBS and adjusted to pH 4.

In all the compounds measured, the voltammetric profile depended on the pH of the solution (**Table 5.1**). It has been accepted that oxidation of polyphenols in phosphate buffers mimics

physiological conditions (Filipiak, 2001). A neutral pH resulted for all the compounds measured in a shift of all oxidation peaks towards lower potentials and agreed well with the observations made by others (Blasco *et al.*, 2004). The relationship between oxidation potential and pH is known to be proportional, with slope values (V/pH) differing among different compounds and experimental conditions (Filipiak, 2001). Accordingly, and despite only two different pH being investigated herein, V/pH values ranged for -0.029 for the first oxidation peak of pelargonidin-3-glucoside to -0.057 mV/pH for the second oxidation peak of cyanidin-3-glucoside (**Table 5.2**). Structural changes in anthocyanins in solutions of different pH are well documented (Janeiro and Oliveira-Brett, 2007). For instance, at lower pH values, anthocyanins in solution exist primarily as flavylium cation forms. If the pH is raised (i.e. pH 4), the flavylium form will be deprotonised leading to the formation of quinoidal structures. Further deprontonisation of the quinoidal base can occur at pH values between 6 and 7 with the formation of quinonoid anions (Janeiro and Oliveira-Brett, 2007). The pH dependency observed herein and elsewhere (Van Acker *et al.*, 2000; Filipiak, 2001; Blasco *et al.*, 2004) demonstrates that not only e- but H^+ may be betrothed in the oxidation of polyphenols at inert electrodes. Further experiments should try to elucidate the mechanisms of these reactions.

Table 5.2: Potential pH-dependence at screen printed carbon electrodes of some anthocyanins found in blackcurrant and/or strawberry fruits.

Compound	Peak	Intercept (V)[1]	Slope (V/pH)[1]
Cyanidin-3-glucoside	I	0.342	-0.042
	II	0.877	-0.057
Delphinidin-3-glucoside	I	0.853	-0.032
	II	0.885	-0.053
Pelargonidin-3-glucoside	I	0.347	-0.029
	II	0.866	-0.048

[1]Intercept and slope values are based on two different pH being investigated (pH 4 and 7)

When SWV is applied to samples containing antioxidants, anodic peaks (*E*) may refer to specific compounds while their concentration is proportional to the intensity of the peak (*I*). Accordingly, a very good correlation coefficient was obtained when the sensor was challenged with

increasing concentrations of standard solutions of cyanidin-rutinoside (r > 0.988;data not shown) or when standard additions of pelargonidin-3-glucoside were performed on strawberry juice and the area under P1 was recorded (r > 0.996) (**Figure 5.4**). When comparing the voltammetric profile of pg-3-gluc standard solutions added in either PBS (**Table 5.1**) or strawberry juice (**Figure 5.4**), it was noticed a shift of both P1 and P2 towards more positive potentials when the standard solutions were added in strawberry juice. Similar findings were observed by Aguirre *et al.* (2010) who reported on the importance of the solvent to stabilise the phenolic groups present in the anthocyanins investigated herein. It is therefore plausible to speculate that other compounds commonly present in the strawberry juice may lead to a stabilization of the antioxidants and hence account for the greater potentials required for their oxidation.

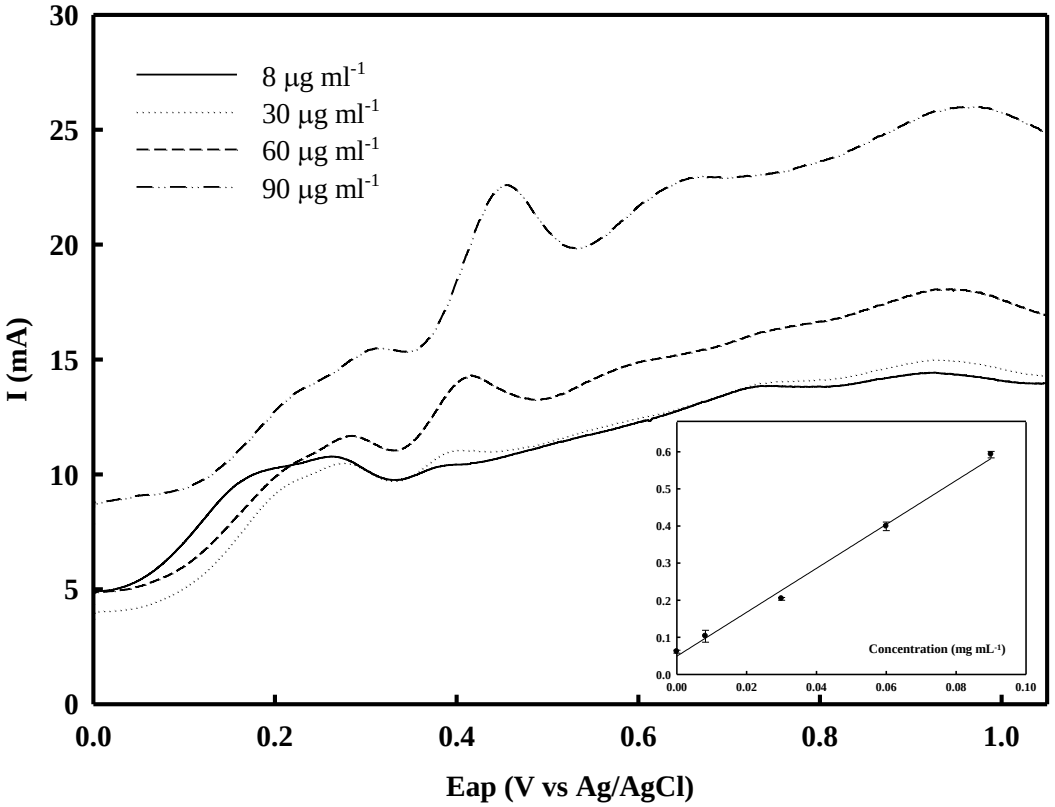


Figure 5.4: Square wave voltammograms of strawberry juice (cv. Symphony) diluted in 0.1 mM PBS and adjusted to pH 4, after standard additions of pelargonidin-3-glucoside solutions. Insert shows calibration curve after standard additions of increasing concentrations of pelargonidin-3-glucoside.

5.3.2 Electrochemical characterization of blackcurrant and strawberry juices

As a result of the increasing applied potential (0 to 1200 mV vs. Ag/AgCl) the electroactive compounds present in the juices were oxidised leading to a characteristic voltammetric profile for each of the samples analysed (**Figure 5.5**). Generally, blackcurrant juices had greater oxidation peaks at lower potentials (< 500 mV) which were indicators of the greatest antioxidant capacities (**Figure 5.5**) of these berries as compared to strawberry fruits (Chapter 3 and 4).

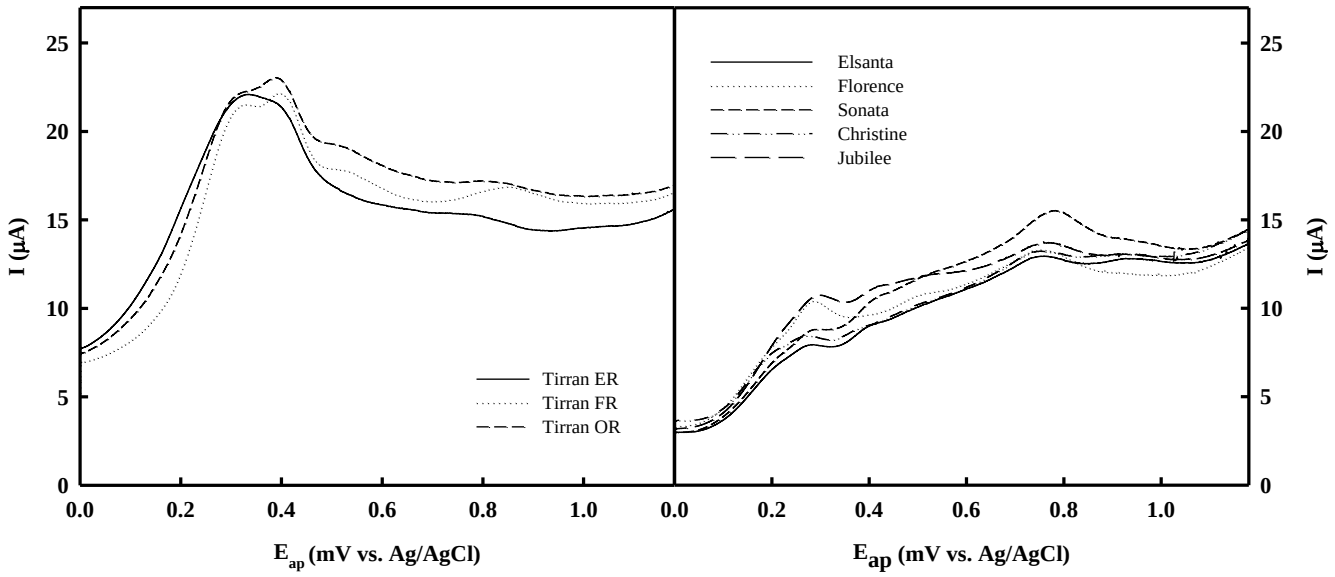


Figure 5.5: Square wave voltammograms of different blackcurrant and strawberry juice samples diluted in 0.1 mM PBS at pH 4. Samples corresponded to juices of blackcurrant cultivars (cv. Ben Tirran) harvested at three different maturities (early ripe (ER), fully ripe (FR) and over-ripe (OR); Chapter 4; section 4.2.2) and strawberry samples from 5 different cultivars (Chapter 3; section 3.3.1).

In both berry-based juices, the first oxidation wave (W1) occurred at ~300 mV, followed by a second one (W2) at ~500 mV and the last (W3) around 800 mV (**Table 5.3**). The area underneath the peak current, corresponding to cumulative response at specific potentials, has earlier been proposed as an indicator of antioxidant capacity of complex mixtures of antioxidants (Chevion *et al.*, 1999; Kilmartin *et al.*, 2001). In the present study, cumulative responses at the end of these oxidation waves are shown in **Table 5.3**. If compared to standard compounds, the broadness of the peaks in either blackcurrant or strawberry fruits was greater than that obtained when working with standard

compounds (**Figure 5.3; Figure 5.5**) which was the result of a combined effect between the different antioxidant compounds with similar formal potentials as found by others (Piljak-Zegarac *et al.*, 2009).

Table 5.3: Oxidation waves[1] and cumulative responses at three oxidation potentials of blackcurrant and strawberry juices and different juice fractions diluted in 0.1 mM PBS (pH 7).

			Cumulative peak areas[2]		
Sample	**Cultivar**	**Max. Peak[3]**	**300**	**500**	**1000**
Strawberry	Christine	790	2.057	3.870	9.780
	Elsanta	790	2.268	4.537	11.602
	Florence	790	2.313	4.618	11.192
	Jubilee	790	2.161	4.788	12.016
	Sonata	790	2.683	5.350	13.459
Blackcurrant	Ben Tirran (ER)[4]	310	4.006	8.01	15.743
	Ben Tirran (FR)	400	3.365	7.505	15.78
	Ben Tirran (OR)	400	3.781	8.11	16.802
	Ben Dorain	320	3.865	7.646	17.798
	Ben Gairn	310	3.543	8.326	15.004
Strawberry (cv. Elsanta)	F1	> 900	0.318	0.562	2.574
	F2	110	0.760	1.273	3.105
	F3	210	1.162	2.174	5.776
Blackcurrant (cv. Ben Tirran)	F1	> 900	0.779	1.283	4.817
	F2	190	2.256	3.528	7.475
	F3	300	2.038	3.775	8.168

[1]Oxidation waves rather than peaks are considered given that different antioxidant compounds may have similar formal potentials and contribute for the same oxidation wave; [2]Cumulative peak areas were calculated considering the response across several potentials and results expressed as μC mmol sample^{-1}; [3]Potential at which the peak with the greatest intensity was recorded. [4]ER: Early ripe; FR: Fully ripe and OR: Over-ripe berries.

To further understand the electrochemical behaviour of the samples analysed, anthocyanins were separated from non-anthocyanin polyphenols and other antioxidant compounds by SPE. AC from the different fractions was then measured electrochemically and using the standard FRAP assay (section 5.2.4.1). Regardless of the quantification method used, anthocyanins represented most of the antioxidants present in blackcurrant fruits, with AC values 2-fold greater than that of other

polyphenolic compounds (F1) and/or water soluble compounds (F2), including ascorbate. In strawberry fruits both anthocyanin and other phenolic compounds (F2 and F3, respectively) were the fractions showing greater AC followed closely by ascorbate and other water soluble compounds (**Figure 5.6**). Several authors (Chapter 3; Crespo *et al.*, 2010) have reported the poor correlation between anthocyanin concentration in strawberry fruits and AC, which is not surprising given that these compounds accounted for no more than 38 ± 5 % of the fruit AC. In contrast, the blackcurrant-anthocyanin fraction accounted for > 55 % of the total fruit AC, and hence anthocyanin concentrations measured by HPLC correlated with FRAP values (**Figure 5.6)** and agreed well with others (Chapter 4; Kahkonen *et al.*, 2003).

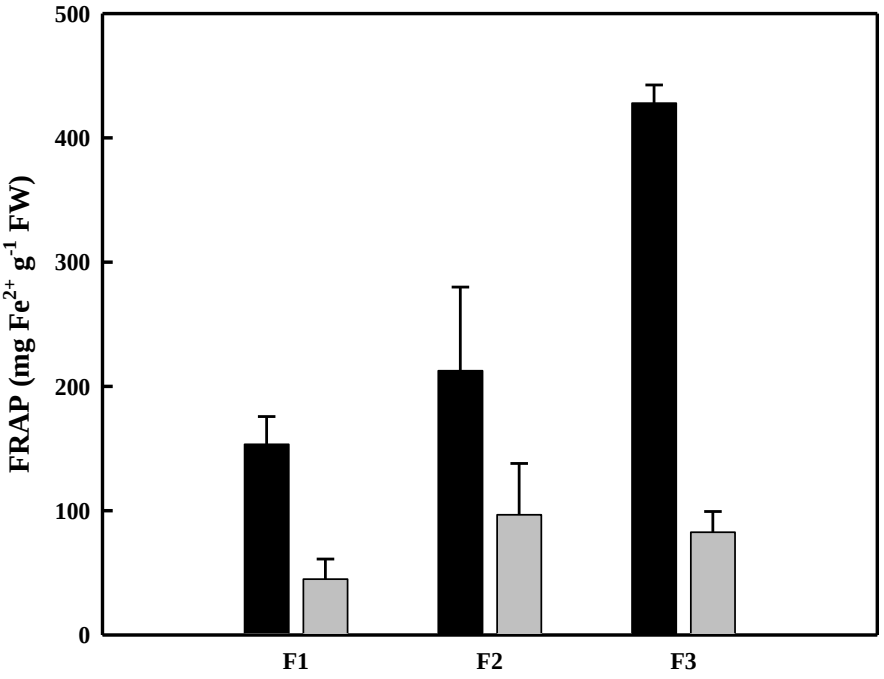


Figure 5.6: Antioxidant capacity measured by the FRAP assay from different strawberry (▬; cv. Elsanta) and blackcurrant (▬;cv. Ben Tirran) fractions. F1: Organic acids and sugars; F2: Non-anthocyanins polyphenolic fraction and F3: Anthocyanin fraction. Error bar indicate standard deviation for n=3.

The voltammograms for each fraction differed considerably between blackcurrant and strawberry fruits. Fraction 1, which consider together a variety of water-soluble compounds, including ascorbate showed for both samples a first oxidation wave at ~ 200 mV followed by a second oxidation

at ~900 mV (**Figure 5.8**). The greater intensity of the peak maxima at ~200 mV in blackcurrants as compared to strawberry fruits, clearly reflected the differences up to 6-fold in ascorbate concentrations between these fruits (Chapter 3 and 4; Rubinskiene *et al.*, 2006; Walker *et al.*, 2006; Terry *et al.*, 2007; Tulipani *et al.*, 2008). Even though ascorbate standard solutions were not assessed in the present study, it is known that oxidation of the ene-diol of ascorbic acid occurs at low potentials (Piljac-Zegarac *et al.*, 2010). Such low potential demonstrates that ascorbic is a highly potent antioxidant with one the most powerful reducing abilities (Piljac-Zegarac *et al.*, 2010). In diluted blackcurrant juice, the oxidation wave for fraction 2 overlapped that from fraction 1 (~ 200 mV), whereas for the same fraction in strawberry juice the oxidation wave occurred at potentials as low as ~110 mV. Differences in the polyphenolic composition, besides anthocyanin, of blackcurrant and strawberry fruits are well known (Hakkinen *et al.*, 1999; Anttonen and Karjalainen, 2002; Rubinskiene *et al.*, 2006; Aaby *et al.*, 2007). For instance, strawberry fruits are rich sources of ellagic acid and ellagitannins (**Figure 5.7**) which contain several pyrogallol groups and hence may be oxidised at very low potentials (Aaby *et al.* 2004) (**Figure 5.8**). In fact, in the conditions described by Aaby *et al.* (2004), ellagic acid and its glycosides had dominant oxidation potentials at ~300 mV with no further oxidation, whereas ellagitanins started to be oxidised at 100 mV but had dominant oxidation potential at the end of 800 mV. In contrast, no studies so far, have reported high concentration of ellagic acid or ellagitannins in blackcurrant berries and therefore it is reasonable to hypothesize that the observed oxidation wave at ~ 100 mV in F2 of strawberry juices was associated with ellagitannins.

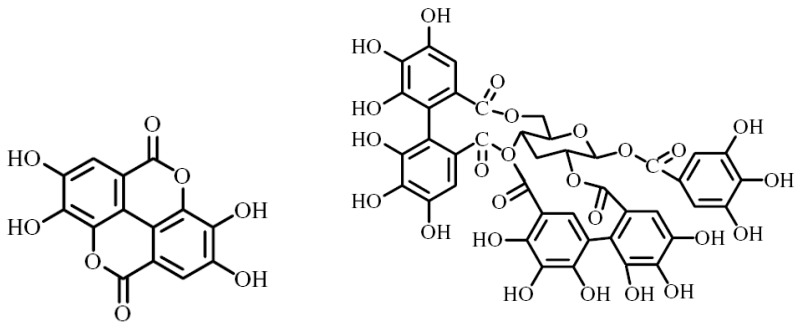


Figure 5.7: Chemical structure of ellagic acid and ellagitannins found in strawberry fruits.

Nevertheless, blackcurrants contain a pool of phenolic compounds (Zadernoswky *et al.*, 2005) which may account for the observed oxidation wave in the non-anthocyanin polyphenolic fraction (F2; **Figure 5.8**). As expected, the anthocyanin fraction (fraction 3) for blackcurrant berries was

characterised by several oxidation waves at ~200, ~250, ~400 and ~700 mV which generally overlapped each other. The complexity observed in this study may be related with not only the four major anthocyanins commonly found in blackcurrants (*viz.* cyanidin-3-glucoside, cyanidin-3-rutinoside, delphindin-3-glucoside and delphinidin-3-rutinoside) but also to other anthocyanins present in lesser amounts (Appendix B; Frøytlog *et al.,* 1998; Häkkinen *et al.,* 1999; Slimestad and Soldheim, 2002; Manhita *et al.,* 2006; Anttonen *et al.,* 2006; Rubinskiene *et al.,* 2006; Jordheim *et al.,* 2007). Further sample separation steps or more complex procedures would be required to simplify these voltammograms and draw any relationship with actual anthocyanin concentrations. As mentioned earlier, oxidation waves below 400 mV in F3 may be attributed to the OH groups of the anthocyanin beta ring whereas oxidation at higher former potentials would be most probably ascribed to the oxidation of the monophenol or meta-diphenols on the A ring of these compounds (Kilmartin *et al.,* 2001). The voltammogram of fraction 3 for strawberry juices mimicked that shown in **Figure 5.4** but with a shift to lower oxidation potentials, and mainly three oxidation waves at ~250, ~450 and ~800 mV.

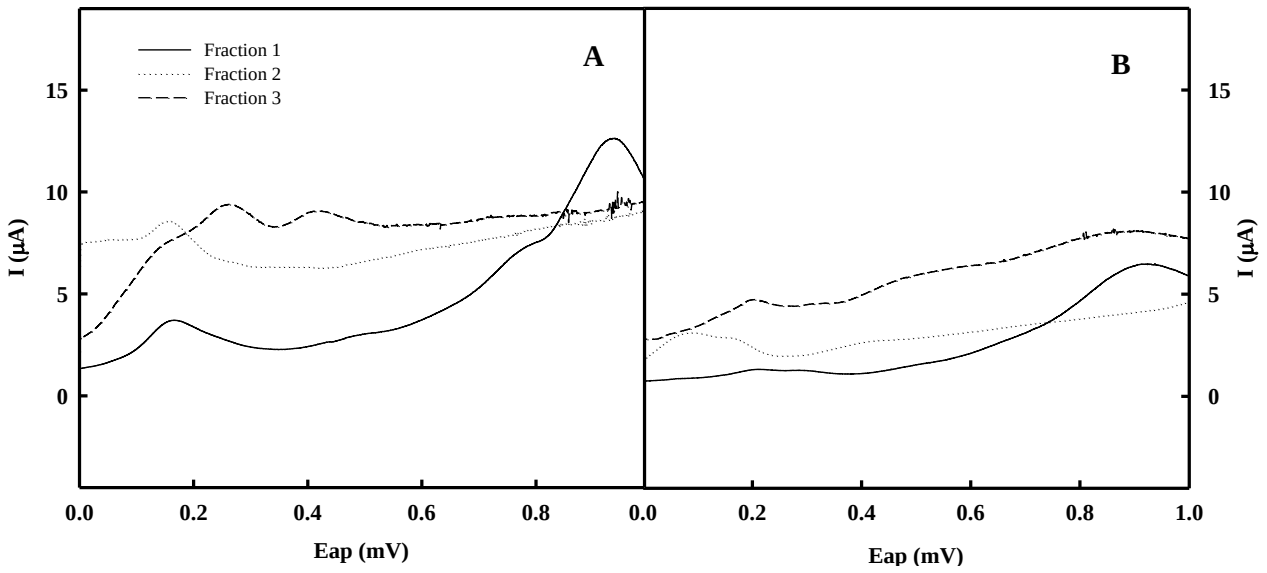


Figure 5.8: Square wave voltammograms of different blackcurrant (cv. Ben Tirran; A) and strawberry juice (cv. Elsanta; B) fractions (viz. fraction 1: water soluble compounds including ascorbic acid; fraction 2: non-anthocyanin polyphenolic compounds; fraction 3: anthocyanins) diluted in 0.1 mM PBS at pH 7.

5.3.3 Towards a sensor for rapid determination of antioxidants and antioxidant capacity: comparison with standardized antioxidant capacity assays

Contradictions between the values given by different antioxidant assays or between antioxidant assays and sample composition have been extensively reported (Chapter 4; Zulueta *et al.*, 2009; Crespo *et al.*, 2010) and may be attributed to different reaction kinetics between the diverse antioxidants present, especially in complex samples containing an array of antioxidant compounds (Zulueta *et al.*, 2009). Both FRAP and Folin-Ciocalteu assays are ET-based methods involving a redox reaction with the antioxidant (Prior *et al.*, 2005). To overcome the limitations of the current antioxidant assays, electrochemisty is now among the most important approaches in the analysis of antioxidants in food and other biological samples (Blasco *et al.*, 2007), given that electrochemical methods have the same conceptual principle as those exhibited by antioxidants in real *in vitro* systems. Previous studies (Aaby *et al.*, 2004; Blasco *et al.*, 2004; Aaby *et al.*, 2005; Pyo *et al.*, 2004; Piljac-Zegarac *et al.*, 2009) showed that antioxidant capacity is generally well correlated with electrochemical response, especially if considering cumulative responses up to potentials of 800 or 1000 mV (Blasco *et al.*, 2007). Likewise, Q_{500} and Q_{1000} values agreed well ($r > 0.69$) with the results obtained by either the Folin-Ciocalteu or the FRAP assay when assessing AC of blackcurrant or strawberry fruits (**Figure 5.9**).

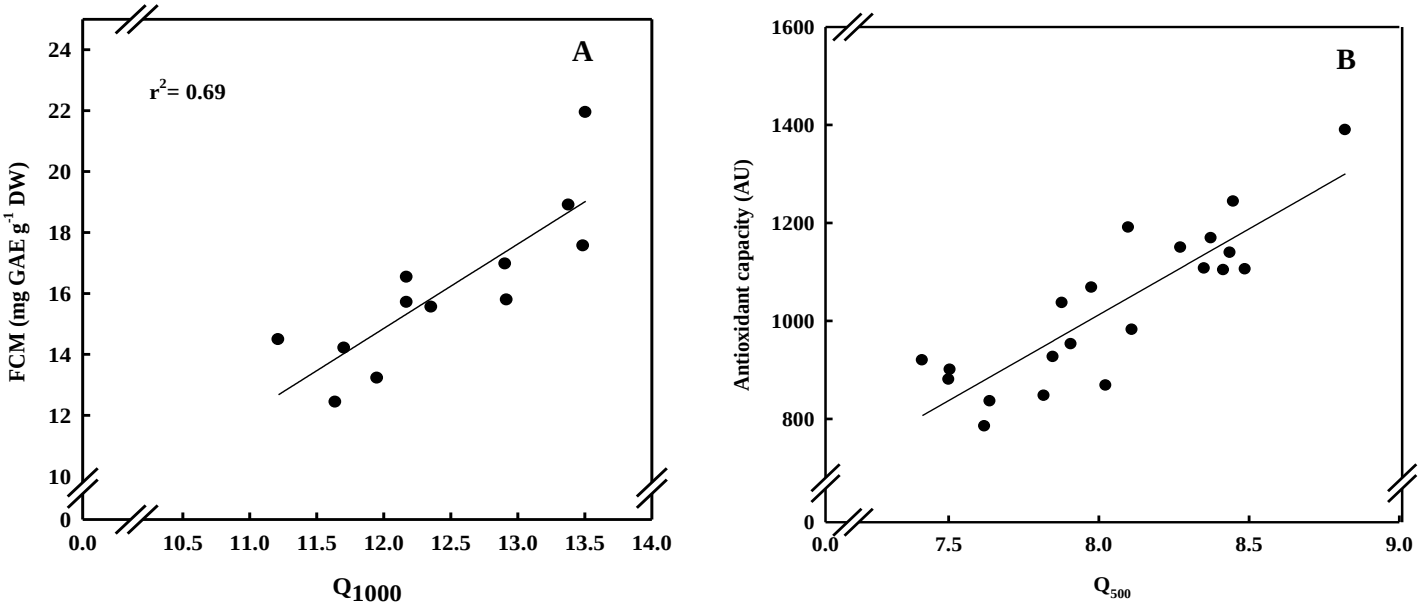


Figure 5.9: (A) Correlation between cumulative responses at 1000 mV (Q_{1000}) and total phenolics concentrations in strawberry fruits. (B) Correlation between cumulative responses at 500 mV (Q_{500}) and antioxidant capacity (arbitrary units; AU) of blackcurrant samples.

In contrast, Q_{300} values were poorly correlated with AC values (data not shown) as was also observed by Kilmartin and Hsu (2003) when assessing AC in different tea extracts by CV at carbon electrodes. In the former study, the authors (Kilmartin and Hsu, 2003) suggested that the poor correlation between both parameters was due to the relatively high concentrations of other phenolics (F2; **Figure 5.8**), commonly present in complex samples like berries (Hakkinen *et al.*, 1999), with low antioxidant capacity and which will not contribute to Q_{300} values. Indeed, partitioning of different antioxidant fractions in both berry-based juices, thereby simplifying the samples, considerably improved the correlation between the values obtained by SWV and those given by the standard FRAP assay ($r > 0.91$; $P < 0.01$) (**Figure 5.10**).

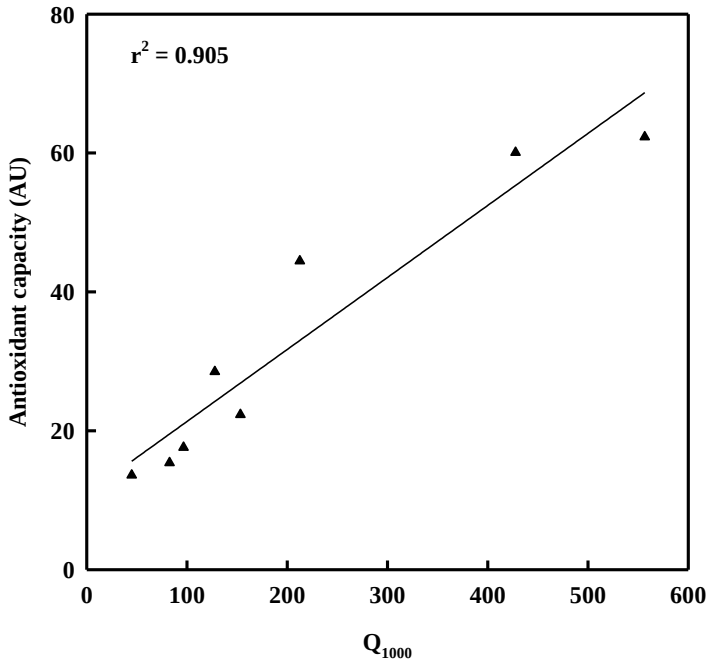


Figure 5.10: Correlation between sensor cumulative response at 1000 mV (Q_{1000}) and antioxidant capacity (AU), measured by the FRAP assay, in different blackcurrant and strawberry fractions. Partitioning of anthocyanin and non-anthocyanin antioxidants was done as described in materials and methods section.

The proposed antioxidant sensor was however not only able to discriminate between samples differing in their AC but also to measure specific antioxidants (*viz.* ascorbate and anthocyanins). It has been described that ascorbic acid in strawberry fruits accounted for ca. 24% of the cumulative area at 300 mV when measured by HPLC coupled to coulometric detection (Aaby *et al.*, 2007). The relatively poor contribution of ascorbic acid observed by Aaby *et al.* (2007) may explain the low correlation

between Q_{300} and AsA detailed herein and strongly suggests that other antioxidants, most probably phenolic acids are responsible for most of the reducing ability of strawberry juices at potentials below 300 mV. Even though the oxidation of ascorbate at inert electrodes occurs generally at low potentials, the results showed that ascorbic acid concentrations in both strawberry and blackcurrant fruits could be accurately predicted by Q_{500} and Q_{1000} values (**Figure 5.11**). The correlation was even improved when considering Q_{500} values and AsA concentrations of both blackcurrant and strawberry juices (r^2 = 0.88; AsA (mg g^{-1} DW) = -7.4 + 2*Q_{500} (μC)).

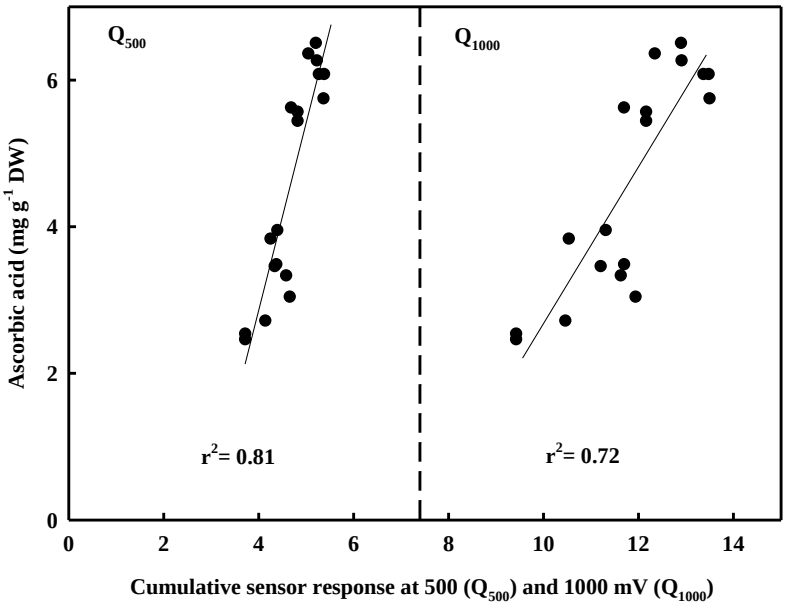


Figure 5.11: Correlation between sensor cumulative responses at 500 (Q_{500}) and 1000 mV (Q_{1000}) with AsA concentrations of strawberry fruits from different cultivars.

In the same way, individual or total anthocyanin concentrations in blackcurrant berries from different cvs. and maturity stages were correctly estimated using the signal given by the sensor, especially if considering cumulative responses up to 500 mV (**Figure 5.12**).

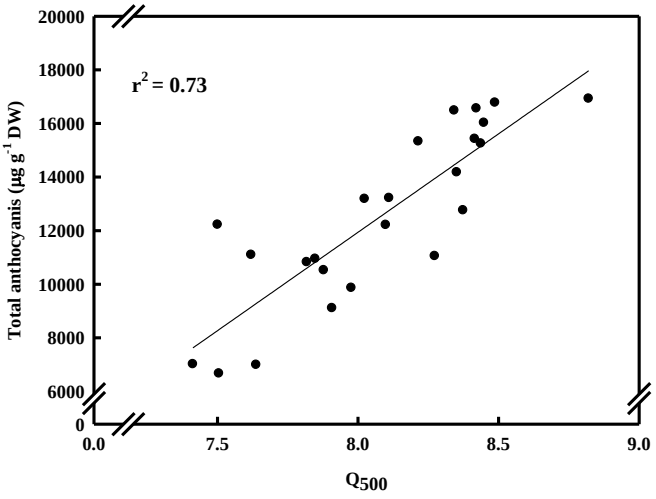


Figure 5.12: Correlation between total anthocyanin concentrations, measured by HPLC and Q500 values. The anthocyanin profile for the different blackcurrants investigated was earlier detailed in Chapter 4 (section 4.4.2.4).

5.4 Conclusions and future work

Blackcurrant and strawberry fruits contain a characteristic pool of antioxidants which when investigated electrochemically led to a specific voltammetric profile for each of the samples investigated. Regardless of the cultivar or degree of maturity, blackcurrant juices were richer sources of antioxidants than strawberry juices and generally presented a more complex electrochemical profile. Partitioning of the different samples revealed that anthocyanins were for both berry-based juices the major contributor to the antioxidant capacity of the fruits, followed by other phenolic-type compounds and ascorbate. Using screen-printed electrodes combined with SWV, the mechanisms of electron transfer of several anthocyanin standards were examined. It was demonstrated that multiple hydroxyl groups on the B-ring like in the pyrogallol group (i.e. delphinidin) were easily oxidised than two (i.e. catechol; cyanidin) or single hydroxyl substituents (i.e. pelargonidin). Hydroxyl groups in the B-ring did not vary their oxidation peak due to the glycosylation of the molecule in the A-ring but seemed to be stabilised when other antioxidants were present in solution.

A new electrochemical approach was also discussed in the context of developing a disposable sensor to measure antioxidant capacity in berry juices. The proposed sensor allowed for fast and easy discrimination of the main class of antioxidants present in both berry types but more importantly was able to discriminate between samples based on their antioxidant capacity as measured by standardised

assays (*viz.* FRAP and Folin-Ciocalteu). Sensor cumulative responses at formal potentials of 500 (Q_{500}) and 1000 mV (Q_{1000}) correlated well with the AC of the fruits as well as with anthocyanin and ascorbate concentrations in the juices. The proposed methodology would accomplish most of the criteria recommend for the development of standardised AC assays described by Prior *et al.* (2005) and would be of biological relevance since electrochemical techniques use the same principle to that exhibited by antioxidants in real biological systems. Besides, the use of screen-printed disposable sensors would cover the main drawback of electrochemical methods which involves the deactivation of the electrode, after single measurements, due to the formation of a polymeric film produced by the coupling of electrogenerated phenoxy radical.

Given the importance of the sample matrix and characteristics (*viz.* pH, presence of other electrochemically active compounds), further standard compounds should be investigated, preferably after standard additions in berry-based juices and hence allow a better identification of these compounds in real samples. In order to have a better understanding of the antioxidant profile of berry-based juices, phenolic compounds rather than anthocyanins, with formal oxidation peaks at low potentials, should be quantified and values correlated with sensor cumulative responses at 300 mV (Q_{300}).

CHAPTER 6

DEVELOPMENT OF A GLUCOSE BIOSENSOR FOR RAPID ASSESSMENT OF STRAWBERRY FRUIT QUALITY:

UNDERSTANDING THE RELATIONSHIP BETWEEN BIOSENSOR RESPONSE AND FRUIT COMPOSITION

6.0 CHAPTER SIX

Development of a glucose biosensor for rapid assessment of strawberry fruit quality: understanding the relationship between biosensor response and fruit composition

6.1 Introduction

The compositional analysis of fruit and vegetables is extremely important for defining eating quality of fresh produce. In strawberry fruit, non-structural carbohydrates, including glucose, fructose and sucrose, account for more than 80% of the total soluble solids (Perkins Veazie *et al.*, 1995; Terry *et al.*, 2007; Tulipani *et al.*, 2008; Crespo *et al.*, 2010), and are intimately related to taste (Pérez *et al.*, 1997; Terry *et al.*, 2007). In this context, perceived sweetness is directly related to sugar concentration in strawberry fruit and may be used as an index of consumer acceptability (Keutgen and Pawelzik, 2008). However, sugar concentration is often confused by some with total soluble solids (TSS) as determined by refractometry, which is one of the parameters ubiquitously used during standard quality control (QC) of strawberry fruit, yet it has been shown that TSS is often poorly correlated with sugar concentration in strawberry (Pérez *et al.*, 1997) and some other horticultural crops (Chope *et al.*, 2006; Somboonkaew *et al.*, 2008; Appendix A). More accurate techniques such as high performance liquid chromatography (HPLC) (Terry *et al.*, 2007; Pérez *et al.*, 1997; Crespo *et al.*, 2010), gas chromatography (GC) (Medeiros and Simoneit, 2007) or enzymatic test kits (Capasso *et al.*, 1996) are available for quantifying sugars; however, they tend to be time-consuming and costly, hence they are not appropriate to be routinely applied in the fresh produce industry for standard daily QC.

The development of analytical methods that are low cost, easy to operate and portable would be of great importance for berry fruit in particular and indeed for the overall fresh produce industry. In this perspective, biosensors may fulfill most of the abovementioned requirements and therefore may provide the food industry with a promising alternative to standard QC, not only by enhancing the relevance and extent of tests carried out, but also by measuring specific analytes which are key indicators of produce quality and consumer acceptability (Terry *et al.*, 2005). Screen-printed electrodes were earlier discussed (Chapter 5) in the context of developing a disposable sensor to measure

antioxidant capacity and individual antioxidants for soft fruits. Similarly, biosensors for glucose determination using glucose oxidase (GOx) have already been applied to some other fresh produce types or beverages (Miertus *et al.*, 1998; Palmisano *et al.*, 2000; Jawaheer *et al.*, 2003; Lupu *et al.*, 2004; Alonso Lomillo *et al.*, 2005; Abayomi, 2007). This said, most of these have not yet been commercialized, due in part, to the high variability and complexity of the food or fruit juice matrices. Strawberries are not an exception, since great variability exists in the composition of fruits of different cultivars (Tulipani *et al.*, 2008) or from fruit derived from plants grown under different agronomic conditions (Chapter 3; Keutgen and Pawelzik, 2007a; Keutgen and Pawelzik, 2007b; Terry *et al.*, 2007a). Moreover, Chapter 5 showed that strawberry fruits are rich sources of electrochemically active compounds (*viz.* ascorbate (AsA), phenolic acids, anthocyanins; Aaby *et al.*, 2005; Aaby *et al.*, 2007) which easily undergo oxidation or reduction at the surface of the electrode under relatively low operating potentials (<300 mV). Accordingly, the aim of the present chapter was to develop and optimize, using experimental design methodology (Alonso-Lomillo *et al.*, 2005), an amperometric GOx-based biosensor for rapid quantification of glucose in strawberry juice. Screen-printed carbon electrodes (SPCE), on this occasion, mediated with Meldolas Blue (MaB+) were chosen based on previous work carried out by Abayomi (2007) and due to the known versatility of this mediator towards both oxygenase and dehydrogenase enzyme formats (**Figure 6.1**; Kulys *et al.*, 1994; Vasilescu *et al.*, 2003; Abayomi *et al.*, 2006; Abayomi and Terry, 2007). Finally, the applicability of the constructed GOx-based biosensor was tested with eight different strawberry cultivars (Chapter 3; section 3.3.1), previously characterised for their content in main sugars, organic acids, total phenolics and antioxidant activity, to further understand the relationship between the biosensor response and sample composition.

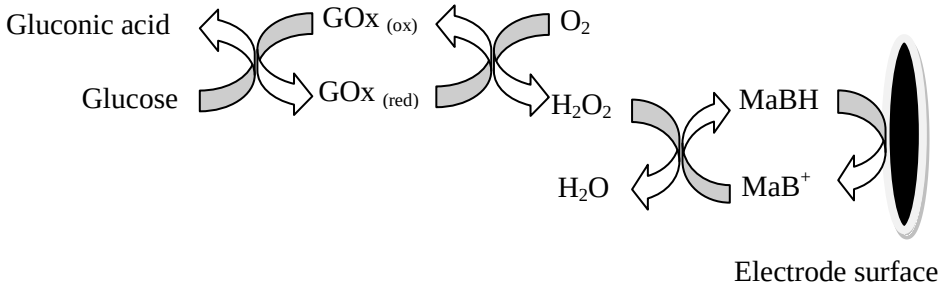


Figure 6.1: Enzymatic reaction at the working electrode in the presence of MaB+ as a mediator.

6.2 Materials and methods

6.2.1 Biosensor development and optimization

6.2.1.1 Reagents.

All reagents including glucose oxidase (EC 1.1.3.4; GOx) derived from *Aspergillus niger,* were purchased from Sigma (Dorset, UK) unless otherwise stated. The chemicals used were of analytical grade and solutions were prepared with MilliQ water (Millipore Inc.; $\sigma = 18M\ \Omega\ cm^{-1}$). Stock solutions of glucose (10 mM) were prepared daily in 0.1 M sodium phosphate buffer at optimum pH and left to equilibrate for *ca*. 4h before measurements started.

6.2.1.2 Screen-printed electrodes and instrumentation.

Screen-printed electrodes (SPE) (C2030519D5, Gwent Electronic Materials Ltd. GEM, Gwent, UK) were used for all measurements (Abayomi *et al.*, 2006; Abayomi *et al.*, 2007). The electrodes were screen-printed in a two electrode configuration comprising a generic carbon mediated with MaB+ working electrode (28 mm^2) and a combined Ag/AgCl reference/counter electrodes onto a PVC substrate. Similar plain carbon non-mediated electrodes were earlier described in Chapter 5 (Figure 5.1). Electrochemical measurements were conducted as described earlier (Chapter 5; section 5.2.3).

6.2.1.3 Biosensor Optimization.

The amperometric response (current density) obtained with an enzyme-based biosensor is a function of several experimental variables including applied potential (E_{ap}), units (U) of enzyme loading (C_{GOx}), pH of the buffer solution and concentration of the mediator. In this context, Eap, C_{GOx} and pH were optimized by means of a 2^3 central composite design (CCD), using standard glucose solutions (10 mM) prepared in 0.1 M sodium phosphate buffer and adjusted to optimum pH (Alonso Lomillo *et al.*, 2005). In the present study, selected levels for each variable to be optimized were chosen corresponding the plus (+), minus (-), and central (0) points and based on results obtained during preliminary experiments; (Eap: 300, -200 and 50 mV; pH: 8, 4 and 6; C_{GOx}: 15, 5 and 10 U, respectively)

Enzyme immobilization was achieved by simply depositing a known volume of GOx solution made up in 0.1 M sodium phosphate buffer at pH 5.7 onto the surface of the working electrode

(Abayomi *et al.*, 2006; Abayomi and Terry, 2007). Electrodes were then left to air dry for 4h at room temperature and subsequently stored at 4 ºC overnight until used the following day.

6.2.1.4 Biosensor calibration and selectivity.

Prior to the analysis of real samples, the GOx-based biosensor was calibrated against glucose standard solutions ranging from 0 to 50 mM made up in 0.1 M phosphate and adjusted to optimum pH values. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3 or 10 times the standard deviation of the biosensor response to glucose standard (5 mM), respectively (Muñoz *et al.*, 2008). The selectivity of the biosensor in the presence of other sugars found in strawberry (*viz*. fructose and sucrose) extracts was also assessed by adding known concentrations (0, 5, 10 and 20 mM) of the above mentioned sugars to the glucose standard solutions.

6.2.2 Characterisation of strawberry fruits

6.2.2.1 Plant Materials.

The plant materials used for this experiment were those described earlier (Chapter 3; section 3.3.1). Briefly, eight different strawberry cvs. (*viz*. Christine, Elsanta, Flamenco, Florence, Jubilee, Sonata, Symphony and Pearl) were selected according to individual differences in sugar/acid taste profiles (H. Duncalfe, *personal communication*). Plants were grown under standard commercial practices and supplied by H&H Duncalfe (Cambs., UK) or other local suppliers.

6.2.2.2 Total soluble solids (TSS), objective colour and sample preparation.

Upon arrival at the laboratory, the objective colour of 10 fruits per cv. was measured using a Minolta DP-400 colorimeter (Minolta Co. Ltd., Japan) with an 8 mm light-path aperture (Terry *et al.*, 2007b). The mean of three readings at 3 equidistant points around the equatorial axis was recorded and the lightness (L*), chroma (colour saturation; C*) and hue angle (H°) automatically calculated (Terry *et al.*, 2007b). In addition, TSS was measured for each cultivar (cv.) in triplicate using a digital Palette PR-32α refractometer (Atago, Japan). Afterwards, samples (*ca*. 1 kg) were immediately snap-frozen in liquid nitrogen and kept at -40 ºC until subsequent analysis. A portion of the fresh-frozen samples was directly used for biosensor measurement and sugar determination by HPLC on a fresh weight basis whereas another 150 g for each cv. were freeze-dried in an Edwards Modulyo freeze drier (W. Sussex, UK) and treated as described earlier (Chapter 3; section 3.3.2) prior to being used for additional sugar

and organic acid determination by HPLC, total phenolics (TP) and antioxidant capacity (AC) measurements.

6.2.2.3 HPLC determination of sugars and organic acids.

Sugar determination on a FW basis was performed on diluted strawberry homogenates (1:20 (w/v) in phosphate buffer at optimum pH) whereas extraction and quantification of both sugar and organic acid on a DW basis was performed as reported previously for strawberry and other soft fruits (*Chapter 3; Section 3.4.4*).

6.2.2.4 Determination of total phenolics and antioxidant capacity.

Total phenolics (TP) were extracted by dissolving 150 mg of freeze-dried powder in 3 mL of 80% (v/v) aqueous ethanol solvent and held in a water-bath for 2 h at 70 ºC, mixing every 20 min. The clear solution obtain after microfiltration was analyzed according to the Folin-Ciocalteu Method (FCM). Antioxidant capacity (AC) was also measured on freeze-dried samples using the ferric reducing ability of plasma (FRAP) (Benzie and Strain, 1996). Further details on the methodology used were described earlier (*Chapter 3; section 3.4.6*).

6.2.3 Electrochemical measurements

Amperograms were obtained by depositing 20 µL of electrolyte solution (0.1 M KCl in the previously described sodium phosphate buffer) on the electrode surface, applying the optimum potential (+300 mV) and letting a steady current to be reached (t = 100s) before depositing 20 µl of standard or diluted (1:20; w/v) strawberry homogenate (Abayomi *et al.*, 2006; Abayomi and Terry, 2007). A total time of 200s was required for each amperometric measurement. Finally, the electrochemical response to different glucose concentrations in strawberry juices was compared against glucose content determined by HPLC.

6.2.4 Data analysis

Data analysis including central composite design (CCD) was carried out using Statgraphics centurion (trial version XV; Statpoint technologies, Warrenton, USA) and statistical analysis using the same software than in earlier chapters (Genstat for Windows Version 9.1.0.147). Least significant difference values (LSD; $P = 0.05$) were calculated as described in earlier chapters. Variations among biosensor responses or main treatments were plotted in SigmaPlot 9.0. Tests for correlations between

mean values for analyte concentrations were made using Spearman's Rank Correlation. Correlations are presented with the Spearman's Correlation Coefficient (r) and *P* value based on a two-tailed test.

6.3 Results and discussion

6.3.1 Sensor characterisation and optimization of the biosensor response.

The electrochemical characterisation of MaB-mediated electrodes was initially assessed in the absence of the enzyme by means of cyclic voltammetry (Bucur *et al.*, 2005). The stability of the transducer was tested by performing multiple scans between -1V and 0V vs Ag/AgCl reference electrode at a scan rate of 0.05 V/s in 0.1 M PBS (pH 5.7 and 7) (**Figure 6.2**). A slight increase in the intensity of the anodic and cathodic peak was observed for approximately 10 min, corresponding to the oxidoreduction of the mediator over the surface of the electrode. Afterwards, the intensity of the peaks was stabilised. The same procedure was done with the electrodes containing 10U of GOx to assess the effect of enzyme loading on the electrochemical behaviour of the transducer. No additional response was observed when 10U or 20U of GOx were immobilised onto the surface of the working electrode.

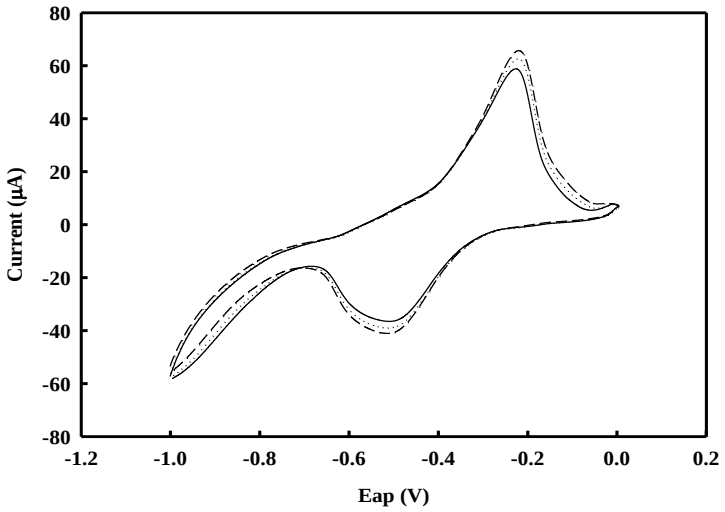


Figure 6.2: Electrochemical characterisation of the MaB+ modified electrodes in the absence of enzyme GOx between -1.0 and 0 V vs Ag/AgCl reference electrode at a scan rate of 0.05 V/s in 0.1 M sodium phosphate buffer (pH 7). Different lines indicate measurements conducted with different set of SPCEs.

E_{ap}, pH of the buffer/electrolyte solution and units of enzyme loading (C_{Gox}) in the enzyme cocktail and subsequently deposited onto the surface of the working electrode were optimized by means of experimental design methodology, using a central composite design (CCD-2^3). The use of similar approaches for the optimization of several parameters that may affect sensor or biosensor responses has satisfactorily been applied in the past (Domínguez Renedo *et al.*, 2004; Alonso Lomillo *et al.*, 2005; Burgoa Calvo *et al.*, 2007). CCD encompasses numerous advantages, as compared to more traditional approaches (Kincl *et al.*, 2005), since it allows optimization of each variable simultaneously and considers the influence of the interaction between variables in the final biosensor response. Analysis of variance (ANOVA) of the optimization procedure revealed that neither enzyme loading (C_{Gox}), within the range studied, nor the interactions between this variable with pH or Eap significantly affected the signal given by the biosensor (**Table 6.1**). In contrast, Eap (P = 0.005) and pH of the buffer solution (P = 0.007) were crucial variables in terms of maximizing the biosensor response (current density). The *P* value for the lack-of-fit (P = 0.055) was higher than 0.05, therefore the quadratic model proposed by the optimization process ($I\ (\mu A) = 0.315 - 21.763*Eap - 0.45*pH + 3.406*Eap^2 + 5.004*Eap*pH + 0.113*pH^2$), excluding non-significant variables, was appropriate for the experimental data at the 95% confidence level. The model clearly reflected that, within the range tested, greater pH and applied potential values resulted in greater current densities. Given the information provided by the optimization process and based on results obtained during preliminary experiments optimum values were fixed at +300 mV (vs Ag/AgCl), pH of 7.2, C_{Gox} = 20U. The use of higher potentials may have increased the level of interference caused by certain electroactive compounds, easily oxidized and commonly found in fresh produce matrices (Terry *et al.*, 2005; Abayomi and Terry, 2006). Moreover, pH values over 8 may have seriously affected the stability of the enzyme (Pazzur and Kleppe, 1964).

Table 6.1: ANOVA of the central composite design used for the GOx-based biosensor optimisation.

Effects	S.S.	D.F	M.S	F_{Ratio}	P-Value
E_{ap}	69.859	1	69.868	180.78	0.0055[a]
pH	53.047	1	53.047	137.27	0.0072[a]
C_{Gox}	0.443	1	0.4427	1.15	0.3965
E_{ap}* E_{ap}	0.511	1	0.511	1.32	0.3692
E_{ap}*pH	50.089	1	50.089	129.62	0.0076[a]
E_{ap}*C_{GOx}	0.024	1	0.024	0.06	0.8247
pH*pH	2.303	1	2.303	5.96	0.1347
pH*C_{GOx}	0.233	1	0.233	0.6	0.5184
C_{GOx}*C_{GOx}	0.356	1	0.356	0.92	0.4384
Lack-of-fit	34.811	5	6.962	18.02	0.0534
Pure error	0.773	2	0.386		

r^2 = 0.832; r^2 (adjusted for d.f.) = 0.616; [a] significant factor at α=0.05; Statistic Durbin-Watson = 1.34669 (P=0.208)

6.3.2 Biosensor selectivity and linear range.

The GOx-based biosensor responded positively to increasing concentrations of glucose in 0.1 M phosphate buffer (pH 7.2) at an applied potential of +300 mV (versus Ag/AgCl). Although the linear range, up to 12 mM, was not affected by the optimization process, the signal given by the biosensor ((µAs) = 0.629 x glucose concentration (mM) + 0.1594) (**Figure 6.3**) was increased by as much as 3-fold in comparison with preliminary experiments and the reproducibility of the signal was considerably improved (data not shown). Although using other electrode formats, similar ranges of concentrations were reported by others when developing glucose sensors for food applications (Jawaheer *et al.*, 2003; Lupu *et al.*, 2004). LOD and LOQ values using 20U of GOx was equivalent to *ca*. 0.01 and 0.1 mg Glucose g^{-1} FW, respectively. In agreement with previous studies, using similar SPC electrode configurations (Abayomi, 2007), virtually no response was obtained with the addition of fructose and sucrose in solutions, confirming the selectivity of the GOx-based biosensor towards the glucose substrate.

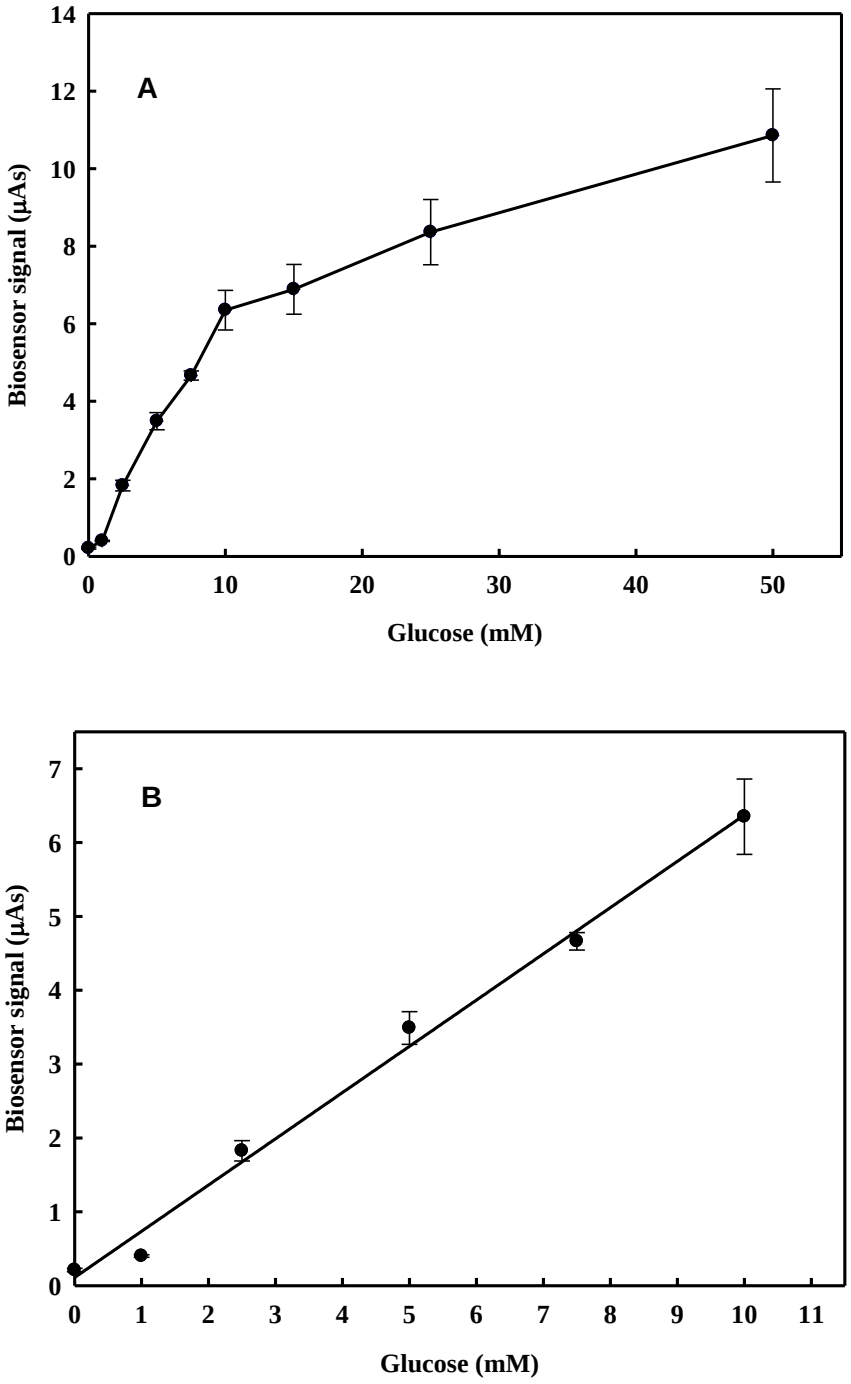


Figure 6.3: Calibration of GOx-mediated biosensor using glucose standards in phosphate buffer pH 7.2. Eap = +300 mV (vs Ag/AgCl); 10 units GOx. Standard deviation bars are from the mean of 5 measurements. **(A)** Biosensor response up to glucose concentrations of 50 mM. (**B)** Biosensor response within the linear range (up to 10 mM glucose), (µAs) = 0.626 x glucose concentration (mM) + 0.110); $P < 0.001$. $r^2 = 0.99$.

6.3.3 Characterization of strawberry fruits.

Characterization of potential biosensor interferences and target components in the different strawberry cultivars analyzed was achieved by previously reported methods (Terry *et al.*, 2007; Giné Bordonaba and Terry, 2008; Crespo *et al.*, 2010). Overall, values for all target analytes significantly differed between cultivars but were in the range of those found by others (Terry *et al.*, 2007; Pérez *et al.*, 1997; Keutgen and Pawelzik, 2008; Tulipani *et al.*, 2008) as well as values reported in earlier chapters (Chapter 3; section 3.5.3). Accordingly, variations in taste and health-related components of fruits from different cultivars or from plants grown under different conditions have been extensively reported in strawberry or other soft fruits (Chapter 3; Chapter 4). From the different cultivars analyzed, berry weight ranged from 15.49 to 23.68 g and significantly differed between cultivars; smaller fruits were obtained from cvs. Christine and Symphony whereas fruits from cv. Jubilee were the larger fruits and had greater dry matter as a proportion of fresh weight (14.1 g DW 100 g^{-1} FW) (**Table 6.2**).

Objective colour of fruits from different cultivars was significantly different (**Table 6.2**) and hence, in agreement with that shown by others (Chapter 3; section 3.5.3; Sacks and Shaw, 1994; Capocasa *et al.*, 2008). Lower H° indicates higher redness and accordingly fruits from cv. Florence (H^o = 29.76) had deeper red coloration than the rest of cultivars analyzed (H^o_{mean} = 38.91). Similar objective colour values were recorded for cvs. Flamenco and Christine with fruits characterized by having high chroma and lightness values (C* = 54 and L* = 44, respectively).

Table 6.2: Variations in weight (W) characteristics, total soluble solids and objective color (a) of eight UK-grown strawberry cultivars.

Cultivar	W (g)	TSS	L*	C*	Hº
Christine	16.13	9.53	44.01	54.83	37.68
Elsanta	17.49	9.17	37.31	45.32	36.97
Flamenco	22.70	11.63	44.44	54.95	54.95
Florence	21.12	8.87	31.79	43.74	29.76
Jubilee	23.09	9.17	39.53	50.82	34.92
Pearl	23.68	9.27	35.94	46.48	46.48
Sonata	21.01	9.1	34.03	43.72	32.99
Symphony	15.49	8.57	38.17	50.18	37.58
Mean	20.09	9.41	38.15	48.75	38.91
LSD (P < 0.05)	5.422	0.171	3.479	6.521	3.427

[a] L* is lightness, C* is chroma and Hº is hue angle.

In strawberry fruits, non-structural carbohydrates are the main components of dry matter. In agreement with that previously reported (Terry *et al.*, 2007; Cordenunsi *et al.*, 2003; Keutgen and Pawelzik, 2008), results showed that sugars accounted for 6 to 8% of the total fresh matter. Fructose, glucose and sucrose were the three main sugars found in the different strawberry cultivars investigated. Both total and individual sugar values differed between cultivars (**Table 6.3**). Strawberry cv. Jubilee fruit had as much as 1.3-fold higher sugar content than the mean value of the other cultivars analyzed. Both glucose and fructose were present in all cultivars at similar proportions (*ca*. 1:1); glucose content ranged from 15.30 to 29.74 mg g^{-1} FW whereas fructose varied from 17.21 to 32.06 mg g^{-1} FW, depending on the type of extraction used. In this context, sugar concentration was measured from either directly diluted (1:20; w/v) fruits or from the methanol-based extracts as described by Terry *et al.* (2007). In all cases, sugar content was greater in those samples extracted with aqueous methanol as compared to water (**Table 6.3**). Glucose, fructose and sucrose concentrations were on average 1.3, 1.1 and 1.5-fold greater in the methanolic extracts than in the directly measured strawberry juice. This said, sugar content was not only affected by the extraction used, but also by the interaction between extraction method and cultivar. Indeed the use of a methanol-based solvent was more efficacious in extracting sugars in certain cultivars (viz. Christine, Flamenco and Jubilee) than in others, indicating that measured sugar content was not only dependent on the extraction used but also by the sample matrix. As a result of the differences in the amount of sugars extracted between the different

methodologies used, the sugar/acid ratio was significantly greater for samples extracted with methanol, (7.5) than for those directly measured from diluted homogenates (5.7; relative units) (**Table 6.3**). Concomitant to this, Terry *et al.* (2007) and values from chapter 3 (section 3.5.3) described greater amounts of sucrose as compared to the earlier values reported in the literature for strawberry fruits (Pérez *et al.*, 1997;Keutgen and Pawelzik, 2007; Keutgen and Pawelzik, 2008). Fructose and glucose are nearly twice as soluble in methanol-based than in aqueous-based solvents while sucrose is almost three times more soluble (Davis *et al.*, 2007) and therefore a short methanol-based extraction may have enhanced the solubility of these sugars, especially sucrose, into the extraction solution. Implications from these findings revealed that not only the type of extraction but also the solvent used and the matrix effect are key factors for accurate investigation of sugars in strawberry fruits. Similarly, Davis *et al.* (2007) found that not only the total sugar content was affected, but also the ratio of glucose, fructose and sucrose within and across different onion cultivars was dependent on the extraction procedure. Consequently, comparison of sugar content in strawberry fruits from different studies should be treated with some caution.

Table 6.3: Effect of extraction procedure (*viz*. direct determination on diluted 1:20 (w/v) strawberry homogenates (Aqueous) or methanol-based extraction as described by Terry *et al.* (2007) (MeOH)) on measured sugar concentration (mg g^{-1} FW) and the sugar acid ratio of eight UK-grown strawberry cultivars.

	Sucrose		**Glucose**		**Fructose**		**Total**		**Sugar/Acid**	
Cultivar	Aqueous	MeOH	Aqueous	MeOH	Aqueous	MeOH	Aqueous	MeOH	Aqueous	MeOH
Christine	17.97	27.05	17.53	21.74	24.06	32.11	59.56	85.89	4.71	7.02
Elsanta	30.48	35.72	18.74	26.71	20.81	27.53	70.03	89.93	7.48	9.60
Flamenco	19.19	37.00	17.83	22.41	20.98	23.12	58.00	82.51	5.56	7.92
Florence	10.43	23.79	19.98	22.10	24.76	22.82	55.17	68.69	7.47	9.31
Jubilee	28.35	42.23	22.59	27.77	28.37	29.71	79.31	99.70	7.23	9.31
Pearl	28.65	34.32	15.69	20.01	17.96	23.48	62.30	79.73	6.12	7.92
Sonata	18.67	21.54	15.30	23.11	19.34	25.23	53.31	69.85	3.45	4.52
Symphony	10.82	22.06	18.34	22.68	21.68	23.84	50.84	68.54	3.32	4.47
Mean	**20.57**	**30.46**	**18.25**	**23.32**	**22.15**	**25.98**	**61.07**	**80.61**	**5.67**	**7.48**
LSD (P < 0.05)	**1.147**		**1.002**		**1.577**		**2.71**		**0.498**	

As mentioned earlier, acids are also important contributors to the characteristic taste of strawberry fruits (Cordenunsi *et al.*, 2004). Greater amounts of citric and malic were found in cv. Symphony (10.38 and 3.34 mg g-^{1} FW, respectively) (**Figure 6.4**) whereas highest ascorbic acid levels were found in cv. Flamenco (0.75 mg g-^{1} FW) (**Figure 6.5**). In addition to AsA, other electrochemically active compounds present in strawberry fruits were measured by means of standardised antioxidant capacity assays. In Chapter 5 (section 5.3.5) the high correlation between TP and FRAP values and the presence of antioxidants, determined electrochemically, was demonstrated. TP values ranged from 1.51 to 3.34 mg GAE g^{-1} FW with cvs. Christine and Flamenco having the highest values (**Figure 6.5**). Antioxidant capacity of strawberry fruits as measured by the FRAP assay also differed between cultivars and in this case, cv. Florence (17.63 ± 1.14 mg Fe^{2+} g^{-1} FW) and Symphony (15.17 ± 0.79 mg Fe^{2+} g^{-1} FW) had the greatest antioxidant capacity (**Figure 6.5**). No significant correlations were observed between TP and AsA indicating that other compounds rather than ascorbate were responsible for the antioxidant capacity of the fruits investigated herein, and hence supporting earlier findings (Chapter 5; section 5.3.2).

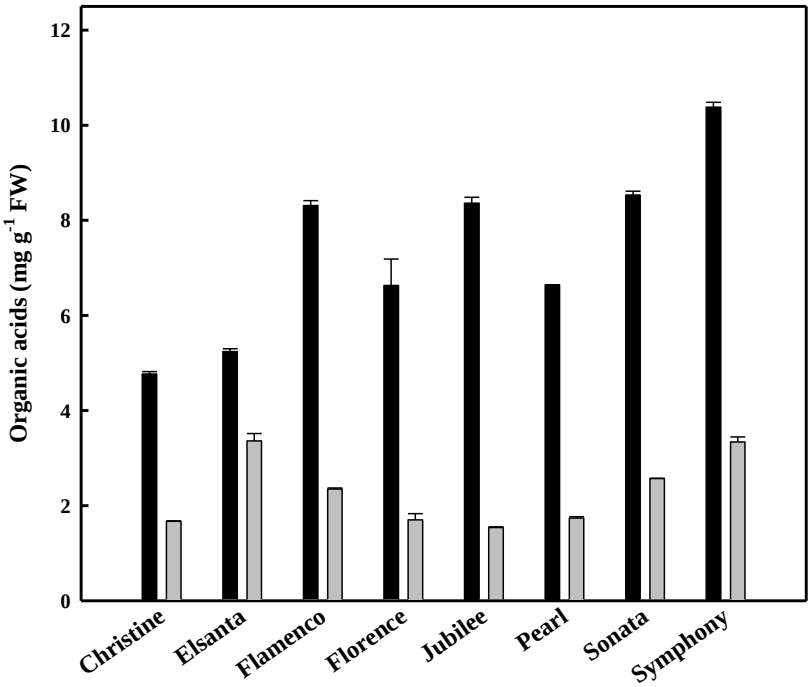


Figure 6.4: Organic acid content (citric (▬) and malic (▬)) from eight different strawberry cultivars. LSDCitric (P = 0.03) = 0.37; LSDMalic (P = 0.01) = 0.142. Values are average of 3 measurements ± standard deviation (SD).

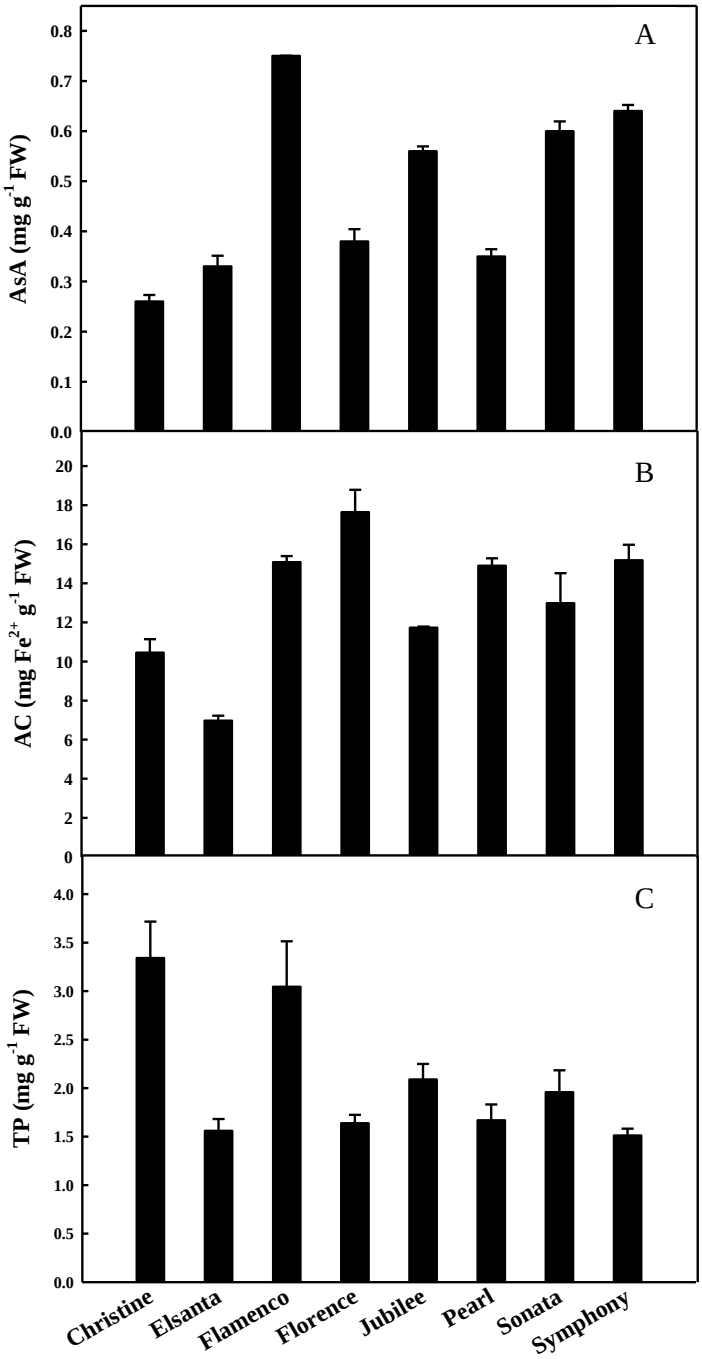


Figure 6.5: Characterization of possible biosensor interferences from eight different strawberry cultivars. (A) Ascorbic acid (mg g^{-1} FW), (B) Antioxidant activity as measured by the FRAP assay (mg Fe^{2+} g^{-1} FW) and (C) total phenolics measured by the Folin-Ciocalteu assay (mg GAE g^{-1} FW). LSDAsA (P = 0.03) = 0.027; LSDAC (P = 0.01) = 1.369; LSDTP (P = 0.01) = 0.429. Values are average of 3 measurements ± SD.

6.4.4 GOx-based biosensor performance with strawberry samples.

After optimization of biosensor performance and characterization of the different samples, the response of the biosensor and the associated background signal was tested by challenging the biosensor with serial dilutions of two strawberry samples (cv. Elsanta and Symphony). Results showed that a dilution of 1:20 (w/v) was best for minimizing the background signal, as represented by the standard deviation, given by each sample (**Figure 6.6**) and agreed well with earlier findings on other fresh-produce types (Abayomi *et al.*, 2007).

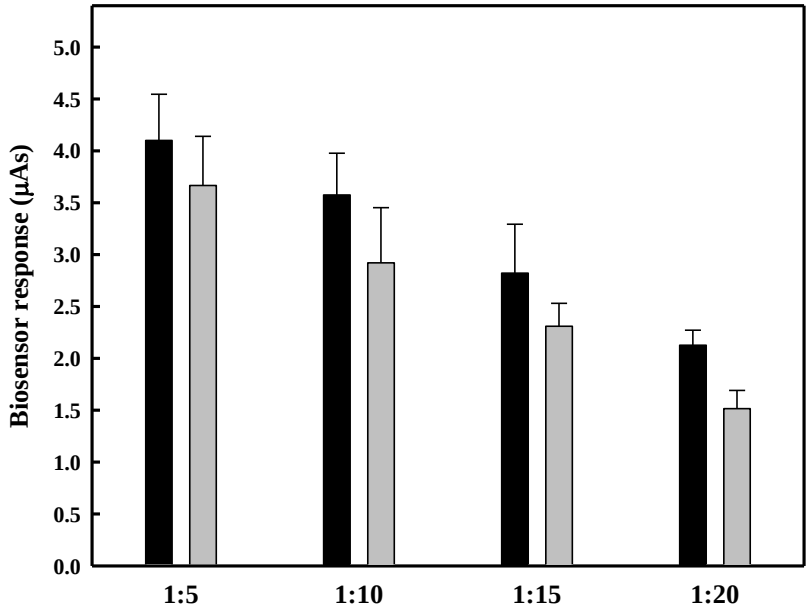


Figure 6.6: Effect of serial dilutions (5, 10, 15 and 20; w/v) of strawberry homogenates (cvs. Elsanta (■) and Symphony(■), in 0.1M sodium phosphate buffer (pH 7.2) on the signal and related background noise given by the biosensor operating under optimum conditions. Values are average of 5 measurements ± SD.

Then, the constructed GOx-based biosensor was tested under optimum experimental conditions against strawberry juices from eight cultivars with known differences in their sugar acid profile. Results indicated that, although the glucose concentrations determined using the biosensor were significantly lower than those obtained using HPLC, there was a strong positive correlation (r^2=0.73; P<0.001) (**Figure 6.6**) between the signal given by the biosensor and known concentrations of glucose in strawberry juice, regardless of the type of extraction used. In contrast, no correlations were found between TSS values, and known glucose concentrations or total sugars when the same samples were

analyzed (**Figure 6.7**). As already reported for strawberry (Appendix A; Pérez *et al.*, 1997, Terry *et al.*, 2005) and other horticultural crops (Chope *et al.*, 2006; Somboonkaew and Terry, 2008) TSS values did not satisfactorily discriminate between those cultivars having high or low sugar content. For instance, cv. Jubilee which had as much as 1.4-fold higher glucose or 1.5-fold greater sugar content than cv. Sonata (**Table 6.3**) had a similar TSS value. The limitations of TSS measurements when determining sugar content of fruits, mainly due to interferences caused by other compounds with refractive properties are described in detail elsewhere (Appendix A). Accordingly, unlike TSS, the constructed GOx biosensor was able to discriminate and rank different cultivars based on their glucose content when compared to known concentrations measured by HPLC. Lower glucose concentrations as determined by the biosensor are not unusual and therefore a correction factor (*ca*. 2-fold) could be adopted. Discrepancies in the glucose content between both methods could be explained either by the specificity of the enzyme towards the β-D-glucose anomer (Pazur and Kleppe, 1964; Jawaheer *et al.*, 2003) or by a simple matrix effect. Glucose in fruits is present in both its α and β form (Jawaheer *et al.*, 2003). Recently, Kwack *et al.* (2008) reported almost a one to one ratio between both anomers in strawberry fruits (cv. Maehyang); this said, no information is available about variations in this ratio between other cultivars. In other fruits such as pineapple the percentage of α-D-glucose was 5 times greater than that of β-D-glucose (Kermasha *et al.*, 1987). Jawaheer *et al.* (2003), reported on the incorporation of mutarotase enzyme within the enzyme cocktail to measure total-D-glucose in different tropical fruits. Such strategy was investigated in this work, but the different steps involved in the preparation of the electrode enzyme were kept as simple as possible and accordingly it was decided not to include the mutarotase enzyme into the cocktail mixture. Applying the appropriate correction factors to equal sugar concentrations given by HPLC or sensor measurements, the biosensor error range from 7.2% (cv. Sonata) to -0.62% (cv. Pearl). Since the ratio between glucose and fructose (*ca*. 1: 1) in strawberry fruit seems to be consistent regardless of the cultivar or agronomic conditions in which the plants are grown (Terry *et al.*, 2007; Keutgen and Pawelzik, 2008; Crespo *et al.*, 2010) determination of glucose by means of the biosensor described herein would not only reflect glucose content but also could be adopted as an estimative measure of fructose content and therefore perhaps perceived sweetness in strawberry fruits.

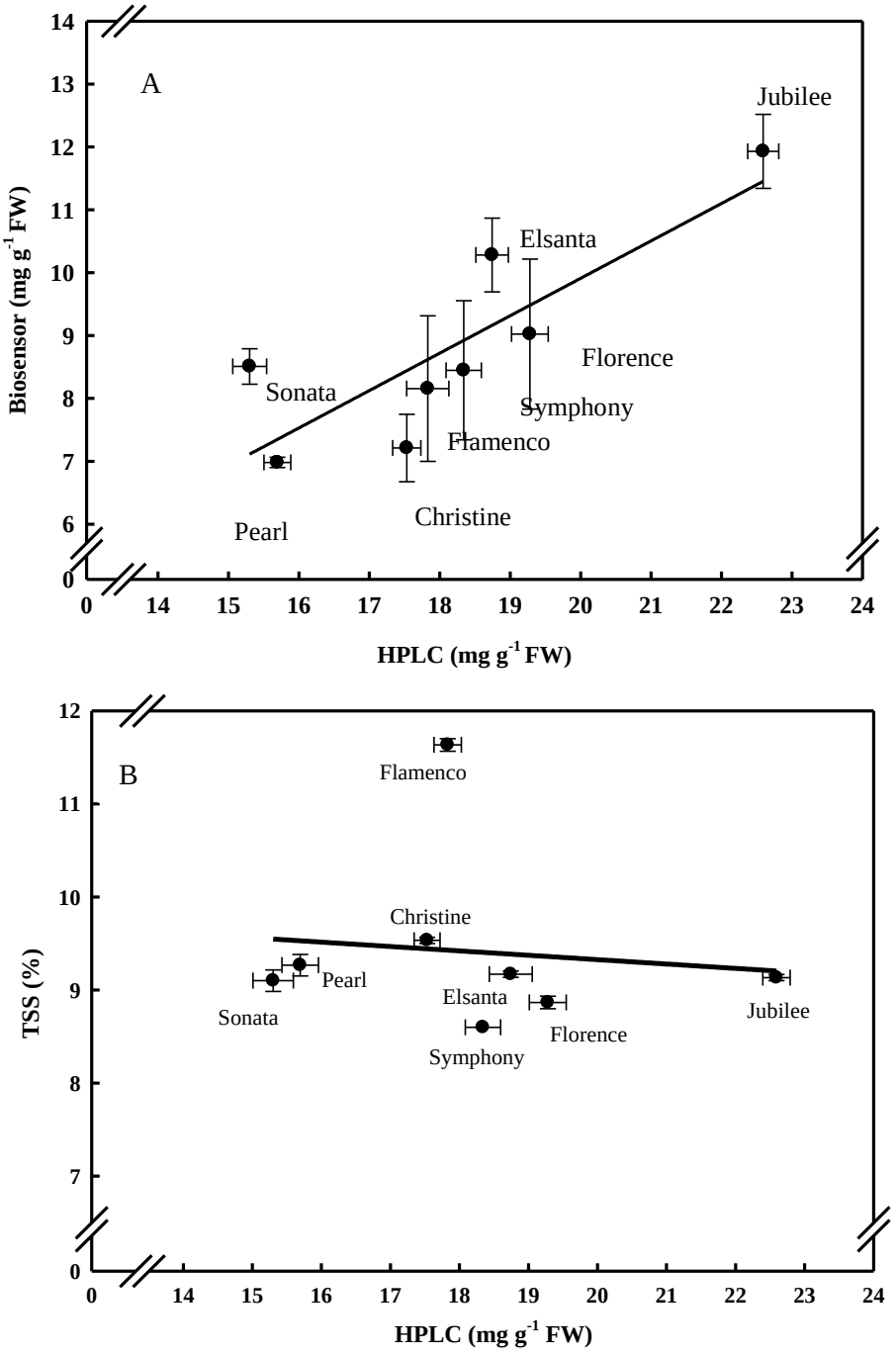


Figure 6.7: (A) GOx-based biosensor response to diluted strawberry homogenates (1:20; w/v) from eight different strawberry cultivars (viz. Christine, Elsanta, Flamenco, Florence, Jubilee, Sonata, Symphony and Pearl) verified against HPLC determination. $r^2 = 0.732$; $P < 0.001$. (B) No correlation was observed between glucose or total sugar content and TSS values.

One of the main challenges when designing a biosensor for food aplications is that food samples contain certain compounds, other than the target analyte, that may easily undergo oxidation or reduction at the electrode surface under the selected operating potential (Lupu *et al.*, 2004; Terry *et al.*, 2005). Such electrochemically active compounds are known to be present in strawberry fruits in relatively high concentrations (viz. ascorbate (Terry *et al.*, 2007; Crespo *et al.*, 2010) anthocyanins and other phenolic compounds (Häkkinen and Törrönen, 2000; Aaby *et al.*, 2005; Aaby *et al.*, 2007; Terry *et al.*, 2007). Indeed, ascorbate and polyphenols are reducing agents and their electrochemical properties are due to their ability to donate electrons (Aaby *et al.*, 2004). These compounds and their electrochemical behaviour at screen-printed carbon electrodes was described in detail earlier (Chapter 5; section 5.3.1). When developing biosensors for fresh produce, previous knowledge of the sample is required in order to fully understand the relationship between the biosensor response and sample composition. In the present study, TP and antioxidant capacities of the fruits were measured by means of the already established Folin-Ciocalteu and FRAP assays, respectively. Both assays may give an overall indication of the concentration of electrochemically active compounds present in the different cvs. analysed since both assays are based on radical or electron scavenging capacities (Chapter 5; section 5.3.3). Values for TP and AC described herein were in agreement with those found in the literature (Terry *et al.*, 2007; Keutgen and Pawelzik, 2008; Tulipani *et al.*, 2008). No correlation was found between the GOx-based biosensor signal and either TP or FRAP values, indicating that under the conditions imposed in this study the biosensor acted free of interference. This said, cultivars having high amounts of ascorbate (**Figure 6.5**) (*viz.* Florence and Flamenco) gave higher variability when tested with the biosensor which may have been due to a matrix effect (**Figure 6.7**). Higher concentration of phenolics or other antioxidant compounds may facilitate the formation of a polymeric film during their oxidation onto the electrode surface and hence lead to a lower reproducibility of the results (Romani *et al.*, 2000).

For the successful application of the developed biosensor several strategies were undertaken through this work to reduce the possible number of interferences. Firstly, the SPCE were mediated with MaB+ based on previous studies (Abayomi *et al.*, 2006; Abayomi and Terry, 2007) and due to the known versatility of this mediator towards both oxygenase and dehydrogenase enzyme formats (Vasilescu *et al.*, 2003). Mediators are used in biosensor technology to replace O_2 as an electron acceptor and allow biosensor performance at much lower operating potentials, hence limiting possible interferences cause by other electrochemically active species found in many food matrices (Terry *et al.*, 2005). Indeed, Chapter 5 showed that some electrochemically active compounds commonly present in

strawberry juices were oxidised at low potential (< 300 mV) at the surface of non-mediated electrodes with the same configuration than those described herein. Accordingly, the use of MaB+ may have increase the signal to noise ratio by favouring the enzymatic reaction at low applied potentials. Secondly, as described earlier (**Figure 6.6**), by performing a 1 to 20 dilution of the strawberry homogenate the background signal given by the samples was minimized considerably. A similar approach was undertaken by others (Lupu *et al.*, 2004; Abayomi *et al.*, 2006; Abayomi and Terry, 2007) when designing biosensors for the detection of target analytes in wine or onions.

6.5 Conclusions and directions for future work

Generally, results from this study have provided further evidence for the existent variability in the composition of strawberry fruits from different cultivars commonly found in the UK market. In addition, and for the first time, it has been demonstrated that a GOx-based biosensor could be used to measure glucose in strawberry fruits and therefore provide growers and retailers with a promising alternative to TSS thus improving quality control (Terry *et al.*, 2005). Analysis time was reduced by 40-fold as compared to conventional HPLC, and therefore, the GOx-based biosensor could be used for screening of large data sets in breeding programmes. The proposed prototype biosensor would enhance the relevance of the analysis carried by measuring specific analytes which are key indicators of strawberry quality and consumer acceptability.

Future research should aim at adding functionality to this prototype biosensor by bolting on addition capabilities to measure other target analytes in strawberry fruit and other fresh produce types to further improve routine quality control. Moreover, the storage stability of the GOx-based biosensor should be studied in future experiments to determine the suitability of the sensor for commercial applications.

The results and methodologies detailed in this chapter have been published as:

Giné Bordonaba, **J**. and Terry, L.A. (2009). Development of a glucose biosensor for rapid assessment of strawberry fruit quality: relationship between biosensor response and fruit composition. *Journal of Agricultural and Food Chemistry*, 57, 8220-8226.

CHAPTER 7

TOWARDS A SENSOR ARRAY FOR IMPROVED SOFT FRUIT QUALITY CONTROL:

SIMULTANEOUS DETERMINATION OF GLUCOSE, ASCORBIC AND MALIC ACID

7.0 CHAPTER SEVEN

Towards a sensor array for improved-soft fruit quality control: simultaneous determination of glucose, ascorbic and malic acid

7.1 Introduction

In previous chapters, it has been discussed that determination of sugar and acid content in berries may be of paramount importance for QC purposes in the soft fruit industry (Chapter 6; section 6.1). Sugars and acid concentrations of berries varied between cultivars (Chapter 3; section 3.5.3; Tulipani *et al.*, 2008; Zheng *et al.*, 2009; Crespo *et al.*, 2010), changed as fruit ripened (Rubinskiene *et al.*, 2006; Chapter 4, section 4.5.2) and the appropriate balance between them may define optimum harvest maturity of the fruit as well as overall fruit quality (Pérez *et al.*, 1997).

Nowadays, consumers are demanding improved quality, with important repercussions to those producers who cannot meet the current demand of the market with guaranteed consistency. Thereby, producers must carefully monitor the quality of fruit through all the stages of production, storage and transport (Jawaheer *et al.*, 2003) to ensure certain quality standards. Nevertheless, very few techniques are commercially available which may allow producers and retailers to monitor fruit quality *in situ* with enough accuracy (Terry *et al.*, 2005). Given their simplicity, portability and accuracy to measure target analytes directly related to quality and consumer acceptance, biosensors are perfect candidates to achieve better quality control in the soft fruit industry (Tothill, 2001; Terry *et al.*, 2005). Yet, only one study refers to the potential application of a sensor array to measure simultaneously different quality markers of fruits (*viz*. glucose, sucrose and ascorbate) using a screen-printed electrode array (Jawaheer *et al.*, 2003). Consequently the work within the present chapter is aimed at developing a new sensor array platform to monitor main sugars and acids in both blackcurrant and strawberry fruits as means of improving soft fruit quality control. It was therefore decided to adopt a common format in terms of electrode design, working potential, method used for signal detection, enzyme stabilisation, and sample preparation which would enable an easy fabrication and operation of a final biosensor array device for improved soft fruit quality control.

In the particular case of both blackcurrant and strawberry fruits, the main sugars and acids, and thereby potential candidate markers for fruit quality, include fructose, glucose and sucrose and citric, malic and ascorbic acid, respectively (**Table 7.1**).

Table 7.1: Summary of sugars and organic acid concentration (mg g^{-1} FW) found in blackcurrant and strawberry fruits from different cultivars.

	Blackcurrant*	Strawberry*
	(mg g^{-1} FW)	
Glucose	60.5	24.5
Fructose	64.2	28.3
Sucrose	40.5	32.4
Citric	44.6	9.2
Malic	6.4	2.5
Ascorbic	3.7	0.2

*Concentrations correspond to mean values for different cultivars and fruits harvested at ripe stage (Chapter 4; Exp.4.1 and 4.2). For strawberry fruits values are mean between deficit and fully irrigated plants (Chapter 3; Exp. 3.1 and 3.2).

7.2 Choice of analytes for biosensor development

From the above-mentioned sugars and acids (**Table 7.1**), the selection of appropriate analytes to be incorporated into the sensor array was carried out from a practical and economic point of view and it is summarised below:

7.2.1 Glucose

Biosensors for glucose determination using glucose oxidase have been already applied in other fresh produce or food types (Miertus *et al.*, 1998; Jawaheer *et al.*, 2003; Lupu *et al.*, 2004; Alonso Lomillo *et al.*, 2005; Abayomi, 2007). Previous experiments within this thesis (Chapter 6) developed a disposable prototype glucose biosensor using glucose oxidase (GOx) immobilised onto mediated Meldolas Blue screen printed electrodes. Mediators are used to bring down the working potential required for hydrogen peroxide breakdown (Maines *et al.*, 1996; Jawaheer *et al.*, 2003; Terry *et al.*, 2005). For this chapter, the glucose sensor was also based on GOx upon which, at the chosen working potential vs. Ag/AgCl, hydrogen peroxide will be catalytically oxidised at a platinised carbon electrode (Newman *et al.*, 2005).

7.2.2 Fructose and Sucrose

The development of glucose sensors is well established and relies on relatively simple technology, whereas fructose sensors have not been studied in such detail and are expensive due to the cost-prohibitive nature of the enzyme required (i.e. fructose dehydrogenase, FDH) and complexity of the electrode systems. For instance, Trivedi *et al.* (2009) reported on the development of a FDH sensor using platinum-based screen printed graphite electrode with a ferricyanide mediator as the electron acceptor. Others (Montañez-Soto *et al.*, 2006) showed that FDH could be incorporated onto a tetrathiofulvalene-tetracyanoquinodimethane organic conducting salt and included in a polymeric matrix of epoxy resin and graphite powder. It is therefore evident that the complexity of the fructose sensors and the high associated costs for its mass production were beyond the objectives of this chapter. Sucrose biosensors, on the other hand, have been mainly developed through relying on invertase, mutarotase and glucose oxidase (Schwedt and Stein, 1994; Thomas, 1998; Guemas *et al.*, 2000; Jawaheer *et al.*, 2003; **Figure 7.1**). Few studies have even reported on the use of mediated electrodes based on sucrose phosphorylase and electrocatalytic oxidation of NADH (Maestre *et al.*, 2001).

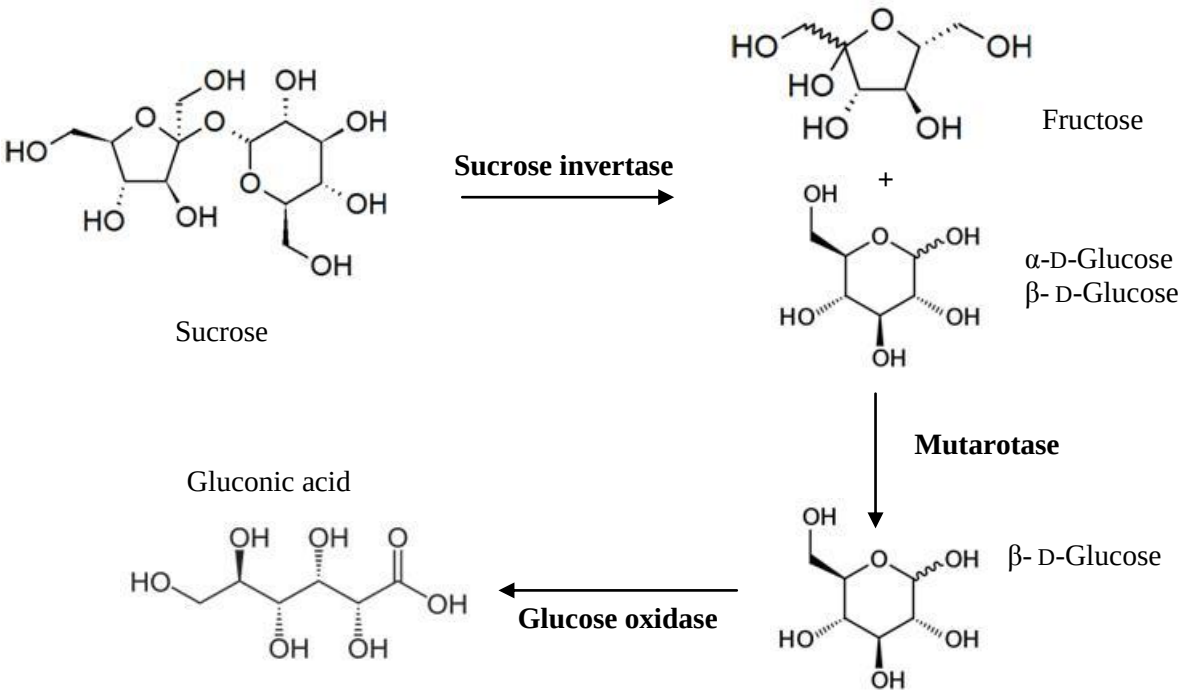


Figure 7.1: Conversion of sucrose into glucose and further oxidation to gluconic acid corresponding to the common scheme used for the fabrication of sucrose biosensors.

Nevertheless, earlier chapters (Chapter 3 and Chapter 4; sections 3.4.3and 4.5.1, respectively) agreed well with that found in the literature (Boccorh *et al.*, 1999; Terry *et al.*, 2007a; Tulipani *et al.*, 2008; Crespo *et al.*, 2010; Zheng *et al.*, 2009) since a quasi 1 to 1 ratio for glucose and fructose was encountered for both blackcurrant and strawberry fruits. Besides, it was demonstrated that glucose concentrations determined by the GOx-based sensor described earlier (Chapter 6; section 6.4.4) correlated well not only with real glucose concentrations in the fruits but with total sugar concentrations. Accordingly, no sugars other than glucose were targeted for detection by the proposed biosensor array.

7.2.3 Citric

Citric acid is the predominant organic acid found in most berries followed by malic and ascorbic acid, respectively. The determination of citric acid is mainly achieved by gas chromatography, high performance liquid chromatography, or enzymatic methods (Flores *et al.*, 1970; Macrae, 1982; Pérez *et al.*, 1997; Terry *et al.*, 2007a; Tulipani *et al.*, 2008; Crespo *et al.*, 2010). Biosensors which individually measured these compounds have already been developed (Kim, 2006). Citric acid determination essentially relies on a combination of enzymes including citrate lyase (CL), malate dehydrogenase (MDH) and lactate dehydrogenase (LDH). Nevertheless, due to the low specific activity of citrate lyase, vast amounts of this enzyme need to be immobilised onto the sensor generally leading to a poor global performance (Jawaheer *et al.*, 2003).

7.2.4 Malic

Malic acid concentrations in both blackcurrant and strawberry fruits may be as high as 6.90 and 3.7 mg g^{-1} FW, respectively, in fully ripe fruits (Chapter 3 and Chapter 4; sections 3.4.3 and 4.5.1, respectively). Besides, significant changes in malic acid concentrations are observed during berry ripening for blackcurrant (Table 3.4) and other fruits (Arif *et al.*, 2002). In theory, two different approaches may be chosen when developing a malic acid biosensor; the first one uses malic enzyme (ME) which catalyses the following reaction:

$$L-malic\ acid + NADP^+ \overset{ME}{\leftrightarrow} pyruvic\ acid + NADPH + CO_2 + H^+$$

The second option uses malate dehydrogenase (MDH) and works according to the following reaction:

$$L-malic\ acid + NAD^+ \overset{MDH}{\longleftrightarrow} oxalacetic\ acid + NADH + H^+$$

The main advantages and drawbacks of both malic enzymes were earlier reported by Arif *et al.* (2002). In the present chapter, ME was chosen for the determination of malic acid not only to measure optimum berry quality (i.e. sugar/acid ratio) but also optimum maturity.

7.2.5 Ascorbic

Ascorbic acid (AsA), a form of vitamin C, is a water-soluble antioxidant with a variety of health-promoting properties (Appendix D). Thus, the development of inexpensive, fast and easier methods for the determination of AsA is very important not only for the food but for the pharmaceutical industry (Civit *et al.*, 2008). Different methods are reported for the determination of AsA in different foodstuffs, mainly using chromatography (Terry *et al.*, 2007a; Giné Bordonaba and Terry, 2008; Giné Bordonaba and Terry, 2009), spectrophotometry (Dürüst *et al.*, 1997), capillary electrophoresis (Wu *et al.*, 2007) or more recently electrochemical methods (Pournaghi-Azar *et al.*, 2002; Guanghan *et al.*, 1994). Indeed, Chapter 5 showed a positive correlation between AsA concentrations of both blackcurrant and strawberry and the electrochemical index Q_{500} (section 5.5.3). The use of biosensors for selective determination of AsA in foodstuffs has also been reported in single (Civit *et al.*, 2008) or array configurations of disposable screen-printed electrodes (Jawaheer *et al.*, 2003). Without the need of enzymes, ascorbic acid may be easily oxidised at the surface of the working electrode (**Figure 7.2**).

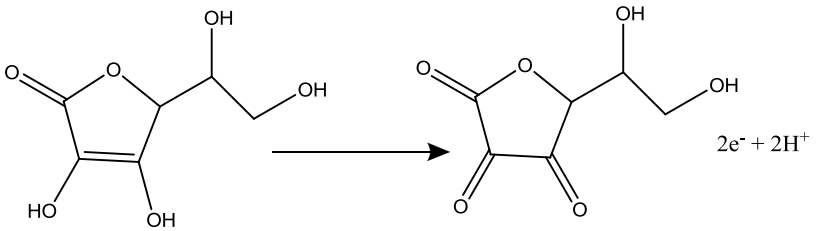


Figure 7.2: Ascorbic acid oxidation scheme at screen carbon electrodes.

Accordingly, the present chapter describes the development of a sensor array for the simultaneous determination of glucose, AsA and malic acid in soft fruits as means of improving soft fruit quality control.

7.3 Materials and methods

7.3.1 Plant materials

Blackcurrant samples from three different cultivars and harvested at different maturities were chosen due to known differences in their sugar and organic acid profile (Chapter 4; section 4.5.2). Similarly, strawberry samples from six different cultivars were grown under commercial practices and supplied by H.H. Duncalfe (Chapter 3; section 3.3) or grown under normal or deficit irrigation conditions at the glasshouse facilities at Cranfield. The sugar and acid profile of the commercial strawberry samples was detailed in Chapter 6 (section 6.3.3) whereas results from fruits grown at Cranfield were earlier presented in Chapter 3 (section 3.5.3)

7.3.2 Reagents

Sugar and organic acid standards, NADP and NADPH were obtained from Sigma-Aldrich and prepared in buffer–electrolyte as required. Unless otherwise stated, the buffer electrolyte consisted of NaH_2PO_4/Na_2HPO_4 (100 mM) adjusted to optimum pH values. Buffer–electrolyte solutions also contained 0.1 M KCl and were prepared daily before analysis. Malic enzyme (malate dehydrogenase, EC 1.1.1.40) from chicken liver (specific activity 19.6 units mg^{-1}), glucose oxidase (EC 1.1.3.4; GOx) derived from *Aspergillus niger* and ascorbate oxidase (EC 1.10.3.3) were also purchased from Sigma-Aldrich. The chemicals used were of analytical grade and solutions were prepared with MilliQ water (σ = 18M Ω cm^{-1}).

7.3.3 Electrodes and electrochemical measurements

The screen-printed electrode array was designed by Prof. Dave Cullen at Cranfield University and fabricated in Gwent Electronic Materials Ltd. (GEM, Gwent, UK). Each electrode within the array was screen-printed in a two electrode configuration through five different printing layers (**Figure 7.3**). The first layer (A) consisted of generic carbon ink, followed by a second layer (B) comprising the insulating ink. The third layer (C) provided the Ag/AgCl reference/counter layer and the fourth screen printing procedure (D) provided the catalytic carbon (platinised carbon). All the layers were screen-printed onto a PVC substrate (E).

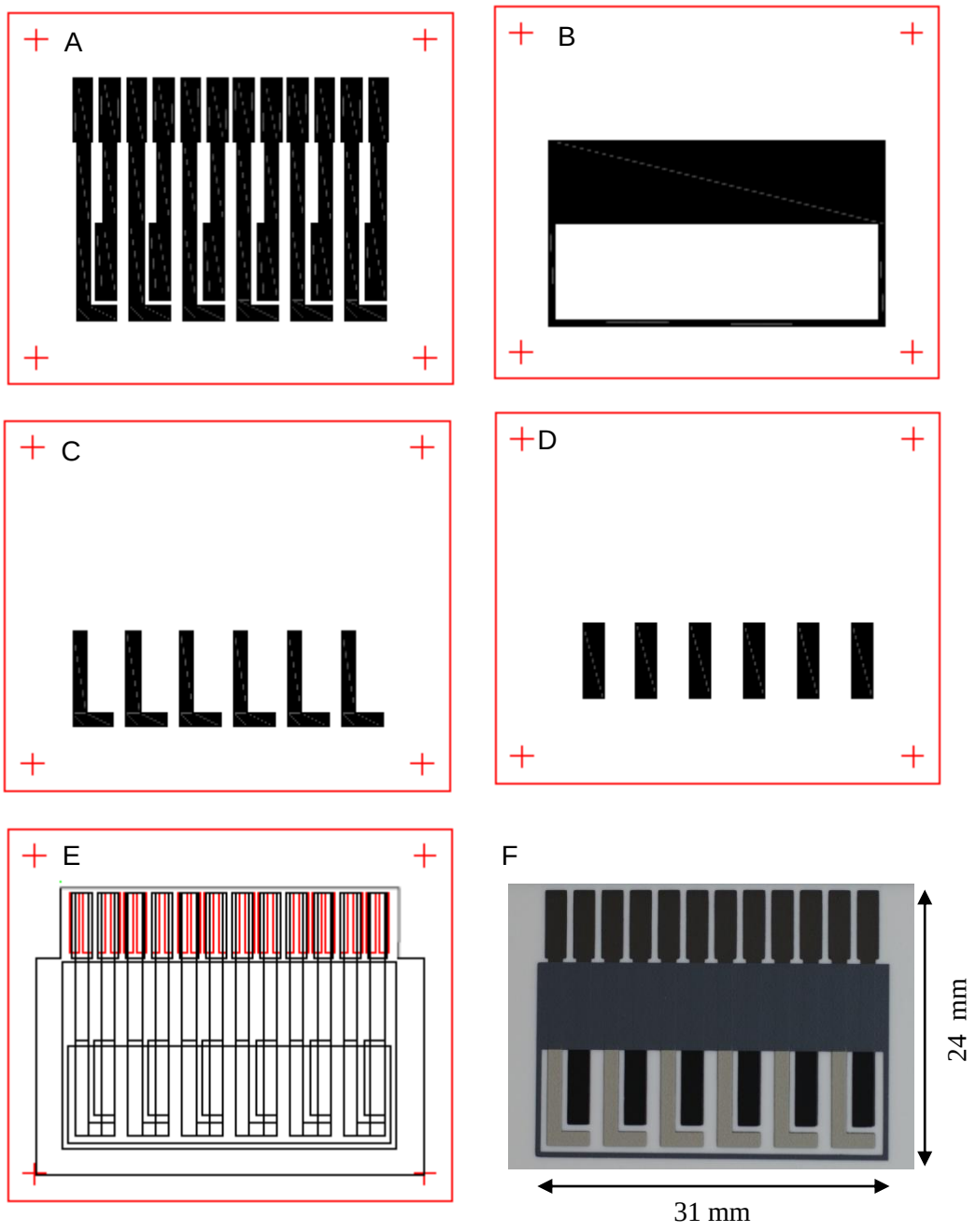


Figure 7.3: Schematic representation of the different layers involved during the screen printed procedure of the multi-electrode platform.

Electrochemical measurements were performed using the same equipment described in Chapter 5 and Chapter 6 (sections 5.2.3 and 6.2.1.2, respectively) yet in this case the electrodes were connected to a Palmsens multiplexer (Palm Instruments BV, The Netherlands). All amperograms and step-

amperograms were obtained using, in this case, PSlite PalmsensPC software which improved the earlier version of the software described elsewhere (Chapter 5; section 5.2.3).

7.3.4 Biosensor construction and optimization

7.3.4.1 GOx-based biosensor

Several experimental variables that affect the GOx-based biosensor response were optimised by means of a 2^3 central composite design as described earlier (Chapter 6; section 6.2.1.3). In this case the optimum applied potential was chosen based on preliminary experiments by comparing the signal-to-noise ratio of glucose and potential interferences (i.e. AsA). In order to increase the sensitivity and stability of the GOx electrode, pectin was used to immobilize and stabilize the enzyme (Jawaheer *et al.*, 2003) (**Figure 7. 4**). Selected levels for each experimental variable (Glucose loading (CGOx), pH of the buffer/pectin matrix and pectin concentration) were based on results obtained during preliminary experiments (**Figure 7.9**).

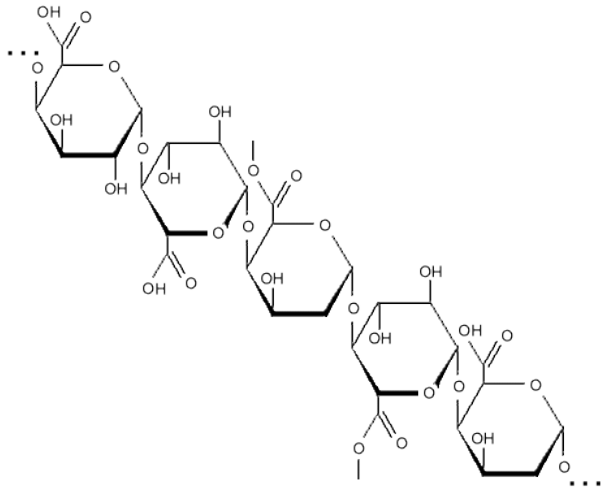


Figure 7.4: Chemical structure of pectin, a complex polysaccharide consisting mainly of esterified D-galacturonic acid resides in an α-(1-4) chain.

Enzyme immobilisation was finally achieved by depositing optimum GOx on a 2.5% (w/v) pectin and 0.1M phosphate buffer onto the surface of the working electrode. Electrodes were left to dry for 4h at 23ºC in a Sanyo incubator and subsequently stored at 4ºC until use the following day (Abayomi *et al.*, 2006 and Abayomi, 2007).

7.3.4.2 Bare electrodes

Bare electrodes were initially assessed to determine AsA concentrations in both standard and sample solutions. Bare electrodes were treated as those described for the determination of glucose but without enzyme or pectin as stabilising agent.

7.3.4.3 Malic acid sensors

ME was the enzyme of choice for the construction of the malic acid biosensor and agreed with earlier works by Arif *et al.* (2002). The signal given by the malic acid sensor relies on the generation of NADPH which is measured amperometrically and related to malic acid concentrations. Preliminary experiments studied the optimum potential for the determination of NADPH using step amperometry within the range of -600 to +600 mV. Under the same imposed conditions a wide range of interferent compounds commonly present in both blackcurrant and strawberry fruits were investigated (*viz.* ascorbic acid, glucose, malic, fructose, citric).

Enzyme immobilisation was achieved following the same procedure as for GOx-based sensors. However, the active site of malic enzyme is thought to be a sulfhydryl group, which can be stabilised using 2-mercaptoethanol (2-MCE). ME also requires trace amounts of divalent cations such as Mg^{2+} or Mn^{2+}. Accordingly and based on earlier works (Arif *et al.*, 2002), the ME stock solution used in this study contained 0.5 mM 2-MCE and 10 mM $MnCl_2$.

7.3.5 Sample preparation

Sample preparation was based on that previously described (Abayomi *et al.*, 2006; Chapter 6; section 6.2.2.2) with some modifications. From both blackcurrant and strawberry samples, 50 g of berries were mixed with 500 mL of buffer electrolyte solution (1:10 w/v). The mixture was homogenised using a domestic blender for approximately 40 seconds and the homogenate obtained was filtered as described earlier (Chapter 5; section 5.2.2; **Figure 7.5**). The pH of the resulting solution was adjusted to optimum values using a using a JENWAY 3020 pH meter (Jenway, Essex, UK). The filtrate solution (ca. 30 mL) obtained from each sample was then divided in two aliquots (15 mL each one). One of the aliquots was directly used for glucose and ascorbic acid determination using the biosensor array. To the second aliquot, 1.5 mL containing 200 U mL^{-1} of ascorbate oxidase was added and samples were incubated for 30 min at room temperature (~22ºC) prior to further analysis using the biosensor array platform. Given the electrochemical activity of AsA (Chapter 5; section 5.3.3),

ascorbate oxidase treatments were performed to assess the influence of major interferent compounds on the biosensor response.

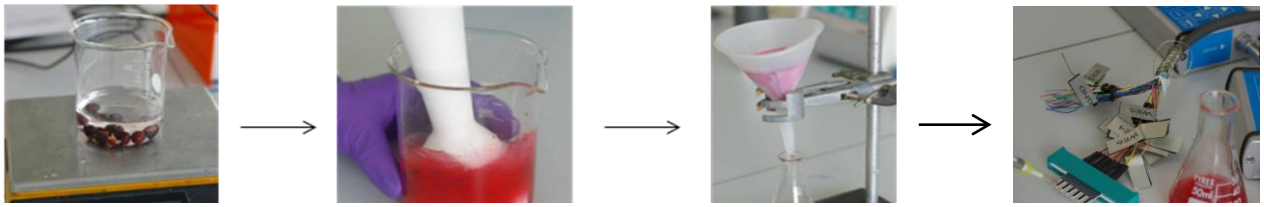


Figure 7.5: Schematic representation of the different steps involved in sample preparation prior to biosensor measurement.

7.3.6 Characterisation of samples by HPLC

Extraction and quantification of both sugar and organic acid was performed on freeze-dried material of the same samples used for biosensor measurements. HPLC determinations were carried out as reported previously for strawberry and other soft fruits (*Chapter 3; Section 3.4.4*).

7.3.7 Data analysis

Data analysis including central composite design (CCD) was carried out using Statgraphics centurion (trial version XV) and statistical analysis using Genstat for Windows Version 9.1.0.147. Given the importance of the sample matrix on the biosensor response, LOD and LOQ values were calculated as described earlier (Chapter 6; section 6.2.4) but, in this case, after standard additions of the target analytes in strawberry samples (cv. Florence). Statistical tests were calculated as described in earlier chapters and variations among biosensor responses or main treatments were plotted in SigmaPlot 9.0 (Chapter 6; section 6.2.4).

7.4 Results and discussion

7.4.1 GOx-based biosensors

In the current experiment, the applied potential (Eap = 0.3 V vs. Ag/AgCl) was chosen in order to provide a substantially higher signal for glucose as well as to limit the response of the biosensor to possible interferents present in the samples (**Figure 7.6**). Ascorbic acid is known to be a major interferent which is easily oxidized even under low potentials resulting in overestimated signals when other analytes are measured (Aaby *et al.*, 2004; Piljac-Zegarac *et al.*, 2008). The ability to

electrochemically measure AsA and other antioxidant compounds using screen-printed electrodes (viz. anthocyanins, phenolic acids) was shown in earlier chapters (Chapter 5; section 5.3.1) and agreed well with others (Aaby *et al.*, 2004; Blasco *et al.*, 2007; Piljac-Zegarac *et al.*, 2008). At the platinised screen printed carbon electrodes (vs. Ag/AgCl), AsA (2.5 mM) oxidation occurred at all range of formal potentials but was greater below 200 mV. In contrast, the signal given by the GOx-based sensor to glucose standard solutions (5 mM) increased gradually as the applied potential was increased. Although different electrode configurations were used, the results presented herein agreed with the findings from earlier chapters (Chapter 6; section 6.3.2) and with those reported by others (Lupu *et al.*, 2004; Abayomi *et al.*, 2006). A final applied potential of 300 mV was therefore chosen for further experiments where the signal given by the biosensor to known glucose concentrations was *ca*. 4-fold higher than those obtained by AsA.

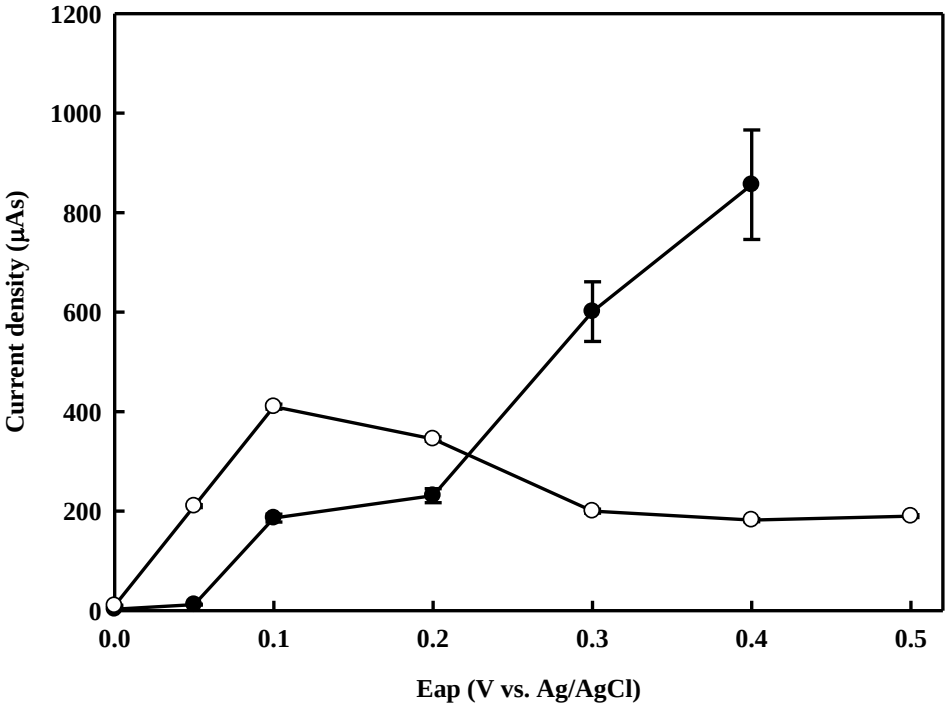


Figure 7.6: GOx-based biosensor response to glucose 5 mM (●) and AsA 2.5 mM (○) standard solutions using phosphate buffer (0.1 M, pH 6.3) as supporting electrolyte solution.

Similar ranges of potentials have been previously used by others (Jawaheer *et al.*, 2003; Gonzalo-Ruiz *et al.*, 2004; Lupu *et al.*, 2004) when developing glucose sensors for food quality applications. At this applied potential, H_2O_2, a product from the reaction between glucose oxidase and

glucose, is indirectly measured after being catalytically oxidized at a metalized carbon electrode (Newman *et al.*, 2005) according to the following reaction:

$$\text{Metal } (OH)_2 + H_2O_2 \longrightarrow \text{Metal} + H_2O + O_2$$
$$\text{Metal} + 2H_2O \longrightarrow \text{Metal } (OH)_2 + 2H^+ + 2e^-$$

With the overall reaction,

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^-$$

Enzyme stabilisation is considered one of the main problems in the development of commercial biosensors (Gibson, 1999; McAteer *et al.*, 1999; Jawaheer *et al.*, 2002; Jawaheer *et al.*, 2003). In order to improve enzyme stabilization and therefore biosensor signal and stability, the natural polysaccharide pectin (Jawaheer *et al.*, 2003) was also studied as a possible enzyme entrapment and preservative matrix and the results were compared with those obtained for GOx electrodes without stabilizing agent (**Figure 7.7**). The entrapment of GOx into the polysaccharide pectin resulted in significantly higher signals as compared to non-entrapped enzymes for the applied potentials ranging from 0.2 to 0.4 V. Additionally the reproducibility of the signal given by the GOx-based biosensor, as indicated by the error bars, was significantly improved within the same range of applied potentials. The advantages of using pectin for the stabilisation of GOx and other enzyme based biosensors were earlier studied by Jawaheer *et al.* (2002). For instance, the high sugar concentration found in this polysaccharide it is known to stabilise the enzyme thereby extending operational lifetime (Jawaheer *et al.*, 2002)

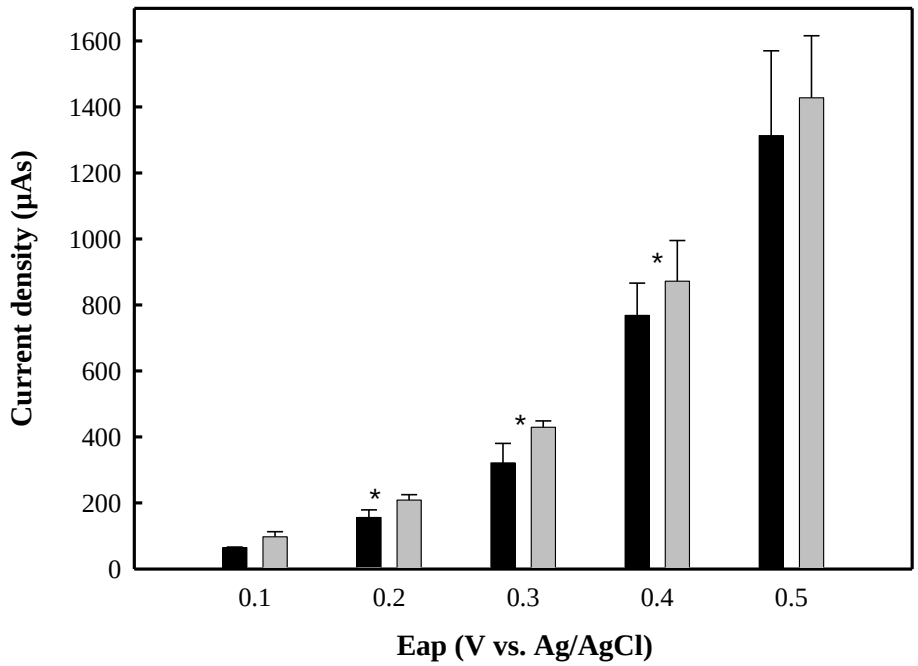


Figure 7.7: Response given by the GOx-based biosensor at different applied potentials (E_{ap} vs Ag/AgCl) using (▬) pectin (3.5%; w/v) with PBS (0.1 M, pH 6.3) or (▬) PBS (0.1 M, pH 6.3). Standard error bars from the mean of 3 measurements. * indicates significant differences at $P < 0.05$.

In the present study, the use of pectin as a stabilising agent not only improved the reproducibility and the current density given by the sensor (**Figure 7.7**) but also reduced the signal given by possible interferents such as AsA (Figure 7.8). Indeed the signal given by the GOx-based sensor to AsA (1mM) was nearly half than that given for bare electrodes (**Figure 7.8**). Pectin deposition onto the surface of the electrode may limit the response of AsA and other possible interferent compounds by acting as a non-selective membrane and hence limiting the diffusion of these compounds to the surface of the working electrode.

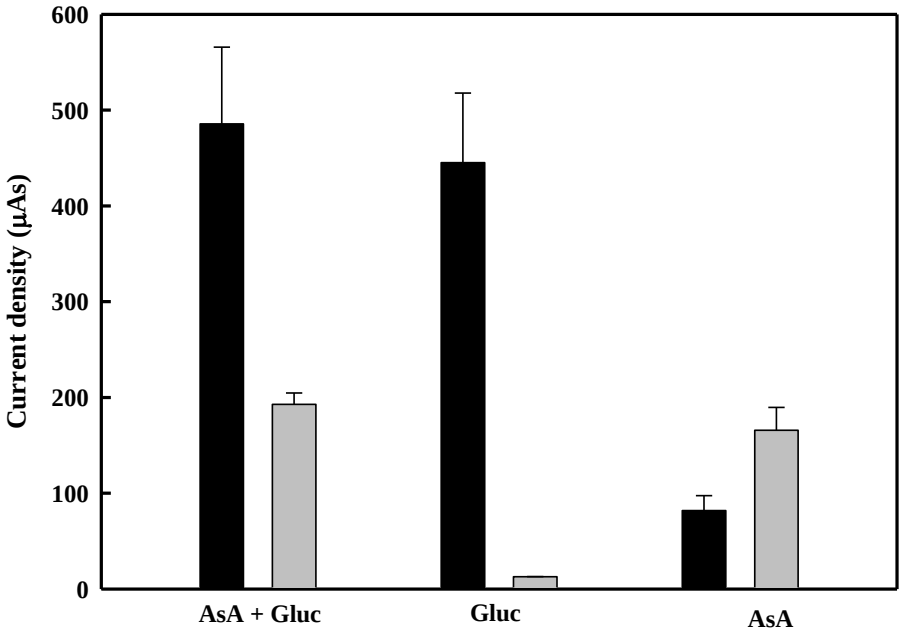


Figure 7.8: Current density of GOx-based (■) and bare electrodes (■) to standard glucose (Gluc; 5 mM), ascorbic acid (AsA; 1mM) and gluc + AsA solutions under optimum operating conditions (*viz.* 0.3 V vs. Ag/AgCl).

Given the advantages offered by pectin as an entrapment and enzyme stabilising agent, different pectin concentrations (%; w/v), together with the pH of the buffer/pectin solution and enzyme loading (C_{GOx}) onto the surface of the working electrode were optimized by means of central composite design (2^3) (**Figure 7. 9**). The advantages of using this experimental methodology approach were described earlier (Chapter 6; section 6.3.1).

	1	2	3	4	5	6	7	8	9
pH	5.5	4	7	3	5.5	8	4	7	5.5
%Pectin (w/v)	1	2	2	3.5	3.5	3.5	5	5	6
TSS	9.8	9.6	10.3	9.5	9.9	11.8	9.9	10.5	10.1

Figure 7.9: Combination of pectin concentration (2-5 %; w/v) and pH of the buffer electrolyte solution (from 4 to 7) tested for the optimisation of the GOx-based biosensor using central composite design (2^3). Selected levels for GOx concentration (CGOx) were from 5 to 10 U based on preliminary experiments.

Statistical analysis from the CCD (2^3) showed that neither enzyme loading (C_{Gox}), within the range studied, nor the concentration of pectin influenced the response of the biosensor ($P>0.05$; data not shown). However, the pH of the buffer/electrolyte solution as well as the interactions between the different variables were crucial factors in terms of the biosensor performance. Optimum biosensor signals were obtained when pH 7.2, 2.5 % pectin (w/v) and 10U GOx were chosen, and hence such values were used for further biosensor experiments. Using a similar approach, optimum Eap and pH values were comparable to those described earlier (Chapter 6; section 6.3.1) and agreed well with those found in the literature (Alonso-Lomillo *et al.*, 2004)

Under optimum conditions the biosensor responded positively to increasing concentrations of glucose in standard solutions at 0.3V (**Figure 7.10**), with a linear range from 0 to 15 mM and hence comparable to that reported by others (Chapter 6; section 6.3.2; Lupu *et al.*, 2004; Abayomi, 2007) or *ca*. 3-fold higher than reported for others (Jawaheer *et al.*, 2003) when developing a glucose sensor to be incorporated into a sensor array. LOD and LOQ values for the GOx-sensor were 0.011 and 0.037 mg glucose g^{-1} FW and hence comparable to those described in earlier chapters (Chapter 6; section 6.3.2).

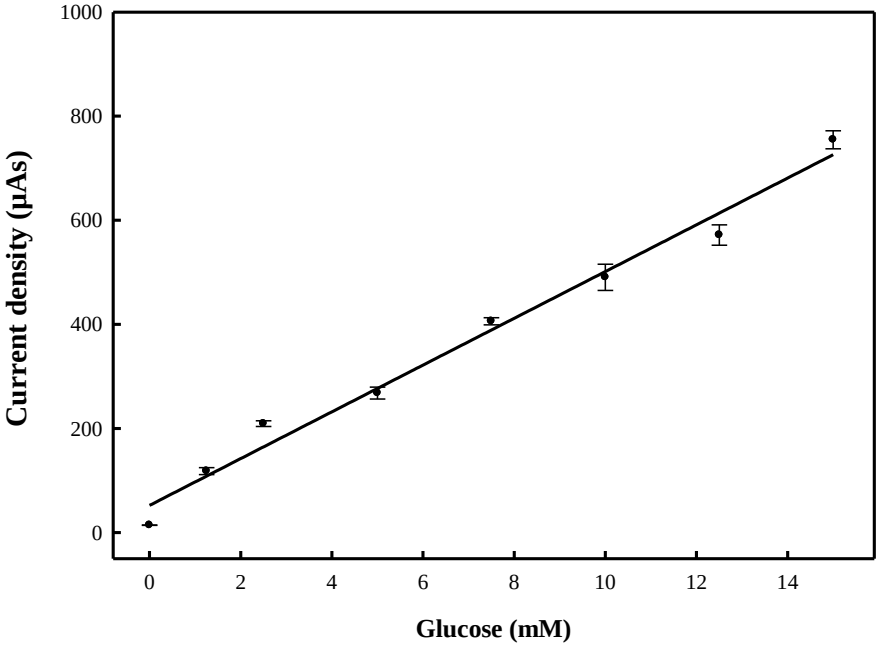


Figure 7.10: Calibration of glucose oxidase (GOx) biosensor using glucose standards in phosphate buffer under optimised conditions (Eap = 0.3V; 10 U GOx; 2% Pectin (w/v) ; pH 7.2). Standard error bars from the mean of 5 measurements. Response within the linear range (0 – 15 mM); µAs = 59.6519 + 42.84 x glucose concentration (mM); P <0.001; r^2 = 0.979; Adjusted r^2 = 0.975.

The selectivity of the GOx-based sensor was also tested by measuring current density produced from other major sugars present in both blackcurrant and strawberries samples (fructose and sucrose). As observed in earlier experiments (Chapter 6; section 6.3.2), but using different sensor configurations, no response was obtained with the addition of these sugars in solution confirming the selectivity of the enzyme glucose oxidase towards the substrate glucose as well as the non-electrochemical activity of these sugars (data not shown).

7.4.2 Bare electrodes performance

At the optimum pH and applied potential described for the glucose biosensor, bare electrodes responded positively to increasing concentrations of ascorbic acid from 0 to 75 mM (**Figure 7.11**). At fixed applied potentials, AsA is easily oxidised onto the surface of the electrodes resulting in an increase of the current that is proportional to the concentration of this analyte (Civit *et al.*, 2008). LOD and LOQ values were equivalent to 12 and 36 µM, respectively. Civit *et al.* (2008) reported LOD values as low as 0.86 µM when working with screen-printed carbon electrodes (SPEs) modified with

an *o*-aminophenol (*o*-AP) film selective for the detection of AA. Others have reported LOD values similar to those described herein using poly(*N*-methylaniline)-modified Pt electrodes (Brazdziuviene *et al.*, 2007) and copper hexacyanoferrate deposited on carbon electrodes (Pauliukaite *et al.*, 2005).

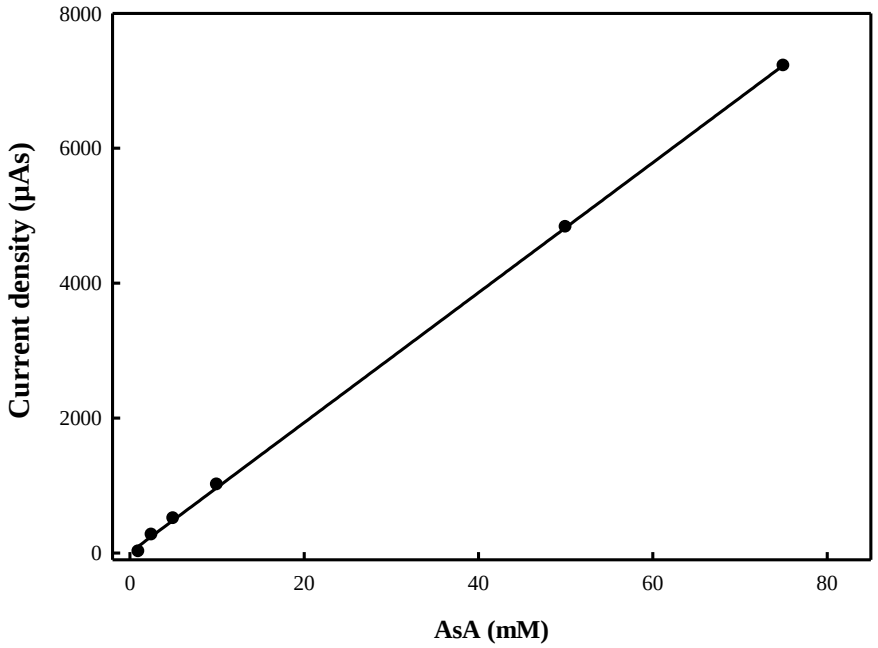


Figure 7.11: Direct determination of ascorbic acid (AsA) on bare platinised-carbon electrodes at 0.3 V, using phosphate buffer (0.1 M, pH 7.2) as supporting electrolyte solution. Standard error bars from the mean of 5 measurements. Linear biosensor response from the range 0 to 75 mM; µAs = 96.82 x AsA (mM); $P < 0.001$; $r^2 = 0.999$.

Differences in the reproducibility of both the GOx-based and bare electrodes highlighted that the lower reproducibility observed in the GOx-based sensors was due to the enzyme and the enzyme immobilisation procedure rather than the electrode itself. The enzyme immobilisation procedure described herein is based on surface adsorption in which the enzyme is directly deposited onto the surface of the working electrode by means of ionic interactions, hydrogen binding and Van der Waals forces (Abayomi *et al.*, 2006). Accordingly, several factors including pH and the ionic strength of the sample solution may detrimentally affect the stability of the enzyme (Gónzalo-Ruiz, 2006). Thus said, this type of immobilisation is simple and is low cost as compared to cross-linking, encapsulation and/or covalent binding approaches, and therefore appropriate for mass-production of disposable sensors (Gónzalo-Ruiz, 2006). Attempts to improve enzyme immobilisation and hence sensor reproducibility

have been extensively reported (Appleton *et al.*, 1997; Jawaheer *et al.*, 2002; Jawaheer *et al.*, 2003; Loose and Setford, 2006). Pectin was observed to improve the biosensor performance herein and elsewhere (Jawaheer *et al.*, 2003). Nevertheless, other parameters are known to detrimentally affect sensor performance. For instance, and albeit using different electrode configurations, Xue *et al.* (2006) and other workers (Chen *et al.* (2006) have already showed that GOx reaches maximum activity around 40 ˚C and that fluctuations in room temperature (~22ºC) during measurements may result in different sensor performance (Abayomi *et al.*, 2006).

7.4.3 Malic acid sensor

In the present work, ME was chosen thus disregarding the need for added reactive species that may be necessary to forward the reaction of MDH (Arif *et al.*, 2002). In addition, optimum pH for MDH has been reported to be substantially higher than that of ME, and consequently leading to major sample preparation steps in order to adequate the pH of the berry juice solutions (Matsumoto *et al.*, 1996). The selectivity of the sensors towards NADPH and other common analytes was assessed using step amperometry within the range of -600 to +600 mV. For all analytes, an increase in the potential resulted in greater current responses. The highest responses were observed when working with ascorbic acid which was in agreement with earlier experiments (section 7.4.2) and the results reported by Arif *et al.* (2002). Similarly, NADPH gave higher current responses when working with potentials above 0.3V, with levels higher than those observed for ascorbic acid (**Figure 7.12**).

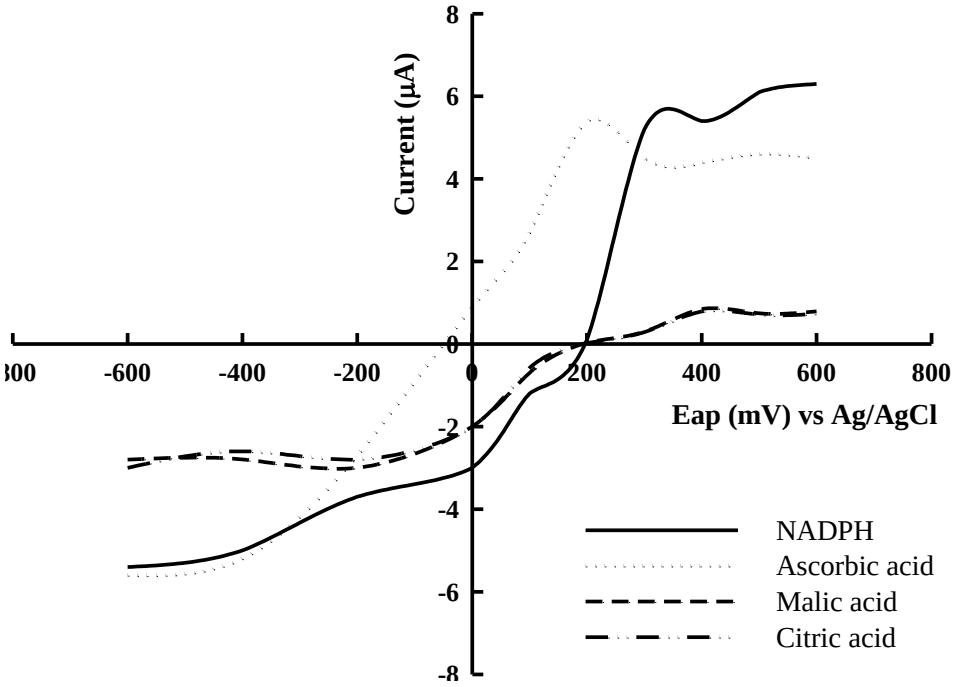


Figure 7.12: Current versus potential profile for NADPH co-factor and a range of possible organic acid interferents measured on platinised screen printed electrodes. Values are means of triplicate measurements

Based on the results obtained, 0.3 V vs. Ag/AgCl was confirmed as the optimum applied potential for the determination of NADPH and hence the prototype malic sensor. In this case, the signal to noise ratio when comparing the NADPH and AsA strongly suggested that prior elimination of AsA would be necessary when analysing real blackcurrant and strawberry samples. However, after enzyme immobilization onto the sensor surface following different strategies, no generation of NADPH was observed, and hence no signal was obtained from the sensors. Such findings revealed that the malic acid enzyme had probably deteriorated, and hence was not catalyzing the conversion of L-malic acid into pyruvate and NADPH. Under such conditions, and taking into account the high cost of the enzyme, the development of this sensor was not continued. Given the poor stability and sensitivity of either ME or MDH, future work should address novel alternatives for the construction of malic acid biosensors.

7.4.4 Simultaneous determination of ascorbate and glucose in soft fruits

Both the GOx-based and ascorbate sensors, included in the prototype sensor array, showed their ability to measure simultaneously specific compounds with enough precision at an applied potential of 0.3 V vs. Ag/AgCl. Considering the 6 electrode configuration of the screen-printed array (**Figure 7.3**), three electrodes were used to monitor glucose whereas the other electrodes were used for the simultaneous detection of AsA. Direct deposition of blackcurrant and strawberry juice from different samples onto the surface of bare electrodes resulted in an increase in the current density directly proportional to the concentration of AsA in the samples. In the particular case of blackcurrant fruits, the sensor response was strongly correlated (r^2 = 0.902; P < 0.01) to known AsA concentrations measured by HPLC (**Figure 7.13**). Nevertheless, AsA concentrations determined by the prototype sensor were for most samples slightly higher than those found by HPLC which may be related to the presence of other electroactive compounds in berry fruits which may easily be oxidised at the surface of the electrode under the conditions described herein (Chapter 5; section 5.3.3; Terry *et al.*, 2005; Abayomi *et al.*, 2006).

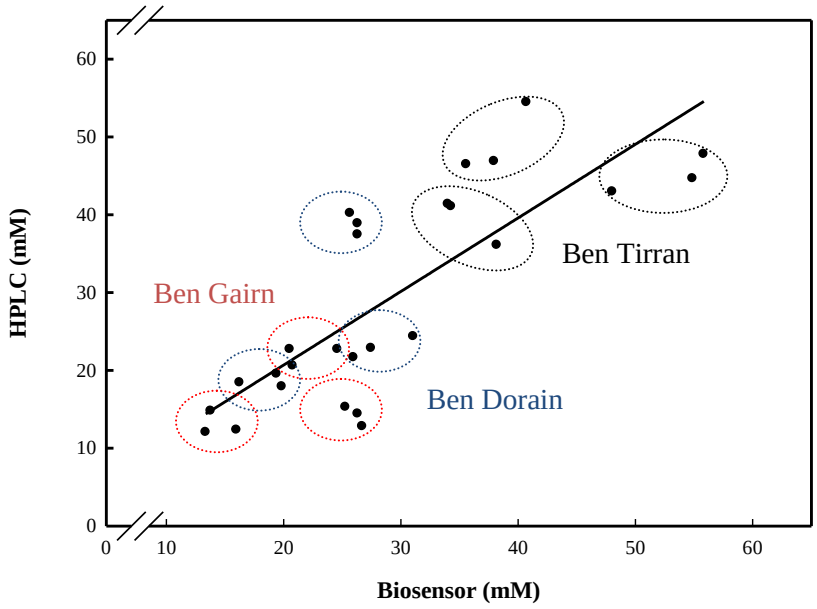


Figure 7.13: Comparison between AsA determination on bare screen-printed electrodes (Eap = 0.3 V vs. Ag/AgCl; using 0.1 M PBS at pH 7.2) and HPLC from three different blackcurrant cultivars at three different maturity stages. All measurements were performed in triplicate (n = 27). r^2 = 0.902; P < 0.001.

Similar findings were observed for strawberry fruits ($r > 0.71$; $P < 0.001$) from different cultivars or from fruits obtained from plants grown under different conditions. The lower correlation observed for strawberry fruits may be attributed to the lower concentration of AsA found in these berries as compared to blackcurrant (Aaby *et al.*, 2007; Zheng *et al.*, 2009; **Figure 7.14**) and hence the relatively greater influence of other electrochemically active compounds in the response of the sensor. Although different sensor configurations were used between experiments the results presented herein mimicked those found in earlier chapters where Q_{500} values were better correlated to AsA concentrations from blackcurrants than from strawberries (Chapter 5; section 5.3.3).

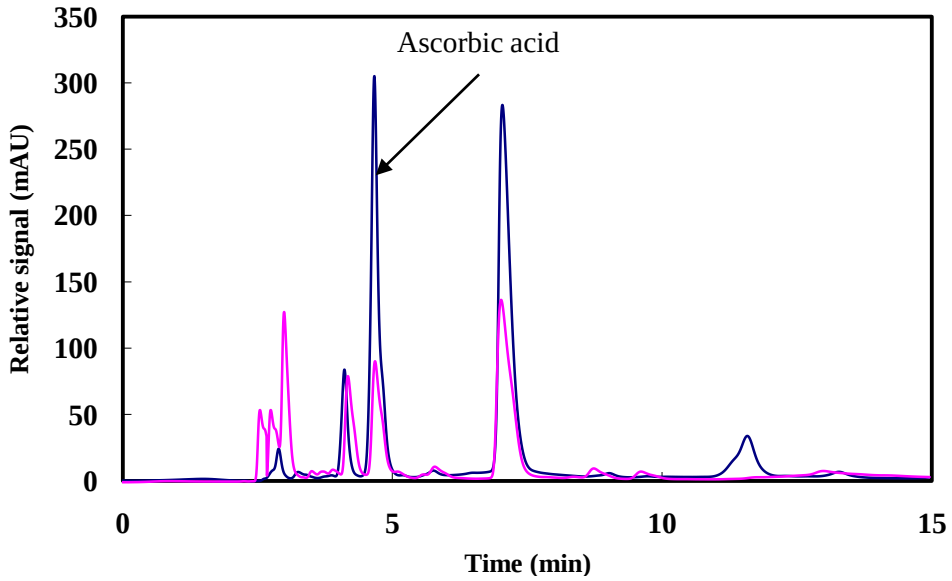


Figure 7.14: Chromatographic profile of main organic acids including ascorbic acid, in both blackcurrant (—) and strawberry (—) fruits. AsA concentrations in both blackcurrant and strawberry fruits were detailed earlier (Chapter 3 and Chapter 4; sections 3.3.3 and 4.5.2.1, respectively)

Prior elimination of the AsA was investigated in order to reduce the interferences caused by this compound on the response of the GOx-based or other enzyme sensors to blackcurrant juice. Different strategies can be adopted for the elimination of AsA. For instance, AsA can be oxidised to an electrochemically inert form, dehydroascorbic acid, by ascorbate oxidase (Anzai *et al.*, 1998). Thus, if juice samples were allowed to react with ascorbate oxidase for a period of time long enough to convert most ascorbic acid into the inert oxidised form, this major interferent would be eliminated or greatly reduced. Indeed, elimination of AsA was satisfactorily achieved by pretreating the samples with AsA oxidase for periods no longer than 15 min (**Figure 7.15**).

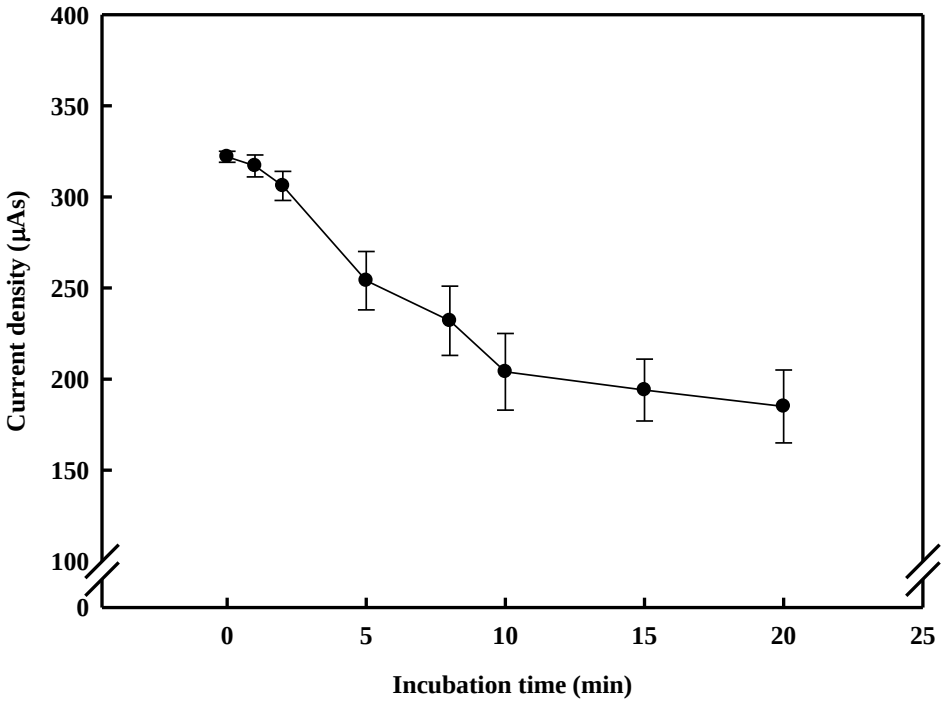


Figure 7.15: Current density generated by blackcurrant samples cv. Ben Dorain (OR) on bare-screen printed carbon electrodes (Eap = 0.3 V vs. Ag/AgCl) after different incubation times with Ascorbic acid oxidase (200 U). Error bars indicated standard deviation for n = 3.

After AsA elimination from the sample (cv. Ben Dorain) the signal given by bare electrodes to blackcurrant juice was *ca*. 50% lower than initial values. The current density difference before and after sample incubation with AsA oxidase was nearly 130 µAs. If considering the calibration curve for ascorbate shown earlier (**Figure 7.11**), 130 µAs is equivalent to ~13 mM hence ca. 2.29 mg g^{-1} FW which strongly agrees with values found for the same sample by standard HPLC measurements (2.09 mg g^{-1} FW). Other strategies for ascorbic acid elimination may be blocking the AsA from reaching the electrode surface by covering the electrode area with selective permeable membranes (*viz*. cellulose acetate, chitosan-pectin, etc.) (Gorton *et al.*, 1991; Jawaheer *et al.*, 2003) or dilution of the samples (Lupu *et al.*, 2004; Abayomi *et al.*, 2006; Chapter 6; section 6.4.4).

After enzymatic elimination of AsA in blackcurrant juices, there was a strong correlation between the signal given by the biosensor and known glucose concentrations determined by HPLC (**Figure 7.16**). Generally, the GOx-based biosensor positively ranked different blackcurrant solutions from different cultivars and from fruits of different maturities according to increasing glucose content as determined by HPLC(Chapter 4; section 4.5.2.1). On the contrary, poor correlations were observed

between TSS and known glucose or total sugar concentrations in both blackcurrant and strawberry fruits (**Figure 7.16**) and hence agreed well with earlier findings (Chapter 6; section 6.4.4; Appendix 1) or that reported by others for berries and other horticultural crops (Pérez *et al.*, 1997; Chope *et al.*, 2006; Soomboonkaew and Terry, 2008). Even though sugar concentrations significantly differed between cultivars and degrees of maturities, values for TTS were quite similar (~15 ºBrix) between the different samples investigated.

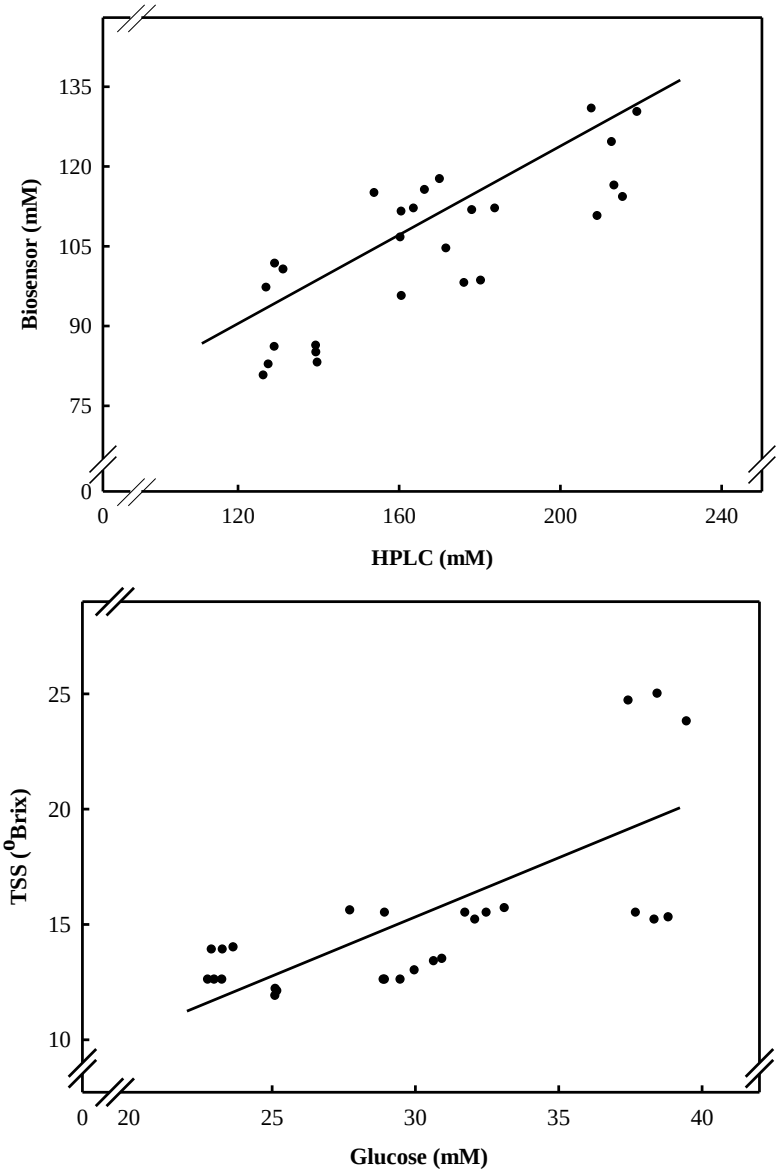


Figure 7.16: GOx biosensor response to blackcurrant juices from three different cultivars at three different maturity stages verified against HPLC determination of glucose. $r^2 = 0.77$; $P < 0.01$. (B) TSS (ºBrix) of juices from three different blackcurrant cultivars at three different maturity stages compared against known glucose concentrations determined by HPLC. $r^2 = 0.62$; $P < 0.001$. All measurements were performed in triplicate (n = 27).

The response of the biosensor was also assessed with strawberry samples and similarly to the results presented above, there was a good correlation ($r^2 = 0.68$; $P < 0.001$) between the signal given by the biosensor and known glucose and AsA concentrations, respectively. Such correlation was slightly improved when the samples were pretreated with AsA oxidase (**Figure 7.17**). A similar correlation between the GOx-based sensor response and glucose content as measured by HPLC were earlier reported (Chapter 6; section 6.3.3).

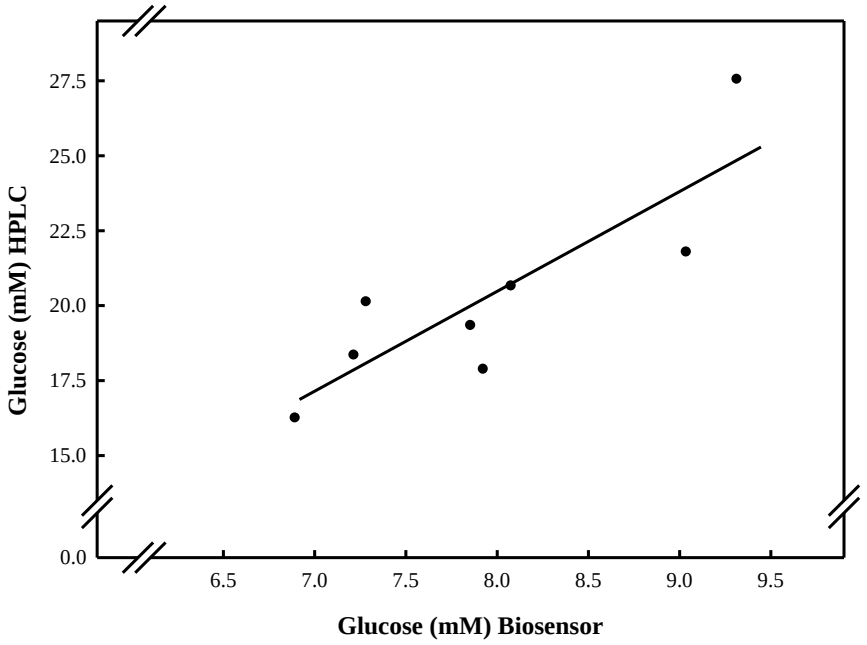


Figure 7.17: GOx biosensor response to pre-treated juices from four different strawberry cultivars (viz. Christine, Elsanta, Florence and Sonata) grown under two different irrigation regimes (Chapter 3; section 3.5.3) against HPLC determination of glucose. $r^2 = 0.73$; $P < 0.01$. Given values represent means of at least duplicate measurements.

Sweetness is an important parameter in strawberry fruits and other berries which can characterise the acceptance of the fruits by consumers (Keutgen and Pawelzik, 2008). The proposed sensor was hence not only able to discriminate between samples based on their glucose content but also due to their calculated sweetness (**Figure 7.18**).

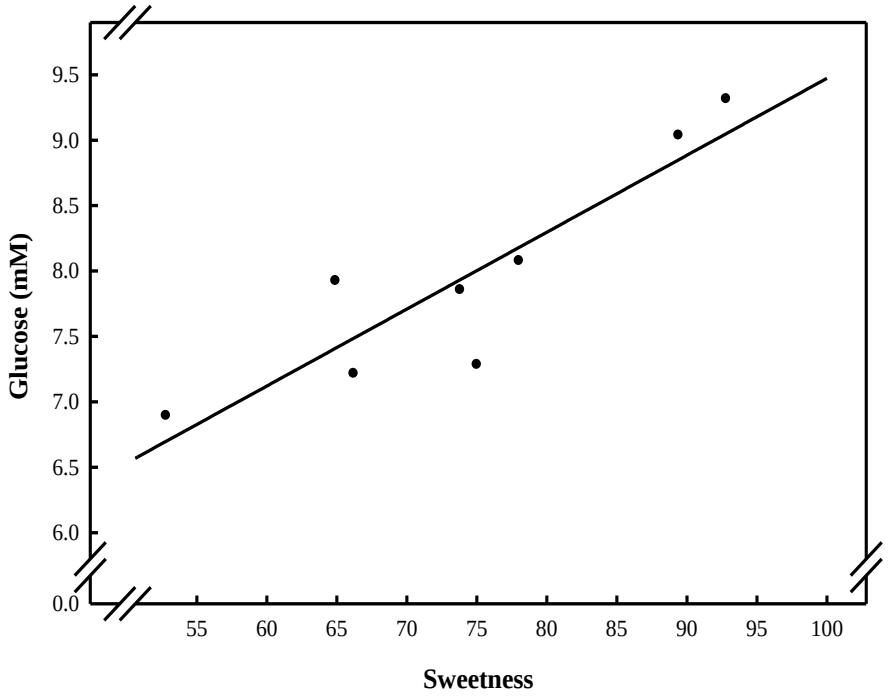


Figure 7.18: GOx biosensor response to pre-treated strawberry juices from four different cultivars (viz. Christine, Elsanta, Florence and Sonata) grown under two different irrigation regimes (Chapter 3; Table 3.6) against sweetness ($r^2 = 0.81$; $P < 0.001$). Given values represent means of at least duplicate measurements.

7.5 Conclusions and future work

The present chapter has shown the possibility of developing a common format for the fabrication of a screen-printed electrode array to measure simultaneously different markers of strawberry and blackcurrant fruit quality. From an economical and practical point of view, glucose, ascorbic and malic acid were initially selected as appropriate markers for strawberry and blackcurrant quality and hence incorporated into the sensor array. Both the GOx-based and ascorbate sensors have shown their ability to measure specific compounds with enough precision and comparable to that found by standard HPLC. The performance of the glucose sensor was considerably improved when the enzyme was immobilised onto a pectin matrix and agreed well with findings by others (Jawaheer *et al.*, 2002). LOD values for the GOx-sensor were similar to those reported in Chapter 6 and were comparable to values found in the literature (Jawaheer *et al.*, 2003; Lupu *et al.*, 2004; Abayomi, 2007). Given the high concentration of AsA present in the samples investigated herein, elimination of this

compound by enzymatic inactivation was highly recommended to improve the performance of the GOx-based sensor. Besides, the information given by the GOx-based sensor could be use to determine not only glucose concentrations but total sugar and a theoretical sweetness index which, in agreement with earlier chapters (Chapter 6), could therefore provide growers, breeders and retailers with a promising alternative to TSS thus improving quality control. In addition, since sweetness in berry fruits is intimately related to consumer acceptability, the signal given by the biosensor could be used to discriminate between fruits tentatively according to consumer preference. This said, future experiments should compare the information given by the sensor with perceived sweetness by an expert taste-panel. The proposed sensor array allows for simultaneous determination of glucose and ascorbate, representing a first attempt to determine the balance and acids within the fruit thereby becoming a powerful tool to discriminate between fruits based on their optimum maturity and appropriate eating quality. Preliminary experiments also showed that the same electrode format could be adapted to measure malic acid concentrations in freshly-squeeze and diluted blackcurrant and strawberry juices. Nevertheless, future studies should investigate alternatives to improve the stability of this type of sensor as well as to reduce the associate costs of fabrication.

In summary, the unity of the different sensors proposed herein should ease the eventual fabrication and operation of a final hand held device that would provide the soft fruit industry with a promising alternative to standard QC.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.0 CHAPTER EIGHT

General discussion and conclusions

8.1 Introduction

The UK soft fruit industry shows strong potential for growth as consumers become increasingly aware of the health and dietary benefits of berry consumption. There is a plethora of research which is being currently undertaken to demonstrate the benefits of berries, and different berry bioactives, for fighting and preventing the incidence of certain diseases (Seeram, 2008; Appendix A4 and A5). Nevertheless, consumer demand is still principally governed by fruit appearance together with a desirable taste and flavour, which in the case of berries seems to be partly related to the balance between sugar and acids within the fruit (Pérez *et al.*, 2007). Prospects for the production of new and more tasteful berries may be achieved not only through genetic improvement and release of new varieties but also through changing the current cultivation systems, as well as boosting the development of growers and breeders diagnostics tools for better quality control. Accordingly this thesis has been structured and divided in two differentiated parts dealing with either quality improvement of both blackcurrant and strawberry fruits by means of different pre-harvest and postharvest strategies as well as improving soft fruit quality control by using biosensor technology.

8.2 General discussion and directions for future work

Sugar and organic acids are key compounds determining the quality of berries, especially in terms of consumer acceptance (Cordenunsi *et al.*, 2003). Major sugars and acids found in both blackcurrant and strawberry fruits comprise glucose, fructose and sucrose together with citric, malic and ascorbic acid (Chapter 3 and Chapter 4; section 3.5.3 and 4.5.1.1, respectively; Pérez *et al.*, 1997; Cordenunsi *et al.*, 2003; Terry *et al.*, 2007; Keutgen and Pawelzik, 2008; Tulipani *et al.*, 2008; Zheng *et al.*, 2009; Crespo *et al.*, 2010).

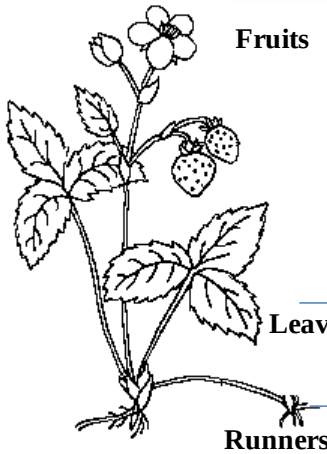
Results from Chapter 3 showed the existing variability in the composition of strawberry fruits of different cultivars, growing locations as well as due to different pre-harvest or growing conditions. Indeed, earlier evidence already demonstrated the role that certain pre-harvest conditions had on strawberry fruit quality (Terry *et al.*, 2007; Tulipani *et al.*, 2008; Crespo *et al.*, 2010), although most of the information available referred to the health-related composition of the fruits (Häkkinen and Torrönen, 2000; Wang and Zheng, 2001; Wang *et al.*, 2002; Recamales *et al.*, 2005; Keutgen and Pawelzik, 2007: Hargreaves *et al.*, 2008) rather than compounds directly related to taste, and hence consumer acceptance. In the context of climate change where growers are under increasing pressure to demonstrate that their water abstractions are reasonable and environmentally justified (Ferreres and Soriano, 2007; Terry *et al.*, 2007), deficit irrigation and related strategies were investigated as a potential strategy to improve strawberry fruits quality (Chapter 3). Earlier works reported that DI was commercially nonviable due to substantial reductions in fruit size and yield (Blatt, 1984; Krüger *et al.*, 1999; Liu *et al.*, 2007). The results presented in Chapter 3 verified that DI on strawberry plants can reduce berry size and yield for certain cultivars, but more importantly can have a marked effect on both fruit physiology and biochemistry (**Table 8.1**). Time from anthesis to fruit harvest was generally longer in plants grown under drought stress (Chapter 3; section 3.5.1) thereby suggesting that DI could be used as a strategy to extend fruit availability thus potentially matching variable consumer demand as affected by weather. The concentration of certain compounds directly related to fruit quality (*viz*. sugar and acids, sweetness and antioxidants) were generally greater in fruits from DI treated plants. In detail, fructose content in cvs. Elsanta and Sonata plants as well as glucose content in Elsanta were significantly greater in DI-treated plants as compared to plants kept at or near field capacity (Chapter 3; **Table 3.6**). Drought stress also affected strawberry acid composition with lower concentrations generally found in fruits from DI-treated plants. Application of DI conditions at later fruit development stages (Exp. 3.5) resulted in little or non-significant changes in sugar or acid concentrations of fruits from the same cultivars (Chapter 3; **Table 3.6**). It is known that both sugars and organic acids originate from photosynthetic assimilates. As an example, strawberries accumulate sugars during the ripening process by translocation from leaves to fruits (Villareal *et al.*, 2010). Sucrose is transported from the leaves to the berry vacuoles where it is accumulated mainly as glucose and fructose. Invertase (P-fructosidase; EC 3.2.1.26) levels in the vacuoles, the enzyme responsible for catalyzing the conversion of sucrose to its monosaccharide constituents, regulate the final concentration of these sugars into the fruit (Davies *et al.*, 1996).

Changes in glucose and fructose but not sucrose as a result of DI agreed well with other studies (Terry *et al.*, 2007). In the former study, the authors concluded that lower concentrations of sugars in fruits that received more water were most probably caused by a dilution effect (Terry *et al.,* 2007). Others (Awang *et al.*, 1995) associated higher soluble solids in strawberry fruits grown under salinity stress to restricted vegetative growth and shift of photoassimilates to fruits. The possible mechanism for increased sugar and lower acid content in fruits submitted to DI were discussed in detail (Chapter 3; section 3.5.3). It was hypothesised that changes in respiratory metabolism and trade-off in resource allocation occurred in the plants which in turn led to an osmotic adjustment and hence accumulation of solutes in DI-treated fruits. Although no evidence exists for strawberry fruits, a process of osmotic adjustment has been reported for other cultivated species grown under drought stress (Mahajan and Tuteja, 2005). Differences in the osmotic potential between sucrose and glucose or fructose may partially explain the differential effect of DI for each major sugar found in strawberry fruits. Nevertheless, it is also possible that levels of invertase, which was not measure in the studies described herein, may be up-regulated by DI and hence partly account for the breakdown of sucrose into glucose and fructose observed herein. To corroborate whether there was a trade off in resource allocations, sugar and organic acid concentrations were concurrently measured in both leaves and fruits (Exp. 3.6). No other studies thus far have investigated sugar and organic acid concentrations of both leaves and strawberry fruits. Evidence was shown that DI did not directly affect sucrose concentrations in leaf tissues or fruits but rather resulted in differences in channelling sucrose degradation products to different carbon pools such as monosaccharides and organic acids or even to other structural carbohydrates and C-building blocks.

The levels of antioxidant compounds in fruits and other plant tissues were also greater in DI as compared to plants kept at or near field capacity, which may be the result of *in situ* synthesis of antioxidants thereby limiting the amount of ROS generated by the plant under stress conditions. Nonetheless, anthocyanin concentrations, the main polyphenolic compounds found in strawberry fruits, were not significantly affected by DI treatments but were enhanced after foliar application of MeJa. However, if taking together the results presented herein and in earlier studies (Terry *et al.*, 2007), and considering the physiological similarities between ABA and MeJA, an initial hypothesis can be drawn. That is that changes in anthocyanin concentrations as a result of different pre-harvest conditions may be modulated by the levels of these natural hormones in the fruit, and hence highlighting the importance of these hormones on secondary plant metabolism probably by increasing PAL activity. Others have already shown that ABA-treated strawberry fruits had increased PAL activity and higher

anthocyanin concentrations (Jiang and Joyce, 2003). Overall, it may be anticipated that drought-generated ROS participate in stress signalling both directly (i.e. *in situ* synthesis of AsA and other antioxidants) and indirectly interacting with stress hormones (i.e. ABA and MeJa) and subsequently leading to increase antioxidant concentrations. Future studies should assess changes in endogenous stress hormones (i.e. ABA and MeJa) and depict any relationship with changes in primary and secondary strawberry metabolites. The observed changes in sugars and antioxidant compounds as a result of drought stress (Exp. 3.1, 3.5 and 3.6) would also corroborate the newly hypothesised association between sugars and sugar-like compounds with antioxidants, as an integrated redox system quenching ROS and contributing to stress tolerance, especially in plant tissues with high soluble sugar concentrations (i.e. fruits) (Rosa *et al.*, 2009; Bolouri-Moghaddam *et al.*, 2010).

Table 8.1: Morphological and biochemical changes on strawberry plants grown under drought stress conditions. The possible mechanisms underlying each change are hypothesised based on the findings from Chapter 3.



	Change[1]	Possible mechanism
Flower	Increase of time to anthesis and slower fruit maturation	Trade off between vegetative growth and propagation (survival strategy)
Fruits	Smaller size, lower yield and high dry matter content. Lower chroma (C*) and lower redness (Hº). Higher glucose and fructose and generally higher acid content. Greater antioxidants (i.e. AsA) and AC	Concentration effect by either limitation of water uptake or enhanced import of solutes into the fruit. Artefact of colour measurements due to smaller fruits and lower distance between achenes. Changes in respiratory metabolism and trade-off in resource allocation -----> Osmotic adjustment -----> Up-regulation of invertase levels. *In situ* synthesis of antioxidants thereby limiting the amount of ROS -----> Integrated redox system between sugars, antioxidants and stress hormones (i.e ABA)
Leaves	Increased sugar and AsA content	Reduced sugar transport from leaves to fruits mediated by inhibition of stomata opening and better transpiration control. *In situ* synthesis of antioxidants (i.e. AsA)
Runners	Lower runners biomass	Trade off between vegetative growth and propagation (survival strategy)
Roots	Restricted root growth	Adaptation to water availability and possible association with hormone signalling under drought stress

[1]The observed changes caused by DI were in all cases genotype-dependent

As compared to strawberry fruits, little information was available which described changes in sugar and acid concentrations in blackcurrant fruits due to different pre-harvest conditions (*viz.* genotype, growing location, degree of ripeness, etc.). Hence the initial approach within this thesis was

to assess the variations in the biochemical profile of blackcurrant berries from a wide range of cultivars (Exp. 4.1 and 4.3) as well as to investigate changes during ripening (Exp. 4.2 and 4.3) or postharvest storage (Exp. 4.4). Experiments were repeated with berries from subsequent years and different growing locations with the aim to also examine changes due to locations and different harvest years rather than only genotypic differences. Accordingly, the work presented in Chapter 4, represented an attempt to explore the genotypic variation in the major taste- and health-related compounds from a wide range of UK-grown fresh blackcurrant berries (Exp. 4.1). Wide variations in the biochemical profile of the target analytes examined were observed and thus, it is clear that the differences in blackcurrant cultivars play an important role in determining fruit composition. Sugar concentrations in blackcurrants were greater than that observed for strawberry fruits, as was also observed for the monosaccharide/disaccharide ratio. Although any comparison between different species should be made with some caution (Davies *et al.* 1996), blackcurrant berries may have a different or additional mode of sugar transport that accounts for their high loading, as was also reported by Davies *et al.* (1996) when comparing grapes and other species. Besides, differences in invertase levels and enzyme activity between berries may account for the greater m/d ratios encountered in these fruits. Comparison in the sugar accumulation patterns of both blackcurrant and strawberry fruits during ripening may lead to a better understanding of sugar metabolism in soft fruits.

Increasing evidence demonstrates the negative correlation between intake of naturally rich products in antioxidants, such as blackcurrants, and the incidence of certain cancers and chronic diseases (Appendix D). Blackcurrant berries were found to be an exceptional source of health-promoting compounds (i.e. AsA, anthocyanins) and agreed well with others (Koeppen and Hermann, 1977; Viola *et al.*, 2000; Walker *et al.*, 2006). Concentrations for both AsA and anthocyanins (Chapter 4; Table 4.3 and Figure 4.3) were over 5-fold greater than that found for strawberry fruits. The application of chemometric techniques was demonstrated to be a suitable tool to compare and assess the variability between the biochemical profiles of different blackcurrant genotypes and maturities at harvest. Indeed, chemometric data analysis revealed that the determination of health-related compounds alone was more suitable to discriminate between different genotypes than using other major components such as citric acid and non-structural carbohydrates (section 4.5.1.4). Differences in cultivars having high AsA or anthocyanin concentrations were especially emphasized by using principal component analysis. Among other agronomical traits, current blackcurrant breeding programmes are focused on the selection of genotypes with either high anthocyanin and/or ascorbic acid content (Brennan, 2005; Walker *et al.*, 2006). Therefore, given the results from Exp. 4.1, it is

assumed that if both biochemical traits are considered individually, current breeding strategies may tend to increase the existent variability between blackcurrant fruits from different genotypes. Taken as a whole, the results from initial experiments (Exp. 4.1) may help better to inform breeding programmes in the selection of appropriate cultivars based on their biochemical profile. Besides, chemometric techniques could perhaps be employed as an additional tool to breeding but based on biochemistry rather than genomics.

Succeeding experiments (Exp. 4.2 and 4.3), showed that both taste- and health-related compounds not only change between genotypes but drastically change during the last stages of berry ripening, with differences in the maturity of harvested berries representing the main source of variation in the composition of fruits. Sugar and acid concentrations increased and declined, respectively, as fruit ripened and agreed well with earlier studies for other berries (Wang *et al.*, 2003; Rubinskiene *et al.*, 2006). Greater monosaccharide content in fully or over ripe fruits was most probably attributed to greater invertase levels in mature fruit and hence hydrolysing sucrose into glucose and fructose which is commonly associated with increase respiratory metabolism.. For most cultivars, AsA and malic concentrations were greater in berries prior to reaching the fully ripe stage. Changes in AsA concentrations during ripening have been earlier associated with ascorbinoxidase levels within the fruit, which tend to be lower at initial ripening stages (Rubinskiene *et al.*, 2006). Others have shown that the AsA accumulation in blackcurrant berries differs between cultivars (Viola *et al.*, 2000) and happens for the period of fruit expansion via the L-galactose pathway and low rates of turnover (Hancock *et al.*, 2007). In contrast to strawberry fruits (Sweetman *et al.*, 2009), blackcurrant berries went through a noticeable decrease in malic acid content during ripening thereby highlighting the importance of this metabolite in becoming available for catabolism through different pathways during the ripening process (Sweetman *et al.*, 2009). It is also noteworthy that within the relatively short window of commercial maturity, there were also large variations in the concentrations of blackcurrant health-promoting compounds rather than ascorbate. For instance, individual anthocyanin concentration increased gradually as fruit ripened for all the cultivars investigated which was in agreement with earlier works on blackcurrants (Rubinskiene *et al.*, 2006) and other berry fruits (Carbone *et al.*, 2009). Differences in anthocyanin accumulation pattern between different cultivars were related, beyond genotypic distinctions, to differences in the ripening period for each cultivar and hence influenced by agroclimatic conditions during the same period. Indeed, soil and water status, light incidence and temperature are

known to affect anthocyanin concentrations in several berries (Terry *et al.*, 2007; Wang *et al.*, 2009; Zheng *et al.*, 2009; Crespo *et al.*, 2010).

Blackcurrants are mainly consumed as frozen or thermally processed products (Brennan, 2005); however with consumers increasingly aware of the health-promoting properties of these berries, the market for fresh blackcurrants is likely to grow. As for many soft fruits, the quality of blackcurrants declines dramatically after harvest if storage conditions are not optimal. Nonetheless, little information exists regarding the effect of postharvest practices on the quality and nutritional value of blackcurrants. Hence Exp.4.4, studied the temporal variations in blackcurrant taste- and health-related compounds during storage at different temperatures. When berries were harvested at the ER stage, they never reached the same taste-related composition or nutritional quality of those harvested at the FR stage regardless of storage conditions and time. Greater temperatures resulted in higher respiration rates which in turn led to lower sugar and organic acid (i.e. malic acid) concentrations. However, other mechanisms rather than just simple respiratory metabolism were most probably responsible to the decrease in sugar content during blackcurrant storage. For instance, sugars in their activated form are common substrates in *de novo* synthesis of anthocyanins and other antioxidants. In accordance with that mentioned earlier for strawberry fruits, it is also possible that there is an association between sugars and antioxidants, as an integrated system quenching ROS and contributing to stress tolerance, in this particular case, to greater temperatures. No other studies thus far, have detailed the effect that storage temperatures, ranging from 1 to 20ºC have on blackcurrant composition, and hence, results from the postharvest experiment (Exp. 4.4) may provide suitable information on the storage conditions required to maximize the quality of blackcurrant berries specially addressing individual requirements for each of the different blackcurrant-based products available in the market. As an example, lower glucose concentrations may be a desirable attribute for the production of blackcurrant beverages since this sugar is the preferred substrate for the Maillard reactions that occur during conventional juice processing (Boccorh *et al.*, 1998). Accordingly, storage of FR harvested berries at lower temperatures may be desired by limiting the rise in glucose concentration during storage. Future work should mimic the experiments presented herein but in subsequent growing seasons to assess the interaction of climactic conditions, genotype and maturity stage on the postharvest behaviour of blackcurrant berries.

Chapter 3 and Chapter 4 helped to better understand the biochemical composition of both blackcurrant and strawberry fruits thereby identifying potential quality markers that could be incorporated into the prototype biosensor array for improved soft fruit quality control (QC). Biosensors for food application have been continuously developed over the last decade (Miertus *et al.*, 1998;

Palmisano *et al.*, 2000; Arif *et al.*, 2002; Jawaheer *et al.*, 2003; Lupu *et al.*, 2004; Alonso Lomillo *et al.*, 2005; Terry *et al.*, 2005; Abayomi *et al.*, 2006; Abayomi and Terry, 2007), yet, most of these (apart from Abayomi and Terry's work) have not been commercialized due, in part, to the high variability and complexity of the food or fruit juice matrices. Accordingly, the different blackcurrant and strawberry trials conducted during this thesis also aided at: first, generating a wide range of samples for future validation of the prototype biosensor and, secondly, understanding the relationship between biosensor response when applied to real samples. To date, the refractometer which measures total soluble solids (TSS) is the only hand-held instrument currently used by industry to routinely assess 'compounds' related to organoleptic quality for a wide range of fruits. Results presented in this thesis (Chapter 3, 4 and 6; Appendix A) agreed with those reported elsewhere (Pérez *et al.*, 1997) and showed that TSS however does not correlate well with sugar content in both blackcurrant and strawberry fruits and hence highlighted the limitations that current QC techniques have on determining both blackcurrant and strawberry quality.

Both blackcurrant and strawberry fruits are among the richest sources of antioxidants, especially anthocyanins (Aaby *et al.*, 2007; Terry *et al.*, 2007) and other phenolic-type compounds as well as ascorbic acid (Viola *et al.*, 2000; Walker *et al.*, 2006; Tulipani *et al.*, 2008). Many antioxidants exhibit inherent electroactivity, acting as reductants in solutions (Kilmartin *et al.*, 2001) and may lead to overestimated signals when other target analytes (*viz*. sugars and acids) need to be determined electrochemically. Although acting as possible interferences, it is certain the role that dietary antioxidants play in maintaining an optimum oxidative balance within the body, and hence, it is not surprising that the analysis of antioxidants or antioxidant capacity (AC) in different foodstuffs and beverages has become a very active area of research (Prior *et al.*, 2005). Accordingly, Chapter 5 aimed, firstly, to study the electrochemical behaviour of natural antioxidants and blackcurrant and strawberry fruit juices on screen-printed carbon electrodes and secondly to assess the relationship between the electrochemical signals and the composition of the fruit juices with particular emphasis to their antioxidant capacity. For all biosensor experiments, screen-printing technology was chosen given that this technique offers the possibility for mass production and can allow a certain degree of miniaturisation (Alonso-Lomillo *et al.*, 2006). Both freshly squeezed berry juices contained a characteristic pool of antioxidants which when investigated electrochemically led to a specific voltammetric profile for each of the samples investigated. Regardless of the cultivar or degree of maturity, blackcurrant juices were richer sources of antioxidants than strawberry juices and generally presented a more complex electrochemical profile which agreed well with the literature (Aaby *et al.*,

2004; Terry *et al.*, 2007; Giné Bordonaba and Terry, 2008; Piljac-Zegarac *et al.*, 2008). Partitioning of the different samples revealed that anthocyanins were for both berry juices the major contributor to the antioxidant capacity of the fruits, followed by other phenolic-type compounds and ascorbate, respectively. In Chapter 5, a new electrochemical approach was also discussed in the context of developing a disposable sensor to measure antioxidant capacity in berry juices. No other studies have investigated the applicability of screen-printed electrodes using a similar approach to that described herein (Blasco *et al.*, 2007). The proposed sensor allowed for fast and easy discrimination of the main class of antioxidants present in both berry types but more importantly was able to discriminate between samples based on their antioxidant capacity as measured by standardised assays (*viz.* FRAP and Folin-Ciocalteu). Sensor cumulative responses at formal potentials of 500 (Q_{500}) and 1000 mV (Q_{1000}) vs. Ag/AgCl correlated well with the AC of the fruits as well as with anthocyanin and ascorbate concentrations in the juices. The proposed methodology would accomplish most of the criteria recommend for the development of standardised AC assays described by Prior *et al.* (2005) and would be of biological relevance since electrochemical techniques use the same principle to that exhibited by antioxidants in real biological systems. Besides, the use of screen-printed disposable sensors would overcome the major drawback of electrochemical methods: deactivation of the electrode, after single measurements, due to the formation of a polymeric film produced by the coupling of electrogenerated phenoxy radical.

After investigating the electrochemical behaviour of soft fruit juices on screen-printed electrodes, it was demonstrated, for the first time, that a GOx-based biosensor could be used to measure glucose in strawberry fruits and therefore provide growers and retailers with a promising alternative to TSS thus improving quality control (Chapter 6). In this work, especial emphasis was given to the experimental design methodology for the optimisation of several variables that directly affect biosensor performance. Hence, pH of the buffer/electrolyte solution, enzyme loading and applied potential were optimised by means of a central composite design (2^3) as described elsewhere (Alonso-Lomillo *et al.*, 2006). After optimisation LOD and LOQ values, equivalent to 0.01 and 0.03 mg glucose per g of fresh fruit, were in the range of those reported by others (Jawaheer *et al.*, 2003; Abayomi, 2007). Analysis time was reduced by 40-fold as compared to conventional HPLC, and therefore, the GOx-based biosensor could be used for screening of large data sets in breeding programmes with a comparable accuracy to that obtained by HPLC (r^2 =0.73). Under the operational conditions chosen, the GOx-based biosensor acted interference-free and had a comparable performance to that reported by others (Jawaheer *et al.*, 2003; Lupu *et al.*, 2004), even though no studies thus far have validated the

performance of similar sensors with a range of samples (i.e. different cultivars) as the ones described in this work. Indeed, total phenolics and antioxidant capacities of the fruits were measured by means of the already established Folin-Ciocalteu and FRAP assays, respectively. Both assays were extensively discussed in Chapter 5 (section 5.3.3) and may give an overall indication of the concentration of electrochemical active compounds present in the different cvs. analysed (Prior *et al.*, 2005; Blasco *et al.*, 2007). No correlation was found between the GOx-based biosensor signal and either TP, FRAP or even AsA values. In this context, several strategies were undertaken to limit the amount of interferences. Firstly, the screen-printed carbon electrodes were mediated with MaB+ based on previous studies (Abayomi *et al.*, 2006; Abayomi and Terry, 2007) and relying on the known versatility of this mediator towards both oxygenase and dehydrogenase enzyme formats (Vasilescu *et al.*, 2003). Mediators are used in biosensor technology to replace O_2 as an electron acceptor and allow biosensor performance at much lower operating potentials, hence limiting possible interferences cause by other electrochemically active species found in many food matrices (Terry *et al.*, 2005). Secondly, by performing a 1 to 20 dilution of the strawberry homogenate the background signal given by the samples was diminished considerably. A similar approach was undertaken by others (Lupu *et al.*, 2004; Abayomi *et al.*, 2006; Abayomi and Terry, 2007) when designing biosensors for the detection of target analytes in wine or onions. The proposed prototype biosensor would enhance the relevance of the analysis carried by measuring specific analytes which are key indicators of strawberry quality and consumer acceptability.

Chapter 7, aimed at adding functionality to the prototype biosensor by bolting on addition capabilities to measure simultaneously other target analytes not only in strawberry but also blackcurrant fruits and hence represent a competitive advantage *vs.* standard routine quality control. Indeed, results from Chapter 7 showed the possibility of developing a common format for the fabrication of a screen-printed electrode array to measure simultaneously different markers of strawberry and blackcurrant fruit quality. Rather than mediators, platinised carbon electrodes were chosen for the construction of the prototype biosensor array. At the specific applied potential (+0.3 V vs. Ag/AgCl), H_2O_2, a product from the reaction between the enzymes and the target analytes, is indirectly measured after being catalytically oxidized at a metalized carbon electrode (Newman *et al.*, 2005). From an economical and practical point of view, glucose, ascorbic and malic acid were selected as appropriate markers for strawberry and blackcurrant quality and hence incorporated into the sensor array. The choice of quality markers was in agreement with Jawaheer *et al.* (2003) but incorporated malic acid due to the importance of this acid in the ripening process of blackcurrants (Chapter 4:

section 4.5.2.1) and other fruits (Arif *et al.*, 2002). Both the GOx-based and ascorbate sensors showed their ability to measure simultaneously specific compounds with enough precision at an applied potential of +0.3 V vs. Ag/AgCl. The performance of the glucose sensor was considerably improved when the enzyme was immobilised onto a pectin matrix and agreed well with findings by others (Jawaheer *et al.*, 2003). Given the high concentration of AsA present in the samples, especially blackcurrants, elimination of this compound by enzymatic inactivation was satisfactorily performed to improve the performance of the GOx-based sensor. Due to the quasi constant ratio between the different sugars in both berries, the information given by the GOx-based sensor could be used to determine not only glucose ($r^2 = 0.77$ when comparing biosensor signal with HPLC) concentrations but total sugar ($r^2 = 0.73$) and even theoretical sweetness index ($r^2 = 0.81$) which, in agreement with earlier chapters (Chapter 6), could therefore provide breeders, growers and retailers with a promising alternative to TSS thus improving quality control. Besides, since sweetness in berry fruits is intimately related to consumer acceptability (Keutgen and Pawelzik, 2008), the signal given by the biosensor could be used to discriminate between fruits tentatively according to consumer preference. This said, future experiments should compare the information given by the sensor with perceived sweetness by an expert taste-panel rather than only using the actual biochemical composition of the fruits. Combining the information given by the GOx-based and AsA sensor is a first attempt to determine the balance between sugars and acids within the fruit thereby becoming a powerful tool to discriminate between fruits based on their optimum maturity and appropriate eating quality. Preliminary experiments also showed that the same electrode format could be adapted to measure malic acid concentrations in both berry juices and agreed with the findings by Arif *et al.* (2002). Nevertheless, future studies should investigate alternatives to improve the stability of this type of sensor as well as to reduce the associate costs of fabrication attributed to the cost-prohibitive nature of the malic enzyme.

To conclude, this research project represents an important step in improving quality and quality control for soft fruits. Blackcurrant and strawberries were the target crops given their importance for the process and fresh soft fruit industry, respectively. Manipulating the taste- and health-related composition of the fruits was possible by means of different pre-harvest and postharvest strategies. Deficit irrigation in strawberry fruits seems to be a potential alternative to attain fruits with increase quality and nutritional value. Besides, this research has demonstrated for the first time the possibility of replacing the standard QC techniques ubiquitously employed by the soft industry for determining sugar and acid concentrations (*viz.* TSS and TTA) with a more rapid and 'fit for purpose' method using

amperometric-based biosensors. The unity of the different sensors proposed in Chapter 7 should ease the eventual fabrication and operation of the final hand held device. Given the considerable differences between both berry types investigated in this work, it is also likely that results would apply or at least be transferrable to other soft fruits. Introduction of multianalyte biosensor platforms would transform QC in the soft fruit industry by enabling rapid and *in situ* analysis of soft fruit quality traits intimately related to consumer acceptance.

8.3 General conclusions

The project objectives were set out in Chapter 1, Section 1.2.2. A brief summary of the conclusions of the project in terms of the objectives is shown below.

- *To increase the knowledge of the biochemical composition in strawberry and blackcurrant berries especially focusing on both taste- and health-related compounds (viz. sugar and organic acids ratio).*

The biochemical composition of both blackcurrant and strawberry fruits was investigated in detail in several experiments (Chapter 3 and Chapter 4). Methods for determination of both taste- (*viz*. sugars and organic acids) and health-related compounds (*viz*. ascorbate, ellagic acid, anthocyanins, antioxidants) were developed, optimised and validated with a range of samples (Chapter 3, Chapter 4 and Appendix A2). Most of the biochemical components significantly differed between genotypes, as fruit ripens or as a result of different pre-harvest and postharvest strategies. In strawberries, for instance, deficit irrigation and related strategies led, for certain cultivars, to marked changes in sugar and acid metabolism resulting in fruits with increase sugar/acid ratios. In blackcurrants, the same sugar/acid was observed to slightly increase as fruit ripened and changes were also noticed during postharvest storage or when comparing fruits grown at different locations or years. Postharvest storage at greater temperatures generally led to greater concentration in the health-related composition (i.e. anthocyanins) of blackcurrant fruits.

- *To assess genotypic differences in quality traits among different UK-grown blackcurrant and strawberry fruits.*

Different strawberry (> 15) and blackcurrant (> 20) genotypes were investigated through this research. Chemometric data analysis was used as an exploratory tool to assess the existing variability in the composition of fruits from different genotypes. Differences in minor components (*viz*. AsA, anthocyanins) rather than sugars or acids accounted for most of the variability between the samples (Chapter 4). The composition of UK-grown strawberry fruits grown under different conditions was also compared to that of Swiss or Spanish fruits through different collaborative trials (Chapter 3; section 3.5.1).

- *To develop of a prototype multianalyte-sensor device which uses a thick-film screen-printed biosensor array for real-time in-situ objective determination of the principal sugars and organic acid content in soft fruit as a means of improving QC.*

Glucose, ascorbic and malic acid were selected as appropriate markers for strawberry and blackcurrant quality and hence incorporated into the sensor array. A common sensor format combined with the proposed sample preparation described in this work allowed for rapid and simultaneous determination of the target analytes. The proposed multianalyte sensor would enable the replacement of standard QC techniques ubiquitously employed by the soft industry in a more targeted approach to discriminate between fruits based on taste and consumer acceptance.

- *To explore the application of screen-printed biosensor technologies for determining health-related compounds increasingly demanded by consumers.*

Disposable screen-printed carbon electrodes were investigated for the development of rapid sensor to measure antioxidant capacity in berry juices. The proposed sensor allowed for fast and easy discrimination of the main class of antioxidants present in both berry types but more importantly was able to discriminate between blackcurrant and strawberry samples from different cultivars and/or degrees of maturity based on their antioxidant capacity and individual antioxidants as measured by standardised assays.

- *Provide the UK soft fruit industry with a sustainable competitive advantage. Allow growers and breeders to improve productivity and reduce economical losses by enabling them to self-test fruit, enabling harvest time optimisation and improvements in postharvest management.*

The constructed prototype sensor array could be used to determine not only glucose concentrations but total sugar and a theoretical sweetness index and therefore provide breeders, growers and retailers with a competitive alternative to TSS thus improving quality control. Besides, it was demonstrated that the proposed biosensor could be used by growers and breeders to discriminate between fruits according to consumer preference. Similarly, combining the information given by the GOx-based and AsA sensor may be a first attempt to determine the balance and acids within the fruit thereby becoming a powerful tool to discriminate between fruits based on their optimum maturity and appropriate eating quality.

CHAPTER 9

LITERATURE CITED

9.0 CHAPTER NINE

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APPENDIX A

UNDERSTANDING THE RELATIONSHIP BETWEEN TOTAL SOLUBLE SOLIDS AND SUGAR CONTENT IN DIFFERENT FRUITS

Appendix A

Understanding the relationship between total soluble solids and sugar content in different fruits

A.1 Introduction

The taste of many fruits and vegetables (FAV) is based, in part, on the ratio between concentration of sugars and acids. In certain fruits and vegetables sugar content may account for *ca*. 80 % of the total soluble components and hence often used as an indicator of fruit ripeness (Perez *et al.*, 1997) and overall fruit quality (**Table A.1**). Sugar are intimately related to perceived sweetness and hence to consumer acceptability of many fruits (Azondanlou *et al.*, 2003; Keutgen and Pawelzik, 2008).

The analytical methodologies used for the analysis of sugars in foodstuffs have evolved drastically over the past years and are still in constant development. Chromatographic techniques including both HPLC and GC have replaced earlier methods based on traditional wet-chemistry and enzymatic assays. Indeed the HPLC technique is the official AOAC International method for routine analysis of sugars (AOAC, 1995) and several studies have focused on development of HPLC methods for sugar analysis in different fruits (Perez *et al.*, 1997; Davis *et al.*, 2007; Terry *et al.*, 2007; Downes and Terry, 2010). Refractive index detection (RID) is commonly the detection system of choice based on the principle that a light beam is refracted differently depending on the substance to which it goes through it. However, most of these methods are costly, time-consuming and require extensive sample preparation, making them unsuitable for being routinely applied by growers or industry as standard quality control procedures (Terry *et al.*, 2005). Instead, most of the fruit industry still relies on total soluble solids (TSS) measurements, generally expressed as ºBrix and measured using a portable hand-held refractometer, due to the believed relationship between sugar content and TSS values. The principle behind this type of instrument is similar to that of RID, however, due to no sample preparation steps, samples that are high in salt, mainly sodium or potassium chloride, or free amino acid content may give overestimated results (Chope *et al.*, 2006). In these lines, the present study aimed

to study the relationship between actual sugar content and TSS values for different fruits (*viz.* blackcurrant, litchi, mango and strawberry) and further to discuss whether or not TSS values may be a valuable tool for standard quality control procedures in the fruit industry.

Table A.1: Sugar concentrations in mango, litchi blackcurrant and strawberry fruits (mg g^{-1} FW) from different cultivars.

	Sucrose	Glucose	Fructose	Ref
Mango (cv.)				
Mahajanaka	29.1	2.2	30.5	Saranwong *et al.* (2004)
Alphonso	0.2	3.8	6.1	Yashoda *et al.* (2006)
Malgova	10.12	17.75	33.23	Thanaraj *et al.* (2009)
Willard	8.9	18.7	22.2	
Kamutha Colomban	11.7	2.2	22.6	
Ampalavi	17.3	7.4	38.9	
Vellai Colomban	18.5	2.1	25.5	
Litchi (cv.)				
Feizixiao	142 (total sugar concentrations)			Wang *et al.* (2007)
Nuomici	170 (total sugar concentrations)			
Mauritius	35	17.5	18.2	Somboonkaew and Terry, 2010
Blackcurrant				
Mean of 17 UK-grown cvs.	14.3	51.7	61.6	Chapter 4; section 4.5.1.1
Mortti	9.8	24.0	32.0	Zheng *et al.* (2009)
Ola	9.8	24.0	33.0	
Melalahti	8.2	28.2	28.0	
Strawberry (cv.)				
Mean of 5 cvs grown under FI conditions	24.9	17.0	18.5	Chapter 3; section 3.4.3
Elsanta (2005-2007)	19.0	19.2	20.8	Chapter 3; section 3.5.4
Antea	13.4	13.1	15.5	Crespo *et al.*, 2010
Asia	16.0	17.0	18.7	
Clery	12.5	16.6	18.4	
Matis	6.8	16.0	18.1	

A.2 Materials and methods

A.2.1 Reagents

Sugar and organic acid standards including glucose, fructose and sucrose and ascorbic, citric and malic acid were of analytical grade and purchased from Sigma-Aldrich unless otherwise stated.

A.2.2 Plant materials

Blackcurrant (*Ribes nigrum* L.), litchi (*Litchi chinensis* Sonn.), mango (*Magnifera indica* L.) and strawberry (*Fragaria* x *ananassa* Duch.) fruits from different origins and submitted to different postharvest conditions were used in this study. The above-mentioned fruits were selected based on their known differences in actual sugar concentrations (**Table A.1**) and availability in the laboratory.

A.2.3 TSS measurements and sample preparation

The weight of fruits was recorded and TSS, expressed as ºBrix, was measured for each sample using a digital Palette PR-32 alpha refractometer (Atago, Japan). Subsequently fruits were individually snap frozen in liquid nitrogen and stored briefly at -40°C before being freeze-dried in an Edwards Modulyo freeze drier (W. Sussex, UK) for variable lengths depending on the nature of the fruit at 0.15 mBar. Lyophilized samples were then ground in a pestle and mortar, weighed and returned to the freezer until use.

A.2.4 Extraction and quantification of sugars

Sugars were extracted using 62.5% (v/v) aqueous methanol as the extraction solvent and following the methodology described elsewhere (Chapter 3; section 3.3.4; Terry *et al.* 2007). Sugar content in the different fruit extracts was determined using an Agilent 1200 series HPLC binary pump system (Agilent, Berks, UK), equipped with an Agilent refractive index detector (RID) G1362A. Extracts (20 μL) were diluted (1:10), and injected into a Rezex RCM monosaccharide Ca^{+} column of 300 mm x 7.8 mm diameter, 8 μm particle size (Phenomenex, CA; Part no. 00H-0130-K0) with a Carbo-Ca^{2+} guard column of 4 mm x 3 mm diameter (Phenomenex,; Part no. AJ0-4493). The mobile phase used was HPLC grade water at a flow rate of 0.6 mL min^{-1} (Terry *et al.*, 2007). Temperature of the optical unit in the detector was set up at 35ºC. The presence and abundance of fructose, glucose and sucrose were automatically calculated by comparing sample peak area to standards (0.025-2.5 mg mL^{-1}) using ChemStation Rev. B.02.01.

A.2.5 Study of possible interferences on TSS measurements

Standard solutions of sugars and organic acids, as well as mixtures, were prepared at different concentrations ranging from 0 to 100 mg ml^{-1} and TSS from the resulting solutions measured using the same digital Palette PR-32 alpha refractometer as described earlier. Linear calibration curves ($y = ax + b$) were generated for each compound and *a* values used to estimate a calculated TSS value (TSSc).

A.3 Results and discussion

A.3.1 Sugar concentrations of different fruits

Sucrose, glucose and fructose were the main sugars detected in all the fruits (**Figure A1**) although the proportion of each sugar considerably varied among different fruits and agreed well with the information given in **Table A.1**. In mango fruits the proportion of each sugar strongly differed among genotypes. Fructose was the main sugar found in mango, followed by sucrose and generally little or trace amounts of glucose. Litchi on the other hand, had greater sucrose concentrations and a quasi one to one ratio between glucose and fructose. Similar findings were observed for blackcurrant and strawberry fruits with glucose and fructose concentrations being comparable. Sucrose concentration in strawberry fruits was nearly equal to glucose and fructose, but strongly influenced by differences among cultivar, whereas blackcurrant fruits had generally a higher monosaccharide/disaccharide ratio. The similarities and differences in the sugar profile from the different samples investigated is shown in **Figure A.1**. Although any comparison between sugar concentration between different species should be made with some caution (Davies *et al.*, 1996), it is possible that for instance blackcurrant fruits had a different or additional mode of sugar transport than strawberries accounting for not only sugar differences but differences in the monosaccharide/disaccharide ratio. Besides, differences in invertase levels and enzyme activity among different fruits may be responsible for differences in the m/d ratio. In these lines, invertase levels in litchi fruit are likely to be much lower than those encountered in blackcurrants.

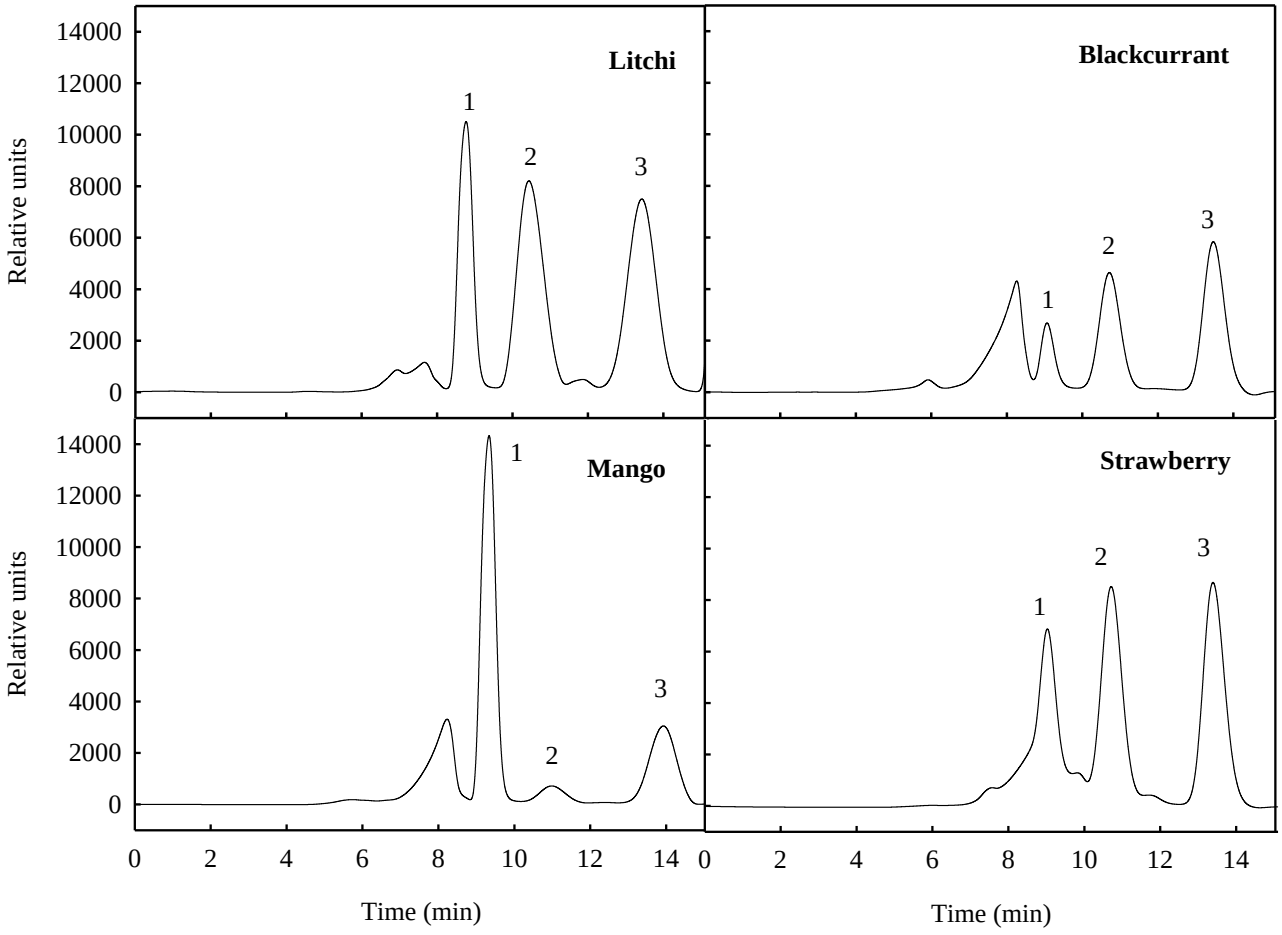


Figure A.1: Chromatographic profile of main sugars found in litchi (cv. Mauritius), mango (cv. Willard), blackcurrant (cv. Ben Dorain) and strawberry (cv. Sabrosa)

A.3.2 Relationship between sugar content and TSS values

Even though sugars account for ca. 80 % of the total soluble solids found in most of the samples described in this work (Pérez *et al.*, 1997; Yashoda *et al.*, 2006; Thanaraj *et al.*, 2009), TSS values, expressed as ºBrix, did not correlate well with sugar concentrations, either individual or total, for any of the samples investigated. For instance, in mango samples from different cultivars TSS values did not correlate with sugar concentrations on a dry (r^2 = 0.08; Table A.2) or fresh (r^2 = 0.12) weight basis and agreed well with that found in the literature (Thanaraj *et al.*, 2009).

Table A.2: Total sugar concentrations (mg g^{-1} DW) and TSS values for mango fruits from different cultivars.

Mango cvs.		Total Sugars (mg g-1 DW)	TSS
Willard	**I**	503.67	15.9
	II	584.16	14.7
	III	549.58	17.4
Karutha Colomban	**I**	656.23	17.2
	II	605.33	20.5
	III	621.77	16.4
Malgova	**I**	548.30	16.2
	II	569.38	13
	III	572.39	14.8

Similar findings were observed for litchi fruits (data not shown; Somboonkaew and Terry, 2008) and strawberry fruits (**Figure A.2**).

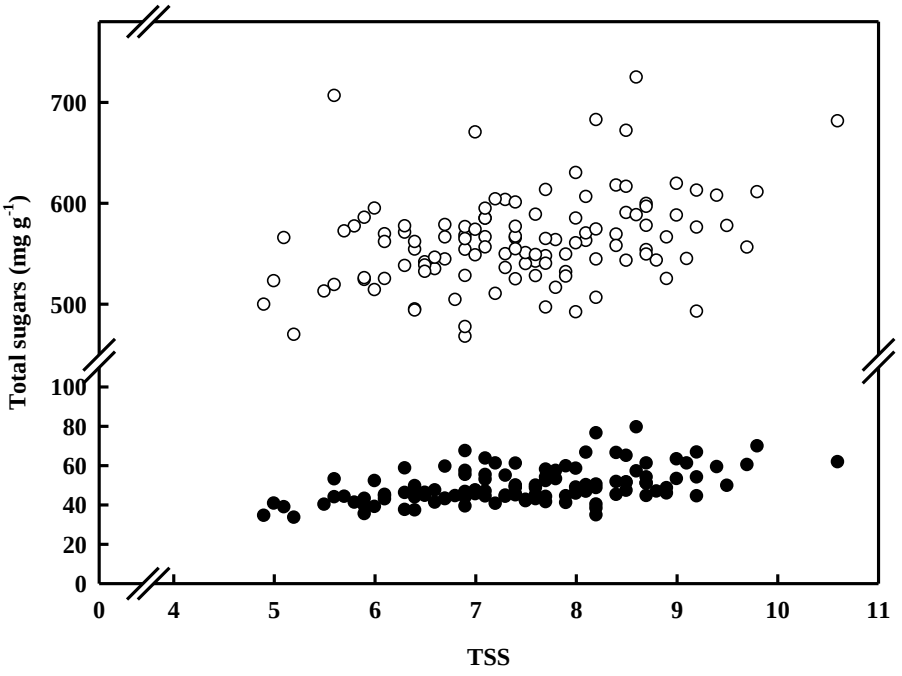


Figure A.2: Correlation between TSS and total sugar concentrations both on a dry and fresh weight basis of strawberry fruits cv. Sabrosa.

To further understand the relationship between actual sugar concentrations and TSS values the influence of each major sugar and acid on TSS values, measured by a hand held refractometer, was

investigated. The refractive properties of either glucose, fructose or sucrose on solution were very similar. For instance, 50 mg ml^{-1} of any of the above-mentioned sugars on solution resulted on TSS values of 5 ºBrix (**Figure A.3**).

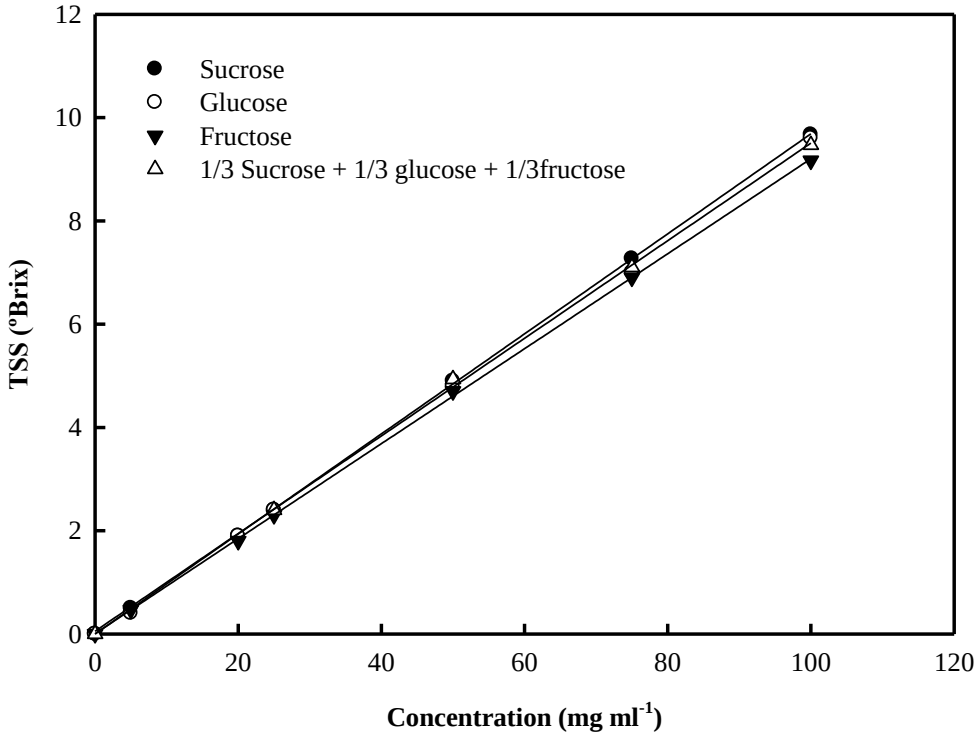


Figure A.3: Linear relationships between sugar concentrations ranging from 0 to 100 mg ml^{-1} and TSS values.

Thereby, a theoretical TSS value (TSSc) could be calculated assuming that sugars represent most of the soluble solids in the samples investigated (Pérez *et al.*, 1997) and given the linear relationships described earlier (**Figure A.3**):

$$TSSc = 0.097*Sucrose + 0.095*Glucose + 0.092*Fructose$$

Calculated TSS values were then compared against real TSS measurements for each of the strawberry samples investigated. As expected, both values were poorly correlated (**Figure A.4**) given that other compounds commonly present in any of the fruits investigated may have refractive properties and hence account for part of the TSS readings (Pérez *et al.*, 1997; Chope *et al.*, 2006).

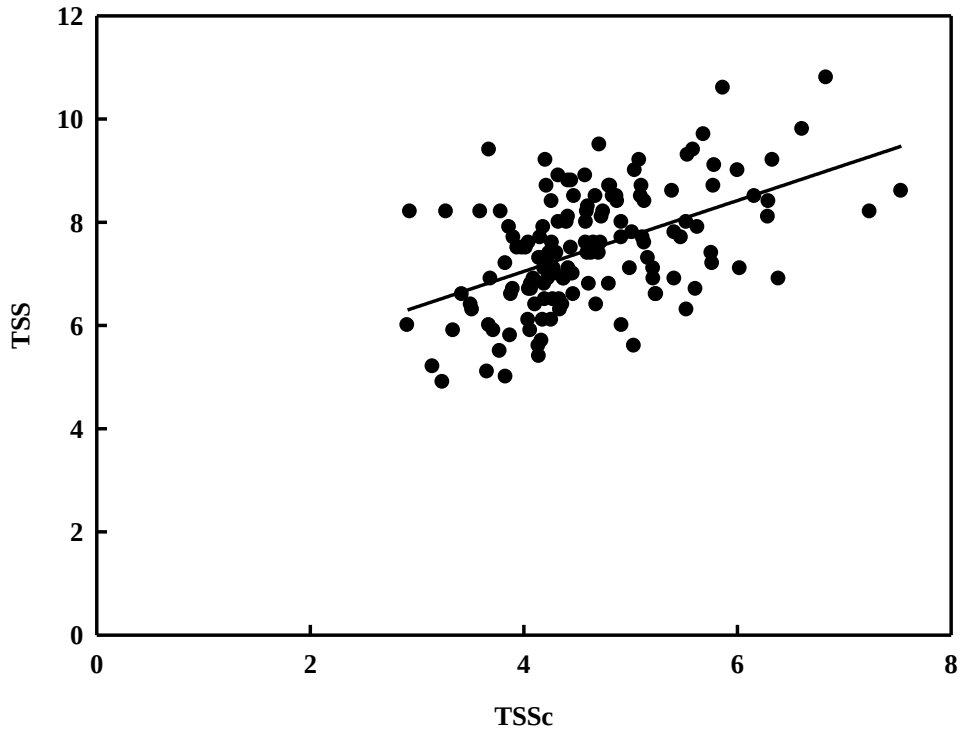


Figure A.4: Relationship between experimental TSS and calculated TSS values.

Accordingly, the influence of major organic acids such as citric and malic on the response of the hand held refractometer was investigated following the same procedure than that described for sugar compounds.

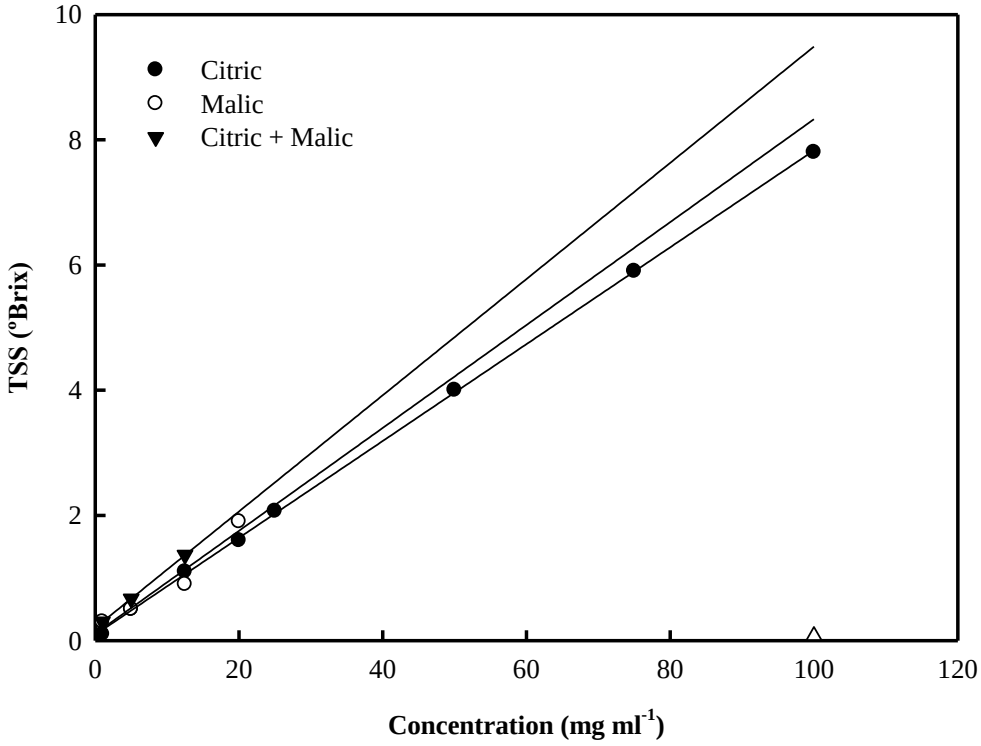


Figure A.5: Linear relationships between organic acid (citric, malic or combinations of both) and TSS values.

Finally, TSSc values recalculated considering the influence of each organic acid.

$$TSSc_2 = 0.097*Sucrose + 0.095*Glucose + 0.092*Fructose + 0.079*Citric + 0.095* Malic$$

Although the relationship between TSS and the newly calculated TSSc values ($TSSc_2$) was significantly improved, there was still a poor correlation between both parameters for all fruit types, indicating that other compounds rather than sugars and organic acid were responsible for the TSS experimental values obtained in this study. Blackcurrant fruits were the samples showing a better correlation between TSS and TSSc ($r^2 = 0.52$; **Figure A.6**).

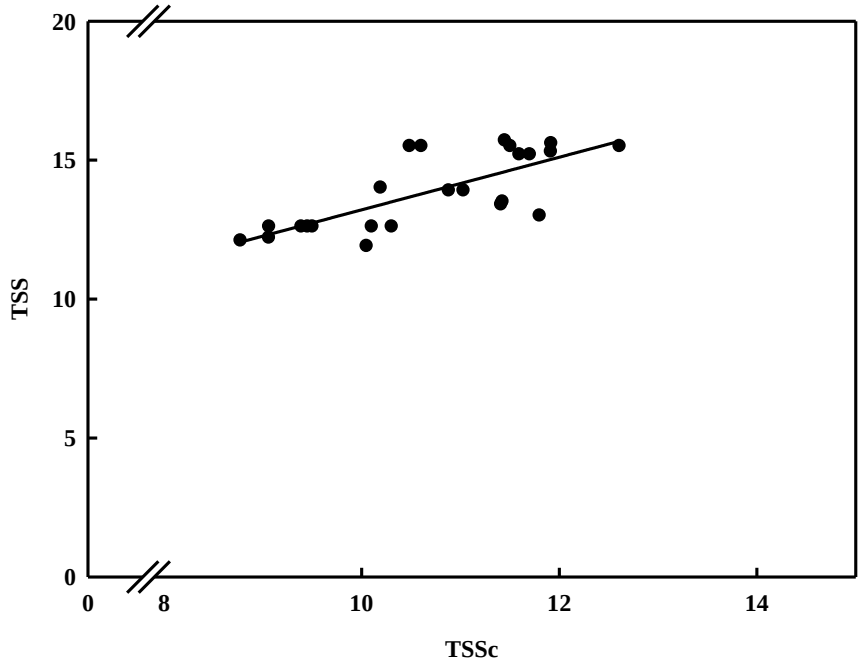


Figure A.6: Relationship between experimental TSS and calculated TSS values (TSSc2)

A.4 Conclusions

There was a poor correlation between TSS values and actual sugar or total sugar and total acid concentrations for all the fruits investigated. Other compounds rather than sugars and organic acids acids are responsible for the refractive properties of fruit juices when analysed by means of a hand held refractometer.

APPENDIX B

A NEW ACETONITRILE-FREE MOBILE PHASE FOR HPLC-DAD DETERMINATION OF ANTHOCYANINS IN BLACKCURRANT AND STRAWBERRY FRUITS:

A COMPARISON AND VALIDATION STUDY

Appendix B

A new acetonitrile-free mobile phase for HPLC-DAD determination of individual anthocyanins in blackcurrant and strawberry fruits: a comparison and validation study

B1. Introduction

Quality of berries, including strawberry and blackcurrant fruits, is partially characterised by traits such as colour and taste. Among the compounds responsible for the characteristic colour of both blackcurrant and strawberry fruits are the so called polyphenolic compounds, specifically anthocyanins; the type of polyphenols which are quantitatively most important (Lopes da Silva *et al.*, 2007). Anthocyanins are among the most important fruit pigments visible to the human eye, and in the particular case of blackcurrant and strawberry fruits are responsible for their purple and red colour at maturity, respectively. The anthocyanidins are the basic constituent of anthocyanins consisting of an aromatic ring (C_6) bonded to an heterocyclic ring (C_3) containing oxygen and bonded with a third aromatic ring (C_6) (Castañeda-Ovando *et al.*, 2009) (**Figure B1**). The differences between anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars attached to the molecule, the position of these sugars and the nature and number of aliphatic or aromatic acids attached to the sugars (**Figure B1**). To date more than 500 anthocyanins made up of 23 different anthocyanidins have been reported. This said, just six are commonly present in fruits, vegetables (FAV) and indeed flowers (*viz*. cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin) (**Figure B1**). Berries, including strawberries and blackcurrants, are undoubtedly one of the richest sources of anthocyanins with reported concentrations up to 180 and 400 μg g^{-1} FW, respectively (Terry *et al.*, 2007; Chapter 4, section 4.5.1.2). Increasing interest in the anthocyanin concentration of several FAV is, however, not only due to their potential as natural colorants but also to their associated health-promoting properties. Despite the enormous variability between individuals, estimated consumption of anthocyanins in the United States is in the order of 12.5 mg day^{-1} per person (Wu et al., 2006; McGhie & Walton, 2007), whereas consumption in Europe has been estimated as *ca*. 20 mg day^{-1} per person (Andersen, 2002) or even greater in certain European countries such as Finland with values of 82 mg day^{-1} per person (Wu *et al.*, 2006). Nowadays, and regardless of the poor bioavailability of these

compounds (Nielsen *et al.*, 2003) increasing evidence suggest that anthocyanins, as natural antioxidants, exert anticarcinogenic, antiinflamatory, vasoprotective and anti-obesity effects when tested *in vitro* or *in vivo* (Prior, 2004).

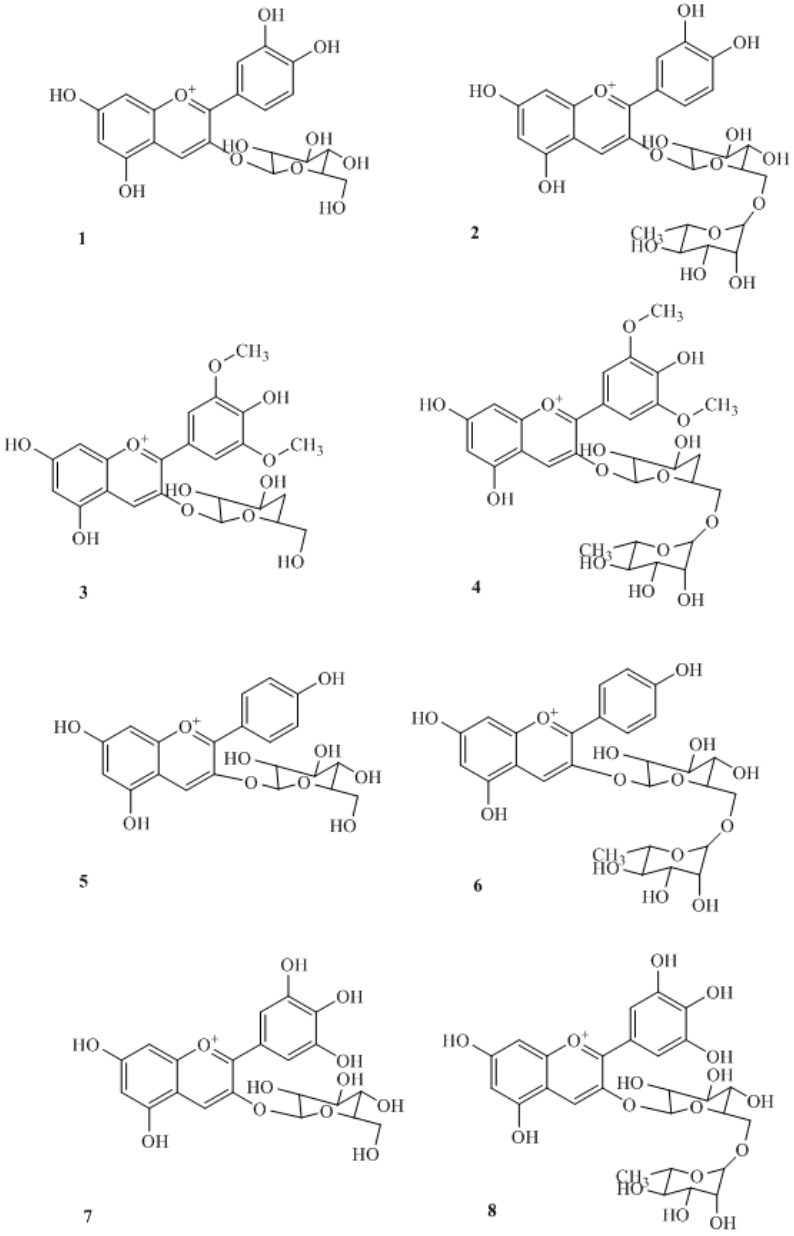


Figure B1: Chemical structure of main anthocyanins found in blackcurrant and strawberry fruits: (1) Cyanidin-3-glucoside; (2) Cyanidin-3-rutinoside; (3) Malvidin-3-glucoside; (4) Malvidin-3-rutinoside; (5) Pelargonidin-3-glucoside; (6) Pelargonidin-3-rutinoside; (7) Delphinidin-3-glucoside; (8) Delphinidin-3-rutinoside

As a result of the suggested health-related properties of anthocyanins, the number of studies trying to extract, identify and quantify these compounds from different food sources has steadily increased in recent years. Generally, these polar pigments are extracted using aqueous mixtures of either ethanol, methanol or acetone (Kahkonen *et al.*, 2001); this said, discrepancies exist in whether acetone or methanol based solvents are more efficient for the extraction of these compounds from different FAV (Chapter 4; section 4.5.2.1). Separation and quantification of anthocyanins is generally achieved on reversed phase HPLC coupled to different detection systems (Lee *et al.*, 2008) (*viz.* Photo diode array (PDA), mass spectrometry (MS)) and use mostly acetonitrile as the mobile phase of choice due to its elution strength, low viscosity, and good miscibility with water (**Table B1**). Nevertheless, the scientific community recently faced a worldwide shortage of this solvent due, in part, to the global economic downturn and consequently the recent near collapse of the automotive industry from which acetonitrile is obtained as a by-product in the production of acrylonitrile. Restrictions over the use of acetonitrile and consequent dramatic price increases resulted in standard elution of anthocyanins being problematic as no viable replacement was really available.

In this context, the aim of the present study was to clarify, using a simple single-step extraction procedure, whether methanol- or acetone-based solvents were more efficient for extracting anthocyanins from strawberry fruits, and further to study the potential of a methanol-based mobile phase that could readily replace the widely used acetonitrile for the chromatographic separation and identification of main anthocyanins in both blackcurrant and strawberry fruits. Hence, different HPLC operating conditions including two different columns and two different mobile phases were tested. The anthocyanin content of different strawberry and blackcurrant cultivars was examined with the most suitable method.

Table B1: Examples of extraction and quantification methods found in the literature, from 2001 onwards, for the identification of main anthocyanins in both blackcurrant and strawberry fruits.

Berry type	Extraction (solvent and conditions)	Quantification (method used)	Stationary phase	Mobile phase	Anthocyanin concentration reported	Reference
Strawberry (cvs. Earliglow and Kent/ Allstar and Honeoye)	Twice with 80% acetone + evaporated at 35 ºC and dissolved in acidified water (3% FA) passed through a C18 Sep-pak cartridge (Waters) to separate sugars and acids. Anthocyanins recovered with acidified methanol (3% FA)	HPLC-DAD (510 nm)*	Novapak C18 (150 x 3.9 mm, 4 μm) (Water)	A: 2.5% FA* in H_20 B: ACN*	**cya-3-gluc:** 4.5-34 μg g^{-1} FW, 561–1153 μg g^{-1} DW, 18.5-45.8 μg g^{-1} juice **pg-3-gluc**: 275.6-847.1 μg g^{-1}FW, 2124-3669 μg g^{-1}DW, 291.3-945.1 μg g^{-1} juice **cya-3-gluc-succinate:** 1.31-5.62 μg g^{-1}FW, 37-75μg g^{-1} DW, 6.1-19.8 μg /g juice, **pg-3-rut:** 24.7-50.9 μg g^{-1} juice **pg-3-gluc-succinate:** 45.6-95.9 μg g^{-1} FW, 323-681 μg g^{-1} DW, 62.2-224.5 μg g^{-1} juice according to cultivar and soil supplement or growing temperature	Wang & Zheng (2001) Wang & Lin (2003) Wang *et al.* (2003)
Strawberry (cvs. Camarosa, Dorit, Chandler, Osmanli and their hybrids)	10 ml acetone/water (1:4, v/v) + 0.1 ml TFA, 1 h	HPLC-DAD	Nucleosil C18 analytical column (150 x 4.6mm, 5 μm) (Supelco)	A: 2.5% FA in H_20 B: 2.5% FA in ACN	**cya-3-gluc:** 4.5-17.5 μg g^{-1} FW **pg-3-gluc:** 53.3-441.0 μg g^{-1} FW	Kosar *et al.* (2004)
Strawberry (cvs. Honeoye, Jonsok and Polka)	4 x ethyl acetate + HCl 2M + methanol		Li ChroCART Purospher RP-18e (125 x 3mm, 5 μm) (Merck)	A: in 5% FA in H_20 B: ACN	**cya-3-gluc:** 22 μg g^{-1} FW **pg-3-gluc**: 248 μg g^{-1} FW **pg-3-rut:** 12 μg g^{-1} FW **pg-3-mlgluc**: 25 μg g^{-1} FW **pg-3-succinylgluc**: 45 μg g^{-1} FW	Määttä-Riihinen *et al.* (2004)
Strawberry (cvs. Senga Sengana, Dukat, Elkat, Selva, Elsanta and Kent)	1 ml HCl in 1 l methanol, 15 min	HPLC-DAD	Li ChroCART Purospher RP-18e (125 x 3mm, 5 μm) (Merck)	A: 4.5% FA B: 80% ACN and 20% solvent A	**cya-3-gluc**: 6.3-27.2 μg g^{-1} FW **pg-3-gluc**: 202.8-331.9 μg g^{-1} FW **pg-3-arab**: 2.3-8.4 μg g^{-1} FW	Skupien & Oszmianski (2004)
Blackcurrant (cv. Öjebyn)	three times with 70% aqueous acetone with 0.01 M HCl, (20 min + 2 x 10 min, 0-4°C)	HPLC-DAD	Hypersil ODS (60mm x 4.6mm, 3 μm) (Agilent)	A: 5% FA in H_2O (v/v) B: ACN	**delp-3-gluc:** 303-391 μg g^{-1}FW **delp-3-rut:** 808.69-1174.39 μg g^{-1} FW **cya-3-gluc:** 151.31-267.77 μg g^{-1}FW **cya-3-rut:** 844.69-947.24 μg g^{-1}FW according to the cultivation system	Anttonen & Karjalainen (2006)

Strawberry (cv. not specified)	methanol (0.1% HCl) and acetone: water (7:3, v/v)	HPLC-DAD, LC-ESI-MS* and LC ESI-MS/MS*	Symmetry C-18 (250 x 4.6nm, 5 μm) (Waters)	A: 1% FA in H_20 B: ACN	NQ	Seeram *et al.* (2006)
Strawberry (cv. Senga Sengana)	triple extraction with acetone and 10 min sonication	HPLC-DAD ESI-MS	Betasil C 18 (250 x 2.1 mm, 5 μm) (Thermo Hypersil)	A: acetic acid/ H_20 (2:98, v/v) B: acetic acid/ ACN/ H_20 (2:50:48, v/v/v)	NQ	Aaby *et al.* (2007a)
Strawberry (cv. Senga Sengana)	triple extraction with acetone and 10 min sonication	HPLC-DAD (500 nm),	Betasil C 18 (250 x 2.1 mm, 5 μm) (Thermo Hypersil)	A: phosphoric acid/acetic acid/ H_20 (1:10:89, v/v/v) B: ACN	**cya-3-gluc**: 19.0 μg g^{-1} FW **pg-3-gluc:** 400 μg g^{-1} FW **pg-3-rut:** 16.3 μg g^{-1} FW **5-carboxypyrano-pg-3-gluc:** 2.4 μg g^{-1} FW **pg-3-mlgluc:** 65.9 μg g^{-1} FW **pg-3-acetylgluc:** 3.9 μg g^{-1} FW	Aaby *et al.* (2007b)
Strawberry (cvs. Camarosa, Ventana, Aromas, Diamante and Medina)	methanol with 0.1% HCl	HPLC-DAD	C-18 Novapack (300 x 3.9, 5 μm) (Waters)	A: ACN/FA / H_20 (3:10:87) B: ACN/FA / H_20 (50:10:40)	**pg- der 1:** 0.18-1.0 μg g^{-1} FW **cya-3-gluc:** 0.64-5.30 μg g^{-1} FW **pg-3-gluc:** 73.69-165.66 μg g^{-1} FW **pg-3-rut:** 3.24-23.92 μg g^{-1} FW **pg derivative 2:** 0.18-1.30 μg g^{-1} FW **pg-acetylglucosid:** 0.55-3.62 μg g^{-1} FW according to the cultivar/system	Hernanz *et al.* (2007)
Blackcurrant and strawberry (cvs. not specified)	2 steps with 80% methanol 2% HCl and 1 step with 50 MeOH 1% HCl each 1 min at room temperature	HPLC-DAD	Li ChroCART Purospher RP-18e (125 x 3mm, 5 μm) (Merck)	A: 8.5% FA in H_20 B: ACN/MeOH* (85:15, v/v)	**cya (as sum of all derivatives):** 13-50 μg g^{-1} FW in strawberries and 993 μg g^{-1} FW in blackcurrants **pg (as sum of all derivatives):** 294-510 μg g^{-1} FW in strawberries and 1017 μg g^{-1} FW in blackcurrants	Koponen *et al.* (2007)
Strawberry (cvs. Eris, Oso Grande, Carisma, Tudnew and Camarosa)	MeOH 0.1% HCl, overnight at 3-5 °C Removing of sugar and non polar substances on a C-18 SepPak Vac 3 cc cartridge (Waters). Anthocyanins were recovered with MeOH:0.1%TFA (95:5)	HPLC-DAD (520 nm)	AQUA C18 (150 x 4.6, 5 μm) (Phenomenex)	A: 0.1% TFA* in H_20 B ACN	**cya-3-gluc**: 10-41 μg g^{-1} FW **pg-3-gluc**: 162-468 μg g^{-1} FW	Lopez da Silva *et al.* (2007)
Strawberry (cv. Elsanta)	MetOH: H_20:HCl (70:29.5:0.5; v/v/v) for 1.5 h at 35 ºC	HPLC-DAD (520 nm)	Alltech Allosphere ODS-1 (250 x 4.6 mm, 5 μm) (Alltech)	A: 1% phosphoric acid and 10% acetic acid (v/v) in H_20 B: ACN	**cya-3-gluc**: 2.15 μg g^{-1} FW **pg-gluc deriv**: 34.99 μg g^{-1} FE **pg-3-gluc**: 125.99 μg g^{-1} FW	Terry *et al.* (2007)

Blackcurrant (17 UK-grown cultivars)	MetOH: H_20:HCl (70:29.5:0.5; v/v/v) for 1.5 h at 35 ºC	HPLC-DAD (520 nm)	Alltech Allosphere ODS-1 (250 x 4.6 mm, 5 μm) (Alltech)	A: 1% phosphoric acid and 10% acetic acid (v/v) in H_20 B: ACN	**cya-3-gluc**: 68.81 μg g^{-1} FW **cya-3-rut**: 546.21 μg g^{-1} FW **dp-3-gluc**:133.06 μg g^{-1} FW **dp-3-rut**: 605.23 μg g^{-1} FW	Giné Bordonaba & Terry (2008)

NQ: no quantification; ACN: acetonitrile; MeOH: methanol; FA: formic acid; TFA: trifluoroacetic acid; HPLC: high perfomance liquid chromatography; DAD: diode array detector; LC: liquid chromatography; ESI: electospray Ionisation; MS: mass spectrometry; MS/MS: tandem mass spectrometry.

B.2 Material and Methods

B.2.1 Plant materials.

Strawberry fruits from three different cultivars (*viz*. Antea, Clery and Matis) with known differences in their anthocyanin profile (Crespo *et al.*, unpublished) were used for method comparison and validation with a previous method carried out on strawberry fruits (Terry *et al.*, 2007). Fruits were grown in Conthey, Switzerland (46°12' N, 7°18' E) using conventional agronomic practices. Blackcurrant berries from cultivars Ben Dorain, Ben Gairn and Ben Tirran were supplied by GlaxoSmithKline Plc. Bushes were grown in Norfolk, UK (52º39' N, 0º 54' E) under standard commercial practices (Rob A. Saunders, *pers. comm.*). All plant materials, at fully ripe stage, were snap-frozen in liquid nitrogen and stored briefly at -40°C before being freeze-dried in an Edwards Modulyo freeze drier (W. Sussex, UK) for 3 days at 0.015kPa. Lyophilized samples were then ground in a pestle and mortar, weighed and returned to the freezer until further analysis.

B.2.2 Anthocyanin extraction.

Individual anthocyanins were extracted as described elsewhere (Terry *et al.*, 2007; Giné Bordonaba & Terry, 2008) by mixing 150 mg of freeze-dried sample with 3 ml of 70% (v/v) methanol and 0.5% (v/v) HCl in HPLC-grade water. The choice of extraction solvent was based on recently published work (Chapter 4; section 4.5.1.2) and was compared to an acidified acetone aqueous extraction solvent (70% acetone (v/v) and 0.5% (v/v) HCl in HPLC-grade water) (Awika *et al.*, 2004; Antonnen and Karjalainen, 2006) using strawberries as a model fruit. The slurry obtained was held at 35ºC in a water bath with constant shaking for 1.5h; mixing the samples every 15 min. Finally, the flocculate obtained was filtered through a 0.2 μm Millex-GV syringe dri**v**en filter unit (Millipore Corporation, MA) and the clear extract analyzed by HPLC.

B.2.3 Extraction recovery.

The recovery values for the different anthocyanins naturally occurring in several berries (*viz*. delphinidin 3-O-glucoside (delp-3-glu), cyanidin 3-O-glucoside (cya-3-gluc), cyanidin 3-O-rutinoside (cya-3-rut), pelargonidin-3-glucoside (pg-3-glu), malvidin-3-glucoside (malv-3-gluc)) were investigated. Briefly, blackcurrant and strawberry extracts were prepared in triplicate, as described previously, and the anthocyanin content determined from the calibration curve obtained with standards. The same freeze-dried sample (in triplicate) was then spiked (standard addition) with known concentrations for each standard and extracted following the same procedure as described earlier. The

recovery (%) was calculated as the ratio [(anthocyanin concentration in the spiked extract – anthocyanin concentration naturally present in the extract)/(spiked anthocyanin concentration)]

B.2.4 HPLC measurements.

The anthocyanin profile of blackcurrant and strawberry fruits was determined using an Agilent 1200 series HPLC binary pump system (Agilent, Berks., UK) equipped with a Agilent 1200s DA G1315B/G1365B photodiode array with multiple wavelength detector. Strawberry diluted (1:5 v:v) extracts were injected (10 μL) into either a Zorbax Eclipse XDB-C18 column of 250 mm x 4.6 mm diameter, 5 μm particle size with an XDB-C18 guard column of 12.5 mm x 4.6 mm diameter (Stationary Phase (SP) 1) or an Alltech Allsphere ODS-1 column of 250 mm x 4.6 mm diameter, 5 μm particle size (Alltech; Part no. 778357) with an Alltech Allsphere ODS-1 guard column of 7.5 mm x 4.6 mm diameter (Part no. 96402) (SP 2) (Terry *et al.*, 2007). The different mobile phases (MP) tested consisted of 2% (v/v) acetic acid in HPLC-grade water (A) and 2% (v/v) trifluoroacetic in methanol (B) (MP1) or that previously described by Terry *et al.* (2007) (MP2); 1% (v/v) phosphoric acid (Acros Organics, Leics., UK) and 10% (v/v) acetic acid (Fischer Scientific, Leics., UK) in HPLC-grade water (A) and acetonitrile (B). The gradient conditions for MP1 was 0-10min, 2-20% B; 10-20min, 20-25% B; 20-25min, 25-35% B; 25-35min, 35-75% B. Flow rate was, in both cases, set up at 1 mL min^{-1} and the column temperature set at either 35ºC or 40ºC using an Agilent G1316A thermostated column compartment for SP1 and SP2, respectively. Temperature of the autosampler was set up at 4ºC using an Agilent G1330B cooled autosampler.

After comparison of the different HPLC operating conditions, validations studie were performed by means of the optimum combination of MP and SP described herein. In all cases, eluted anthocyanins from the different berry extracts (*viz*. blackcurrant and strawberry) were detected at 520 nm and the presence and quantity of each anthocyanin calculated by comparing peak area with standards delp-3-glu, cya-3-gluc, cya-3-rut, pg-3-glu and malvidin-3-glucoside (malv-3-gluc); Extrasynthèse, Lyon, France) using Agilent ChemiStation Rev. B.02.01 (Terry *et al.*, 2007; Chapter 4; section 4.3.3). Further validation of the method was performed by comparing total anthocyanins concentrations obtained by HPLC and those recorded by the pH differential method as described by Lee *et al.* (2008).

B.2.5 Lower limit of detection (LOD) and quantification (LOQ).

For each of the methods compared in the present study, LOD and LOQ values were calculated as the amount of each individual anthocyanin required to give a signal to noise ratio of 3 to 1 and 10 to 1, respectively (Muñoz *et al.*, 2008).

B.2.6 Data analysis.

All statistical analyses were carried out using Genstat for Windows Version 9.1.0.147 (VSN International Ltd., Herts., UK). Data were subjected to analysis of variance (ANOVA) tests. Least significant difference values (LSD; $P = 0.05$) were calculated for mean separation using critical values of t for two-tailed tests. Correlations between experimental variables were made using Spearman's Rank Correlations and, if required, presented as Spearman's Correlation Coefficient (r) and P value based on a two-tailed test. Unless otherwise stated, significant differences were $P < 0.05$.

B.3 Results and Discussion

B.3.1 Sample extraction.

The extraction of key analytes in complex matrices such as FAV is often regarded as one of the main problems when developing any analytical method. Anthocyanins are polar molecules, and hence the most common solvents used in their extraction are aqueous mixtures of ethanol, methanol or acetone (Kahkonen *et al.*, 2001). Acidification of the extraction solvent is a common step performed in order to maintain the anthocyanins in their flavylium form (Gómez Alonso *et al.*, 2007); by lowering the pH of the extraction solution the degradation of non-acylated anthocyanins is also prevented (Kong *et al.*, 2003). Recent studies done with blackcurrant berries (Chapter 4; section 4.5.1.2; Giné Bordonaba and Terry, 2008) demonstrated that within the solvents tested (*viz.* aqueous methanol or ethanol and water), 70 % (v/v) methanol resulted in greater total anthocyanins as compared to the rest of the solvents tested. This said, acetone was not assessed in the above-mentioned study and therefore it is difficult to state whether acetone or methanol may be more efficient in extracting these pigments from berries. In the present work, the methanol-based solvent used in earlier works (Terry *et al.*, 2007) was compared against 70% (v/v) acetone (Awika *et al.*, 2004; Antonnen and Karjalainen, 2006). Under the conditions imposed in this study, the methanol-based solvent extracted best the anthocyanins present in both blackcurrant and strawberry freeze-dried powders. Specifically, the methanol extract had at least 1.1-fold greater amounts of cya-3-gluc, pg-3-gluc and other pg-derivatives found in strawberry fruits (**Figure B2**). Similar finding were observed by Awika *et al.* (2004) who reported that acidified

methanol was twice as efficient at extracting anthocyanins from black sorgum than 70 % (v/v) aqueous acetone. As compared to other extraction methods (Table 1), which can be tedious and cumbersome, the procedure described herein was based on a simple stepped solid-liquid extraction, yet still achieved complete colour removal of the freeze-dried material. The calculated extraction recovery for each individual anthocyanin, after standard addition, ranged from 74 to 96% depending on the food matrix (*viz*. strawberry and blackcurrants). Specifically, malv-3-gluc (94%), pg-3-gluc and cya-3-gluc (86%) were extracted better if compared to dp-3-gluc (81%) which may be the result of the hydroxylation of the B ring. In addition, results indicated that the sugar moiety attached to the different anthocyanidins investigated may play a role in determining its solubility in the extraction solution and hence affecting the efficacy of the extraction since the recovery of cya-3-rut (74 or 77%) was lower than that of cya-3-gluc (86 and 83%) for strawberry and blackcurrant fruits, respectively.

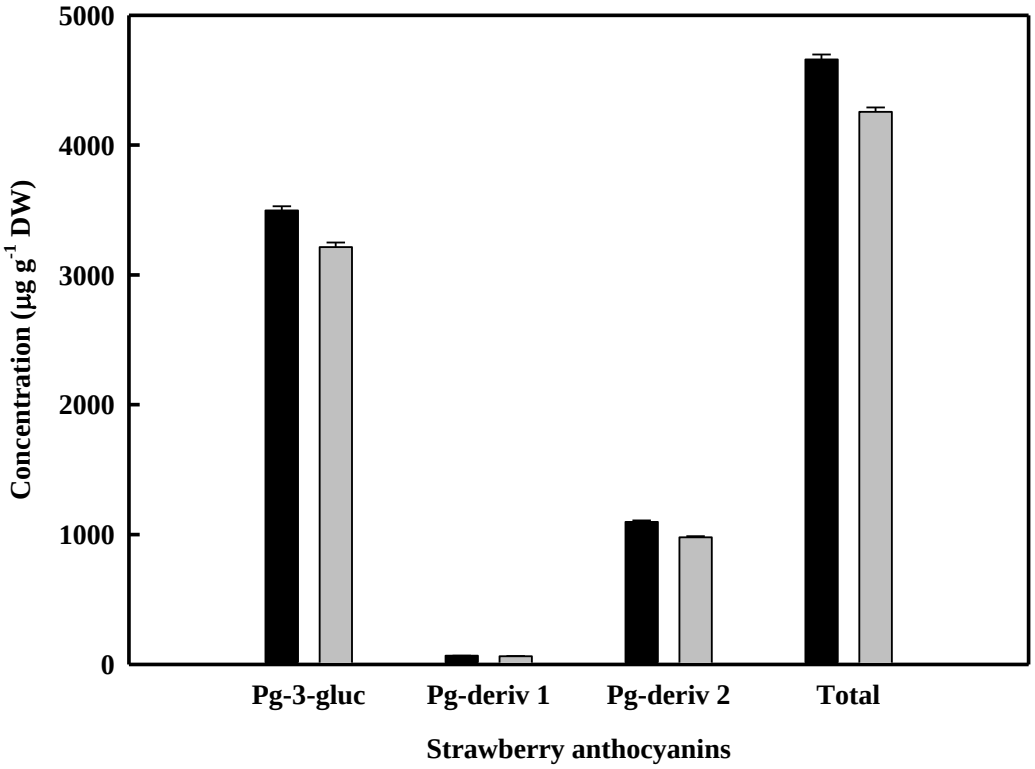


Figure B2: Anthocyanin concentration of strawberry samples extracted with either (▬) 70:29.5:0.5 (Methanol:H_2O:HCl; v/v) or (▬) 70:29.5:0.5 (Acetone:H_2O:HCl; v/v). Values are means for n = 3 ± SD.

B.3.2 HPLC method comparison.

As described earlier (Lee *et al.*, 2008), anthocyanins will respond differently when analysed under different HPLC conditions. In this study, the elution order of the individual anthocyanins was similar in all HPLC operating conditions tested with delp-3-gluc, being the first eluted anthocyanin followed by cya-3-gluc, cya-3-rut, pg-3-gluc and malv-3-gluc, respectively (**Figure B3**), but retention times depended on the different MP and SP considered. Overall, peaks were eluted earlier when using acetonitrile as the MP regardless of the column which may be the result of solvation effects and the differential ability of acetonitrile and methanol to influence hydrogen bonding between analytes and polar groups on the SP. The advantages of using ACN for the separation of fruit pigments have been reported extensively (Muñoz *et al.*, 2008) and it is undoubtedly the most common HPLC solvent used for separation and identification of anthocyanins in berries and other food and vegetables (**Table B1**). However, in the past year the scientific community has faced a worldwide shortage of this solvent, mainly caused by the near collapse of the industrial sectors where acetonitrile is commonly obtained as a by-product during the production of acrylonitrile, resulting in prices of this solvent soaring drastically. Even though the availability of this solvent seems now to be partially restored, the debate still exists on whether or not the continuous supply of acetonitrile is guaranteed and reliable. Both methanol and acetonitrile share many favourable properties for its use in reverse phase HPLC (*viz.* complete miscibility with water, low viscosity, excellent UV transmission and low chemical reactivity) (Ribeiro *et al.*, 2004). Hence, the replacement of acetonitrile by methanol, without affecting HPLC performance, could currently lead to significant cost reductions both in terms of solvent usage and disposal. Despite both organic solvents being toxic, some studies have shown the greater toxicity of acetonitrile as compared to methanol when tested on model organisms (Barahona-Gomariz *et al.*, 1994). In addition, the disposal of acetonitrile still requires special attention since its combustion results in the generation of highly toxic hydrogen cyanide (HCN) (Ribeiro *et al.*, 2004).

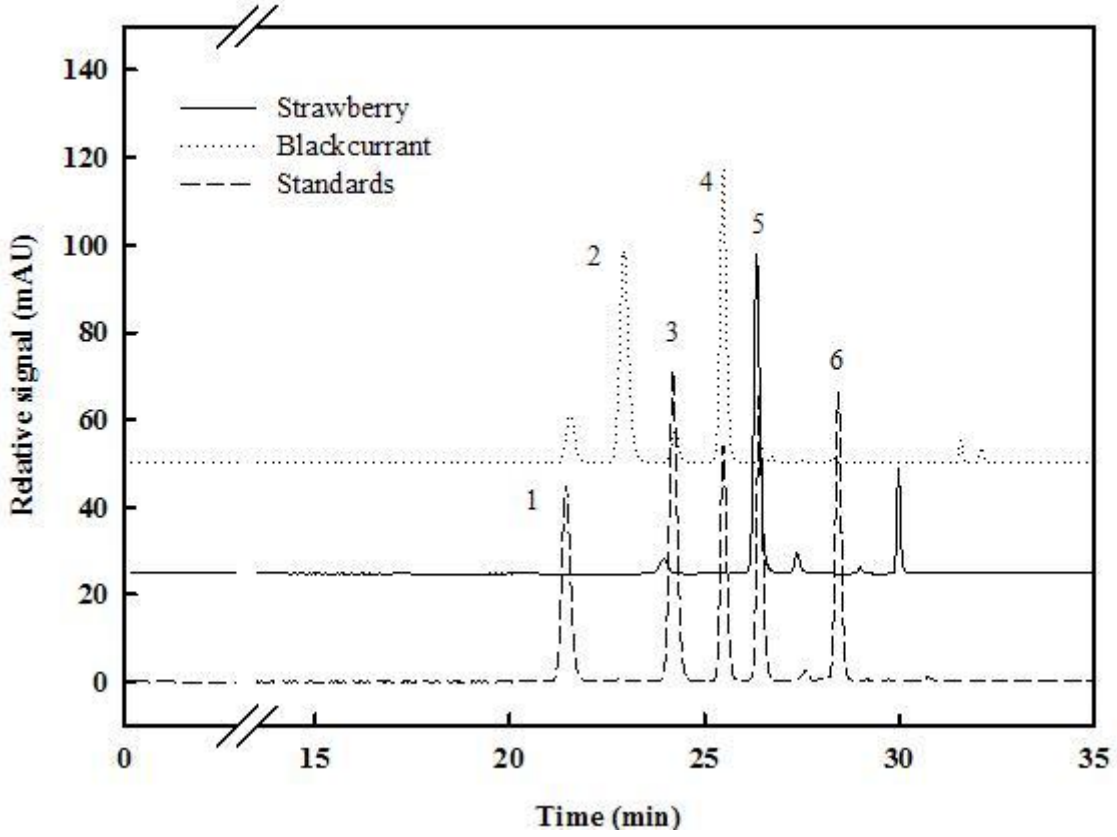


Figure B3: Chromatographic profile of anthocyanins found in blackcurrant and strawberry fruits detected with the methanol-based mobile phase and using a Zorbax Eclipse XDB-C18 column and anthocyanin external standards (viz. cya-3-gluc (peak 3), cya-3-rut (peak 4), dp-3-gluc (peak 1),dp-3-rut (peak 2), malv-3-gluc (peak 6) and pg-3-gluc (peak 5)).

From the different HPLC columns evaluated, SP1 (Zorbax Eclipse XDB-C18) showed generally better resolution, improved peak symmetry and better peak separation than that of SP2 (Alltech Allosphere ODS-1). Indeed, some minor anthocyanins, identified as pg-3-derivatives on the basis of their spectra (**Figure B4-b**), could not be eluted when combining SP2 and the methanol-based mobile phase. Among the properties of each column investigated herein, SP1 had lower pore diameter (7 nm) and higher ligand concentration (2.4 μmols m^{-2}) than that of SP2 (8 nm, 1.6 μmols m^{-2}) and hence accounting for the better peak resolution of this column. Gilroy *et al.* (2004) did a detailed comparison between different column selectivities, including those investigated in this study, and found that SP1 had higher hydrophobicity, lower hydrogen-bond acidity and greater column cation-exchange activity, among others, than that of SP2; this said, the costs of SP1 are nearly double than those of SP2.

It is noteworthy that all these characteristics may be crucial in determining the resolution of different anthocyanin peaks under the separation conditions used in this study. When comparing results from the different experimental conditions tested, results clearly indicated that differences existed in the response factor for each method resulting in discrepancies in the final amount of anthocyanins being detected (**Table B2**). Accordingly, recent work (Lee *et al.*, 2008) has already stated that absorbance not recorded at individual peaks λ_{max} and solvent effect from changing gradients would account for the variation of the response of individual anthocyanins to each method and analytical conditions used.

Table B2: Concentration of anthocyanins (*viz*. cyanidin-3-glucoside (cya-3-gluc), pelargonidin-3-glcuoside (pg-3-gluc), and pelargonidin derivatives (pg-deriv); µg g^{-1} DW) of three strawberry cultivars determined using either an acetonitrile- or methanol-based mobile phase (MP) and two different HPLC columns (Zorbax Eclipse XDB-C18 (SP1) or Alltech Allsphere ODS-1 (SP2)).

Cultivar	SP*	MP	cy-3-glc	pg-3-glc	pg-deriv 1	pg-deriv 2	Total
Antea	1	Methanol	114.3	2016.0	60.0	-	2190.3
	1	Acetonitrile	118.6	2474.5	74.5	-	2667.6
	2	Methanol	113.1	2257.2	0.0	-	2370.4
	2	Acetonitrile	116.0	1755.6	34.7	-	1906.2
Clery	1	Methanol	n.d.	3199.2	57.0	1005.2	4261.4
	1	Acetonitrile	n.d.	3544.9	66.1	1060.9	4672.0
	2	Methanol	n.d.	3211.1	0.0	958.3	4169.4
	2	Acetonitrile	n.d.	2779.0	50.4	648.7	3478.1
Matis	1	Methanol	90.8	1895.2	53.6	281.7	2321.3
	1	Acetonitrile	84.9	2013.3	57.4	285.5	2441.1
	2	Methanol	87.1	1868.3	0.0	237.9	2193.3
	2	Acetonitrile	108.2	1709.2	33.4	167.8	2018.5
LSD (P<0.05)			14.69	25.05	3.67	5.63	26.89

*SP1: Zorbax Eclipse XDB-C18 column; SP2: Alltech Allsphere ODS-1; - Anthocyanin non-detected

In the present study, cultivar was the main individual source of variation for each individual anthocyanin, followed by MP, and the SP but to a lesser extent. Moreover, there was a strong interaction between MP, SP and sample for each individual anthocyanin which highlighted the importance of considering not only the experimental conditions but also the type of sample when developing any HPLC method. Generally, the use of acetonitrile as a MP (MP2) resulted in greater amounts of anthocyanins detected, especially the major strawberry anthocyanin pg-3-gluc (1.12-fold greater pg-3-gluc concentrations were detected using acetonitrile as compared to methanol). This said, the accuracy and repeatability of the measurements, especially for minor anthocyanins, was comparable or even improved when using methanol as MP (MP1) (**Table B3**, **Table B4**), and in all cases values obtain with either the acetonitrile- or methanol-based mobile phases were well correlated ($r > 0.95$). The limit of detection (LOD) and limit of quantification (LOQ) for each of the studied anthocyanins was calculated as the amount of compound required to give a signal to noise ratio of 3:1 and 10:1, respectively (Muñoz *et al.*, 2008). When using SP1 with the methanol-based MP (MP1), LODs ranged from 0.25 to 1.01 µg ml^{-1} for the different anthocyanins tested whereas LOQs ranged from 0.83 to 3.36 µg ml^{-1} (Table 3). LOD and LOQ values were comparable among the different MP tested, and therefore the proposed methanol-based method described herein was proved to be sensitive enough to determine the anthocyanin composition of selected berries with reported concentrations ranging from 180 to 1350 µg g^{-1} FW (Clifford, 2000; Terry *et al.*, 2007; Chapter 4; section 4.5.1.2). A good correlation was also obtained when comparing total anthocyanin concentrations as determined by the methanol-based HPLC method and the standardised pH differential method ($r > 0.88$) and hence the method described herein had similar performance than that earlier reported by Lee *et al.* (2008) (data not shown).

The precision of the different methods used was further assessed by evaluating the stability of the retention times from several standard injections performed on alternate days (Muñoz *et al.*, 2008). The relative standard deviation (RSD) values from the different retention times were in all cases lower than 2% (data not shown).

Table B3: Performance parameters including limit of detection (LOD), limit of quantification (LOQ) and relative standard deviation (RSD) of the individual strawberry anthocyanins (*viz*. cyanidin-3-glucoside (cya-3-gluc), pelargonidin-3-glcuoside (pg-3-gluc), and pelargonidin derivatives (pg-deriv); µg g^{-1} DW) eluted under different HPLC mobile phases (acetonitrile- or methanol-based) and two stationary phases (Zorbax Eclipse XDB-C18 (SP1) or Alltech Allsphere ODS-1 (SP2))

·	LOD (µg ml^{-1})				LOQ (µg ml^{-1})				R.S.D (%)			
	Acetonitrile		Methanol		Acetonitrile		Methanol		Acetonitrile		Methanol	
	SP1	SP2	SP1	SP2	SP1	SP2	SP1	SP2	SP1	SP2	SP1	SP2
Cya-3-gluc	0.249	0.403	0.247	4.599	0.828	1.344	0.823	15.332	1.292	1.243	0.945	17.601
Pg-3-gluc	1.009	4.359	0.854	5.842	3.364	14.532	2.847	19.475	0.311	0.85	0.15	1.042
Pg-deriv 1	0.403	1.641	0.341	-	1.344	5.469	1.138	-	1.321	16.382	2.122	-
Pg-deriv 2	0.368	1.512	0.311	1.198	1.227	5.042	1.039	3.992	0.336	3.005	0.369	1.679

- Anthocyanin non-detected

Table B4: Parameters of the different calibration graphs (y = ax +b; s = standard error) used to quantify target standard anthocyanins (*viz.* cyanidin-3-glucoside (cya-3-gluc), cyanidin-3-rutinoside (cya-3-rut), pelargonidin-3-glcuoside (pg-3-gluc) and malvidin-3-glucoside (malv-3-gluc)) using different mobile phases (acetonitrile- or methanol-based) and HPLC columns (SP).

	SP*	MP	a	s_a	b	s_b^2	r^2
Cya-3-gluc	1	Methanol	3911.366	13.6501	-5.805	21.6669	1
	1	Acetronitrile	4010.349	27.6319	67.259	43.8602	0.9999
	2	Methanol	3852.975	8.3001	9.043	13.1749	1
	2	Acetonitrile	4454.798	2.6352	13.173	4.1829	1
Cya-3-rut	1	Methanol	2533.561	1.7632	-18.945	2.7988	1
	1	Acetronitrile	2606.504	8.1672	28.737	12.9639	1
	2	Methanol	1864.902	4.677	17.097	7.4238	1
	2	Acetonitrile	3066.913	15.70468	-26.945	24.9281	0.9999
Pg-3-gluc	1	Methanol	2990.629	1.9604	-24.041	3.1180	1
	1	Acetronitrile	2506.456	8.1584	54.226	12.9498	1
	2	Methanol	3252.496	1.9755	-22.991	3.1357	1
	2	Acetonitrile	3373.605	17.27515	-29.639	27.4209	0.9999
Malv-3-gluc	1	Methanol	2805.757	10.2227	-77.937	16.2266	0.9999
	1	Acetronitrile	2838.974	12.1479	32.523	19.2823	0.9999
	2	Methanol	2493.687	3.7328	-13.236	5.9251	1
	2	Acetonitrile	3000.941	118.7738	-41.617	188.5299	0.9953

* SP1: Zorbax Eclipse XDB-C18 column; SP2: Alltech Allsphere ODS-1.

B.3.3 Anthocyanin content in selected berries.

Differences in anthocyanin concentrations among cultivars have been extensively reported for both strawberry and blackcurrant berries (Lopes da Silva *et al.*, 2007; Chapter 3; section 3.5.4). Using the methanol-based method, anthocyanin content in the different strawberry samples investigated ranged from 2.19 to 4.26 mg g^{-1} DW and was strongly affected by cultivar (**Table B2**). Similarly, significant differences were observed for the different blackcurrant cultivars with on average, concentrations 3.5-fold higher than those reported for strawberry fruits (**Table B5**). Values for total anthocyanins in strawberry fruits ranging from 150-350 µg g^{-1} FW (*ca*. 1.50 - 3.50 mg g^{-1} DW) were described earlier by Clifford (2000).

Table B5: Concentration of main anthocyanins (*viz*. delphinidin-3-glucoside (dp-3-gluc), delphinidin-3-rutinoside (dp-3-rut), cyanidin-3-glucoside (cya-3-gluc), cyanidin-3-rutinoside (cya-3-rut); µg g^{-1} DW) identified in blackcurrant from different cultivars using the methanol-based mobile phase (MP1) and Zorbax Eclipse XDB-C18 as stationary phase (SP1).

Cultivar	Dp-3-gluc	Dp-3-rut	Cya-3-gluc	Cya-3-rut	U1[1]	U2[1]	U3[1]	U4[1]	Total
Ben Dorain	0.939	4.310	0.490	4.850	0.059	0	0	0	10.649
Ben Gairn	2.286	5.217	0.974	4.817	0.065	0.056	0	0	13.416
Ben Tirran	1.442	7.064	0.573	6.519	0.097	0.071	0.220	139.96	16.126
Mean	1.556	5.530	0.679	5.396	0.074	-	-	-	13.397

[1]Unidentified peaks

Strawberry fruits from cv. Clery had as much as 2-fold higher anthocyanin content on a DW basis than that of cv. Antea and Matis. Similarly, blackcurrant berries from cv. Ben Tirran had 1.2- and 1.5-fold greater anthocyanin content than cvs. Ben Gairn and Ben Dorain, respectively. In addition to variations in the total anthocyanin content, differences also existed in the anthocyanin profile of diverse cultivars for both strawberry and blackcurrant fruits. For instance, strawberries from cv. Clery were characterised by the absence of cya-3-gluc whereas strawberries from cv. Antea did not have one of the pg-derivatives identified in the rest of strawberry fruits. Likewise, the chromatograms from blackcurrant berries of cv. Ben Tirran showed eight clear peaks as compared to seven and six for fruits from cultivars Ben Gairn and Ben Dorain, respectively. Most of the studies conducted thus far investigating the anthocyanin profile of blackcurrant berries, have concluded that four major anthocyanins (*viz*. cya-3-gluc, cya-3-rut, delp-3-gluc and delp-3-rut) constitute almost 90% of the total anthocyanin content of blackcurrants (Häkkinen *et al.*, 1999; Manhita *et al.*, 2004; Rubinskiene *et al.*, 2005; Anttonen and Karjalainen, 2006; Jordheim *et al.*, 2007). Other anthocyanins including peonidin-3-rutinoside and malvidin-3-glucoside have also been detected (but in lesser amounts) in a number of studies conducted on blackcurrant berries (Frøytlog *et al.*, 1998; Slimestad and Soldheim, 2002). In the present study, peaks 5, 6, 7 and 8 (**Table B5**; **Figure B4**) for blackcurrant samples and peaks 3 and 4 from strawberry fruits were identified based on information provided by the characteristic UV-spectra of each peak and comparison with the literature. In the particular case of strawberry fruits, peaks 3 and 4 were identified as pg-3-derivatives on the basis of their spectra (**Figure B4-b**). The elution order of the pg-3-derivatives and their occurrence in strawberry fruits as reported by others (Aaby *et al.*, 2007; Hernanz *et al.*, 2007), strongly suggests peak 3 to be pg-3-rutinoside. Peaks 7 and 8 from blackcurrant berries were most probably peonidin-3-glucoside, peonidin-3-rutinoside, based on their UV-spectra and earlier works by others (Anttonen and Karjalainen, 2006; Jordheim *et al.*, 2007).

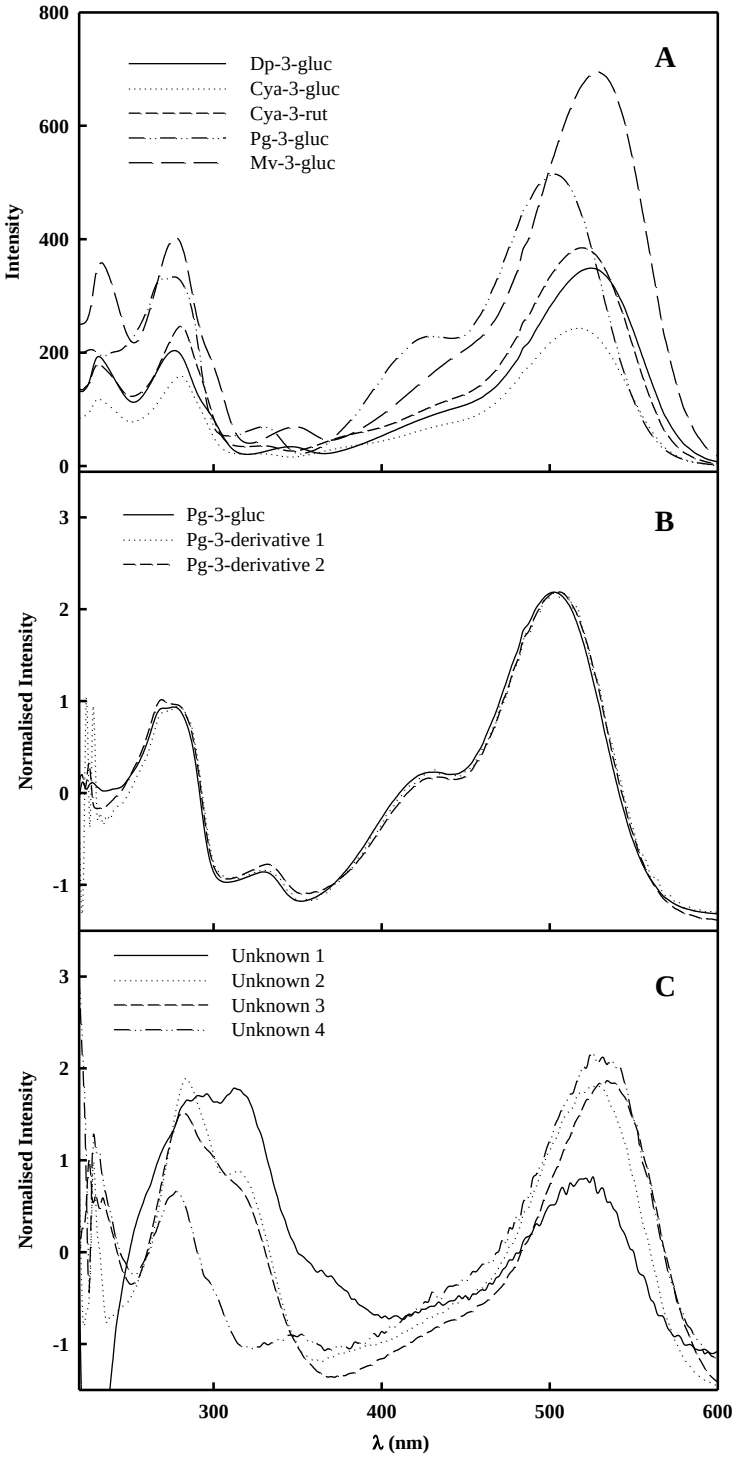


Figure B4: UV-spectra (**A**) of anthocyanin standards ((*viz*. cyanidin-3-glucoside (cya-3-gluc), cyanidin-3-rutinoside (cya-3-rut), pelargonidin-3-glcuoside (pg-3-gluc) and malvidin-3-glucoside (malv-3-gluc)), (**B**) of Pg-3-gluc and pelargonidin derivative anthocyanins from strawberry fruits and (**C**) of unknown anthocyanins from blackcurrant berries.

The anthocyanin composition of strawberry fruits has been the target of several studies (**Table B1**). Probably one of the most detailed studies on the anthocyanin profile of strawberry fruits is that conducted by Lopes da Silva *et al.* (2007) in which 25 different anthocyanins within five different strawberry cultivars were detected. Terry *et al.* (2007) identified (using just standard HPLC) only three major anthocyanins (*viz*. cya-3-gluc, pg-derivative and pg-3-gluc at concentrations of 2.16, 33.56 and 121.54 μg g^{-1} FW, respectively) in strawberry cv. Elsanta fruit grown under glasshouse and subjected to full or deficit irrigation. Indeed, in the same work the authors demonstrated that differences even existed in anthocyanin content between primary and secondary fruit from the same primary truss. In addition to fruit position on the cymose, the anthocyanin profile differs spatially with different tissues/locations in the fruit (Gil, Holcroft & Kader, 1997). For instance, Aaby *et al.* (2005) showed different anthocyanin profiles in receptacle tissue and achenes from two different strawberry cultivars (Totem and Puget Reliance) whereas Gil *et al.* (1997) reported the absence of cya-3-gluc in strawberry internal tissue (cv. Selva). Both cvs. Totem and Puget Reliance had pg-3-gluc as the main anthocyanin in the flesh whereas similar amounts of this anthocyanin and cya-3-gluc were detected in the achenes of both cultivars. Based on earlier findings and corroborated by the results from this study it is important to note the relevance of not only detailing extraction method, sample nature (freeze-dried or fresh), elution and stationary phase used, but also specifying cultivar, maturity at harvest, fruit position, tissue fraction (within the same fruit), and growing conditions when comparing anthocyanin concentrations from different berries.

B.4 Conclusions

Despite the differences in absolute concentrations between the different HPLC operating conditions tested, the methanol-based HPLC-DAD method proposed herein allows for proper elution and the accurate quantification of major anthocyanins in selected berries (*viz*. blackcurrants and strawberries) with comparable accuracy to that when using acetonitrile as a mobile phase. Concentrations of individual anthocyanins obtained with either the acetonitrile or methanol-based mobile phases were in all cases well correlated ($r > 0.95$) and, hence, given the recent shortage of acetonitrile, and the current prices of this solvent, the optimised and validated method described herein, especially in combination with the proposed simple extraction procedure, may be a feasible alternative for rapid screening of major anthocyanins in both strawberry and blackcurrant berries. Methanol-based

elution could thus not only be used as a fallback method if problems with acetonitrile supply reoccur, but also as a replacement in its own right.

APPENDIX C

STATISTICAL TABLES

APPENDIX C

Statistical tables

C.1. ANOVA tables for Chapter 3

Table C.1-C.40: Effect of deficit or full irrigation on the daily (C.1 = Day 1; C.40 = Day 40) water volume of the growing media of five different strawberry cultivars (Exp.3.1). Water treatments started when the majority of secondary strawberry fruits from the primary truss were at flower initiation stage.

Table C.1

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	283027.	70757.	8.43	<.001
Treatment	1	24288.	24288.	2.89	0.097
Cultivar.Treatment	4	61482.	15371.	1.83	0.142
Residual	40	335861.	8397.		
Total	49	704659.			

Table C.2

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	166973.	41743.	13.09	<.001
Treatment	1	14484.	14484.	4.54	0.039
Cultivar.Treatment	4	43495.	10874.	3.41	0.017
Residual	40	127530.	3188.		
Total	49	352482.			

Table C.3

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	108347.	27087.	8.47	<.001
Treatment	1	44940.	44940.	14.06	<.001
Cultivar.Treatment	4	106402.	26601.	8.32	<.001
Residual	40	127861.	3197.		
Total	49	387550.			

Table C.4

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	235943.	58986.	14.97	<.001
Treatment	1	64584.	64584.	16.39	<.001
Cultivar.Treatment	4	155369.	38842.	9.86	<.001
Residual	40	157589.	3940.		
Total	49	613485.			

Table C.5

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	117166.	29292.	3.08	0.027
Treatment	1	81043.	81043.	8.51	0.006
Cultivar.Treatment	4	127607.	31902.	3.35	0.019
Residual	40	380834.	9521.		
Total	49	706651.			

Table C.6

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	213622.	53406.	6.33	<.001
Treatment	1	162792.	162792.	19.30	<.001
Cultivar.Treatment	4	103293.	25823.	3.06	0.027
Residual	40	337320.	8433.		
Total	49	817027.			

Table C.7

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	481701.	120425.	14.95	<.001
Treatment	1	246964.	246964.	30.65	<.001
Cultivar.Treatment	4	309026.	77257.	9.59	<.001
Residual	40	322273.	8057.		
Total	49	1359964.			

Table C.8

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	380612.	95153.	10.28	<.001
Treatment	1	303421.	303421.	32.79	<.001
Cultivar.Treatment	4	375008.	93752.	10.13	<.001
Residual	40	370141.	9254.		
Total	49	1429182.			

Table C.9

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	490333.	122583.	13.77	<.001
Treatment	1	374459.	374459.	42.07	<.001
Cultivar.Treatment	4	366467.	91617.	10.29	<.001
Residual	40	356035.	8901.		
Total	49	1587293.			

Table C.10

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	369382.	92346.	8.78	<.001
Treatment	1	292919.	292919.	27.84	<.001
Cultivar.Treatment	4	296252.	74063.	7.04	<.001
Residual	40	420817.	10520.		
Total	49	1379369.			

Table C.11

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	525975.	131494.	8.53	<.001
Treatment	1	467738.	467738.	30.36	<.001
Cultivar.Treatment	4	420337.	105084.	6.82	<.001
Residual	40	616286.	15407.		
Total	49	2030336.			

Table C.12

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	761710.	190427.	11.66	<.001
Treatment	1	738112.	738112.	45.21	<.001
Cultivar.Treatment	4	456882.	114220.	7.00	<.001
Residual	40	653095.	16327.		
Total	49	2609799.			

Table C.13

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	958840.	239710.	17.17	<.001
Treatment	1	837995.	837995.	60.02	<.001
Cultivar.Treatment	4	268721.	67180.	4.81	0.003
Residual	40	558470.	13962.		
Total	49	2624025.			

Table C.14

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	733089.	183272.	14.51	<.001
Treatment	1	957174.	957174.	75.77	<.001
Cultivar.Treatment	4	269729.	67432.	5.34	0.002
Residual	40	505310.	12633.		
Total	49	2465303.			

Table C.15

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	729707.	182427.	15.16	<.001
Treatment	1	975246.	975246.	81.04	<.001
Cultivar.Treatment	4	353951.	88488.	7.35	<.001
Residual	40	481392.	12035.		
Total	49	2540297.			

Table C.16

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	186422.	46605.	9.84	<.001
Treatment	1	292460.	292460.	61.72	<.001
Cultivar.Treatment	4	114576.	28644.	6.05	<.001
Residual	40	189538.	4738.		
Total	49	782996.			

Table C.17

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	201971.	50493.	8.74	<.001
Treatment	1	319680.	319680.	55.36	<.001
Cultivar.Treatment	4	99991.	24998.	4.33	0.005
Residual	40	230978.	5774.		
Total	49	852620.			

Table C.18

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	145885.	36471.	4.27	0.006
Treatment	1	255470.	255470.	29.88	<.001
Cultivar.Treatment	4	108697.	27174.	3.18	0.023
Residual	40	341944.	8549.		
Total	49	851996.			

Table C.19

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	315183.	78796.	7.97	<.001
Treatment	1	579318.	579318.	58.56	<.001
Cultivar.Treatment	4	240764.	60191.	6.08	<.001
Residual	40	395678.	9892.		
Total	49	1530944.			

Table C.20

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	414743.	103686.	11.38	<.001
Treatment	1	779002.	779002.	85.51	<.001
Cultivar.Treatment	4	309157.	77289.	8.48	<.001
Residual	40	364382.	9110.		
Total	49	1867284.			

Table C.21

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	753577.	188394.	11.87	<.001
Treatment	1	1138540.	1138540.	71.72	<.001
Cultivar.Treatment	4	699869.	174967.	11.02	<.001
Residual	40	634991.	15875.		
Total	49	3226978.			

Table C.22

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	1150977.	287744.	13.58	<.001
Treatment	1	2035758.	2035758.	96.07	<.001
Cultivar.Treatment	4	253540.	63385.	2.99	0.030
Residual	40	847614.	21190.		
Total	49	4287889.			

Table C.23

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	1227649.	306912.	11.41	<.001
Treatment	1	2024072.	2024072.	75.26	<.001
Cultivar.Treatment	4	118396.	29599.	1.10	0.370
Residual	40	1075828.	26896.		
Total	49	4445946.			

Table C.24

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	943706.	235926.	10.85	<.001
Treatment	1	2414723.	2414723.	111.03	<.001
Cultivar.Treatment	4	174027.	43507.	2.00	0.113
Residual	40	869911.	21748.		
Total	49	4402367.			

Table C.25

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	1062797.	265699.	10.15	<.001
Treatment	1	2882400.	2882400.	110.15	<.001
Cultivar.Treatment	4	108112.	27028.	1.03	0.402
Residual	40	1046755.	26169.		
Total	49	5100064.			

Table C.26

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	691657.	172914.	10.05	<.001
Treatment	1	3031214.	3031214.	176.17	<.001
Cultivar.Treatment	4	237234.	59309.	3.45	0.016
Residual	40	688261.	17207.		
Total	49	4648367.			

Table C.27

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	580235.	145059.	12.70	<.001
Treatment	1	3066155.	3066155.	268.44	<.001
Cultivar.Treatment	4	277516.	69379.	6.07	<.001
Residual	38	434036.	11422.		
Total	47	4259044.			

Table C.28

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	623047.	155762.	13.29	<.001
Treatment	1	3692403.	3692403.	315.07	<.001
Cultivar.Treatment	4	343231.	85808.	7.32	<.001
Residual	38	445335.	11719.		
Total	47	4990505.			

Table C.29

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	503574.	125894.	6.07	<.001
Treatment	1	4258383.	4258383.	205.38	<.001
Cultivar.Treatment	4	183751.	45938.	2.22	0.076
Residual	38	787895.	20734.		
Total	47	5593498.			

Table C.30

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	319639.	79910.	3.01	0.030
Treatment	1	4142449.	4142449.	156.12	<.001
Cultivar.Treatment	4	56799.	14200.	0.54	0.711
Residual	38	1008259.	26533.		
Total	47	5348694.			

Table C.31

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	425548.	106387.	6.42	<.001
Treatment	1	4538030.	4538030.	273.93	<.001
Cultivar.Treatment	4	195866.	48967.	2.96	0.032
Residual	38	629512.	16566.		
Total	47	5585366.			

Table C.32

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	422545.	105636.	8.34	<.001
Treatment	1	5159757.	5159757.	407.38	<.001
Cultivar.Treatment	4	175743.	43936.	3.47	0.016
Residual	38	481293.	12666.		
Total	47	6023508.			

Table C.33

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	268885.	67221.	3.64	0.013
Treatment	1	5054610.	5054610.	273.66	<.001
Cultivar.Treatment	4	64546.	16137.	0.87	0.489
Residual	38	701875.	18470.		
Total	47	5872146.			

Table C.34

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	192613.	48153.	3.69	0.012
Treatment	1	4135544.	4135544.	317.13	<.001
Cultivar.Treatment	4	45988.	11497.	0.88	0.484
Residual	38	495536.	13040.		
Total	47	4710607.			

Table C.36

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	160955.	40239.	2.56	0.055
Treatment	1	4481240.	4481240.	285.64	<.001
Cultivar.Treatment	4	36364.	9091.	0.58	0.679
Residual	35	549095.	15688.		
Total	44	4634709.			

Table C.37

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	119612.	29903.	1.63	0.189
Treatment	1	4878109.	4878109.	265.88	<.001
Cultivar.Treatment	4	25149.	6287.	0.34	0.847
Residual	35	642139.	18347.		
Total	44	4999812.			

Table C.38

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	585541.	146385.	3.09	0.028
Treatment	1	3315229.	3315229.	70.02	<.001
Cultivar.Treatment	4	235345.	58836.	1.24	0.311
Residual	35	1657253.	47350.		
Total	44	5178947.			

Table C.40

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	4	215547.	53887.	1.57	0.208
Treatment	1	5079664.	5079664.	148.46	<.001
Cultivar.Treatment	3	35353.	11784.	0.34	0.793
Residual	28	958026.	34215.		
Total	36	4684824.			

Table C.41-C.54: Effect of deficit or full irrigation on the daily (C.41 = Day 1; C.54 = Day 14) water volume of the growing media of six different strawberry cultivars (Exp.3.5). Water treatments started when the majority of strawberry fruits from the primary truss were at white stage.

Table C.41

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	5	164242.	32848.	1.53	0.205
treatment	1	41126.	41126.	1.92	0.175
cultivar.treatment	5	77677.	15535.	0.72	0.610
Residual	36	772862.	21468.		
Total	47	1055906.			

Table C.42

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	5	211642.	42328.	1.63	0.177
treatment	1	384492.	384492.	14.81	<.001
cultivar.treatment	5	234594.	46919.	1.81	0.136
Residual	36	934516.	25959.		
Total	47	1765245.			

Table C.44

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	5	337261.	67452.	2.31	0.064
treatment	1	506147.	506147.	17.35	<.001
cultivar.treatment	5	301119.	60224.	2.06	0.093
Residual	36	1050466.	29180.		
Total	47	2194992.			

Table C.46

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	5	328364.	65673.	1.94	0.115
treatment	1	341870.	341870.	10.08	0.003
cultivar.treatment	5	272080.	54416.	1.60	0.186
Residual	33	1119340.	33919.		
Total	44	2055631.			

Table C.47

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	5	592455.	118491.	2.35	0.061
treatment	1	379378.	379378.	7.52	0.010
cultivar.treatment	5	211332.	42266.	0.84	0.532
Residual	35	1765941.	50455.		
Total	46	2902275.			

Table C.48

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	5	409855.	81971.	3.20	0.018
treatment	1	828451.	828451.	32.30	<.001
cultivar.treatment	5	329172.	65834.	2.57	0.045
Residual	34	872031.	25648.		
Total	45	2426788.			

Table C.50

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	5	622966.	124593.	5.96	<.001
treatment	1	1510526.	1510526.	72.22	<.001
cultivar.treatment	5	114546.	22909.	1.10	0.380
Residual	35	732039.	20915.		
Total	46	2970992.			

Table C.51

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	5	690766.	138153.	4.60	0.003
treatment	1	2291628.	2291628.	76.25	<.001
cultivar.treatment	5	124278.	24856.	0.83	0.539
Residual	35	1051926.	30055.		
Total	46	4142684.			

Table C.52

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	5	715641.	143128.	4.04	0.005
treatment	1	1202911.	1202911.	33.91	<.001
cultivar.treatment	5	148013.	29603.	0.83	0.534
Residual	35	1241463.	35470.		
Total	46	3304109.			

Table C.53

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	5	460876.	92175.	4.65	0.002
treatment	1	902831.	902831.	45.57	<.001
cultivar.treatment	5	108574.	21715.	1.10	0.379
Residual	36	713248.	19812.		
Total	47	2185529.			

Table C.54

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cultivar	5	566040.	113208.	3.06	0.021
treatment	1	1274986.	1274986.	34.47	<.001
cultivar.treatment	5	113951.	22790.	0.62	0.688
Residual	36	1331518.	36987.		
Total	47	3286494.			

Table C.55: Effect of deficit or full irrigation and foliar application of MeJa on the water holding characteristics of the growing media of three trawberry cultivars (Exp.3.6). Water treatments started when the majority of secondary strawberry fruits from the primary truss were at green stage. MeJa

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		3428280.	1714140.	46.44	<.001
Irrigation	1		27739522.	27739522.	751.59	<.001
MeJa	1		50424.	50424.	1.37	0.243
Day	28		12325106.	440182.	11.93	<.001
Cv.Irrigation	2		3571763.	1785881.	48.39	<.001
Cv.MeJa	2		2077314.	1038657.	28.14	<.001
Irrigation.MeJa	1		1101129.	1101129.	29.83	<.001
Cv.Day	56		1283457.	22919.	0.62	0.986
Irrigation.Day	28		2645042.	94466.	2.56	<.001
MeJa.Day	28		1371823.	48994.	1.33	0.122
Cv.Irrigation.MeJa	2		2152991.	1076496.	29.17	<.001
Cv.Irrigation.Day	56		1155216.	20629.	0.56	0.996
Cv.MeJa.Day	56		1022767.	18264.	0.49	0.999
Irrigation.MeJa.Day	28		235461.	8409.	0.23	1.000
Cv.Irrigation.MeJa.Day	56		704974.	12589.	0.34	1.000
Residual	682	(14)	25171158.	36908.		
Total	1029	(14)	84900163.			

Table C.56-C60.: Effect of deficit or full irrigation on organic acid concentration (on a DW and FW basis) and sugar/acid ratio of secondary fruits from different cultivars (Exp.3.1).

Table C.56: AsA

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		9.119	3.040	1.26	
Cv	4		36.995	9.249	3.84	0.006
Treat	1		8.125	8.125	3.37	0.069
Cv.Treat	4		32.035	8.009	3.32	0.014
Residual	95	(12)	228.934	2.410		
Total	107	(12)	308.929			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		0.18036	0.06012	1.92	
Cv	4		0.48944	0.12236	3.90	0.006
Treat	1		0.00545	0.00545	0.17	0.678
Cv.Treat	4		0.35677	0.08919	2.84	0.028
Residual	94	(13)	2.94785	0.03136		
Total	106	(13)	3.84506			

Table C.57: Citric

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		540.1	180.0	0.41	
Block.*Units* stratum						
Cv	4		12767.8	3192.0	7.25	<.001
Treat	1		196.7	196.7	0.45	0.506
Cv.Treat	4		4283.0	1070.8	2.43	0.053
Residual	95	(12)	41839.1	440.4		
Total	107	(12)	58515.9			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		1.606	0.535	0.08	
Block.*Units* stratum						
Cv	4		117.015	29.254	4.29	0.003
Treat	1		42.501	42.501	6.24	0.014
Cv.Treat	4		42.016	10.504	1.54	0.197
Residual	94	(13)	640.460	6.813		
Total	106	(13)	824.688			

Table C.58: Malic

DW

Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3		265.17	88.39	1.15	
Block.*Units* stratum						
Cv	4		3426.91	856.73	11.12	<.001
Treat	1		458.40	458.40	5.95	0.017
Cv.Treat	4		1246.76	311.69	4.05	0.005
Residual	95	(12)	7319.26	77.04		
Total	107	(12)	12290.76			

FW

Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3		5.144	1.715	1.47	
Block.*Units* stratum						
Cv	4		47.685	11.921	10.22	<.001
Treat	1		0.035	0.035	0.03	0.863
Cv.Treat	4		6.070	1.518	1.30	0.276
Residual	94	(13)	109.688	1.167		
Total	106	(13)	162.511			

Table C.59: Total acids

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		11.508	3.836	0.39	
Block.*Units* stratum						
Cv	4		198.642	49.660	4.99	0.001
Treat	1		45.977	45.977	4.62	0.034
Cv.Treat	4		60.283	15.071	1.52	0.204
Residual	94	(13)	934.642	9.943		
Total	106	(13)	1219.881			

FW

Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3		1574.4	524.8	2.02	
Block.*Units* stratum						
Cv	4		7538.1	1884.5	7.27	<.001
Treat	1		3152.7	3152.7	12.16	<.001
Cv.Treat	4		2358.9	589.7	2.27	0.067
Residual	93	(14)	24115.8	259.3		
Total	105	(14)	37670.3			

Table C.60: Sugar/acid ratio

Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3		9.210	3.070	1.00	
Block.*Units* stratum						
Cv	4		85.754	21.438	6.97	<.001
Treat	1		2.779	2.779	0.90	0.344
Cv.Treat	4		31.677	7.919	2.58	0.043
Residual	91	(16)	279.760	3.074		
Total	103	(16)	401.869			

Table C.61-C64.: Effect of deficit or full irrigation on colour and weight characteristics of secondary fruits from different cultivars (Exp.3.1).

Table C.61: Chroma (C*)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		13.94	4.65	0.24	
Block.*Units* stratum						
Cv	4		4356.79	1089.20	57.08	<.001
Treat	1		330.72	330.72	17.33	<.001
Cv.Treat	4		250.05	62.51	3.28	0.012
Residual	209	(18)	3988.18	19.08		
Total	221	(18)	8479.12			

Table C.62: Lightness (L*)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		8.60	2.87	0.27	
Block.*Units* stratum						
Cv	4		1922.34	480.58	45.13	<.001
Treat	1		59.94	59.94	5.63	0.019
Cv.Treat	4		280.97	70.24	6.60	<.001
Residual	209	(18)	2225.81	10.65		
Total	221	(18)	4272.19			

Table C.63: Hue angle (Hº)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		116.95	38.98	2.37	
Block.*Units* stratum						
Cv	4		1734.94	433.73	26.32	<.001
Treat	1		181.08	181.08	10.99	0.001
Cv.Treat	4		398.69	99.67	6.05	<.001
Residual	209	(18)	3444.65	16.48		
Total	221	(18)	5725.65			

Table C.64: Berry weight

Source of variation	d.f.		s.s.	m.s.	v.r.	F pr.
Block stratum	3		43.762	14.587	2.48	
Block.*Units* stratum						
Cv	4		316.208	79.052	13.47	<.001
Treat	1		278.045	278.045	47.36	<.001
Cv.Treat	4		201.586	50.396	8.58	<.001
Residual	209	(18)	1203.478	5.871		
Total	221	(18)	1981.118			

Table C.65-C67.: Effect of deficit or full irrigation on sugar concentration (on a DW and FW basis) of secondary fruits from different cultivars (Exp.3.1).

Table C.65: Fructose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		17215.7	5738.6	8.43	
Block.*Units* stratum						
Cv	4		11494.4	2873.6	4.22	0.003
Treat	1		309.9	309.9	0.46	0.501
Cv.Treat	4		6026.0	1506.5	2.21	0.073
Residual	94	(13)	63970.8	680.5		
Total	106	(13)	96805.4			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		143.57	47.86	2.20	
Block.*Units* stratum						
Cv	4		399.49	99.87	4.59	0.002
Treat	1		451.21	451.21	20.73	<.001
Cv.Treat	4		361.13	90.28	4.15	0.004
Residual	94	(13)	2023.78	21.76		
Total	106	(13)	3316.37			

Table C.66: Glucose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		257.39	85.80	3.11	
Block.*Units* stratum						
Cv	4		372.03	93.01	3.37	0.013
Treat	1		298.34	298.34	10.82	0.001
Cv.Treat	4		352.79	88.20	3.20	0.017
Residual	94	(13)	2563.77	27.57		
Total	106	(13)	3736.58			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		23463.4	7821.1	8.79	
Block.*Units* stratum						
Cv	4		7996.1	1999.0	2.25	0.070
Treat	1		662.8	662.8	0.75	0.390
Cv.Treat	4		10958.7	2739.7	3.08	0.020
Residual	94	(13)	83602.7	889.4		
Total	106	(13)	122849.6			

Table C.67: Sucrose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		315.12	105.04	1.33	
Block.*Units* stratum						
Cv	4		4126.49	1031.62	13.08	<.001
Treat	1		310.83	310.83	3.94	0.050
Cv.Treat	4		366.33	91.58	1.16	0.333
Residual	93	(14)	7335.46	78.88		
Total	105	(14)	11977.09			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		30608.	10203.	2.76	
Block.*Units* stratum						
Cv	4		145622.	36406.	9.86	<.001
Treat	1		5929.	5929.	1.61	0.208
Cv.Treat	4		24936.	6234.	1.69	0.159
Residual	94	(13)	347067.	3692.		
Total	106	(13)	531411.			

Table C.68-C72.: Effect of deficit or full irrigation on colour and weight characteristics of secondary fruits from different cultivars (Exp.3.5).

Table C.68: Chroma (C*)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		67.75	22.58	1.26	
Block.*Units* stratum						
CV	5		869.38	173.88	9.73	<.001
Treat	1		0.28	0.28	0.02	0.901
CV.Treat	5		109.11	21.82	1.22	0.302
Residual	152	(25)	2716.61	17.87		
Total	166	(25)	3636.66			

Table C.69: Lightness (L*)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		31.04	10.35	0.68	
Block.*Units* stratum						
CV	5		946.19	189.24	12.50	<.001
Treat	1		26.56	26.56	1.75	0.187
CV.Treat	5		200.28	40.06	2.64	0.025
Residual	152	(25)	2302.03	15.14		
Total	166	(25)	3344.46			

Table C.70: Hue angle (Hº)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		21.58	7.19	0.70	
Block.*Units* stratum						
CV	5		1120.83	224.17	21.91	<.001
Treat	1		7.46	7.46	0.73	0.394
CV.Treat	5		114.26	22.85	2.23	0.050
Residual	152	(25)	1555.38	10.23		
Total	166	(25)	2652.92			

Table C.71: Berry weight

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		12.342	4.114	1.03	
Block.*Units* stratum						
CV	5		97.604	19.521	4.88	<.001
Treat	1		143.645	143.645	35.92	<.001
CV.Treat	5		49.648	9.930	2.48	0.034
Residual	165	(12)	655.756	3.999		
Total	179	(12)	942.119			

Table C.72: Dry matter content

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		0.0029410	0.0009803	2.52	
Block.*Units* stratum						
CV	5		0.0110483	0.0022097	5.67	<.001
Treat	1		0.0041043	0.0041043	10.53	0.001
CV.Treat	5		0.0030982	0.0006196	1.59	0.166
Residual	165	(12)	0.0638961	0.0003896		
Total	179	(12)	0.0838048			

Table C.73-C78.: Effect of deficit or full irrigation on sugar concentration (on a DW and FW basis) of secondary fruits from different cultivars (Exp.3.5).

Table C.73: Fructose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		2391.8	797.3	1.28	
Block.*Units* stratum						
CV	5		54963.3	10992.7	17.60	<.001
Treat	1		291.9	291.9	0.47	0.495
CV.Treat	5		7011.0	1402.2	2.25	0.053
Residual	147	(30)	93054.1	624.5		
Total	161	(30)	144270.8			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		114.14	38.05	1.17	
Block.*Units* stratum						
CV	5		1381.79	276.36	8.47	<.001
Treat	1		130.78	130.78	4.01	0.047
CV.Treat	5		397.64	79.53	2.44	0.037
Residual	147	(30)	4798.44	32.64		
Total	161	(30)	6452.01			

Table C.74: Glucose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		2344.4	781.5	1.46	
Block.*Units* stratum						
CV	5		45453.2	9090.6	17.02	<.001
Treat	1		259.2	259.2	0.49	0.487
CV.Treat	5		4444.3	888.9	1.66	0.147
Residual	149	(28)	79571.9	534.0		
Total	163	(28)	121194.9			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		52.20	17.40	0.58	
Block.*Units* stratum						
CV	5		1322.95	264.59	8.79	<.001
Treat	1		115.12	115.12	3.82	0.052
CV.Treat	5		279.47	55.89	1.86	0.106
Residual	147	(30)	4426.89	30.11		
Total	161	(30)	5878.09			

Table C.75: Sucrose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		12831.	4277.	1.12	
Block.*Units* stratum						
CV	5		384094.	76819.	20.13	<.001
Treat	1		3309.	3309.	0.87	0.353
CV.Treat	5		13189.	2638.	0.69	0.631
Residual	149	(28)	568692.	3817.		
Total	163	(28)	899340.			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		387.83	129.28	1.33	
Block.*Units* stratum						
CV	5		8875.91	1775.18	18.23	<.001
Treat	1		114.91	114.91	1.18	0.279
CV.Treat	5		149.02	29.80	0.31	0.909
Residual	147	(30)	14312.31	97.36		
Total	161	(30)	22015.72			

Table C.76: Calculated sweetness

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		2199.4	733.1	1.16	
Block.*Units* stratum						
CV	5		15179.7	3035.9	4.79	<.001
Treat	1		2654.9	2654.9	4.19	0.043
CV.Treat	5		4067.9	813.6	1.28	0.274
Residual	147	(30)	93196.2	634.0		
Total	161	(30)	114273.8			

Table C.77: monosaccharide/disaccharide ratio

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		6.518	2.173	1.05	
Block.*Units* stratum						
CV	5		128.443	25.689	12.43	<.001
Treat	1		0.177	0.177	0.09	0.770
CV.Treat	5		9.304	1.861	0.90	0.483
Residual	147	(30)	303.791	2.067		
Total	161	(30)	416.989			

Table C.78: Total sugar concentrations

DW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3		10070.	3357.	0.78	
Block.*Units* stratum						
CV	5		51860.	10372.	2.40	0.040
Treat	1		8228.	8228.	1.90	0.170
CV.Treat	5		4451.	890.	0.21	0.960
Residual	147	(30)	643930.	4322.		
Total	161	(30)	704960.			
FW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block stratum	3		909.1	303.0	1.10	
Block.*Units* stratum						
CV	5		7575.1	1515.0	5.50	<.001
Treat	1		1081.7	1081.7	3.93	0.049
CV.Treat	5		1404.9	281.0	1.02	0.408
Residual	147	(30)	40509.0	275.6		
Total	161	(30)	50075.2			

Table C.79-C82.: Effect of deficit or full irrigation and preharvest application of MeJa on anthocyanin concentration (DW basis) of secondary fruits from different cultivars (Exp.3.5).

Table C.79: Cyanidin-3-glucoside

DW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		0.0028464	0.0014232	12.02	<.001
Irrigation	1		0.0003883	0.0003883	3.28	0.074
MeJA	1		0.0005663	0.0005663	4.78	0.032
CV.Irrigation	2		0.0005218	0.0002609	2.20	0.118
CV.MeJA	2		0.0028934	0.0014467	12.22	<.001
Irrigation.MeJA	1		0.0002191	0.0002191	1.85	0.178
CV.Irrigation.MeJA	2		0.0002963	0.0001481	1.25	0.292
Residual	73	(23)	0.0086451	0.0001184		
Total	84	(23)	0.0141469			

Table C.80: Pelargonidin-3-glucoside

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		26.3057	13.1528	22.87	<.001
Irrigation	1		0.2144	0.2144	0.37	0.543
MeJA	1		6.5732	6.5732	11.43	0.001
CV.Irrigation	2		4.3441	2.1720	3.78	0.027
CV.MeJA	2		28.9877	14.4938	25.20	<.001
Irrigation.MeJA	1		0.2923	0.2923	0.51	0.478
CV.Irrigation.MeJA	2		1.1089	0.5544	0.96	0.386
Residual	73	(23)	41.9802	0.5751		
Total	84	(23)	87.7432			

Table C.81: Pelargonidin-derivative 1

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		0.051552	0.025776	23.69	<.001
Irrigation	1		0.003728	0.003728	3.43	0.068
MeJA	1		0.005841	0.005841	5.37	0.023
CV.Irrigation	2		0.012093	0.006047	5.56	0.006
CV.MeJA	2		0.032186	0.016093	14.79	<.001
Irrigation.MeJA	1		0.006813	0.006813	6.26	0.015
CV.Irrigation.MeJA	2		0.009622	0.004811	4.42	0.015
Residual	73	(23)	0.079418	0.001088		
Total	84	(23)	0.175189			

Table C.82: Pelargonidin-derivative 2

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		1.525222	0.762611	98.17	<.001
Irrigation	1		0.030480	0.030480	3.92	0.051
MeJA	1		0.004745	0.004745	0.61	0.437
CV.Irrigation	2		0.060499	0.030249	3.89	0.025
CV.MeJA	2		0.009642	0.004821	0.62	0.540
Irrigation.MeJA	1		0.036536	0.036536	4.70	0.033
CV.Irrigation.MeJA	2		0.073494	0.036747	4.73	0.012
Residual	72	(24)	0.559321	0.007768		
Total	83	(24)	2.062954			

Table C.83-C86.: Effect of deficit or full irrigation and preharvest application of MeJa on organic acid concentration (DW basis) of secondary fruits from different cultivars (Exp.3.5).

Table C.83: AsA

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		1707.7	853.8	7.14	0.001
Irrigation	1		137.6	137.6	1.15	0.287
MeJA	1		197.9	197.9	1.66	0.202
CV.Irrigation	2		620.3	310.1	2.59	0.082
CV.MeJA	2		105.8	52.9	0.44	0.644
Irrigation.MeJA	1		44.3	44.3	0.37	0.545
CV.Irrigation.MeJA	2		619.9	310.0	2.59	0.082
Residual	72	(24)	8606.3	119.5		
Total	83	(24)	10779.2			

Table C.84: Citric

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		1726.7	863.3	2.10	0.130
Irrigation	1		2556.6	2556.6	6.21	0.015
MeJA	1		1896.5	1896.5	4.60	0.035
CV.Irrigation	2		6115.2	3057.6	7.42	0.001
CV.MeJA	2		521.5	260.8	0.63	0.534
Irrigation.MeJA	1		361.0	361.0	0.88	0.352
CV.Irrigation.MeJA	2		2234.7	1117.3	2.71	0.073
Residual	72	(24)	29663.0	412.0		
Total	83	(24)	39345.9			
Variate: Malic_DW						

Table C.85: Malic

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		418.02	209.01	6.86	0.002
Irrigation	1		35.87	35.87	1.18	0.281
MeJA	1		34.24	34.24	1.12	0.293
CV.Irrigation	2		214.56	107.28	3.52	0.035
CV.MeJA	2		102.75	51.37	1.69	0.192
Irrigation.MeJA	1		128.71	128.71	4.23	0.043
CV.Irrigation.MeJA	2		114.05	57.02	1.87	0.161
Residual	72	(24)	2192.38	30.45		
Total	83	(24)	2945.78			

Table C.86: Total organic acids

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		8536.2	4268.1	5.03	0.009
Irrigation	1		3174.5	3174.5	3.74	0.057
MeJA	1		2683.8	2683.8	3.16	0.080
CV.Irrigation	2		13430.3	6715.2	7.92	<.001
CV.MeJA	2		751.9	375.9	0.44	0.644
Irrigation.MeJA	1		1372.0	1372.0	1.62	0.208
CV.Irrigation.MeJA	2		6609.8	3304.9	3.90	0.025
Residual	72	(24)	61076.9	848.3		
Total	83	(24)	83391.3			

Table C.87-C91.: Effect of deficit or full irrigation and preharvest application of MeJa on sugar concentration (DW basis) and the sugar/acid ratio of secondary fruits from different cultivars (Exp.3.5).

Table C.87: Fructose

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		25045.	12522.	4.74	0.012
Irrigation	1		2183.	2183.	0.83	0.366
MeJA	1		171.	171.	0.06	0.800
CV.Irrigation	2		3414.	1707.	0.65	0.527
CV.MeJA	2		2124.	1062.	0.40	0.670
Irrigation.MeJA	1		1230.	1230.	0.47	0.497
CV.Irrigation.MeJA	2		26.	13.	0.00	0.995
Residual	74	(22)	195431.	2641.		
Total	85	(22)	224169.			

Table C.88: Glucose

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		13942.	6971.	6.53	0.002
Irrigation	1		39.	39.	0.04	0.849
MeJA	1		898.	898.	0.84	0.362
CV.Irrigation	2		946.	473.	0.44	0.644
CV.MeJA	2		2759.	1379.	1.29	0.281
Irrigation.MeJA	1		904.	904.	0.85	0.360
CV.Irrigation.MeJA	2		8873.	4437.	4.16	0.019
Residual	74	(22)	78962.	1067.		
Total	85	(22)	99492.			

Table C.89: Sucrose

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		13942.	6971.	6.53	0.002
Irrigation	1		39.	39.	0.04	0.849
MeJA	1		898.	898.	0.84	0.362
CV.Irrigation	2		946.	473.	0.44	0.644
CV.MeJA	2		2759.	1379.	1.29	0.281
Irrigation.MeJA	1		904.	904.	0.85	0.360
CV.Irrigation.MeJA	2		8873.	4437.	4.16	0.019
Residual	74	(22)	78962.	1067.		
Total	85	(22)	99492.			

Table C.90: Total sugars

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		131293.	65647.	9.55	<.001
Irrigation	1		460.	460.	0.07	0.797
MeJA	1		1900.	1900.	0.28	0.601
CV.Irrigation	2		1836.	918.	0.13	0.875
CV.MeJA	2		10050.	5025.	0.73	0.485
Irrigation.MeJA	1		6318.	6318.	0.92	0.341
CV.Irrigation.MeJA	2		20801.	10401.	1.51	0.227
Residual	74	(22)	508888.	6877.		
Total	85	(22)	641209.			

Table C.91: Sugar/acid ratio

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CV	2		95.888	47.944	5.27	0.007
Irrigation	1		34.451	34.451	3.79	0.056
MeJA	1		38.437	38.437	4.23	0.043
CV.Irrigation	2		180.600	90.300	9.93	<.001
CV.MeJA	2		39.487	19.743	2.17	0.121
Irrigation.MeJA	1		9.612	9.612	1.06	0.307
CV.Irrigation.MeJA	2		46.592	23.296	2.56	0.084
Residual	72	(24)	654.721	9.093		
Total	83	(24)	954.513			

Table C.92-C.93: Effect of deficit or full irrigation and preharvest application of MeJa on antioxidant capacity (AC) (on a DW and FW basis) and dry weight characteristics of strawberry leaves.

Table C.92: AC

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		3687.2	1843.6	6.15	0.008
Irrigation	1		62.8	62.8	0.21	0.652
MeJa	1		28.4	28.4	0.09	0.762
Cv.Irrigation	2		83.9	42.0	0.14	0.870
Cv.MeJa	2		424.3	212.1	0.71	0.505
Irrigation.MeJa	1		1514.9	1514.9	5.05	0.036
Cv.Irrigation.MeJa	2		1704.0	852.0	2.84	0.082
Residual	20	(4)	5998.4	299.9		
Total	31	(4)	13315.0			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		449.53	224.77	4.73	0.021
Irrigation	1		0.18	0.18	0.00	0.952
MeJa	1		18.32	18.32	0.39	0.542
Cv.Irrigation	2		27.68	13.84	0.29	0.750
Cv.MeJa	2		87.85	43.93	0.92	0.413
Irrigation.MeJa	1		249.27	249.27	5.25	0.033
Cv.Irrigation.MeJa	2		265.10	132.55	2.79	0.085
Residual	20	(4)	950.31	47.52		
Total	31	(4)	2019.34			

Table C.93: AC

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cultivar	2		2.200	1.100	0.55	0.586
MeJa	1		24.734	24.734	12.34	0.002
Irrigation	1		3.685	3.685	1.84	0.190
Cultivar*MeJa	2		3.152	1.576	0.79	0.469
Cultivar*Irrigation	2		18.323	9.161	4.57	0.023
Irrigation*MeJa	1		0.892	0.892	0.45	0.512
Cultivar*MeJa*Irrigation	2		16.862	8.431	4.21	0.030
Residual	20	(4)	40.075	2.004		
Total	31	(4)	105.919			

Table C.94-C.96: Effect of deficit or full irrigation and preharvest application of MeJa on organic acid concentrations (on a DW and FW basis) of strawberry leaves.

Table C.94: AsA

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		2.8572	1.4286	6.77	0.006
Irrigation	1		3.4396	3.4396	16.29	<.001
MeJa	1		0.1781	0.1781	0.84	0.369
Cv.Irrigation	2		1.5014	0.7507	3.55	0.048
Cv.MeJa	2		0.4904	0.2452	1.16	0.333
Irrigation.MeJa	1		0.1187	0.1187	0.56	0.462
Cv.Irrigation.MeJa	2		0.5642	0.2821	1.34	0.285
Residual	20	(4)	4.2235	0.2112		
Total	31	(4)	10.9027			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		0.34122	0.17061	6.77	0.006
Irrigation	1		0.41077	0.41077	16.29	<.001
MeJa	1		0.02127	0.02127	0.84	0.369
Cv.Irrigation	2		0.17930	0.08965	3.55	0.048
Cv.MeJa	2		0.05857	0.02929	1.16	0.333
Irrigation.MeJa	1		0.01417	0.01417	0.56	0.462
Cv.Irrigation.MeJa	2		0.06738	0.03369	1.34	0.285
Residual	20	(4)	0.50438	0.02522		
Total	31	(4)	1.30204			

Table C.95: Citric

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		175.896	87.948	10.06	<.001
Irrigation	1		3.189	3.189	0.36	0.553
MeJa	1		2.347	2.347	0.27	0.610
Cv.Irrigation	2		19.764	9.882	1.13	0.343
Cv.MeJa	2		0.621	0.310	0.04	0.965
Irrigation.MeJa	1		1.435	1.435	0.16	0.690
Cv.Irrigation.MeJa	2		64.332	32.166	3.68	0.044
Residual	20	(4)	174.848	8.742		
Total	31	(4)	385.510			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		21.006	10.503	10.06	<.001
Irrigation	1		0.381	0.381	0.36	0.553
MeJa	1		0.280	0.280	0.27	0.610
Cv.Irrigation	2		2.360	1.180	1.13	0.343
Cv.MeJa	2		0.074	0.037	0.04	0.965
Irrigation.MeJa	1		0.171	0.171	0.16	0.690
Cv.Irrigation.MeJa	2		7.683	3.841	3.68	0.044
Residual	20	(4)	20.881	1.044		

Total	31	(4)	46.039			

Table C.96: Malic

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		108.01	54.00	0.70	0.510
Irrigation	1		12.41	12.41	0.16	0.693
MeJa	1		30.25	30.25	0.39	0.539
Cv.Irrigation	2		556.22	278.11	3.59	0.047
Cv.MeJa	2		56.78	28.39	0.37	0.698
Irrigation.MeJa	1		130.34	130.34	1.68	0.210
Cv.Irrigation.MeJa	2		14.18	7.09	0.09	0.913
Residual	20	(4)	1551.12	77.56		
Total	31	(4)	2331.08			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		12.898	6.449	0.70	0.510
Irrigation	1		1.483	1.483	0.16	0.693
MeJa	1		3.612	3.612	0.39	0.539
Cv.Irrigation	2		66.425	33.213	3.59	0.047
Cv.MeJa	2		6.781	3.390	0.37	0.698
Irrigation.MeJa	1		15.565	15.565	1.68	0.210
Cv.Irrigation.MeJa	2		1.693	0.847	0.09	0.913
Residual	20	(4)	185.239	9.262		
Total	31	(4)	278.384			

Table C.97-C.99: Effect of deficit or full irrigation and preharvest application of MeJa on sugar concentrations (on a DW and FW basis) of strawberry leaves.

Table C.97: Fructose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		2280.01	1140.01	14.60	<.001
Irrigation	1		834.77	834.77	10.69	0.004
MeJa	1		265.78	265.78	3.40	0.080
Cv.Irrigation	2		230.21	115.10	1.47	0.253
Cv.MeJa	2		70.14	35.07	0.45	0.645
Irrigation.MeJa	1		360.88	360.88	4.62	0.044
Cv.Irrigation.MeJa	2		67.87	33.93	0.43	0.654
Residual	20	(4)	1562.18	78.11		
Total	31	(4)	4853.56			

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		310.09	155.05	13.90	<.001
Irrigation	1		89.23	89.23	8.00	0.010
MeJa	1		14.26	14.26	1.28	0.272
Cv.Irrigation	2		50.19	25.09	2.25	0.131
Cv.MeJa	2		11.46	5.73	0.51	0.606
Irrigation.MeJa	1		44.77	44.77	4.01	0.059
Cv.Irrigation.MeJa	2		3.26	1.63	0.15	0.865
Residual	20	(4)	223.12	11.16		

Total	31	(4)	649.83			

Table C.98: Glucose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		1081.47	540.73	6.92	0.005
Irrigation	1		370.76	370.76	4.75	0.041
MeJa	1		236.05	236.05	3.02	0.098
Cv.Irrigation	2		424.94	212.47	2.72	0.090
Cv.MeJa	2		208.07	104.04	1.33	0.286
Irrigation.MeJa	1		502.23	502.23	6.43	0.020
Cv.Irrigation.MeJa	2		30.97	15.48	0.20	0.822
Residual	20	(4)	1562.24	78.11		
Total	31	(4)	3940.30			

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		147.65	73.83	6.34	0.007
Irrigation	1		32.91	32.91	2.83	0.108
MeJa	1		8.95	8.95	0.77	0.391
Cv.Irrigation	2		90.89	45.45	3.90	0.037
Cv.MeJa	2		35.23	17.62	1.51	0.244
Irrigation.MeJa	1		66.76	66.76	5.74	0.027
Cv.Irrigation.MeJa	2		8.14	4.07	0.35	0.709
Residual	20	(4)	232.82	11.64		
Total	31	(4)	571.33			

Table C.99: Sucrose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		4166.0	2083.0	14.45	<.001
Irrigation	1		205.8	205.8	1.43	0.246
MeJa	1		0.6	0.6	0.00	0.948
Cv.Irrigation	2		64.5	32.2	0.22	0.802
Cv.MeJa	2		151.1	75.5	0.52	0.600
Irrigation.MeJa	1		4.4	4.4	0.03	0.862
Cv.Irrigation.MeJa	2		519.7	259.9	1.80	0.191
Residual	20	(4)	2882.8	144.1		
Total	31	(4)	7392.3			

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		576.92	288.46	13.41	<.001
Irrigation	1		16.23	16.23	0.75	0.395
MeJa	1		14.55	14.55	0.68	0.421
Cv.Irrigation	2		19.38	9.69	0.45	0.644
Cv.MeJa	2		26.36	13.18	0.61	0.552
Irrigation.MeJa	1		0.41	0.41	0.02	0.892
Cv.Irrigation.MeJa	2		93.64	46.82	2.18	0.140
Residual	20	(4)	430.25	21.51		
Total	31	(4)	1096.43			

Table C.100: Effect of deficit or full irrigation and preharvest application of MeJa on runners biomass.

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.	
Cv		2		1810.8	905.4	3.73	0.040
Irrigation		1		1778.1	1778.1	7.32	0.013
MeJa		1		819.2	819.2	3.37	0.079
Cv.Irrigation		2		1945.2	972.6	4.00	0.032
Cv.MeJa		2		238.9	119.4	0.49	0.618
Irrigation.MeJa		1		130.4	130.4	0.54	0.471
Cv.Irrigation.MeJa		2		217.8	108.9	0.45	0.644
Residual		23	(1)	5587.5	242.9		
Total		34	(1)	12429.8			

Table C.100-C.103: Effect of soil-less substrate on strawberry fruit quality

Table C.100: Free Ellagic acid

	DW				
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cv	2	2472.6	1236.3	2.56	0.098	
Substrate	3	1448.7	482.9	1.00	0.410	
Cv.Substrate	6	3819.8	636.6	1.32	0.288	
Residual	24	11604.7	483.5			
Total	35	19345.8				

Table C.101: Total Ellagic acid

	DW				
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cv	2		86868.	43434.	0.09	0.912
Substrate	3		831921.	277307.	0.59	0.627
Cv.Substrate	6		2743511.	457252.	0.98	0.465
Residual	21	(3)	9822156.	467722.		
Total	32	(3)	13115260.			

Table C.102: Ellagitannins

	DW				
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cv	2	792702.	396351.	0.55	0.586	
Substrate	3	2828919.	942973.	1.30	0.297	
Cv.Substrate	6	3439386.	573231.	0.79	0.586	
Residual	24	17404062.	725169.			

Total	35	24465069.

Table C.103: Pelargonidin-derivative 1

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		1488.	744.	0.46	0.638
Substrate	3		17321.	5774.	3.59	0.036
Cv.Substrate	6		9139.	1523.	0.95	0.489
Residual	17	(7)	27372.	1610.		
Total	28	(7)	48503.			

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cv	2		2424.33	1212.17	42.79	<.001
Substrate	3		303.36	101.12	3.57	0.036
Cv.Substrate	6		385.61	64.27	2.27	0.086
Residual	17	(7)	481.56	28.33		
Total	28	(7)	2790.00			

Table C.104-C.109: Year-to-year variation on sugar concentration of strawberry fruits (cv. Elsanta)

Table C.104: Fructose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Year	2		11944.4	5972.2	8.45	<.001
Residual	37	(5)	26150.9	706.8		
Total	39	(5)	36610.0			

Table C.105: Glucose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Year	2		29960.	14980.	14.97	<.001
Residual	37	(5)	37029.	1001.		
Total	39	(5)	63188.			

Table C.106: Sucrose

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Year	2		51283.	25641.	5.37	0.009
Residual	37	(5)	176781.	4778.		
Total	39	(5)	223051.			

Table C.107: Monosaccharide/disaccharide ratio

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Year	2		29.513	14.757	7.24	0.002
Residual	37	(5)	75.408	2.038		
Total	39	(5)	101.325			

Table C.108: Monosaccharide/disaccharide ratio

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Year	2		1268.2	634.1	6.07	0.005
Residual	37	(5)	3866.4	104.5		
Total	39	(5)	4999.9			

Table C.109: Total sugars

			DW			
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Year	2		75198.	37599.	4.03	0.026
Residual	37	(5)	345365.	9334.		
Total	39	(5)	412673.			

C.2. ANOVA tables for Chapter 4

Table C.109-C.109: Variation in sugar concentrations and taste-related characteristics of 17 UK-grown blackcurrant genotypes (Exp. 4.1)

Table C.109: Monosaccharide/disaccharide ratio

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	17	6418.524	377.560	270.58	<.001
Residual	36	50.234	1.395		
Total	53	6468.758			

Table C.110: Fructose

DW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	16	22713.16	1419.57	134.36	<.001	
Residual	34	359.22	10.57			
Total	50	23072.38				
FW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	17	5868.5098	345.2065	364.35	<.001	
Residual	36	34.1090	0.9475			
Total	53	5902.6188				

Table C.111: Glucose

DW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	16	42169.041	2635.565	753.48	<.001	
Residual	34	118.927	3.498			
Total	50	42287.969				
FW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	17	9840.8168	578.8716	1438.03	<.001	
Residual	36	14.4916	0.4025			
Total	53	9855.3084				

Table C.112: Sucrose

DW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	16	44269.083	2766.818	687.89	<.001	
Residual	34	136.754	4.022			
Total	50	44405.837				
FW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	17	4187.8471	246.3439	649.87	<.001	
Residual	36	13.6463	0.3791			
Total	53	4201.4934				

Table C.113: Total sugars

DW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	16	137253.05	8578.32	278.11	<.001	
Residual	34	1048.74	30.85			
Total	50	138301.79				
FW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	17	32444.150	1908.479	638.34	<.001	
Residual	36	107.630	2.990			
Total	53	32551.780				

Table C.114-C.120: Variation in organic acid concentrations of 17 UK-grown blackcurrant genotypes (Exp. 4.1)

Table C.114: AsA

DW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	16	318.39632	19.89977	234.88	<.001	
Residual	34	2.88058	0.08472			
Total	50	321.27689				
FW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	17	42.986771	2.528634	335.48	<.001	
Residual	36	0.271346	0.007537			
Total	53	43.258117				

Table C.115: Citric

DW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	16	29582.027	1848.877	736.87	<.001	
Residual	34	85.309	2.509			
Total	50	29667.336				
FW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
CVs	17	3746.5492	220.3852	888.55	<.001	
Residual	36	8.9290	0.2480			
Total	53	3755.4782				

Table C.116: Malic

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
C1	16	209.69280	13.10580	254.49	<.001	
Residual	34	1.75098	0.05150			
Total	50	211.44377				

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	220.43974	12.96704	264.58	<.001	
Residual	36	1.76435	0.04901			
Total	53	222.20409				

Table C.117: Oxalic

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	16	1.344694	0.084043	24.99	<.001	
Residual	34	0.114334	0.003363			
Total	50	1.459027				

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	0.2203410	0.0129612	45.61	<.001	
Residual	36	0.0102307	0.0002842			
Total	53	0.2305717				

Table C.118: Tartaric

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
C1	16	32646.164	2040.385	501.75	<.001	
Residual	34	138.263	4.067			
Total	50	32784.427				

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	0.2203410	0.0129612	45.61	<.001	
Residual	36	0.0102307	0.0002842			
Total	53	0.2305717				

Table C.119: Total acids

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	5053.0571	297.2387	772.46	<.001	
Residual	36	13.8526	0.3848			
Total	53	5066.9097				

Table C.120: Total acids

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	19.867391	1.168670	480.24	<.001	
Residual	36	0.087606	0.002433			
Total	53	19.954997				

Table C.121-C.125: Variation in individual anthocyanin concentrations of 17 UK-grown blackcurrant genotypes (Exp. 4.1)

Table C.121: Cyanidin-3-glucoside

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	16	430849.1	26928.1	50.51	<.001	
Residual	34	18127.0	533.1			
Total	50	448976.1				

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	40225.94	2366.23	45.25	<.001	
Residual	36	1882.46	52.29			
Total	53	42108.40				

Table C.122: Cyanidin-3-rutinoside

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	16	13596376.	849774.	17.56	<.001	
Residual	34	1645134.	48386.			
Total	50	15241511.				

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	1238732.	72867.	16.41	<.001	
Residual	36	159832.	4440.			
Total	53	1398564.				

Table C.123: Delphinidin-3-glucoside

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
C1	16	705610.	44101.	17.50	<.001	
Residual	34	85683.	2520.			
Total	50	791293.				

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	75321.8	4430.7	18.51	<.001	
Residual	36	8616.0	239.3			
Total	53	83937.7				

Table C.124: Delphinidin-3-rutinoside

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
C1	16	5129596.	320600.	10.81	<.001	
Residual	34	1008749.	29669.			
Total	50	6138345.				

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	421164.	24774.	8.56	<.001	
Residual	36	104135.	2893.			
Total	53	525299.				

Table C.125: Total anthocyanins

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
C1	16	47797421.	2987339.	16.59	<.001	
Residual	34	6120509.	180015.			
Total	50	53917930.				

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	4.19277	0.24663	14.07	<.001	
Residual	36	0.63096	0.01753			
Total	53	4.82372				

Table C.126-C.129: Variation in total anthocyanins and total phenolic concentrations of 17 UK-grown blackcurrant genotypes (measured spectrophotometrically)(Exp.4.1)

Table C.126: Total anthocyanins

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
C1	16	889.3466	55.5842	190.24	<.001	
Residual	34	9.9343	0.2922			
Total	50	899.2810				

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	117.64400	6.92024	183.98	<.001	
Residual	36	1.35414	0.03761			
Total	53	118.99814				

Table C.127: Total phenolics

DW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
C1	16	982.527	61.408	46.35	<.001	
Residual	34	45.041	1.325			
Total	50	1027.568				

FW

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.	
CVs	17	141.0017	8.2942	58.97	<.001	
Residual	36	5.0631	0.1406			
Total	53	146.0648				

Table C.128: Total phenolics/Total anthocyanins (Spectrophotometer)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
C1	16	10.442360	0.652648	103.07	<.001	
Residual	34	0.215289	0.006332			
Total	50	10.657650				

Table C.129: Total phenolics/Total anthocyanins (HPLC)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	10.995923	0.646819	90.99	<.001	
Residual	36	0.255919	0.007109			
Total	53	11.251842				

Table C.130: Variation in pH values of 17 UK-grown blackcurrant genotypes (Exp. 4.1)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CVs	17	0.854417	0.050260	5.80	<.001	
Residual	36	0.311733	0.008659			
Total	53	1.166150				

Table C.131-C.135: Variation in sugar concentrations of three UK-grown blackcurrant cultivars harvested at three different maturities (Exp.4.2)

Table C.131: Fructose

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CV	2	218.9	109.5	0.99	0.390	
Maturity	2	4313.6	2156.8	19.53	<.001	
CV.Maturity	4	2553.2	638.3	5.78	0.004	
Residual	18	1987.6	110.4			
Total						

Table C.132: Glucose

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CV	2	384.8	192.4	1.88	0.182	
Maturity	2	2953.2	1476.6	14.40	<.001	
CV.Maturity	4	1530.7	382.7	3.73	0.022	
Residual	18	1846.2	102.6			
Total	26	6714.9				

Table C.133: Sucrose

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
CV	2	1794.0	897.0	3.83	0.041	
Maturity	2	10027.2	5013.6	21.39	<.001	
CV.Maturity	4	6226.8	1556.7	6.64	0.002	
Residual	18	4219.5	234.4			
Total	26	22267.4				

Table C.134: Total sugars

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Sample	2	4760.2	2380.1	3.10	0.070	
Maturity	2	722.0	361.0	0.47	0.633	
Sample.Maturity	4	11455.8	2864.0	3.73	0.022	
Residual	18	13837.8	768.8			
Total	26	30775.8				

Table C.135: monosaccharide/disaccharide ratio

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Sample	2	41.165	20.582	2.58	0.104	
Maturity	2	387.861	193.931	24.30	<.001	
Sample.Maturity	4	526.185	131.546	16.49	<.001	
Residual	18	143.630	7.979			
Total	26	1098.841				

Table C.136-C.140: Variation in organic acid concentrations of three UK-grown blackcurrant cultivars harvested at three different maturities (Exp.4.2)

Table C.136: Citric

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cultivar	2	16279.4	8139.7	58.65	<.001	
Maturity	2	306.5	153.3	1.10	0.353	
Cultivar.Maturity	4	6641.8	1660.4	11.96	<.001	
Residual	18	2498.0	138.8			
Total	26	25725.7				

Table C.137: Malic

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cultivar	2	4387.475	2193.737	485.40	<.001	
Maturity	2	1336.837	668.419	147.90	<.001	
Cultivar.Maturity	4	84.679	21.170	4.68	0.009	
Residual	18	81.349	4.519			
Total	26	5890.340				

Table C.138: Ascorbic

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Cultivar	2	294.5984	147.2992	380.06	<.001	
Maturity	2	64.1662	32.0831	82.78	<.001	
Cultivar.Maturity	4	54.6542	13.6635	35.25	<.001	
Residual	18	6.9762	0.3876			
Total	26	420.3950				

Table C.139: Total acids

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.	
Sample	2	38831.1	19415.6	98.11	<.001	
Maturity	2	1739.8	869.9	4.40	0.028	
Sample.Maturity	4	9164.7	2291.2	11.58	<.001	
Residual	18	3562.1	197.9			
Total	26	53297.6				

Table C.140: Sugar/Acid ratio

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Sample	2	4.41094	2.20547	53.05	<.001	
Maturity	2	0.13428	0.06714	1.62	0.226	
Sample.Maturity	4	0.98453	0.24613	5.92	0.003	
Residual	18	0.74828	0.04157			
Total	26	6.27804				

Table C.141-C.143: Variation in antioxidant capacities, measured by the FRAP assay, total phenolics and total flavonols of three UK-grown blackcurrant cultivars harvested at three different maturities (Exp.4.2)

Table C.141: AC (FRAP)

DW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Sample	2	218099.	109049.	16.20	<.001	
Maturity	2	39336.	19668.	2.92	0.080	
Sample.Maturity	4	183094.	45774.	6.80	0.002	
Residual	18	121162.	6731.			
Total	26	561691.				

FW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	2	20056.8	10028.4	36.45	<.001	
Maturity	2	14621.9	7310.9	26.57	<.001	
Cultivar.Maturity	4	3725.6	931.4	3.39	0.031	
Residual	18	4952.1	275.1			
Total	26	43356.3				

Table C.142: Total phenolics

DW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	2	10.068	5.034	0.93	0.414	
Maturity	2	52.450	26.225	4.83	0.021	
Cultivar.Maturity	4	245.705	61.426	11.32	<.001	
Residual	18	97.711	5.428			
Total	26	405.934				

Table C.143: Total flavonols

DW						
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Extraction solvent (ES)	1	1.41292	1.41292	55.40	<.001	
Maturity	2	27.86401	13.93201	546.26	<.001	
Cultivar	2	52.63240	26.31620	1031.83	<.001	
ES.Maturity	2	1.13443	0.56722	22.24	<.001	
ES.Sample	2	1.98785	0.99392	38.97	<.001	
Maturity.Cultivar	4	5.58043	1.39511	54.70	<.001	
ES.Maturity.Cultivar	4	0.52114	0.13028	5.11	0.002	
Residual	36	0.91816	0.02550			
Total	53	92.05134				

Table C.144-C.143: Variation in individual anthocyanin concentrations of three UK-grown blackcurrant cultivars harvested at three different maturities (Exp.4.2)

Table C.144: Cyanidin-3-glucoside

		DW				
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cv	2	9.32420	4.66210	179.63	<.001	
Maturity	2	5.37587	2.68794	103.56	<.001	
Cv.Maturity	4	15.50609	3.87652	149.36	<.001	
Residual	18	0.46718	0.02595			
Total	26	30.67334				

Table C.145: Cyanidin-3-rutinoside

		DW				
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cv	2	399.2850	199.6425	972.91	<.001	
Maturity	2	28.0824	14.0412	68.43	<.001	
Cv.Maturity	4	20.3306	5.0826	24.77	<.001	
Residual	18	3.6936	0.2052			
Total	26	451.3916				

Table C.146: Delphinidin-3-glucoside

		DW				
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cv	2	156.47761	78.23880	794.10	<.001	
Maturity	2	8.27159	4.13579	41.98	<.001	
Cv.Maturity	4	54.48386	13.62097	138.25	<.001	
Residual	18	1.77345	0.09852			
Total	26	221.00650				

Table C.147: Delphinidin-3-rutinoside

		DW				
Source of variation	**d.f.**	**(m.v.)**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cv	2	21.6079	10.8040	64.10	<.001	
Maturity	2	72.0423	36.0211	213.70	<.001	
Cv.Maturity	4	123.1219	30.7805	182.61	<.001	
Residual	18	3.0340	0.1686			
Total	26	219.8061				

Table C.148-C.151: Variation in organic acid concentrations of 11 UK-grown blackcurrant cultivars harvested at two different maturities (Exp.4.3)

Table C.148: Citric

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Sample	10	66607.6	6660.8	43.02	<.001	
Maturity	1	221.5	221.5	1.43	0.238	
Sample.Maturity	10	6029.1	602.9	3.89	<.001	
Residual	44	6812.8	154.8			
Total	65	79671.1				

Table C.149: Malic

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Maturity	1	1293.430	1293.430	163.72	<.001	
Sample	10	7908.384	790.838	100.11	<.001	
Maturity.Sample	10	379.160	37.916	4.80	<.001	
Residual	44	347.602	7.900			
Total	65	9928.576				

Table C.150: Ascorbic

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Maturity	1	15.557	15.557	10.51	0.002	
Sample	10	330.430	33.043	22.31	<.001	
Maturity.Sample	10	54.013	5.401	3.65	0.001	
Residual	44	65.155	1.481			
Total	65	465.155				

Table C.151: Total acids

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Maturity	1	899.5	899.5	3.82	0.057	
Sample	10	92335.2	9233.5	39.17	<.001	
Maturity.Sample	10	7024.7	702.5	2.98	0.006	
Residual	44	10372.7	235.7			
Total	65	110632.0				

Table C.152-C.156: Variation in sugar concentrations of 11 UK-grown blackcurrant cultivars harvested at two different maturities (Exp.4.3)

Table C.152: Sucrose

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Maturity	1	6702.74	6702.74	467.11	<.001	
Sample	10	33585.28	3358.53	234.05	<.001	
Maturity.Sample	10	3763.80	376.38	26.23	<.001	
Residual	44	631.37	14.35			
Total	65	44683.19				

Table C.153: Glucose

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Maturity	1	6306.24	6306.24	97.83	<.001	
Sample	10	28934.26	2893.43	44.89	<.001	
Maturity.Sample	10	1936.32	193.63	3.00	0.006	
Residual	44	2836.27	64.46			
Total	65	40013.09				

Table C.154: Fructose

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Maturity	1	14764.7	14764.7	24.70	<.001	
Sample	10	42698.7	4269.9	7.14	<.001	
Maturity.Sample	10	7525.8	752.6	1.26	0.283	
Residual	44	26300.5	597.7			
Total	65	91289.6				

Table C.155: monosaccharide/disaccharide ratio

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Maturity	1	103.5738	103.5738	596.62	<.001	
Sample	10	369.7127	36.9713	212.97	<.001	
Maturity.Sample	10	95.6333	9.5633	55.09	<.001	
Residual	44	7.6385	0.1736			
Total	65	576.5582				

Table C.156: Sugar/acid ratio

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Sample	10	8.96233	0.89623	14.19	<.001	
Maturity	1	0.81318	0.81318	12.87	<.001	
Sample.Maturity	10	2.25027	0.22503	3.56	0.002	
Residual	44	2.77932	0.06317			
Total	65	14.80509				

Table C.157-C.161: Variation in sugar concentrations of blackcurrant berries grown at two different locations.

Table C.157: Sucrose

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Location	1	2456.81	2456.81	82.69	<.001	
Residual	4	118.85	29.71			
Total	5	2575.66				

Table C.158: Fructose

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Location	1	1524.1	1524.1	9.11	0.039	
Residual	4	669.3	167.3			
Total	5	2193.4				

Table C.159: Glucose

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Location	1	1524.81	1524.81	16.80	0.015	
Residual	4	363.05	90.76			
Total	5	1887.87				

Table C.160: Total sugars

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Location	1	16295.6	16295.6	20.95	0.010	
Residual	4	3110.7	777.7			
Total	5	19406.3				

Table C.161: monosaccharide/disaccharide ratio

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Location	1	14.83586	14.83586	589.20	<.001	
Residual	4	0.10072	0.02518			
Total	5	14.93658				

Table C.162-C.166: Variation in organic acid concentrations of blackcurrant berries grown at two different locations.

Table C.162: Citric

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Location	1	2.47	2.47	0.05	0.830	
Residual	4	188.26	47.06			
Total	5	190.73				

Table C.163: Malic

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Location	1	128.203	128.203	55.98	0.002	
Residual	4	9.161	2.290			
Total	5	137.364				

Table C.164: Ascorbic

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Location	1	0.0035	0.0035	0.01	0.917	
Residual	4	1.1569	0.2892			
Total	5	1.1605				

Table C.165: Total acids

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Location	1	96.26	96.26	1.50	0.288	
Residual	4	257.43	64.36			
Total	5	353.68				

Table C.166: Sugar/Acid ratio

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Location	1	0.42039	0.42039	25.29	0.007	
Residual	4	0.06648	0.01662			
Total	5	0.48688				

Table C.167-C.171: Variation in sugar concentrations of blackcurrant berries (cv. Ben Hope) harvested at two different maturities (block) during postharvest storage at different temperature ranges.

Table C.167: monosaccharide/disaccharide ratio

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	647.813	647.813	274.58	<.001
Day	2	194.342	97.171	41.19	<.001
Range	4	66.022	16.505	7.00	<.001
Block.Day	2	50.852	25.426	10.78	<.001
Block.Range	4	27.378	6.845	2.90	0.026
Day.Range	8	19.441	2.430	1.03	0.420
Block.Day.Range	8	16.126	2.016	0.85	0.558
Residual	90	212.339	2.359		
Total	119	1234.313			

Table C.167: Fructose

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	171150.3	171150.3	308.29	<.001
Day	2	54.0	27.0	0.05	0.953
Range	4	1683.7	420.9	0.76	0.555
Block.Day	2	10638.7	5319.4	9.58	<.001
Block.Range	4	2862.7	715.7	1.29	0.280
Day.Range	8	5443.2	680.4	1.23	0.293
Block.Day.Range	8	6374.9	796.9	1.44	0.193
Residual	90	49964.5	555.2		
Total	119	248172.1			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	11323.42	11323.42	276.25	<.001
Day	2	1.69	0.85	0.02	0.980
Range	4	516.80	129.20	3.15	0.018
Block.Day	2	433.31	216.65	5.29	0.007
Block.Range	4	877.36	219.34	5.35	<.001
Day.Range	8	573.43	71.68	1.75	0.098
Block.Day.Range	8	695.85	86.98	2.12	0.042
Residual	90	3689.09	40.99		
Total	119	18110.94			

Table C.168: Glucose

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	21647.1	21647.1	206.87	<.001
Day	2	4348.7	2174.4	20.78	<.001
Range	4	3987.6	996.9	9.53	<.001
Block.Day	2	2030.1	1015.0	9.70	<.001
Block.Range	4	1166.7	291.7	2.79	0.031
Day.Range	8	1318.8	164.8	1.58	0.143
Block.Day.Range	8	2487.7	311.0	2.97	0.005
Residual	90	9417.9	104.6		
Total	119	46404.5			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	1769.07	1769.07	162.74	<.001
Day	2	183.06	91.53	8.42	<.001
Range	4	544.56	136.14	12.52	<.001
Block.Day	2	84.21	42.10	3.87	0.024
Block.Range	4	365.02	91.26	8.39	<.001
Day.Range	8	115.12	14.39	1.32	0.242
Block.Day.Range	8	238.09	29.76	2.74	0.009
Residual	90	978.36	10.87		
Total	119	4277.49			

Table C.169: Sucrose

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	28425.09	28425.09	318.48	<.001
Day	2	16706.45	8353.23	93.59	<.001
Range	4	7406.29	1851.57	20.75	<.001
Block.Day	2	41.95	20.98	0.24	0.791
Block.Range	4	782.45	195.61	2.19	0.076
Day.Range	8	455.36	56.92	0.64	0.744
Block.Day.Range	8	997.06	124.63	1.40	0.209
Residual	90	8032.62	89.25		
Total	119	62847.27			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	1100.921	1100.921	190.48	<.001
Day	2	833.390	416.695	72.10	<.001
Range	4	486.354	121.588	21.04	<.001
Block.Day	2	0.538	0.269	0.05	0.955
Block.Range	4	51.509	12.877	2.23	0.072
Day.Range	8	24.961	3.120	0.54	0.824
Block.Day.Range	8	62.169	7.771	1.34	0.232
Residual	90	520.171	5.780		
Total	119	3080.014			

Table C.170: Total sugars

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	153849.	153849.	147.82	<.001
Day	2	37276.	18638.	17.91	<.001
Range	4	31378.	7845.	7.54	<.001
Block.Day	2	20661.	10330.	9.93	<.001
Block.Range	4	7557.	1889.	1.82	0.133
Day.Range	8	11812.	1477.	1.42	0.200
Block.Day.Range	8	20589.	2574.	2.47	0.018
Residual	90	93672.	1041.		
Total	119	376795.			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	13292.2	13292.2	128.37	<.001
Day	2	1534.5	767.3	7.41	0.001
Range	4	4407.5	1101.9	10.64	<.001
Block.Day	2	867.3	433.7	4.19	0.018
Block.Range	4	2797.6	699.4	6.75	<.001
Day.Range	8	1345.7	168.2	1.62	0.129
Block.Day.Range	8	2214.9	276.9	2.67	0.011
Residual	90	9319.2	103.5		
Total	119	35779.0			

Table C.171: Sweetness

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	58570.6	58570.6	169.48	<.001
Day	2	2283.9	1141.9	3.30	0.041
Temp_range	4	10586.6	2646.6	7.66	<.001
Block.Day	2	3171.2	1585.6	4.59	0.013
Block.Temp_range	4	8593.4	2148.3	6.22	<.001
Day.Temp_range	8	4714.2	589.3	1.71	0.108
Block.Day.Temp_range	8	6900.3	862.5	2.50	0.017
Residual	90	31103.7	345.6		
Total	119	125924.0			

Table C.172-C.176: Variation in organic acid concentrations of blackcurrant berries (cv. Ben Hope) harvested at two different maturities (block) during postharvest storage at different temperature ranges.

Table C.172: Ascorbic

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	412.139	412.139	244.95	<.001
Day	2	116.029	58.014	34.48	<.001
Temp_range	4	9.828	2.457	1.46	0.221
Block.Day	2	10.426	5.213	3.10	0.050
Block.Temp_range	4	18.916	4.729	2.81	0.030
Day.Temp_range	8	35.738	4.467	2.66	0.012
Block.Day.Temp_range	8	21.108	2.638	1.57	0.146
Residual	90	151.428	1.683		
Total	119	775.611			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	16.0536	16.0536	137.46	<.001
Day	2	4.8105	2.4053	20.60	<.001
Temp_range	4	1.1491	0.2873	2.46	0.051
Block.Day	2	0.6893	0.3447	2.95	0.057
Block.Temp_range	4	0.7656	0.1914	1.64	0.171
Day.Temp_range	8	2.1538	0.2692	2.31	0.027
Block.Day.Temp_range	8	2.1184	0.2648	2.27	0.029
Residual	90	10.5108	0.1168		
Total	119	38.2512			

Table C.173: Citric

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	48591.	48591.	2.04	0.157
Day	2	39495.	19747.	0.83	0.440
Temp_range	4	98278.	24569.	1.03	0.396
Block.Day	2	50381.	25191.	1.06	0.352
Block.Temp_range	4	100439.	25110.	1.05	0.384
Day.Temp_range	8	198019.	24752.	1.04	0.414
Block.Day.Temp_range	8	190736.	23842.	1.00	0.442
Residual	90	2145446.	23838.		
Total	119	2871383.			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	1461.	1461.	1.26	0.265
Day	2	1781.	891.	0.77	0.467
Temp_range	4	4700.	1175.	1.01	0.406
Block.Day	2	2218.	1109.	0.96	0.389
Block.Temp_range	4	4811.	1203.	1.04	0.393

Day.Temp_range	8	9130.	1141.	0.98	0.455
Block.Day.Temp_range	8	8898.	1112.	0.96	0.474
Residual	90	104507.	1161.		
Total	119	137507.			

Table C.174: Malic

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	6812.9	6812.9	12.58	<.001
Day	2	4689.6	2344.8	4.33	0.016
Temp_range	4	3799.6	949.9	1.75	0.145
Block.Day	2	1307.2	653.6	1.21	0.304
Block.Temp_range	4	2968.3	742.1	1.37	0.251
Day.Temp_range	8	3717.9	464.7	0.86	0.555
Block.Day.Temp_range	8	3631.4	453.9	0.84	0.572
Residual	90	48752.7	541.7		
Total	119	75679.6			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	310.40	310.40	10.39	0.002
Day	2	234.11	117.06	3.92	0.023
Temp_range	4	210.97	52.74	1.77	0.143
Block.Day	2	67.79	33.90	1.13	0.326
Block.Temp_range	4	143.52	35.88	1.20	0.316
Day.Temp_range	8	202.22	25.28	0.85	0.565
Block.Day.Temp_range	8	198.72	24.84	0.83	0.577
Residual	90	2688.79	29.88		
Total	119	4056.53			

Table C.175: Total acids

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	104506.	104506.	4.25	0.042
Day	2	38795.	19397.	0.79	0.458
Temp_range	4	94821.	23705.	0.96	0.432
Block.Day	2	48692.	24346.	0.99	0.376
Block.Temp_range	4	103620.	25905.	1.05	0.385
Day.Temp_range	8	210635.	26329.	1.07	0.391
Block.Day.Temp_range	8	202218.	25277.	1.03	0.422
Residual	90	2214811.	24609.		
Total	119	3018098.			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	3581.	3581.	2.96	0.089
Day	2	1589.	794.	0.66	0.522
Temp_range	4	4701.	1175.	0.97	0.428
Block.Day	2	2099.	1050.	0.87	0.424
Block.Temp_range	4	4756.	1189.	0.98	0.422

Day.Temp_range	8	9688.	1211.	1.00	0.442
Block.Day.Temp_range	8	9484.	1185.	0.98	0.458
Residual	90	109034.	1211.		
Total	119	144932.			

Table C.176: Sugar/acid ratio

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	13.37465	13.37465	144.90	<.001
Day	2	0.44909	0.22454	2.43	0.094
Temp_range	4	0.78269	0.19567	2.12	0.085
Block.Day	2	0.81081	0.40541	4.39	0.015
Block.Temp_range	4	0.99190	0.24798	2.69	0.036
Day.Temp_range	8	0.97758	0.12220	1.32	0.242
Block.Day.Temp_range	8	0.95651	0.11956	1.30	0.256
Residual	90	8.30704	0.09230		
Total	119	26.65027			

Table C.177: Variation in respiration ratio of blackcurrant berries (cv. Ben Hope) harvested at two different maturities (block) during postharvest storage at different temperature ranges.

Table C.177: Respiration rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	73.6	73.6	0.33	0.570
Day	2	6059.9	3029.9	13.38	<.001
Range	4	39597.1	9899.3	43.72	<.001
Block.Day	2	961.4	480.7	2.12	0.126
Block.Range	4	577.2	144.3	0.64	0.637
Day.Range	8	3847.8	481.0	2.12	0.041
Block.Day.Range	8	907.7	113.5	0.50	0.852
Residual	90	20377.3	226.4		
Total	119	72402.0			

Table C.178-C.185: Variation in individual anthocyanins of blackcurrant berries (cv. Ben Hope) harvested at two different maturities (block) during postharvest storage at different temperature ranges.

Table C.177: % Delphinidin-3-glucoside

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	551.003	551.003	400.87	<.001
Day	2	26.422	13.211	9.61	<.001
range	4	43.555	10.889	7.92	<.001
Block.Day	2	21.125	10.562	7.68	<.001
Block.range	4	27.231	6.808	4.95	0.001
Day.range	8	17.874	2.234	1.63	0.129
Block.Day.range	8	35.237	4.405	3.20	0.003
Residual	90	123.706	1.375		
Total	119	846.153			

Table C.178: % Cyanidin-3-glucoside

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	169.2825	169.2825	324.63	<.001
Day	2	47.9418	23.9709	45.97	<.001
range	4	17.7830	4.4457	8.53	<.001
Block.Day	2	55.4590	27.7295	53.18	<.001
Block.range	4	14.7146	3.6786	7.05	<.001
Day.range	8	20.6712	2.5839	4.96	<.001
Block.Day.range	8	33.5488	4.1936	8.04	<.001
Residual	90	46.9313	0.5215		
Total	119	406.3321			

Table C.179: % cyanidin-3-rutinoside

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	306.826	306.826	50.53	<.001
Day	2	422.926	211.463	34.83	<.001
range	4	233.809	58.452	9.63	<.001
Block.Day	2	111.085	55.543	9.15	<.001
Block.range	4	59.816	14.954	2.46	0.051
Day.range	8	136.448	17.056	2.81	0.008
Block.Day.range	8	88.171	11.021	1.82	0.084
Residual	90	546.486	6.072		
Total	119	1905.567			

Table C.180: % delphinidin-3-rutinoside

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	359.780	359.780	84.84	<.001
Day	2	179.433	89.716	21.16	<.001
range	4	60.766	15.192	3.58	0.009
Block.Day	2	66.207	33.104	7.81	<.001
Block.range	4	5.747	1.437	0.34	0.851
Day.range	8	206.088	25.761	6.07	<.001
Block.Day.range	8	166.770	20.846	4.92	<.001
Residual	90	381.667	4.241		
Total	119	1426.458			

Table C.181: Cyanidin-3-glucoside

		DW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block	1	525111.	525111.	39.21	<.001
Day	2	368805.	184403.	13.77	<.001
range	4	727942.	181985.	13.59	<.001
Block.Day	2	1674315.	837158.	62.51	<.001
Block.range	4	171724.	42931.	3.21	0.016

Day.range	8	459964.	57496.	4.29	<.001
Block.Day.range	8	484192.	60524.	4.52	<.001
Residual	90	1205239.	13392.		
Total	119	5617293.			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	42773.	42773.	41.02	<.001
Day	2	18356.	9178.	8.80	<.001
range	4	18176.	4544.	4.36	0.003
Block.Day	2	74308.	37154.	35.63	<.001
Block.range	4	3550.	888.	0.85	0.497
Day.range	8	12391.	1549.	1.49	0.174
Block.Day.range	8	20129.	2516.	2.41	0.021
Residual	90	93854.	1043.		
Total	119	283538.			

Table C.182: Cyanidin-3-rutinoside

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	350369857.	350369857.	593.80	<.001
Day	2	88863215.	44431607.	75.30	<.001
range	4	10597579.	2649395.	4.49	0.002
Block.Day	2	25602477.	12801239.	21.70	<.001
Block.range	4	6869176.	1717294.	2.91	0.026
Day.range	8	28663785.	3582973.	6.07	<.001
Block.Day.range	8	28574956.	3571869.	6.05	<.001
Residual	90	53104332.	590048.		
Total	119	592645376.			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	22009568.	22009568.	448.27	<.001
Day	2	4883519.	2441760.	49.73	<.001
range	4	268709.	67177.	1.37	0.251
Block.Day	2	1044541.	522271.	10.64	<.001
Block.range	4	854693.	213673.	4.35	0.003
Day.range	8	2241216.	280152.	5.71	<.001
Block.Day.range	8	2440411.	305051.	6.21	<.001
Residual	90	4418878.	49099.		
Total	119	38161537.			

Table C.183: Delphinidin-3-glucoside

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	583961.	583961.	16.31	<.001
Day	2	2453005.	1226502.	34.25	<.001
range	4	1742138.	435535.	12.16	<.001
Block.Day	2	1893508.	946754.	26.44	<.001

Block.range	4	469176.	117294.	3.28	0.015
Day.range	8	530908.	66363.	1.85	0.077
Block.Day.range	8	474245.	59281.	1.66	0.120
Residual	90	3222609.	35807.		
Total	119	11369550.			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	63053.	63053.	26.12	<.001
Day	2	140979.	70490.	29.20	<.001
range	4	72263.	18066.	7.48	<.001
Block.Day	2	87571.	43785.	18.14	<.001
Block.range	4	35252.	8813.	3.65	0.008
Day.range	8	47314.	5914.	2.45	0.019
Block.Day.range	8	44522.	5565.	2.31	0.027
Residual	90	217257.	2414.		
Total	119	708212.			

Table C.184: Delphinidin-3-rutinoside

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	175418414.	175418414.	722.38	<.001
Day	2	8398723.	4199362.	17.29	<.001
range	4	7060725.	1765181.	7.27	<.001
Block.Day	2	14568270.	7284135.	30.00	<.001
Block.range	4	117307.	29327.	0.12	0.975
Day.range	8	4077797.	509725.	2.10	0.044
Block.Day.range	8	8123729.	1015466.	4.18	<.001
Residual	90	21854933.	242833.		
Total	119	239619899.			

FW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	10806786.	10806786.	556.99	<.001
Day	2	461945.	230972.	11.90	<.001
range	4	171468.	42867.	2.21	0.074
Block.Day	2	592010.	296005.	15.26	<.001
Block.range	4	117903.	29476.	1.52	0.203
Day.range	8	754433.	94304.	4.86	<.001
Block.Day.range	8	892154.	111519.	5.75	<.001
Residual	90	1746177.	19402.		
Total	119	15542875.			

Table C.185: Total anthocyanins

DW

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	1	1.119E+09	1.119E+09	600.15	<.001
Day	2	1.963E+08	9.817E+07	52.65	<.001
range	4	4.412E+07	1.103E+07	5.92	<.001
Block.Day	2	1.110E+08	5.552E+07	29.77	<.001

Block.range	4	4.606E+06	1.151E+06	0.62	0.651
Day.range	8	4.456E+07	5.571E+06	2.99	0.005
Block.Day.range	8	6.096E+07	7.620E+06	4.09	<.001
Residual	90	1.678E+08	1.865E+06		
Total	119	1.748E+09			

FW

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Block	1	71178301.	71178301.	447.49	<.001
Day	2	10961418.	5480709.	34.46	<.001
range	4	575265.	143816.	0.90	0.465
Block.Day	2	4439454.	2219727.	13.96	<.001
Block.range	4	1596853.	399213.	2.51	0.047
Day.range	8	5357282.	669660.	4.21	<.001
Block.Day.range	8	6471103.	808888.	5.09	<.001
Residual	90	14315551.	159062.		
Total	119	114895227.			

C.3. ANOVA tables for Chapter 5

Table C.186: Antioxidant capacity measured by the FRAP assay from different blackcurrant and strawberry polyphenolic fractions

Table C.185: Total anthocyanins

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Fraction	3	209139.9	69713.3	71.43	<.001
Sample	1	373812.1	373812.1	383.01	<.001
Fraction.Sample	3	118593.3	39531.1	40.50	<.001
Residual	16	15615.8	976.0		
Total	23	717161.2			

C.4. Regressions for Chapter 5

Regression AsA vs Q500

f=y0+a*x

R	**Rsqr**	**Adj Rsqr**	**Standard Error of Estimate**
0.8453	0.7146	0.6968	0.4106

	Coefficient	**Std. Error**	**t**	**P**	**VIF**
y0	-3.6681	0.9460	-3.8776	0.0013	95.5307<
a	0.5049	0.0798	6.3294	<0.0001	95.5307<

Analysis of Variance:

Uncorrected for the mean of the observations:

	DF	SS	MS
Regression	2	100.9754	50.4877
Residual	16	2.6977	0.1686
Total	18	103.6731	5.7596

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	1	6.7547	6.7547	40.0614	<0.0001
Residual	16	2.6977	0.1686		
Total	17	9.4524	0.5560		

Statistical Tests:

PRESS 3.1561

Durbin-Watson Statistic 0.7256 Failed

Normality Test Passed (P = 0.9010)

K-S Statistic = 0.1300 Significance Level = 0.9010

Constant Variance Test Passed (P = 0.6031)

Power of performed test with alpha = 0.0500: 0.9978

Regression AsA vs Q 1000

f=y0+a*x

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.8995	0.8091	0.7972	0.3358

	Coefficient	Std. Error	t	P	VIF
y0	-3.6881	0.7299	-5.0527	0.0001	85.0485<
a	1.2775	0.1551	8.2357	<0.0001	85.0485<

Analysis of Variance:

Uncorrected for the mean of the observations:

	DF	SS	MS
Regression	2	101.8689	50.9345
Residual	16	1.8042	0.1128
Total	18	103.6731	5.7596

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	1	7.6482	7.6482	67.8263	<0.0001
Residual	16	1.8042	0.1128		
Total	17	9.4524	0.5560		

Statistical Tests:

PRESS 2.1435

Durbin-Watson Statistic 0.7535 Failed

Normality Test Passed (P = 0.7965)

K-S Statistic = 0.1474 Significance Level = 0.7965

Constant Variance Test Passed (P = 0.6986)

Power of performed test with alpha = 0.0500: 0.9999

Regression TP vs Q_{1000}

f=y0+a*x

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.8327	0.6934	0.6628	1.5012

	Coefficient	Std. Error	t	P	VIF
y0	-18.3960	7.2665	-2.5316	0.0298	281.1430<
a	2.7706	0.5826	4.7559	0.0008	281.1430<

Analysis of Variance:

Uncorrected for the mean of the observations:

	DF	SS	MS
Regression	2	3161.9794	1580.9897
Residual	10	22.5372	2.2537
Total	12	3184.5166	265.3764

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	1	50.9767	50.9767	22.6189	0.0008
Residual	10	22.5372	2.2537		
Total	11	73.5139	6.6831		

Statistical Tests:

PRESS 37.1977

Durbin-Watson Statistic 1.9894 Passed

Normality Test Passed (P = 0.8279)

K-S Statistic = 0.1732 Significance Level = 0.8279

Constant Variance Test Passed (P = 0.8863)

Power of performed test with alpha = 0.0500: 0.9485

Regression Total anthocyanins vs Q_{1000}

f=y0+a*x

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.8561	0.7329	0.7208	1695.5831

	Coefficient	Std. Error	t	P	VIF
y0	-46819.4989	7645.6975	-6.1236	<0.0001	487.9859<
a	7344.8857	945.2692	7.7702	<0.0001	487.9859<

Analysis of Variance:

Uncorrected for the mean of the observations:

	DF	SS	MS
Regression	2	3940297386.7172	1970148693.3586
Residual	22	63250043.9472	2875001.9976
Total	24	4003547430.6644	166814476.2777

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	1	173579011.5989	173579011.5989	60.3753	<0.0001
Residual	22	63250043.9472	2875001.9976		
Total	23	236829055.5461	10296915.4585		

Statistical Tests:

PRESS 77501763.1083

Durbin-Watson Statistic 1.1096 Failed

Normality Test Passed (P = 0.9894)

K-S Statistic = 0.0880 Significance Level = 0.9894

Constant Variance Test Passed (P = 0.2996)

Power of performed test with alpha = 0.0500: 1.0000

Regression FRAP vs Sensor (different fractions)

f=y0+a*x

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9511	0.9045	0.8886	6.6529

	Coefficient	Std. Error	t	P	VIF
y0	10.9594	3.7556	2.9182	0.0267	2.5493
a	0.1037	0.0138	7.5383	0.0003	2.5493

Analysis of Variance:

Uncorrected for the mean of the observations:

	DF	SS	MS
Regression	2	11242.8443	5621.4222
Residual	6	265.5675	44.2612
Total	8	11508.4118	1438.5515

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	1	2515.1894	2515.1894	56.8260	0.0003
Residual	6	265.5675	44.2612		
Total	7	2780.7568	397.2510		

Statistical Tests:

PRESS 622.6143

Durbin-Watson Statistic 1.3014 Failed

Normality Test Passed (P = 0.6192)

K-S Statistic = 0.2527 Significance Level = 0.6192

Constant Variance Test Failed (P = <0.0001)

Power of performed test with alpha = 0.0500: 0.9846

C.5. ANOVA tables for Chapter 6

Table C.187-C.190: Weight and colour characteristics of six different strawberry cultivars (Exp. 3.2)

Table C.187: Berry weight

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	7	741.96	105.99	2.87	0.011
Residual	72	2663.46	36.99		
Total	79	3405.41			

Table C.188: Chroma (C*)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	7	745.17	106.45	4.16	0.002
Residual	32	819.82	25.62		
Total	39	1565.00			

Table C.189: Hue angle (Hº)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	7	2282.019	326.003	46.06	<.001
Residual	32	226.487	7.078		
Total	39	2508.506			

Table C.190: Lightness (L*)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	7	693.538	99.077	13.59	<.001
Residual	32	233.327	7.291		
Total	39	926.864			

Table C.191-C.195: Antioxidant capacity, organic acid concentrations and total phenolics of six different strawberry cultivars (Exp. 3.2)

Table C.191: AC (FRAP)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	7	235.6120	33.6589	53.79	<.001
Residual	16	10.0112	0.6257		
Total	23	245.6232			

Table C.192: AsA

DW					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	64.38767	9.19824	363.75	<.001
Residual	16	0.40460	0.02529		
Total	23	64.79226			
FW					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	0.6524607	0.0932087	364.39	<.001
Residual	16	0.0040927	0.0002558		
Total	23	0.6565533			

Table C.193: Citric

DW					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	7305.227	1043.604	237.05	<.001
Residual	16	70.440	4.403		
Total	23	7375.667			
FW					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	73.96515	10.56645	237.47	<.001
Residual	16	0.71192	0.04450		
Total	23	74.67707			

Table C.194: Malic

DW					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	11.737449	1.676778	249.25	<.001
Residual	16	0.107637	0.006727		
Total	23	11.845085			
FW					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	11.737449	1.676778	249.25	<.001
Residual	16	0.107637	0.006727		
Total	23	11.845085			

Table C.195: Total phenolics

DW					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	688.546	98.364	20.38	<.001
Residual	16	77.216	4.826		
Total	23	765.762			

		FW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	10.47726	1.49675	24.36	<.001
Residual	16	0.98299	0.06144		
Total	23	11.46026			

Table C.196-C.200: Sugar concentrations of six different strawberry cultivars extracted in either methanol-base or PBS as extraction solvent (Exp. 3.2)

Table C.196: Fructose

		FW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	383.5502	54.7929	60.95	<.001
Extraction	1	172.0223	172.0223	191.35	<.001
Cultivar.Extraction	7	120.8396	17.2628	19.20	<.001
Residual	32	28.7684	0.8990		
Total	47	705.1804			

Table C.197: Glucose

		FW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	209.4791	29.9256	82.49	<.001
Extraction	1	478.6675	478.6675	1319.50	<.001
Cultivar.Extraction	7	98.3351	14.0479	38.72	<.001
Residual	32	11.6084	0.3628		
Total	47	798.0901			

Table C.198: Sucrose

		FW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	2320.2255	331.4608	696.87	<.001
Extraction	1	1174.1441	1174.1441	2468.54	<.001
Cultivar.Extraction	7	272.8265	38.9752	81.94	<.001
Residual	32	15.2206	0.4756		
Total	47	3782.4167			

Table C.199: Total sugar concentrations

		FW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	4391.699	627.386	235.50	<.001
Extraction	1	4796.946	4796.946	1800.60	<.001
Cultivar.Extraction	7	254.256	36.322	13.63	<.001
Residual	32	85.251	2.664		
Total	47	9528.151			

Table C.200: Sugar acid ratio

		FW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Cultivar	7	145.96563	20.85223	232.36	<.001
Extraction	1	39.43857	39.43857	439.47	<.001
Cultivar.Extraction	7	2.42780	0.34683	3.86	0.004
Residual	32	2.87174	0.08974		
Total	47	190.70374			

C.6. Regressions for Chapter 5

Regression AsA sensor vs HPLC
f=y0+a*x

R	**Rsqr**	**Adj Rsqr**	**Standard Error of Estimate**
0.9617	0.9249	0.9215	0.3934

	Coefficient	**Std. Error**	**t**	**P**	**VIF**
y0	0.3329	0.2230	1.4923	0.1498	7.7156<
a	0.7898	0.0480	16.4657	<0.0001	7.7156<

Analysis of Variance:

Uncorrected for the mean of the observations:

	DF	**SS**	**MS**
Regression	2	381.1066	190.5533
Residual	22	3.4044	0.1547
Total	24	384.5110	16.0213

Corrected for the mean of the observations:

	DF	**SS**	**MS**	**F**	**P**
Regression	1	41.9546	41.9546	271.1189	<0.0001
Residual	22	3.4044	0.1547		
Total	23	45.3590	1.9721		

Statistical Tests:

PRESS 4.1741
Durbin-Watson Statistic 0.7196 Failed
Normality Test Passed (P = 0.9989)
K-S Statistic = 0.0746 Significance Level = 0.9989
Constant Variance Test Failed (P = 0.0030)
Power of performed test with alpha = 0.0500: 1.0000

C.7. ANOVA tables for Appendix B

Table C.201-C.204: Comparison of different mobile and stationary phases for HPLC determination of anthocyanins in blackcurrant and strawberry samples.

Table C.201: Total anthocyanins

		FW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
MP	1	25989.2	25989.2	102.07	<.001
SP	1	1461324.4	1461324.4	5739.20	<.001
Sample	2	28333954.1	14166977.0	55639.31	<.001
MP.SP	1	1366419.2	1366419.2	5366.47	<.001
MP.Sample	2	35505.2	17752.6	69.72	<.001
SP.Sample	2	259418.6	129709.3	509.42	<.001
MP.SP.Sample	2	274002.0	137001.0	538.06	<.001
Residual	24	6110.9	254.6		
Total	35	31762723.5			

Table C.202: Cyanidin-3-glucoside

		FW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
MP	1	123.91	123.91	2.58	0.121
SP	1	61.24	61.24	1.28	0.270
Sample	2	89849.82	44924.91	935.48	<.001
MP.SP	1	162.19	162.19	3.38	0.079
MP.Sample	2	86.05	43.02	0.90	0.421
SP.Sample	2	234.21	117.10	2.44	0.109
MP.SP.Sample	2	384.52	192.26	4.00	0.032
Residual	24	1152.56	48.02		
Total	35	92054.49			

Table C.203: Pelargonidin-3-glucoside

		FW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
MP	1	7253.1	7253.1	32.84	<.001
SP	1	610503.6	610503.6	2763.94	<.001
Sample	2	11619843.4	5809921.7	26303.30	<.001
MP.SP	1	1015257.5	1015257.5	4596.38	<.001
MP.Sample	2	981.0	490.5	2.22	0.130
SP.Sample	2	69217.2	34608.6	156.68	<.001
MP.SP.Sample	2	187545.6	93772.8	424.54	<.001
Residual	24	5301.2	220.9		
Total	35	13515902.5			

Table C.203: Pelargonidin-derivative 1

		FW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
MP	1	2.564E+04	2.564E+04	3636.14	<.001
SP	1	9.629E+04	9.629E+04	13653.50	<.001
Sample	2	5.433E+06	2.716E+06	3.851E+05	<.001
MP.SP	1	4.822E+04	4.822E+04	6837.60	<.001
MP.Sample	2	2.604E+04	1.302E+04	1846.32	<.001
SP.Sample	2	8.135E+04	4.067E+04	5767.11	<.001
MP.SP.Sample	2	5.596E+04	2.798E+04	3967.09	<.001
Residual	24	1.693E+02	7.053E+00		
Total	35	5.766E+06			

Table C.204: Pelargonidin-derivative 2

		FW			
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
MP	1	5327.171	5327.171	1124.95	<.001
SP	1	15635.771	15635.771	3301.85	<.001
Sample	2	371.247	185.623	39.20	<.001
MP.SP	1	2074.838	2074.838	438.15	<.001
MP.Sample	2	189.474	94.737	20.01	<.001
SP.Sample	2	311.802	155.901	32.92	<.001
MP.SP.Sample	2	167.753	83.877	17.71	<.001
Residual	24	113.651	4.735		
Total	35	24191.707			

APPENDIX D

HEALTH-PROMOTING PROPERTIES OF RIBES AND *RUBUS* SPECIES

Appendix D

A review on the health-promoting properties of *Ribes* and *Rubus* species

D.1. Introduction

A great deal of berry fruits which are commercially available in its fresh or processed form belong to the *Ribes* and *Rubus* genera which encompass a different set of species including blackberry (*Rubus spp*.), black raspberry (*Rubus occidentalis* L.; *Rubus leucodermis* Torr. & A. Grey), red raspberry (*Rubus idaeus* L.), blackcurrant (*Ribes nigrum* L.), white and red currant (*Ribes rubrum* L.), arctic bramble (*Rubus arcticus* L.), boysenberries (*Rubus ursinus* x *idaeus*), cloudberries (*Rubus chamaemorus* L.), gooseberry (*Ribes uva-crispa* L.), loganberry (*Rubus loganobaccus* L. H. Bailey), etc. Plants from both *Ribes* and *Rubus* are generally shrubs of small to medium size and generally characterised by giving attractive small fruits rich in potential health-related compounds. As with many other fruits and vegetables they represent an important source of micro and macronutrients including fibre, sugars, vitamins, minerals etc.; however most of their health-promoting properties have been largely associated with their high levels of bioactive compounds (*viz*. ascorbic acid, phenolic acids and flavonoids including anthocyanins) with known antioxidant capacity (**Table 1**). Nowadays, scientific evidence suggests that increase production or ineffective scavenging of reactive oxygen species (ROS) may play a crucial role in the development of certain pathologic conditions, especially cancer or chronic diseases (Wolfe *et al.,* 2008). Consumption of fruits and vegetables are likely to be responsible for decreasing the severity or incidence of these diseases by reducing oxidative stress and modulating signal transduction pathways involved in cell proliferation and survival (Wolfe *et al.,* 2008). In this context and up-to-date, several studies have shown higher antioxidant activity, in cell-free systems, of *Ribes* and *Rubus* fruits when compared to many other food sources. Accordingly, numerous health-promoting properties have been attributed to fruits from either the *Ribes* or *Rubus* genera during the last few decades. Rather than health-related benefits due to single compounds, it is believed that most of their benefits come from the additive or synergistic effect from several bioactives (Seeram, 2008) present in these berries.

Table D1: Nutrient and mineral composition of main *Ribes* and *Rubus* berries.

	Blackcurrant	Blackberries	Raspberries	Redcurrants
%DM	20	12	13	16
Sugars	15.4	9.6	12	14
Organic acids	-	-	-	-
Proteins	1.4	1.4	0.9	1.4
Fiber	-	5	7	4
Vitamin C	85-500	11-28	25	22-53
Anthocyanins	152-400	160	-	1.1-136
Antioxidant activity	-	56.6-71.8	-	40-63

The present chapter aims to describe the different key bioactive compounds of fruits from *Ribes* and *Rubus* species and discuss the latest scientific evidence on the health-promoting properties of these fruits.

D.1.1 Ribes

The genera *Ribes* L. embraces both the shrubs of currants and gooseberries and belongs to the family Grossulariaceae. It includes more than 150 described species of bushes which are native throughout Northern Europe, North America, and Asia and from mountainous areas of northwest Africa and South America (Brennan, 2005). Five main *Ribes* subgenera are grown for their fruit and these include black currants, red currants, white currants, gooseberries and jostaberries (Brennan, 2005). Historically, blackcurrants, for instance, were imported from Holland to England in 1611 by Tradescant (Brennan, 2005). Later on, during the 18th century blackcurrants were domesticated in Eastern Europe and spread over Russia and in the particular case of UK, blackcurrant cultivation was especially encouraged by the British Government during the 2nd World War due in part to its suitability to the UK weather and the high content of vitamin C of the berries, as no other sources of this vitamin were really available. In those dates, the major part of the production was made into blackcurrant syrup or juice whereas nowadays blackcurrants are still the leading *Ribes* crop worldwide and are still mainly processed rather than used fresh due to their strong flavour (Brennan, 1997; Barney and Hummer, 2005). Red currants, on the other hand, are typically grown to be eaten fresh or to be processed into juice and conserves while white currants provide the greatest yields and are freshly consumed and used for baby food processing (Barney and Hummer, 2005). Similarly, gooseberries are mainly cultivated for the fresh market and for the inclusion into jams and pies (Brennan, 2005).

Ribes fruits have been appreciated for centuries as a nutritious food. Berries, including blackcurrants, redcurrants, etc. may be considered as an ancient food in northern Europe. As for other species from the same genera, the use of blackcurrant fruit as an herbal medicine emerged in the middle ages. In the 16th century European herbalists started to recommend *Ribes* berries or their syrups for the treatment of several illnesses including bladder stones and liver disorders, coughs and lung ailments. Thus said, it was not until the eighteenth century when the use of *Ribes* fruits became widespread among European herbalists and physicians. Several berry-derived products were employed for treatment in numerous intestinal conditions, typhoid fever, gout, rheumatism, and for infections of the mouth, skin, and urinary tract.

Economically, the currant and gooseberry production around the world is mainly based in Asia, Europe and Australia. Within Asia, the Russian Federation represents more than 99 per cent of the total production whilst Poland and Germany produce more than 70 per cent of the European crop (**Table D2**). New Zealand represents almost the totality of the crop harvested in Australia; 6,110 tonnes out of the 7,110 tonnes harvested during 2005. Currently, North American acreage for currants and gooseberries is increasing and this is, in part due to both the lifting of the legislation which prohibited blackcurrant cultivation in several States and the release of new resistant varieties. Thus said, the crop has not still reach the popularity it currently has in Europe, and such is reflected by the paucity of research undertaken by the USA with these berries as compared to other berry fruits.

Table D2: Production (1000 tones) for the main producing countries of currants and gooseberries from the European Union.

Country	***1990***	***1995***	***2000***	***2005***
Poland	165.3	196.9	175.3	203.5
Germany	230.7	252.3	246.5	186
United Kingdom	18.9	21.9	13.8	22.4
Austria	25.8	19.7	24.7	21.1
Czech Republic	-	32.5	24.9	18.6
Hungary	23.4	16	16.5	13.4
France	7.5	11.3	8.4	13
Total	***471.6***	***550.6***	***510.1***	***478***

Source: FAOSTAT 2008.

D.1.2 Rubus

Rubus is a broad genera of flowering plants in the family Rosaceae, subfamily Rosoideae. It is found worldwide, except in desert areas but mainly present in the northern hemisphere. The

important cultivated species from the genera include the European red raspberry (*Rubus idaeus* ssp.*idaeus*), the North American red raspberry (*Rubus strigosus* (Michx.)Maxim.), the eastern North American blackberry (*Rubus occidentalis* L.) and the Andean blackberry hybrid (*Rubus glaucus* Benth., *Rubus adenotrichus* Schlech.) (Mertz *et al.*, 2007). Blackberry fruits, for instance, tend to be first green and red to brown red and hard when immature but turn into black-coloured and juicy fruits as the berry ripens (Perkins-Veazie, 2004; Nunes, 2008). Most of the commercial blackberry production occurs in the United States but with appreciable amounts also grown in the United Kingdom and New Zealand (Dai *et al.*, 2007). Within the USA, the pacific coast region produces *ca*. 80% of the total national production. Raspberries are the most important species in the genera *Rubus* although being considered one of the most perishable fruits with the risk of decay, colour darkening and changes in flavour occurring rapidly after harvest (Krüger *et al.*, 2003). Historically, raspberries and other *Rubus* species can be tracked to ancient times. However, the first written mention of raspberries can be found in an English book on herbal medicine dated 1548. Juices and extracts from *Rubus* fruits were extensively used in the 16th century for the treatment of several conditions but mainly for the treatment of infections (Dai *et al.*, 2007).

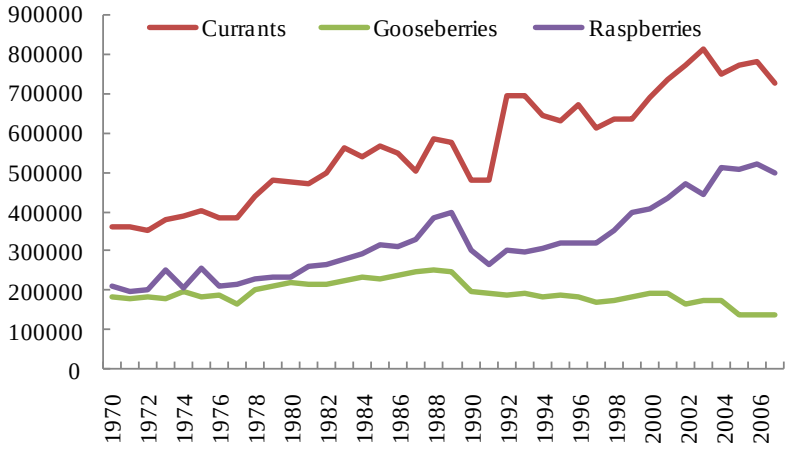


Figure D1: Worldwide production (tonnes) of certain *Ribes* and *Rubus* species from 1970 to 2007 (Data: FAOSTAT 2008).

D.2 Identity and role of bioactives

Ribes and *Rubus* species are characterised by their high anthocyanins and phenolic content as well as other bioactives (e.g. ascorbic acid) which are responsible for ranking berries from these species among the fruits with higher antioxidant capacity. Generally, it is accepted that total phenolic content (TP) and total flavonoids (TF) are well correlated with antioxidant capacity as determined

by any of the standards assays such as FRAP or ORAC (see chapter 19 for further information). For instance, TP and TF of four different raspberry cultivars were well correlated with antioxidant capacity (r^2=0.988 and r^2=0.996) respectively in a study conducted by Liu *et al.* (2002).

D.2.1 Polyphenolic compounds

Phenolic compounds are widely distributed in both *Ribes* and *Rubus* species ranging from simple moieties with an individual hydroxylated aromatic ring to complex polymeric molecules (Harborne, 1995). Phenolics are plant secondary metabolites which are synthesised and accumulate in the plant via endogenous controlled processes or regulated by exogenous factors such as environmental conditions (*viz*. temperature, light) (Dixon and Paiva, 1995). Indeed, the diverse range of phenolics found in berries from *Ribes* or *Rubus* species are responsible for their astringency, bitterness, colour, flavour and also for the oxidative stability of their derived products. Due in part to the high concentration of phenolic compounds of *Ribes* and *Rubus* fruits, a great deal of research has investigated the different polyphenolic fractions of these berries. However, it is important to note that most of this information is focused on blackberries, raspberries and blackcurrants (Zadernowski *et al.*, 2005; Mertz *et al.*, 2007; Giné Bordonaba and Terry, 2008) whereas little is known about other minor *Ribes* and *Rubus* berries.

Generally, polyphenol content may be estimated by adaptations of the standard Foulin Ciocalteu method. Briefly, this method is based on the reduction of a phosphowolframate-phosphomolibdate complex by phenolics compounds resulting in blue reaction products (see chapter 19 for further information) which are then measured spectrophotometrically. By using this method with any of its reported modifications, many papers refer to the higher total phenolic content, expressed as gallic acid equivalents (GAE), of different *Ribes* and *Rubus* fruits as compared to other fruits and vegetables (**Figure D2**). Thus said, values for TP content found in the literature are controversial since variation in the content of total phenolics between berry types is mainly due to differences in cultivar, agroclimatic and growing conditions and finally to differences in the methods used in each study (Giné Bordonaba and Terry, 2008).

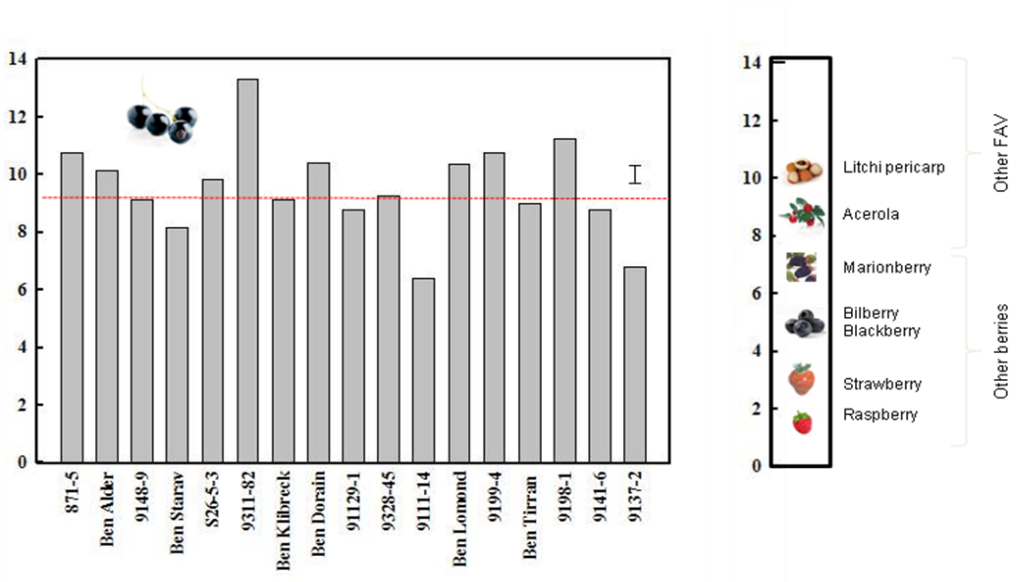


Figure D2: Concentration of total phenolics (mg GAE g^{-1}), measured by the Folin Ciocalteau method, in 17 UK-grown blackcurrant cultivars and compared to other fruits and vegetables (FAV). Results are expressed on a fresh weight (FW) basis and bar indicates LSD value ($P < 0.05$) (Source: based on Giné Bordonaba and Terry, 2008)

D.2.1.1 Flavonoids

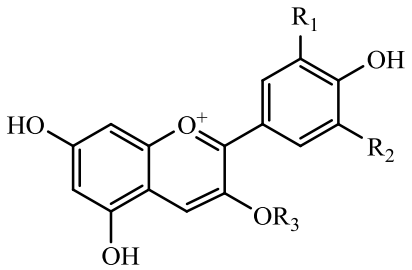
Flavonoids are a group of polyphenolic compounds which can be divided into different subclasses including flavanols, flavonols, flavones, flavanones, isoflavones and anthocyanidins (Pinent *et al.*, 2008). Most berries from *Ribes* and *Rubus* genera are rich sources of these compounds, with blackcurrants, for instance containing *ca*. 10-fold greater flavonol concentrations than other berries (Häkkinen *et al.* 1999). Some of these flavonols (i.e. quercetin) are ubiquitously found in most *Ribes* berries accounting for 46.3, 39.6, 29.8, 14.3 and 10.1% of the total phenolic and flavonol fraction in green gooseberry, redcurrant, blackcurrant, green currant and white currant (Häkkinen *et al.* 1999). In contrast, *Rubus* species including red raspberry, artic bramble and cloudberry had no more than 2.5% of quercetin (**Figure D4**). In the same study, relative concentrations of myricetin and kaempferol ranged from 0 to 9.4% for all the above mentioned *Ribes* and *Rubus* species (Häkkinen *et al.* 1999).

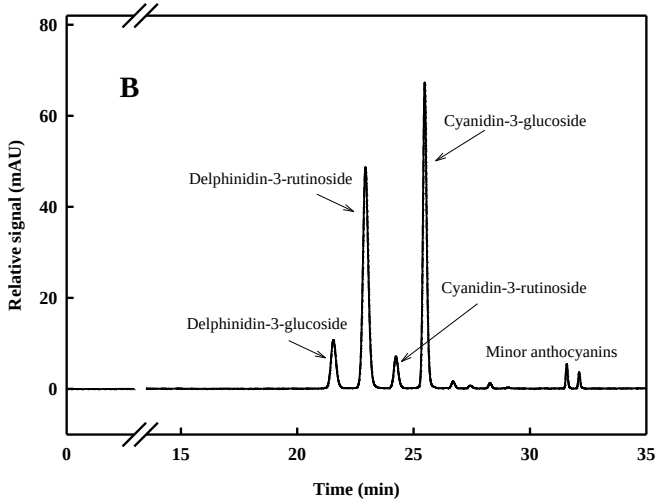
Anthocyanins are considered one of the main plant pigments visible to the human eye. They belong to the flavonoid class and they usually conjugate to form glycosides of polyhydroxy and polymetoxy derivatives of 2-phenylbenzopyrilium or flavylium salts. The differences between different anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars

attached to the molecule, the position of these sugars, usually C3 or less frequently at C5 or C7, and the nature and number of aliphatic or aromatic acids attached to the sugars (**Figure D3**). In fruits, anthocyanins are generally found in the external layers of the skin (hypodermis), and within the skin these compounds are encountered in vacuoles of different sizes. Anthocyanins in berries including raspberry, blackberries and blackcurrants have been extensively studied during the past years not only for their interest as natural colourants but also to their health-promoting properties. Indeed, blackcurrant extracts have hitherto acted as an important model to understand anthocyanin absorption in both humans and animals (Matsumoto *et al.,* 2001; Netzel *et al.,* 2001; Nielsen *et al.,* 2003; Wu *et al.,* 2005). A simple survey of the literature, using any of the available search engines, reveals that the number of articles published referring to blackcurrant anthocyanins have exponentially increased during the last fifteen years (from one in 1991 up to 15 articles in 2008), and similar results can be obtained if searching for other *Ribes* or *Rubus* species. All studies so far, have concluded that four major anthocyanins (**Figure D3**) (*viz*. cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-glucoside and delphinidin-3-rutinoside) constitute almost 90% of the total anthocyanin content of blackcurrants (Häkkinen *et al.,* 1999; Manhita *et al.,* 2006; Anttonen *et al.,* 2006; Rubinskiene *et al.,* 2006; Jordheim *et al.,* 2007; Giné Bordonaba and Terry, 2008). Other anthocyanins including peonidin-3-rutinoside and malvidin-3-glucoside have been also reported, but in lesser amounts in this berry (Frøytlog *et al.,* 1998; Slimestad and Soldheim, 2002; **Figure D3**). In raspberries, cyanidin-3-sophoroside, cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-glucorutinoside and pelargonidin-3-sophoroside as well as pelargonidin-3-glucoside have all been identified (De Ancos *et al.,* 2000; Fan-Chiang and Wrolstad, 2005). Marionberry anthocyanins include cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-glucoside and acylated cyanidin-based anthocyanins (Wu *et al.,* 2004). Anthocyanin concentration in raspberries as determined by the pH differential method (see chapter 19) ranged from 1.7 to 576 μg g^{-1} FW depending on the cultivar (Liu *et al.,* 2002). Blackberries contain cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, pelargonidin-3-glucoside, cyanidin-3-xyloside and malvidin-3-glucoside; cyanidin-3-glucoside being the dominant anthocyanin (Goiffon *et al.,* 1991; Fan-Chiang and Wrolstad, 2005). Indeed, anthocyanin distribution in the *Ribes* and *Rubus* genera is specie dependent. Certain European gooseberry cultivars (*Ribes grossularia* L.) contained up to 10 different anthocyanins, with higher proportion of aromatic acylated anthocyanins especially if compared to other commercially available berries (Jordheim *et al.,* 2007) (**Figure D3**). In blackcurrants and blackberries, anthocyanin content is well correlated with berry colour since the deeper the colour of the fruit the higher the anthocyanin content. Recently, the anthocyanin profile has been proposed as a valuable tool to distinguish between different *Rubus* species (Mertz *et al.,*

2007). Similarly and combining the anthocyanin profile with multivariate data analysis, recent work was able to discriminate between different blackcurrant cultivars (Giné Bordonaba and Terry, 2008). Besides variations between cultivars and degrees of maturities (Rubinskiene *et al.*, 2006; Giné Bordonaba and Terry, 2008; Giné Bordonaba and Terry (unpublished)) anthocyanin content in both *Ribes* and *Rubus* species depends on the harvest season and agroclimatic conditions (Rubinskiene *et al.*, 2006; Fan-Chiang and Wrolstad, 2005). Over the last decade a vast number of publications have referred to the bioavailability or health-related properties of anthocyanins, including those of *Ribes* and *Rubus* fruits. So far anthocyanins including those commonly found in *Ribes* and *Rubus* fruits, exhibited anti-inflamatory, antioxidant, vasomodulatory, and anti-hemoestatic (Rechner & Kroner, 2005) activities when assessed in vitro. In addition, since early 80s it is known the beneficial effect of these compounds on the treatment of retinopathies (Scharrer and Ober, 1981). In earlier works, Matsumoto *et al.* (2001) observed that despite the low bioavailability of these flavonoids, anthocyanins were directly absorbed, distributed to the blood and excreted in urine as their glycosylated forms. Similar findings have been later reported by other authors when working with *Ribes* or *Rubus* berry extracts as anthocyanin sources (Netzel *et al.*, 2001; Mülleder *et al.*, 2002; McGhie *et al.*, 2003; Hollands *et al.*, 2008).

A





Compound	R1	R2	R3	Berry	Reference
Delphinidin-3-glucoside	OH	OH	D-Glucose	Blackcurrant, gooseberry,	Frøytlog *et al.*, 1998 ; Wu *et al.*, 2004;Jordheim *et al.*, 2007; Giné Bordonaba & Terry, 2008
Delphinidin-3-rutinoside	OH	OH	D-Glucose-L-rhamnose	Blackcurrant, gooseberry	Frøytlog *et al.*, 1998 ; Wu *et al.*, 2004; Jordheim *et al.*, 2007; Giné Bordonaba & Terry, 2008
Cyanidin-3-glucoside	OH	H	D-Glucose	Blackcurrant, raspberry, blackberry, boysenberry, marionberry, gooseberry, redcurrant	Goiffon *et al.*, 1991; Frøytlog *et al.*, 1998 ; Cooney *et al.*, 2004 ; Wu *et al.*, 2004; Jordheim *et al.*, 2007; Mertz *et al.*, 2007; Giné Bordonaba & Terry, 2008;
Cyanidin-3-rutinoside	OH	H	D-Glucose-L-rhamnose	Blackcurrant, raspberry, blackberry, boysenberry, marionberry, gooseberry, redcurrant	Goiffon *et al.*, 1991; Frøytlog *et al.*, 1998 ; Cooney *et al.*, 2004 ; Wu *et al.*, 2004; Jordheim *et al.*, 2007; Mertz *et al.*, 2007; Giné Bordonaba & Terry, 2008
Cyanidin-3-arabinoside	OH	H	D-Arabinose	Blackberry	Goiffon *et al.*, 1991
Pelargonidin-3-glucoside	H	H	D-Glucose	Marionberry, blackberry	Goiffon *et al.*, 1991; De Ancos *et al.*, 2000; Proteggente *et al.*, 2002; Wu *et al.*, 2004; Fan-Chiang and Wrolstad, 2005
Pelargonidin-3-rutinoside	H	H	D-Glucose-L-rhamnose	Blackcurrant	Wu *et al.*, 2004

Peonidin-3-rutinoside	OCH_3	H	D-Glucose-L-rhamnose	Blackcurrant, gooseberry	FrØytlog *et al.*, 1998; Slimestad and Soldheim, 2002; Wu *et al.*, 2004; Jordheim *et al.*, 2007;
Peonidin-3-glucoside	OCH_3	H	D-Glucose	Blackcurrant, gooseberry	Wu *et al.*, 2004 ; Jordheim *et al.*, 2007
Malvidin-3-glucoside	OCH_3	OCH_3	D-Glucose	Blackcurrant, blackberry	Goiffon *et al.*, 1991; FrØytlog *et al.*, 1998; Slimestad and Soldheim, 2002; Wu *et al.*, 2004; Jordheim *et al.*, 2007
Cyanidin-3-sophoroside	OH	H	D-Glucose-L-glucose	Raspberry, redcurrant, boysenberry	Cooney *et al.*, 2004; Wu *et al.*, 2004
Cyanidin-3-glucorutinoside	OH	H	D-glucose-L-rhamnosyl-D-glucose	Raspberry, Blackberry	Goiffon *et al.*, 1991; Gónzalez *et al.*, 2003
Pelargonidin-3-sophoroside	H	H	D-Glucose-L-glucose	Raspberry	De Ancos *et al.*, 2000; Proteggente *et al.*, 2002; Fan-Chiang and Wrolstad, 2005
Cyanidin-3-galactoside	OH	H	D-Galactose	Blackberry	Goiffon *et al.*, 1991
Cyanidin-3-arabinoside	OH	H	D-Arabinose	Blackberry	Goiffon *et al.*, 1991
Cyanidin-3-sambubioside	OH	H	D-Xylose-D-glucose	Redcurrant	Wu *et al.*, 2004
Cyanidin-3-xyloside	OH	H	D-Xylose	Blackberry, gooseberry	Goiffon *et al.*, 1991; Wu *et al.*, 2004
Petunidin-3-glucoside	OH	OCH_3	D-Glucose	Blackcurrant	Wu *et al.*, 2004
Delphinidin-3-xyloside	OH	OH	D-Xylose	Blackcurrant	Wu *et al.*, 2004
Petunidin-3-rutinoside	OH	OCH_3	D-Glucose-L-rhamnose	Blackcurrant	Wu *et al.*, 2004
Malvidin-3-rutinoside	OCH_3	OCH_3	D-Glucose-L-rhamnose	Blackcurrant	Frøytlog *et al.*, 1998; Slimestad & Solheim, 2002;

Figure D3: (A) Chemical structure of *Ribes* and *Rubus* anthocyanins and their occurrence in selected berries. (**B**) Chromatographic profile of major blackcurrant anthocyanins identified by HPLC coupled to DAD (Giné Bordonaba *et al.*, 2010)

As mentioned earlier, all flavonoids from *Ribes* and *Rubus* species except flavanols are found in glycosylated forms which clearly affect their absorption (Scalbert & Williamson, 2000). Absorption in the stomach is possible for some flavonoids in their aglycone form but not for their glycosides. It has been postulated that glycosides forms may resist gastric hydrolysis and therefore arrive to the duodenum as intact molecules. Similarly, in the small intestine absorption is limited to aglycones and some of their glucosides. As a result, most flavonoid molecules linked to rhamnose or other glycoside moieties need to be hydrolysed by the colon microflora prior to their absorption (Scalbert & Williamson, 2000). Anthocyanins, though, represent an exception, since intact glycosides have been recovered from urine or identified as the main form in blood. In contrast, there is little evidence of anthocyanin aglycones in human blood or urine (Kay, 2006) which may be related to the poor stability of this compounds in neutral pH conditions. This dichotomy resulted in the mechanisms involved in anthocyanin metabolism and absorption being still not fully understood. For instance, Passamonti *et al.* (2003) suggested that glycosides of anthocyanins may be transported by bilitranslocase at the gastric level whereas Wu *et al.* (2004) proposed that these molecules may be converted into glucuronides by UDP glucose dehydrogenase. Generally, the urinary excretion of anthocyanins reported is very low ranging from 0.016 and 0.13% of dosage within the first 2-8h after consumption (Nielsen *et al.,* 2003). Nevertheless, recent evidence strongly suggests that anthocyanin metabolites may be overlooked with the current identification methods and hence the absorption of these compounds may have been dramatically underestimated (Felgines *et al.*, 2003). In the particular case of blackcurrants, Nielsen et al (2003) studied the absorption and excretion of blackcurrant anthocyanins and found that the rutinoside forms were detected in urine from both Watanabe heritable hyperlipidemic rabbits and healthy humans in higher concentrations (% excretion from 0 to 4h; 0.058±0.033) than the anthocyanin glucosides (0.046±0.043). The authors suggested that this was probably due to the cleveage of the glucoside forms, but not of the rutinosides, in the small intestines by β-glucosidases. Interestingly, blackcurrant berries are especially higher in both cyanindin and delphinidin rutinoside (Giné Bordonaba and Terry, 2008). Other studies have found larger proportions of the delphinidin glycosides as compared to cyanidin glycosides in blood (Matsumoto *et al.,* 2001) or that the concentration of anthocyanin glycosides in plasma increases and decreases more rapidly compared to their respective

rutinoside forms. Similarly, the plasma concentration: dose ratio, in pigs after ingestion of marionberry freeze-dried powder, was greater for cyanidin-3-rutinoside than that of cyanidin-3-glucoside (Wu *et al.,* 2004). Bioavailability and fate of anthocyanins is however also influenced by the food matrix, and up to date few studies have focussed on this issue. Nielsen *et al.* (2003) studied different food matrixes and found than rabbits fed with blackcurrant juice showed higher plasma level of anthocyanins as compared to animals fed with purified anthocyanins in an aqueous citric acid matrix. However, results from the same study show that in human subjects the concentration of anthocyanins in plasma was not affected by the additional ingestion of a high carbohydrate-rich meal (Nielsen *et al.,* 2003). Further research should address the role that the food matrix may have on the bioavailability of anthocyanin and other flavonoids.

D.2.1.2 Phenolic acids

Phenolic acids including hydroxybenzoic or hydroxycinammates acids are non-flavonoids polyphenolic compounds that are of significant importance in berries from the *Ribes* and *Rubus* genera (Häkkinen *et al.,* 1998; Hakkinen *et al.,* 1999) (**Figure D4**). Nevertheless, significant discrepancies exist between different published works found in the literature regarding phenolic concentrations within these berries. Most of these differences may be due to different cultivars or agroclimatic conditions assessed, but also to the techniques used to extract and quantify the different phenolic fractions (Giné Bordonaba and Terry, 2008). Hydroxybenzoic acids have a general structure directly derived from benzoic acid with variations in the hydroxylation or methylation or the aromatic ring whereas hydroxycinammic acids tend to naturally occur as conjugated forms, being esters of hydroxyacids such as quinic, shikimic and tartaric acids or its corresponding sugar derivatives (Shahidi and Naczk, 2006). For instance, ellagic acid (**Figure D4**), a type of hydroxybenzoic acid derivative, is known to be present in both *Ribes* and *Rubus* berries but particularly in raspberries where it accounts for approximately 88% of the total phenolic acids (Häkkinen *et al.,* 1999; Amakura *et al.,* 2000; Olsson *et al.,* 2004). Raspberries and blackberries contain three times more ellagic acid than walnuts and fifteen times more than other fruits and nuts (Tomás-Barberán & Clifford, 2000). Accordingly, ellagic acid concentration in raspberries was reported as 765±142 μg g^{-1} DW while this compound was not detected in any of the other berries analysed (Olsson *et al.,* 2004). Other studies have detected *ca*. 400 μg g^{-1} DW in freeze-

dried raspberries when extracted with methanol (Daniel *et al.*, 1989). In the same study, after acid hydrolysis concentrations raised up to 1900 µg g^{-1} DW, indicating that most of ellagic acid present in raspberries is encountered as ellagitannins (Daniel *et al.*, 1989). Similarly, in raspberries cultivars Zeva, Heritage and Williamet, ellagic acid was detected together with other six ellagic acid derivatives (Tomás-Barberán & Clifford, 2000). The corresponding ellagic acid concentrations in blackberry pulp and seeds were reported as 2.43 mg g^{-1} DW and 3.37 mg g^{-1} DW (Wang *et al.*, 1996). Hydroxicinnamic acids (113±43 µg g-1 DW), quercetin (28 ± 9 µg g^{-1} DW), quercetin-glycosides (99±35 µg g^{-1} DW) and other flavonols (50 ± 34 µg g^{-1} DW) were detected in blackcurrant berries using an HPLC coupled to diode array detector (DAD) by Olsson *et al.* (2004). Häkkinen *et al.* (1999) found large quantities of p-coumaric and caffeic acid in blackcurrant berries when a large set of different berries were screened for their phenolic content. When assessing variation in the phenolic content of different polish-grown small berries, including blackcurrant and blackberries, Zadernowski *et al.* (2005) reported up to fourteen different phenolic compounds. m-Coumaric acid derivatives were the principal phenolic compounds with concentration over three-fold in blackcurrants (1872.9 ± 145 µg g^{-1}DW) than in blackberries (596.6 ± 75.1 µg g^{-1} DW).

Although phenolic acids are the major polyphenols ingested by humans, the bioavailability of these compounds has not yet received the same attention as that of flavonoids (Lafay & Gil-Izquierdo, 2008). The limited information, so far, reveals that, for instance, absorption of ferulic acid (**Figure D4**) takes place mainly in the small intestine with a urinary excretion of 40% of the ingested dose whereas ferulic acid conjugates are principally absorbed in the large intestine (Kern *et al.*, 2003) and with lower recoveries. Gallic acid (**Figure D4**) is fairly well absorbed in the upper part of the gut with urinary excretions ranging from 36-40% of the ingested dose depending very much on the food source (Shahrzad *et al.*, 2001). Similarly, when hydroxycinnamic acids are ingested, they are rapidly absorbed, which indicates an absorption in the upper part of the gut (Lafay & Gil-Izquierdo, 2008).

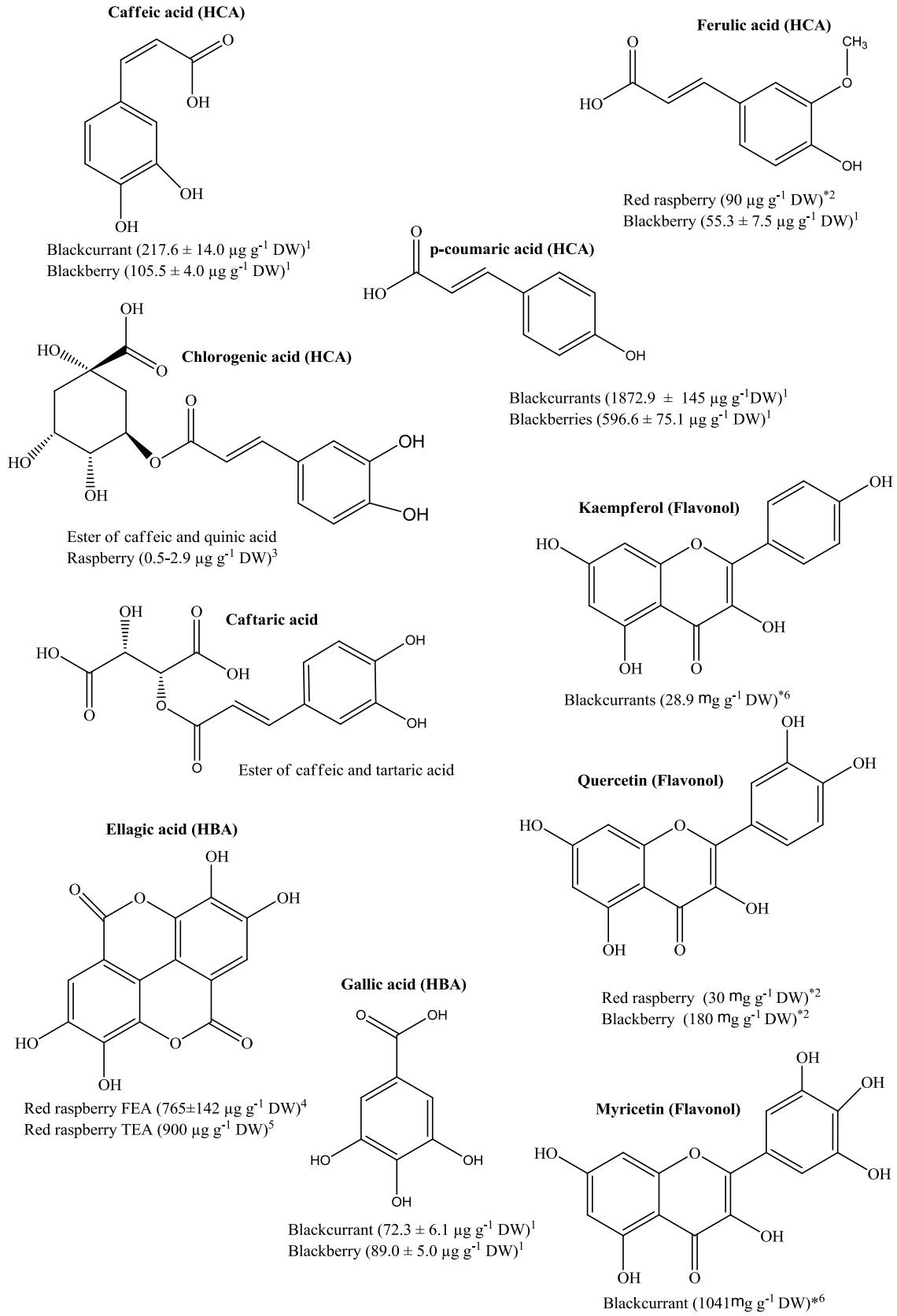


Figure D4: Chemical structure of phenolic acids (hydroxycinnamates (HCA) and hydroxybenzoic acids (HBA)) and flavonols and their reported occurrence in certain *Ribes* and *Rubus* berries. FEA: Free ellagic acid; TEA: Total ellagic acid after acid hydrolysis. [1]Zadernowsky *et al.*, 2005; [2]Jakobek *et al.*, 2009; [3]Zhang *et al.*, 2010;

[4]Olsson *et al.,* 2004 ; [5]Daniel *et al.,* 1989 ; [6]Häkkinen *et al.,* 2000.[*]Values were transformed to dry weight (DW) basis based on 10% dry matter content. Values given for coumaric acid correspond to the m-isomer.

D.2.1.3 Tannins and stilbenes

Tannins are also important component of berry fruits (Szajdek and Borowska, 2008) and are responsible for the astringent taste of some fruits from different *Rubus* and *Ribes* species. Basically, the tart taste of certain berries can be attributed, in part, to the interactions between this type of polyphenols and proteins. Tannins include both condensed non-hydrolysable tannins (*viz*. proanthocyanidins) and hydrolysable tannins (viz. esters of ellagic and gallic acid, also known as ellagitannins and gallotannins, respectively). Although hydrolysable tannins are more rarely encountered in berries (Szajdek and Borowska, 2008), Mertz *et al.* (2007) described two different ellagitanins detected in blackberry extracts. The first one consisting of lamberianin C and the second one tentatively identified as sanguiin H-6, which was previously identified in *Ribes* species by others (Määttä-Riihinen *et al.,* 2003). Recently, McDougall *et al.* (2008) identified similar mixtures of ellagitannin components and ellagic acid in tannin-enriched extract profile from raspberry and cloudberry fruits. Similarly, Nohynek *et al.* (2006) found similar concentrations of ellagitannins in both raspberry and cloudberry, while these components were not detected in blackcurrants.

In blackcurrants, all the cultivars investigated by Wu *et al.* (2004) had a similar proanthocyanidin profile containing both procyanidins and prodelphinidins. In the same study, polymeric proanthocyanidins with degree of polymerisation superior than 10 were the main proanthocyanidin detected (80% of 1.21-1-66 mg g^{-1} FW) (Wu *et al.* 2004). Similarly, earlier works established that average degree of polymerisation in blackcurrant proanthocyanidins was 38.7 (Gu *et al.,* 2003). In a range of gooseberries cultivars and redcurrant, cv. Red lake, proanthocyanidins with high degree of polymerisation (> 10) accounted also for most of the total concentrations of these compounds (0.45 – 1.34 and 60.8 mg g^{-1} FW, respectively) (Wu *et al.* 2004).

Both condensed tannins or hydrolysable tannins show a greater free radical scavenging capacity than vitamin C or other type of polyphenols (Szajdek and Borowska, 2008) and hence their potential role in ameliorating oxidative stress related to many diseases. The bioavailability and metabolism of these types of polyphenols have been extensively study during the last years and results indicate, for instance, that

ellagitannins are primarily metabolised by the intestinal flora rather than being absorbed as such in the human body (Cerdá *et al.,* 2005). Similarly, in vitro studies using human colonic microflora have demonstrated, to certain extent, that polymeric proanthocyanidins were almost completely degraded in 48h (Deprez *et al.,* 2000). Although initial studies showed that proanthocyanidins were metabolised and absorbed in both mice and rats (Santos-Buelga & Scalbert, 2000), more recent studies have failed to corroborate such findings (for the interested reader see the excellent review by Beecher, 2004).

D.2.2 Ascorbic acid (Vitamin C)

Ascorbic acid (AsA) is one of the most important water soluble vitamins. Most plants and animals are able to synthesize this compound, however, apes and human lack the enzymes required for the synthesis of this vitamin and therefore it has to be supplemented mainly through consumption of fruits and vegetables (Naidu, 2003).

Similarly to that described for polyphenolic-type compounds, vitamin C concentration in berries from *Ribes* and *Rubus* species depends on several factors such as genotype, cultivation techniques, agroclimatic conditions, ripeness and postharvest storage and time (Hancock *et al.,* 2007; Pantelidis *et al.,* 2007; Giné Bordonaba and Terry, 2008; Chope, Giné Bordonaba & Terry, unpublished). In blackcurrant, the synthesis and role of ascorbic acid have been recently elucidated (Hancock *et al.,* 2007). Variation in AsA content exist between blackcurrant cultivars (Viola *et al.,* 2000; Giné Bordonaba and Terry, 2008) and such variation has been suggested to be established during initial development stages (Viola *et al.,* 2000). Recently, a wide range of UK-grown blackcurrant cultivars was screened for several quality and health-related components (Giné Bordonaba and Terry, 2008) and concentrations of AsA detected ranged from 1.922 to 5.415 mg g^{-1} FW and therefore were far higher than those found in other common berry fruits where AsA content is commonly < 1 mg g^{-1} FW. Similarly, other studies also found that AsA content in blackcurrant samples was much higher than that of other berries analysed (Remberg *et al.,* 2007). Within other *Ribes* and *Rubus* fruits concentration of AsA, varies from 0.15-0.17 in blackberries, 0.15-0.32 in raspberries, and 0.17-0.21 mg g^{-1} FW in redcurrants (Hägg *et al.,* 1995; De Ancos *et al.,* 2000; Haffner *et al.,* 2002; Benvenuti *et al.,* 2004).

Consumption of products naturally rich in ascorbic acid is associated with multiple health benefits. For instance, both ascorbate and dehydroascorbate delay the initiation of LDL oxidation (Retsky and Frei, 1995) which is a process related to the formation of atherosclerosis. In addition vitamin C plays an important role in the biosynthesis of certain vital constituents (viz. collagen, carnitine, neurotransmitters), and also stimulates immunological resistance and can act as adetoxicant for certain mutagenic and carcinogenic compounds (Coulter *et al.,* 2006). In this context, extensive clinical, animals and in-vitro studies have been conducted during the last decades trying to elucidate such health-promoting properties (for further information see the review by Naidu, 2003). Nevertheless, a study by Olsson *et al.* (2004) failed to demonstrate any prevention on cancer cell proliferation using ascorbate standard alone (Olsson *et al.* 2004). In the same study, a correlation was found between inhibition of cancer cell growth and ascorbic acid content between the different *Ribes* and *Rubus* extracts analysed and therefore the authors speculated that such phenomenon was most probably the result of a synergistic effect of vitamin C with other bioactives present in the extracts studied (Olsson *et al.,* 2004).

D.2.3 Fatty acids

Research over the past two decades have been carried out on the metabolism of polyunsaturated fatty acids (PUFAs), in general, but with special emphasis on that of n-3 fatty acids in particular (Simopoulos, 1999). This is due in part, to the early evidence that reducing the ratio of n-6 to n-3 fatty acids might play a role in decreasing the risk of heart disease and cancer. Nevertheless, numerous health-promoting properties are also reported for certain n-6 fatty acids (Ruiz del Castillo *et al.*, 2002) which are more rarely encountered in nature. Today, it is known that fatty acids are essential for normal growth and development and also may have a crucial role in the prevention and treatment of coronary and metabolic diseases as well as inflammatory and autoimmune disorders and cancer (Simopoulos, 1999). Within the thirty different blackcurrant genotypes studied, Ruiz del Castillo *et al.* (2002) found that gamma-linolenic (n-6) acid ranged from 11-19% of the total fatty acid fraction whereas other fatty acids such as stearidonic and α-linolenic (n-3) acid varied from 2-4% and 10-19%, respectively (Ruiz del Castillo *et al.*, 2002). Few natural products are such rich sources of gammalinolenic acid as blackcurrant seeds. This fatty acid, in particular, is transformed to

dihomogammalinolenic acid (DGLA; 20:3 n-6) which is the intermediate precursor of prostaglandin E_1 which at the same time is recognised for its anti-inflamatory and immunomodulating properties (Leventhal *et al.*, 1994). Supplementation with GLA has shown to be a satisfactory remedy for a diverse range of conditions including rheumatoid arthritis and atopic eczema. Within the *Rubus* genera, blackberries are an exceptionally rich source of omega-3 (α-linolenic acid; n-3) and other polyunsaturated fatty acids (PUFA) owing in part to their numerous and large seeds (Bushman *et al.*, 2004). Seeds, however, tend to pass intact through the alimentary canal and hence any bioactives contained in this part of the fruit may not be assimilated. The preparation of berry extracts may overcome this limitation by enabling a better homogenisation of the different components distributed in the whole fruit rather than specific tissuest. Cold-pressed black raspberry, marionberry and boysenberry seed oil had 32.4, 15.8 and 19.5%, respectively, of α-linolenic acid and 53.0, 62.8 and 53.8%, respectively, of linoleic acid (Parry *et al.*, 2005). In the same study, boysenberry seed oil showed the highest scavenging activity against $DPPH^{\bullet}$ and peroxyl radicals induced by AAPH followed by red raspberry and marionberry (Parry *et al.*, 2005).

D.3 Chemopreventive activity and bioavailability

D.3.1 Introduction

Generally it is accepted that a correct balance between oxidants and antioxidants is synonymous with good health and that alterations to this balance are associated with certain pathologic conditions such as aging, cancer and cardiovascular diseases. In this context, most of the health benefits linked to the intake of berries from *Ribes* and *Rubus* species have been largely linked with the high antioxidant capacity of these fruits when assessed *in-vitro* or in cell-free systems. However, the health-related properties of these berries are may not be limited to the presence of antioxidant compounds. For example, several studies have reported the benefits derived from blackcurrant seed oil (BSO) due to its high content in gammalinolenic acid (Noli *et al.*, 2007) as well as the health-benefits derived from the intake of a blackcurrant polysaccharide fractions (Takata *et al.*, 2005). Moreover, when considering recent studies and taking into consideration the low bioavailability of certain phytochemicals such as flavonoids, it appears that the health benefits associated with these berries may be the result of more complex biological processes rather than their capacity to scavenge free radicals. Williams *et al.* (2004)

suggested that flavonoids may act as modulators of intracellular signalling processes which can modify cellular redox status. Others (Seeram, 2008) suggested the synergistic effect between different berry bioactives as being responsible for many of the reported health-promoting properties.

Fruits and other parts from *Ribes* and *Rubus* plants have been extensively used as remedies for many diseases, and data can be traced back to as early as the 16th century (Dai *et al.*, 2007). Nowadays, there is a plethora of scientific reports available which have described the beneficiary role these berries may have on cardiovascular diseases, brain disfunction and ageing, eye care, urinary tract health, antimutagenic and anticarcinogenic, antibiotic and antiinflamatory processes (**Table D3 and D4**). Some of the most relevant information is summarised in the following sections.

D.3.1 Cancer studies

The anticarcinogenic effects derived from the intake of both *Ribes* and *Rubus* species are well documented (**Table D3**). Bioactive compounds of these berries play different roles in cancer prevention, such as protection against oxidative DNA damage and the formation of DNA adducts, enhancement of DNA repair mechanisms and modulation of signalling pathways involved in different crucial cellular processes (viz. cell proliferation, apoptosis, inflammation, angiogenesis and arrest of the cell cycle) (Stoner *et al.*, 2008). Indeed, ROS-induced DNA damage may be recognised as the possible first step involved in the complex process of carcinogenesis. Several studies (**Table 3**) have demonstrated in-vitro or even in-vivo (using animal models) the effects of *Ribes* and *Rubus* bioactives on the protection against oxidative DNA damage. However, probably most of the information available pertains to *in vitro*-based studies showing the inhibitory effect of berry extracts on different types of cancer cell lines (Liu *et al.*, 2002; Olsson *et al.*, 2004; Han *et al.*, 2005; Ross *et al.*, 2007; McDougall *et al.*, 2008) as well as the ability of extracts to scavenge reactive oxygen species (ROS) (Hecht *et al.*, 2006; Jiao *et al.*, 2005).

Table D3. Reported anticarcinogenic properties of fruit from *Ribes* and *Rubus* species.

Activity	Action	System	Dose	Extract type[1]	Reference
Anticarcinogenic	Inhibition of cancer cell proliferation in a dose dependent manner	HepG2 human liver cancer cells	Extract equivalent to 50mg ml^{-1} of raspberry extracts	Raspberry extracts from four different cultivars (Heritage, Kiwigold, Goldie and Anne)	Liu *et al.*, 2002
Anticarcinogenic	Inhibition of the growth of premalignant and malignant human oral cell lines	Human oral ephitelial cell lines; malignant (83-01-82CA), premalignant (SCC-83-01-82)	50-200 µg ml^{-1} twice over six days	Different fractions from freeze-dried black raspberries extracts (*viz.* ferulic acid, β-sitosterol) from cv. Jewel	Han *et al.*, 2005
Anticarcinogenic	Preventing cell proliferation	Human colon cancer cells (CaCo-2) and Human cervical cancer cells (Hela)	25-75 µg of GAE/mL	Digested[2] raspberry extracts (cv. Glen Ample)	Mc Dougall *et al.*, 2005
Anticarcinogenic	Inhibition of tumor induction by N-nitrosomethylbenzylamine	Mouse epidermal (JB6 C1 41) cells	50 & 100 µg ml^{-1} of bioactive fractions	Different bioactive fractions from freeze-dried black raspberries cv. Jewel	Hecht *et al.*, 2006
Anticarcinogenic	Scavenge ultraviolet induced ·OH and O_2 radicals Decrease the number of malignant and non-malignant skin tomours	In vitro: JB6 Cells In vivo: Mouse model	 3.5 µM C3G $mouse^{-1}$	Cyanidin-3-glucoside from blackberry	Ding *et al.*, 2006
Anticarcinogenic	Inhibition of human colon tumor cell growth Supression of interleukin-12 release	HT 29 Human cancer cells Mouse bone marrow-derived dentritic cells	13.6-49.2 µg anthos ml^{-1} 0-40 µg anthos/ml	Blackberry (cv. Hull) extracts	Dai *et al.*, 2007
Anticarcinogenic	Inhibition of cancer cell proliferation in a dose dependent manner	Human cervical cancer (HeLa) in vitro	17.5 µg ml^{-1} GAE	Ellagitannin rich fraction from raspberry cv. Glen Ample)	Ross *et al.*, 2007
Anticarcinogenic	Preventing cell proliferation	Human colon cancer cells (CaCo-2) and Human cervical cancer cells (Hela)	EC_{50} 25-40 µg polyphenols ml^{-1}	Different polyphenolic fractions from various berry extracts (lingonberry, raspberry, etc)	Mc Dougall *et al.*, 2008

Anticarcinogenic	Inhibition of N-nitrosomethylbenzylamine induced tumours in the rat esophagus	Sprague-Dawley male rats	Different treatments containing either 5% anthocyanins fractions of black raspberry at different concentrations or freeze-dried black raspberry extract	Freeze-dried black raspberry extracts	Wang *et al.*, 2009

[1] Whenever possible sample tissue or the cultivars used are specified. [2] Samples were chemically digested mimicking the conditions that occur in the gastrointestinal tract.

Recently, McDougall *et al.* (2008), when assessing the inhibitory effect of a wide range of berry extracts on human cervical cancer and colon cancer cell lines, found that particularly those from the *Rubus* family were the most effective on preventing cell proliferation. Raspberry extracts from cv. Glen Ample, previously digested in similar conditions than those that occur in the upper gastrointestinal tract, were shown to reduce the population of human HT29 cancer cells in the G1 phase of the cell cycle (Coates *et al.,* 2007). In the same study, the authors observed a protective effect against DNA damage in the HT29 cancer cells due to the same berry extract. In another study, raspberry extracts, from cvs. Heritage, Kiwigold, Goldie and Anne, satisfactorily inhibited, in a dose dependent manner (> 10 mg ml^{-1}), HepG2 cell proliferation (Liu *et al.,* 2002). In this case, the authors could not explain the inhibitory effect as a result of the phenolic/flavonoid fraction of the different extracts investigated, therefore, suggesting that other phytochemicals were most probably involved. Similarly, Ross *et al.* (2007) found that ellagitannin content in raspberry extracts (cv. Glen Ample) was well correlated with the inhibition of cell proliferation and therefore concluded that antiproliferative activity from raspberries was predominantly associated to the content of these bioactives. As in many studies, anthocyanin and other polyphenolic fractions from berries are generally purified from interfering compounds by using solid-phase extraction (As an example, please see **Figure 5.2**).

Seeram *et al.* (2006) also demonstrated the antiproliferative properties of different berry extracts including blackberry, black and red raspberry, yet the applied dose (200 µg ml^{-1}) was probably far higher to what can be supplied in-vivo (Stoner *et al.,* 2008). This said, this seminal work clearly demonstrated the following; (1) significant differences exist between the efficacy of different berry extracts on different cells (oral, breast, colon and prostate human cancer cell lines) and (2) that extracts from black raspberry resulted in a significant induction of cell apoptosis (Seeram *et al.,* 2006). Others (Wu *et al.* 2007; Dai *et al.,* 2007) also concluded that extracts from different berries including blackberry (cv. Hull) inhibited cancer cell proliferation and in specific cases increased certain markers of cancer cells apoptosis. Certain bioactive fractions from black raspberry and blackberry extracts, have also been reported as inhibitors of tumours induced by N-nitrosomethylbenzylamine (Hecht *et al.,* 2006) or potent scavengers of ultraviolet light-induced ·OH and O_2 radicals when assessed in vitro in mouse epidermal JB6 cells.

The health benefits associated with the intake of berry-derived products have also been investigated aiming to develop new food stuffs with added nutritional value. Recently, raspberry seed flour was shown to inhibit cell proliferation in human colon cancer cells (Parry *et al.,* 2006). The authors from the latest study highlighted the potential benefits of these berry-derived products in the formulation of new products. However, in an intervention study, blackcurrant seed press residue, a by-product without specific commercial value, consumed as part of a 250g day^{-1} bread meal (containing 8% of the press residue), failed to reduced, and rather increased, oxidative stress markers in stools and urine of thirty six women (Helbig *et al.,* 2009). Despite serum and stool total tocopherol concentrations were increased as a result of the blackcurrant seed press residue, Helbig and collaborators (2009) pointed out that consumption of ground berry seed may not represent any health advantage.

Although there is still a paucity of information obtained from *in vivo* trials or intervention studies as compared to that from *in vitro* studies, work has also been conducted using both animals and human subjects trying to elucidate the effect of *Rubus* and/or *Ribes* extracts on cancer cell proliferation. For instance, freeze-dried black raspberry extracts (cv. not specified) inhibited tumour-induced development in the rat oesophagus by inhibiting the formation of DNA adducts and reducing the proliferation of prenoplastic cells (Chen *et al.,* 2006). In the same study the authors determined the possible mechanisms of action in which raspberry extracts inhibited tumour development. In addition, a polysaccharide rich fraction from blackcurrant juice was shown to retard tumour growth when tested in Etarlich carcinoma-bearing mice (Takata *et al.,* 2005). Clinical data from human studies also exist, for instance, Stoner's group showed that after consumption of lyophilised black raspberry extract (60 g day^{-1}) in 50 subjects with colorectal cancer and/or polyps, proliferation and angiogenesis biomarkers were diminished as well as apoptosis was enhanced by the same extracts (Stoner *et al.,* 2007). Kresty *et al.,* also showed that lyophilised black raspberry extract (32 or 45 g day^{-1}) diminished urine markers of oxidative stress in patients (n=10) with Barrett's esophagus (Kresty *et al.,* 2006). In contrast to all these positive and encouraging results, there are also cases of human intervention studies that failed to demonstrate the beneficial effects of berry intake (Moller *et al.,* 2004). This dichotomy or contradiction should be appreciated as sometimes the positive benefits of fruit and vegetables are over-exaggerated. Therefore is evident that further research is required at all levels (*in*

vitro, *in vivo* and intervention studies) to understand the mechanisms by which consumption of *Ribes* and *Rubus* fruits may help against cancer.

D.3.2 Cardiovascular and metabolic diseases

In certain pathologic conditions (*viz*. hypertension, diabetes and atherosclerosis), the endothelium-dependent vasorelaxation to different vasodilator agonists is considerably restrained. Such phenomenon is directly associated with a decreased in the release of NO, which certainly is crucial for the regulation of vasomotor tone and structure under certain physiological status. Given this, the development of vasodilator compounds with the ability of restoring NO levels could potentially contribute to the treatments of such cardiovascular diseases. Accordingly, Nakamura *et al.* (2002) showed that blackcurrant concentrate could have an endothelium-dependent vasorelaxation effect when tested *in vitro* with rat thoracic aorta tissues. The authors found that the increased levels of NO were in part one of the mechanisms involved in the vasorelaxation caused by the blackcurrant concentrate. In another study, purified anthocyanins from blackcurrant berries or blackcurrant juice demonstrated an antiatheroesclerotic effect when tested in Watanable heritable hyperlipidemic rabbits (Nielsen *et al.*, 2005) suggesting the potential of these extracts as a prevention of certain cardiovascular conditions. A recent study in which an elderly population was given blackcurrant and other berries drinks showed a statistical significant improvement in oxidative status as measured by plasma antioxidant capacity (McGhie *et al.*, 2007).

There is increasing attention over the positive effect that berry-derived bioactives and specifically anthocyanins have on blood vessels walls (*viz*. vasodilation, permeability, fragility, etc.) (Kähkönen *et al.*, 2003). Concomitant to this, anthocyanins from blackcurrant had considerable antioxidant activity when tested in vitro in lipid environments such as methyl linoleate and human low-density-lipoprotein (Kähkönen *et al.*, 2003). Nevertheless, some of the positive effects from berries of *Ribes* and *Rubus* species on cardiovascular health (**Table D4**) may be associated with the fatty acid composition of the oil obtained from the seeds of those species. Certain disorders, and specifically hypertension, are related to the abnormalities in tissue fatty acid metabolism, due in part to a reduction in desaturase activity. As discussed earlier in this chapter, blackcurrant seed oil (BSO) is a rich source of gammalinolenic acid (GLA), a polyunsaturated fatty acid (PUFA) with known health-related properties. Engler and

Engler (1998) demonstrated already in the last decade, that oil enriched with GLA from blackcurrant had a significant blood pressure-lowering effect when tested in spontaneous hypertensive rats.

Evidence from *in vitro* studies conducted with *Ribes* and *Rubus* species as well as other anthocyanin rich compounds suggest that certain compounds present in these fruits may mitigate certain metabolic diseases (**Table D4**), such as diabetes. Indeed, i*n vitro* studies revealed that flavonoids modified the insulin-secreting capacity of the cells, reduced NaF-induced apoptosis and modulate cell proliferation of β cells (Pinent *et al.,* 2008). β cells are the pancreatic cells responsible to produce and release insulin and hence control blood glucose levels. McDougall *et al.* (2005) showed that when tested *in vitro*, blackcurrant and raspberry polyphenols-rich extracts (with phenolic concentrations ranging from 10 to 1500 µg) had an insulin-like effect since it significantly inhibited both α-amylase and α-glucosidase, compounds responsible for hydrolysing complex carbohydrates into glucose and other simple sugars and hence elevating blood glucose levels. Whereas blackcurrant (cv. Ben Lomond) extracts inhibited α-glucosidase better, α-amylase was more readily inhibited by the raspberry (cv. Glen Ample) extract (McDougall *et al.* 2005). Accordingly, Jayaprakasam *et al.,* (2005) found that specifically cyanidin-3-glucoside and delphinidin-3-glucoside, both anthocyanins commonly present in *Ribes* and *Rubus* species (**Table D4**), were the most effective insulin secretagogues among several anthocyanins tested in vitro. Sugimoto *et al.* (2003) studied the protective effects of major boysenberry anthocyanins (BoAnt) against oxidative stress in streptozotocin (STZ)-induced diabetic rats. Increases in the concentration of plasma oxidative substances and also in the liver were back to the levels of those observed control rats when a diet with this berry anthocyanins was given to the diabetic animals. Accordingly, the authors pointed out that boysenberry anthocyanins were effective in protecting the development of *in vivo* oxidation involved with diabetes. Nevertheless, few *in vivo* studies and clinical data are available yet in order to validate the *in vitro* observations.

D.3.3 Urinary tract health and inhibition of intestinal pathogens

During late 50's and 60's, several studies verified the role of anthocyanins and other polyphenols in altering microbial activity. The results from those studies demonstrated that for instance, anthocyanins had stimulatory as well as inhibitory effects

on microbial growth. More recently, the influence of blackcurrant concentrates or isolated anthocyanins from the same berry on the growth of microorganisms was evaluated (Werlein, *et al.,* 2005). The authors concluded that while the anthocyanin fraction alone did not have significant effects on the growth of the microorganisms studied, blackcurrant extract inhibited *in vitro* the growth of certain microorganisms (*Staphylococcus aureus, Enterococcus faecium*) as well as stimulated the growth of *Saccharomyces cerevisiae* (Werlein, *et al.,* 2005). Finnish researchers have demonstrated in several *in vitro* studies (Puupponen-Pimiä *et al.,* 2001; Puupponen-Pimiä *et al.,* 2005) the inhibitory effect of berry extracts including raspberry, artic bramble and cloudberry, on the growth of both Gram-positive or Gram-negative intestinal pathogens. Recent research on this (**Table D4**), also supports the notion that proanthocyanindins commonly found in *Ribes* and *Rubus* berries prevent the adhesion of certain pathogenic bacteria to uroepithelial cells (Foo *et al.*, 2000).

D.3.4 Aging and brain health

There are numerous motor and cognitive behavioural deficits that occur during aging. Although many of the mechanisms involved remain still unclear, numerous researchers sustain that oxidative stress and inflammation are, in part, involved in the aging process (Shukitt-Hale, 2008; Lau *et al.,* 2006). Indeed, Lau *et al.* (2006) suggested that combinations of antioxidants and anti-inflamatory polyphenols from berries may be key compounds to help preventing, suppressing or inhibiting age-related deficits. Studies conducted on animals showed that supplementation with dietary antioxidants improved cognitive function (Joseph *et al.,* 1998). Even though little research has been conducted with fruits from *Ribes* and *Rubus* on this regard, it is assumed that similar results to those obtained with blueberries or other berries may be attributed to consumption of these fruits. For instance, Shukitt-Hale *et al.* (2009) recently examined the effect of a 2% blackberry-supplemented diet in reversing age-related deficits of aged rats. Results indicated that the blackberry diet not only improved motor performance on various tasks but that blackberry-fed rats had significantly greater working, or short-term, memory performance than the control rats (Shukitt-Hale *et al.* 2009). Another of the few studies conducted on *Ribes* berries is that by McGhie *et al.* (2007) in which the authors assessed the ability of blackcurrant-based drinks to improve measures of oxidative stress and inflammation in an elderly population and observed an improvement in the plasma antioxidant capacity. Nevertheless, after the blackcurrant intake, plasma antioxidant

capacity was the only indicator which improved from a wider range of oxidative stress markers studied. Anthocyanins and other polyphenolic fractions, at concentrations from 100 to 500 μg mL^{-1}, of *Rubus* (boysenberry cv. Riwaka Choice) and *Ribes* (blackcurrant cv. Ben Ard) species have been reported to have protective effect against the cytotoxic or neurotoxic effect of dopamine and amyloid β25-35 in M1 muscarinic receptor-transfected COS-7 brain cells (Ghosh, *et al.,* 2007). Either dopamine or amyloid β_{25-35} have the ability of disrupting Ca^{2+} buffer ability of brain cells, leading to further oxidative stress and cell degeneration associated with ageing. The mechanisms underlying the positive effects of berries and other fruits and vegetables on aging have been recently reviewed by Shukitt-Hale *et al.* (2008) and Joseph *et al.* (2009).

D.3.5 Other health-promoted properties

Blackcurrant seed oil (BSO) supplemented to patients suffering rheumatoid arthritis during a 24-week trial resulted in a significant reduction of signs and symptoms of disease activity (Leventhal *et al.,* 1994). The authors concluded that BSO was a potentially effective treatment for rheumatoid arthritis. Accordingly, other studies conducted in vivo with Sprague-Dawley rats, demonstrated that BSO suppressed significantly both the cellular and fluid phases of inflammation (Tate and Zurier, 1994). In this context later studies showed that purified anthocyanins from blackcurrant and other berries were the responsible for inhibition of nuclear factor-KB, which controls the expression of many genes involved in the inflammatory response, as well as the reduction of pro-inflammatory mediators when tested in healthy adults (Karlsen *et al.*, 2007).

Consumption of blackcurrant berries has also been associated with positive effects against kidney stone formation. Keβler et al (2002) showed that blackcurrant juice could be used as a support treatment and metaphylaxis of uric acid stones due to its alkalising effects when assessed. Crude extracts from wild blackcurrant berries had antiviral effects against influenza virus in a study conducted by Suzutani et al (2003). This antiviral activity was speculated to be related to the interaction between the combining site on the viral envelope and certain constituents, not identified, in the crude extract.

BSO administered to dogs suffering from atopic dermatitis resulted in increased concentration of both gamma-linolenic and dihomo-linolenic acid in the serum of the animals, but more importantnly an improvement of atopic dermatitis was observed (Noli *et al.,* 2007). Other health-promoting properties from *Ribes* or *Rubus* berries relate to the eye vision. Nakaishi *et al.* (2000) demonstrated in a double-blind placebo-controlled cross over study with healthy human subjects that blackcurrant anthocyanins at doses of 12.5, 20 or 50 mg had a positive effect preventing myopic shift during visual tasks and promoted visual recovery. In the same study, oral intake of blackcurrant anthocyanins was found to decrease the dark adaptation threshold in a dose-dependent manner.

Few years later, Matsumoto *et al.* (2006), showed the ocular distribution of anthocyanins (BCAs) in rats and rabbits after oral, intravenous and intraperitoneal administration of anthocyanins isolated from blackcurrants. This study revealed, for the first time, that blackcurrant anthocyanins were absorbed and distributed in ocular tissues as intact forms and pass through the blood-aqueous barriers and blood-retinal barriers in both of the animals investigated. In summary, the above-mentioned studies may have demonstrated that oral intake of purified anthocyanins or anthocyanin-rich extracts from *Ribes* and *Rubus* species may be therapeutically used for the treatment of certain ophtalmological conditions.

Table D4. Miscellaneous of health-promoting properties of *Ribes and Rubus* species reported on the literature.

Activity	Action	System	Dose	Extract type	Reference
Antirheumatoid	Reduction in signs and symptoms of disease activity in rheumatoid arthritis patients	Human subjects	1.05 g day^{-1}	Blackcurrant seed oil (BSO)	Leventhal *et al.*, 1994
Antiinflamatory	Supression of both cellular and fluid phases of inflammation as induced by monosodium urate crystals	Sprague-Dawley rats	ND	BSO (Gammalinolenic and alphalinolenic acid)	Tate and Zurier, 1994
Cardiovascular health	Inhibition of blood pressure (BP) over 40% and reduction in diastolic BP	Human subjects	6 g BSO day^{-1} over 8 weeks period	BSO	Deferne and Leeds, 1996.
Cardiovascular health	Favourable blood pressure lowering effect of gammalinolenic acid	Spontaneus hypertensive rats	11% by weight of BSO	GLA enriched BSO (17% GLA)	Engler *et al.*, 1998
Eye health	Preventing myopic refractory shift during visual tasks and promoting visual recovery	Double-blind, placebo-controlled cross over study with healthy human subjects	12.5, 20 and 50 mg $subject^{-1}$	Blackcurrant anthocyanoside concentrate	Nakaishi *et al.*, 2000
Anti-urolithiasis	Alkalizing effect in urine (greater pH and oxalic acid and citric acid in urine) which could support the metaphylaxis and treatment of urolithiasis	Human subjects	330 ml blackcurrant juice in three loading phases per person	Blackcurrant juice	Keβler *et al.*, 2002
Cardiovascular health	Endothelium-dependent vasorelaxation effect	Thoracic aorta from male Sprague-Dawley rats	10-30 µg ml^{-1} blackcurrant concentrate	Blackcurrant concentrate	Nakamura *et al.*, 2002

Antiviral activity	Anti-Influenza Virus (IV) activity	Confluent monolayers of MDCK cells infected with IV A and IV B.	10-100 µg ml^{-1} IC_{50} = 3.2 µg ml^{-1}	Blackcurrant extract (Kurokarin extract)	Know *et al.*, 2003
Antiatheroesclerosis	Increase plasma cholesterol and LDL cholesterol No effect on plasma cholesterol but lowered very low LDL	Watanable heritable hyperlipidemic rabbits	100 mg anthocyanins 100 g^{-1} std diet 100 g standard diet + blackcurrant drink	Purified anthocyanins from blackcurrant and blackcurrant juice	Finné Nielsen *et al.*, 2005
Antidiabetic	Inhibition of α-amylase and α-glucosidase (insulin-like effects)	In vitro assays	10-1500 µg GAE ml^{-1} (α-amylase assay) 5-150 µg GAE ml^{-1} (α-glucosidase assay)	Blackcurrant	Mc Dougall *et al.*, 2005
Antimicrobial activity	Inhibition of human pathogenic bacteria	Liquid cultures of selected pathogenic bacteria (staphylococcus and Salmonella)	1-5 mg ml^{-1} extracts or 2-10mg ml^{-1} dry berry powder	Raspberry and blackcurrant extracts	Puupponen-Pimiä *et al.*, 2005
Immunoestimulatory effects and anticarcinogenic	Macrophage stimulating activity. Especially interleukin (IL) inducing activity and retardation of tumour growth when tested in vivo	In vitro In vivo: Etarlich carcinoma-brearing mice	Juice 10 ml Kg per body weight	Polysaccharide rich fraction from blackcurrant juice	Takata *et al.*, 2005
Antimicrobial activity	Inhibited growth of Staphylococcus aureus DSM 799 and Enterococcus faecium DSM 2918	Microbial culture	0.22-1.224 mg ml^{-1} anthocyanins	Blackcurrant concentrate, blackcurrant juice and blackcurrant powder	Werlein *et al.*, 2005
Antioxidant effect	Protection against induced H2O2 toxicity and protection of DNA damage from HL-60 human pomyelocitic cells	SH-SY5Y human neuroblastoma cells	Within human physiological range	Anthocyanins and polyphenolic fractions from blackcurrant and boysenberry	Ghosh *et al.*, 2006

Antimicrobial activity	Inhibition of human pathogenic bacteria	Liquid culture of selected microbial pathogens	1 mg ml^{-1} dry berry extract	Raspberry, cloudberry and blackcurrant	Nohynek *et al.*, 2006
Protection against neurotoxic effect	Protective effect and restore the calcium buffering ability of cells subjected to oxidative stress by dopamine and amyloid β_{25-35}	COS-7 cells	100-500 µg ml^{-1}	Anthocyanin and phenolic fractions from blackcurrant	Ghosh *et al.*, 2007
Antioxidant and antiinflamatory	Decreased plasma antioxidant activity and reduced plasma malondialdehyde	Human intervention study in an elderly population	Daily consumption per patient	Blackcurrant and boysenberry drink	Mc Ghie *et al.*, 2007
Antidermatitis	Increased concentration of both gammalinolenic and dihomo-gammalinolenic acid in serum and improvement of atopic dermatitis	Dogs with atopic dermatitis	0.1 ml kg^{-1} Body weight daily	BSO	Noli *et al.*, 2007
Antioberisty	Inhibition of pancreatic lipase activity and hence influence fat digestion and affect energy intake	In vitro using lipase from porcine pancreas type II.	50 µg ml^{-1} GAE	Different polyphenols rich berry extracts including blackcurrants raspberry, lingonberry, etc.	McDougall *et al.*, 2009.

D.4 Effect of preharvest, postharvest and processing

The level of secondary metabolites in plants from *Ribes* and *Rubus* is regulated by both environmental and genetic factors. Plants produce a wide range of bioactive compounds as a result of survival or adaptive strategies. These bioactives compounds, are plant secondary metabolites produced for defence, protection and cell-to-cell signalling as a response of exposure to certain environmental stresses. Although the environmental mechanisms responsible for enhanced-bioactive content in *Ribes* and *Rubus* species remain still unclear, cultivation of plants under certain stress conditions is one of the means to enhance the content of these berry bioactives. In addition, extensive research is being done through several breeding programmes worldwide to develop improved varieties with enhanced content of phytochemicals in combination with low-input cropping systems (Brennan, 2007).

Blackberries, blackcurrants and raspberries, like other berries from the *Ribes* and *Rubus* species, are not only available fresh but are mainly distributed as frozen and thermally processed products (*viz*. jellies, juices, purees, cobblers and pies). For instance, most of the blackcurrant market in UK is designated to the production of blackcurrant juice (e.g. Ribena®). After harvest, fruit quality of both *Ribes* and *Rubus* fruits decline dramatically, making postharvest storage at chilling temperatures (around 0ºC) a requirement for the industry and generally for periods no longer than 3 weeks (Harb, Bisharat, & Streif, 2008). Controlled atmosphere (CA) is also occasionally used to extend storage life, not only for blackcurrants, but for many other perishable berry fruits (Agar *et al.,* 1997; Terry *et al.,* 2009) when prolonged storage is required. However, both nutritional value and quality of berries is known to be negatively affected by postharvest storage conditions. For example, the concentration of ascorbic acid (AsA) in berries tends to decrease with increased storage temperature and time (Roelofs *et al.,* 1993; Agar *et al.,* 1997; Kalt *et al.,* 1999; Häkkinen *et al.,* 2000; Viola *et al.,* 2000; Antunes *et al.,* 2003). In particular, Roelofs *et al.* (1993) showed that AsA content was significantly reduced when redcurrant berries were stored for 25 days at either 1ºC or at fluctuating temperatures between 10 and 20ºC. Similarly, a reduction of 40% in AsA content was observed in blackcurrant berries stored for 10 days at 10 or 20ºC (Viola *et al.,* 2000). Even greater reductions in AsA, up to 50% of the initial content, were reported by Antunes *et al.* (2003) in blackberries stored at 20ºC. Generally, the decline in AsA, and indeed overall acid

concentrations, during storage is accompanied by a darkening of the berry (Chope, Giné Bordonaba & Terry, unpublished). This change in coloration has been related to an increase in anthocyanin concentration (Robbins *et al.,* 1989; Kalt *et al.,* 1999) which occurs in a temperature and time dependent manner. Raspberries stored at 0ºC for 24 days contained 70% more anthocyanins as compared to initial values after harvest (Robbins *et al.,* 1989). Conversely, another study (Chanjirakul *et al.,* 2006) showed that in raspberries stored for 7 or 10 days at 10ºC the concentration of anthocyanins was reduced considerably as compared to initial values before storage. Other bioactives are also affected by storage temperature. Ellagic acid content in red raspberry was reduced by 30% after 9 months of storage at -20ºC in a study conducted by Häkkinen *et al.,* 2000. As mentioned earlier, controlled atmospheres (CA) and in particular those with high CO_2 concentrations may be used to extend shelf life of many berries including blackberry, raspberry and currants (Terry *et al.,* 2009). However, under this storage conditions, berries from *Ribes* (*viz*. black and redcurrants) and *Rubus* (blackberry and raspberry) tend to suffer considerable reductions in their ascorbic acid content (Agar *et al.* 1997). Little research has been conducted on elucidating the effects of CA storage on other common bioactives from *Ribes* and *Rubus* species.

The effect of postharvest processing treatment is also well documented. In all thermally processed blackberry-derived products the concentration of monomeric anthocyanins as well as the antioxidant activity of the products dramatically declined as compared to non-treated products (Hager *et al.,* 2008). In the same study, juice processing resulted in the greatest losses whereas canned products were the least affected by processing. Most of the anthocyanin losses occurred during blanching and enzymatic treatment of blackberry juice (34% loss in total monomeric anthocyanins). In contrast, total phenolic concentration of blackcurrant juices stored at 4ºC tended to decline from day 0 (1919.8 ± 149.5 mg ml^{-1} GAE) to day 15 (1309.6 ± 107.8 mg ml^{-1} GAE) but raised up to initial values after 29 days of storage (Piljac-Žegarac *et al.,* 2009), thus said, antioxidant capacity as measured by the trolox equivalent antioxidant capacity (TEAC) assay was significantly diminished. Postharvest storage of processed blackberry products also resulted in significant losses of monomeric anthocyanins from this berry, but had little or no significant effect on the antioxidant activity of most of the blackberry derived products. Indeed,

processing blackcurrant derived products also dramatic reduced the content of total anthocyanins (0.05-10.3% of the levels in fresh fruit) and did not enhance the urinary yield of anthocyanins in human subjects (Hollands *et al.*, 2008).

D.5 Conclusions and future research needs

Considerable amount of recent studies advocate that a high intake of *Ribes* and *Rubus* fruits may offer a number of health benefits against degenerative diseases and can promote longevity. Based on the survey of the literature presented herein, there is no doubt that most of *Ribes* and *Rubus* berries are particularly rich sources of biologically active compounds (i.e. high levels of anthocyanins, proanthocyanidins, quercetin, myricetin, phenolic acids, etc.). In addition, and for instance, blackcurrants are one of the richest sources of vitamin C, contributing together with bioactive phenolics to the high antioxidant activity of berries. The array of health-promoting from these berries range from the inhibition of development of certain cancers, cardiovascular and metabolic disorders, and inflammation related diseases. Besides, *Ribes* and *Rubus* fruits, may therapeutically be used to treat urinary infections, ophtalmological diseases and even fight against ageing-related conditions. Blackcurrant was recently demonstrated to provide effective neuroprotection against oxidative stress induced neuronal damages in human cell cultures. Among the bioactives of these berries, anthocyanins have received much attention in comparison with other polyphenols or non-polyphenol type compounds and hence further research should clarify the health-promoting properties of *Ribes* and *Rubus* bioactives other than anthocyanins.

As indicated, a majority of *Ribes* berries are consumed as derived products rather than fresh. However, most of the information related to the health-promoting properties refers to fresh berries or purified berry fractions rather than what the consumer normally gets. Only a limited number of studies have shown the detrimental effect of processing on the concentration and bioavailability of certain *Ribes* and *Rubus* bioactives (Hollands *et al.*, 2008).

Despite all the positive effects mentioned earlier, robust animal and human intervention trials are still necessary in order to substantiate any claims of human health benefits.

APPENDIX E

HEALTH-PROMOTING PROPERTIES OF STRAWBERRIES

Appendix E

A review on the health-promoting properties of strawberries

E.1 Introduction

Strawberry fruit (*Fragaria* x *ananassa* Duch.) is one of the most widely consumed fruits worldwide, either as fresh, processed products or even as dietary supplements, and worldwide represent an overall cultivated area greater than 200,000 ha (Liu *et al.*, 2007). As fresh fruits or derived-products they constitute a rich source of diverse bioactives with an array of known health-promoting properties. Recently, within a large set of 1113 food samples obtained from the US Department of Agriculture National Food and Nutrient Analysis Program strawberries were ranked among the top three regarding their antioxidant content (3.584 mmol serving^{-1}) (Halvorsen *et al.*, 2006). In a following study, Wolfe *et al.* (2008) reported that strawberry fruits were amongst the largest suppliers of cellular antioxidant activity from 25 different fruits and vegetables consumed by the American population. In agreement with others, strawberries were also the top source of antioxidants from fruits and vegetables (FAV) in the Scottish population studied by Haleem *et al.* (2008).

E.1.1 The strawberry fruit

The commercial strawberry fruit belongs to the Rosoideae order of the Rosaceae family. Strawberry is a perennial plant with rooting runners that usually bears red fruit once it is developed. Botanically, strawberry fruit is in fact a "false fruit" being described as a modified receptacle with one-seeded fruits or achenes located on the outer surface (Perkins-Veazie, 1995). Whereas most crops were domesticated some 10,000 thousands years ago, the first strawberry species can only be tracked to roman times (approximately 2,200 years ago), were wild species were grown for their appealing flavour and fragrance. Cultivation of strawberry fruits in Europe did not start until many years later, in the XIVth century when first the French but rapidly followed by the English started to see strawberry plants not only as ornamental plants but as an intriguing food source. Towards the end of the XVIth century three European *Fragaria* species were commonly referred to as *F. vesca*

(diploid), *F. moschata* (hexaploid) and *F. viridis* (diploid), and shortly after that a new wild strawberry, discovered in the eastern part of North America (*F. virginiana*; octoploid) was introduced in the European gardens mainly due to its intense fragrance. It was not until later, that another American species, *F. chilonensis* (octoploid), characterised by good size fruits, reached Europe after its discovery in the Pacific coast of the American continents (Medina-Minguez, 2008). Due to the sterility of *F. chilonensis* plants when introduced in Europe, *F. moschata* and *F. viginiana* were rapidly placed between *F. chilonensis* plants, originating a new strawberry hybrid that was later named as *Fragaria x ananassa* by Duchesne in 1780. The generation of such octoploid hybrid, only 150 years ago, was the origin for most of the cultivated species worldwide. Since then, hundreds of strawberry cultivars (*Fragaria* x *ananassa* Duch.) have been grown, with cv. Elsanta being nowadays the predominant cultivar in North Western Europe.

Through history, though, strawberries have not only been appreciated for their particular flavour but also for their medicinal properties. Indeed, in the XIIIth century, when medical books were filled with botanical remedies, a Greek doctor, named Nicholas Myrepsur, detailed the medicinal properties of strawberries for the treatment of several illnesses (Medina-Minguez, 2008). Nowadays, the popularity of strawberries may be attributed to its characteristic taste, which in part is defined by the balance between sugars and acids within the fruits, and its known potential health-promoting properties.

E.1.2. Economic importance

Worldwide, the production of strawberries has grown steadily during the last 40 years having most of its production in the northern hemisphere (> 95%). The USA is, in official numbers, the leading producing nation, followed by Spain, Turkey and the Russian Federation (**Figure E1**). This said, no official statistics are available for the size of the Chinese strawberry industry, even though it is accepted that China is nowadays a direct competitor for most of the major strawberry producing regions with estimated values for the period 2001-2003 of *ca*. 1.5 million tones (Carter *et al.*, 2005). In addition, strawberry production is one of the main parts of the European soft fruit industry, accounting for instance in the UK for a production value of £96m in 2003 (Defra 2003)

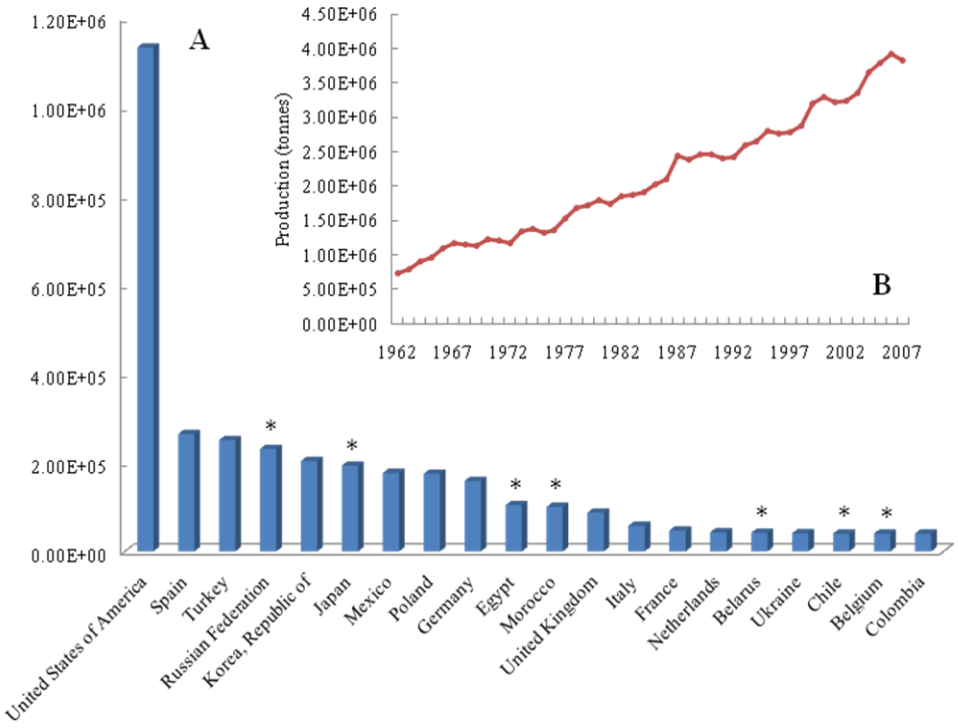


Figure E1: (**A**) Twenty highest strawberry producing countries (tonnes) in 2007. (*) indicate FAO estimates or unoficial figures for production values. (**B**) Worldwide strawberry production over the last forty-five years (Source: FAOSTAT 2009)

E.2 Identity, role and bioavailability of strawberry bioactives

E.2.1 Introduction

Certainly, strawberry fruits have long been recognised as one of the main sources of vitamin C, folic acid, dietary fibre as well as an excellent source of dietary polyphenols in the diet (**Table E1**). Within all types of bioactives present in strawberry fruits, polyphenols are without doubt the ones which have received most attention. In strawberries, the main polyphenols are ellagic acid, ellagic acid glycosides, ellagitannins, gallotannins, flavanols, flavonols, anthocyanins, and coumaroyl glycosides (Hannun, 2004; Zhang *et al.*, 2008) as well as non-polyphenol type such as folate and vitamin C (Tulipani *et al.*, 2009). Up to 40 different phenolic compounds were recently described by Aaby *et al.* (2007) in strawberry fruits cv. Senga Sengana by means of high performance liquid chromatography (HPLC) coupled to different detectors (*viz*. diode array, mass sprectometer, coulometric array). The following sections aim to describe in detail the main bioactive compounds presents in strawberry fruits as well as their bioavailability.

Table E1: Nutrient and bioactive composition of strawberry fruits based on data available in the literature. Values are presented as mg or μg g^{-1} of fresh fruit (FW).

	Concentration	Reference
Dry matter (mg)	53-125	Terry *et al.*, 2007; Tulipani *et al.*, 2008; Giné Bordonaba & Terry, 2009
Fibre (mg)	23	ESHA food database
Sugars (mg)	61.95-110.45	Terry *et al.*, 2007; Giné Bordonaba & Terry, 2009
Glucose	18.01-31.00	
Fructose	22.54-36.10	
Sucrose	21.40-43.35	
Organic acids (mg)	5.96-14.29	Terry *et al.*, 2007; Giné Bordonaba & Terry, 2009
Ascorbate (vitamin C)	0.24-0.74	
Citrate	4.2-10.1	
Malate	1.52-3.45	
Proanthocyanidins (flavanols) (mg)	4.47	Hosseinian *et al.*, 2007
Monomers (epicatechin, B2)	0.31	
Dimers	0.39	
Oligomers	1.55	
Polymers (up to hexamer)	2.22	
Anthocyanins (μg)	66-571	Wang *et al.* 2003; Kosar *et al.* 2004; Määttä-Riihinen *et al.* 2004; Skupien & Oszmianski, 2004; Terry *et al.*, 2007
Cyanidin-3-glucoside	4.5-34	
Pelargonidin-3-glucoside	53-441	
Pelargonidin derivatives	8.4-95.9	
Ellagic acid (μg)	19.9-522	Gil *et al.*, 1997; Häkkinen and Törrönen, 2000; Terry *et al.* (unpublished)
Folate (vitamin B) (μg)	0.13-0.96	Strålsjö *et al.* 2003; Tulipani *et al.*, 2008
Resveratrol (μg)	0.12-2[a]	Wang *et al.*, 2007
Quercetin (μg)	3-40	Gil *et al.*, 1997; Häkkinen and Törrönen, 2000

Kaempferol (µg)	2-13.7	Gil *et al.*, 1997; Häkkinen and Törrönen, 2000
Total phenolics[b]	0.86-3.75	Terry *et al.*, 2007; Giné Bordonaba & Terry, 2009
Antioxidant capacity[c]	6.2-17.8	Terry *et al.*, 2007; Giné Bordonaba & Terry, 2009

[a] values for pulp and achenes, respectively; [b] measured by the Folin-Ciocalteu assay and results expressed as mg gallic acid equivalents (GAE) g^{-1} FW; [c] measured by the FRAP assay and results expressed as µmol Fe^{2+} g^{-1} FW

E.2.2 Flavanols

Flavanols, not to be confused with flavonols are also a type of flavonoid that may play and important role in the prevention of certain pathologies (Santos-Buelga and Scalbert, 2000; Gonzalez-Paramás *et al.*, 2006). *In-vitro* studies have endeavoured to reveal the different biological activities of these compounds (*viz*. antioxidant and scavenger of free radicals, inhibitors of tumour growth and development, antibacterial, etc.; Pascual-Teresa *et al.*, 2000). The content of flavanols in strawberries was described in detail by Pascual-Teresa *et al.* (2000), in which ten different compounds were identified; catechin-(4,8)-catechin (10.1 µg g^{-1} FW), catechin (15.7 µg g^{-1} FW) and epicatechin-3O-gallate (6.6 µg g^{-1} FW) being the main ones. Studies on flavan-3-ols showed that their bioavailability is mainly dependent and intimately linked to the chemical structure of the molecule. For instance, catechin and epicatechin monomers are supposed to be one of the most bioavailable polyphenols with urinary excretions ranging from 1 to 30% of the ingested amounts (Tomás-Barberán, 2008). Nonetheless, further studies are required on other flavan-3-ols to reach any conclusion on their metabolism and differential adsorption in the human body.

Proanthocyanidins are better known as condensed tannins, they are mixtures of oligomers and polymers build up of flavan-3-ol units and principally bond through C4-C8 bond (Gu *et al.*, 2003). Recent evidence suggests that certain proanthocyanins not only occur in red wines but also in several foodstuffs including strawberries (Pascual-Teresa *et al.*, 2000; Fossen *et al.*, 2004; González-Paramás *et al.*, 2006). Proanthocyanidins are supposed to contribute to the *French paradox phenomenon* by exerting several health-promoting properties, such as antioxidant, anti-imflamatory and anticarcinogenic activity (Santos-Buelga and Scalbert, 2000). The proanthocyanidin concentration of strawberries and other berries was recently described by Hosseinian *et al.* (2007). When the whole fruit was considered, strawberry (4.47 mg g^{-1} FW) ranked second after raspberry (5.05 mg g^{-1} FW) for their total proanthocyanidin concentration. In the same study, 0.31, 0.39, 1.55 and 2.22 mg

g^{-1} FW corresponded to monomers, dimers, oligomers and polymers of the total anthocyanidins fractions encountered in the fruit.

E.2.3 Flavonoids

Flavonoids are a type of polyphenolic compounds which can be divided into different subclasses including flavanols, flavonols, flavones, flavanones, isoflavones and anthocyanidins. Within the group of flavonoids probably most attention have been paid to the anthocyanins. These are water soluble flavonoids-type polyphenols widely expressed in the plant kingdom. Anthocyanins have been reported to have anticarcinogenic, antiinflamatory, vasoprotective and antiobesity properties (McGhie and Walton, 2007). In addition recent research suggests that anthocyanins may play a role in enhancing vision and improving memory (Joseph *et al.*, 1998). Recent reports estimate an anthocyanin consumption in the United States of *ca*. 12.5 mg day^{-1} (Wu *et al.*, 2006), or even greater in certain European countries (Mullen *et al.*, 2008). Yet, greater consumption does not always result in greater bioavailability and/or effect. Generally, these polar pigments are extracted using aqueous mixtures of either ethanol, methanol or acetone (Terry *et al.*, 2007; Giné Bordonaba & Terry, 2008); this said, discrepancies exist in whether acetone or methanol based solvents are more efficient for the extraction of these compounds from different FAV (Giné Bordonaba and Terry, 2008). Separation and quantification of anthocyanins is generally achieved on reversed phase HPLC coupled to different detection systems (*viz*. Photo diode array (PDA), mass spectrometry (MS)) and use mostly acetonitrile as the mobile phase of choice due to its elution strength, low viscosity, and good miscibility with water.

Anthocyanin concentration in strawberry fruit varies greatly between cultivars and is also influnced by growing conditions and maturity at harvest (Wang *et al.*, 2000; Lopes da Silva *et al.*, 2007; Terry *et al.*, 2007; Crespo *et al.*, 2010). As compared to other fruits a vast array of information is available detailing the anthocyanin profile and concentration of strawberry fruits, all of them identifying pelargonidin-3-glucoside as the main anthocyanin in strawberry fruits, representing over 80% of the total anthocyanin pool (**Figure E2**).

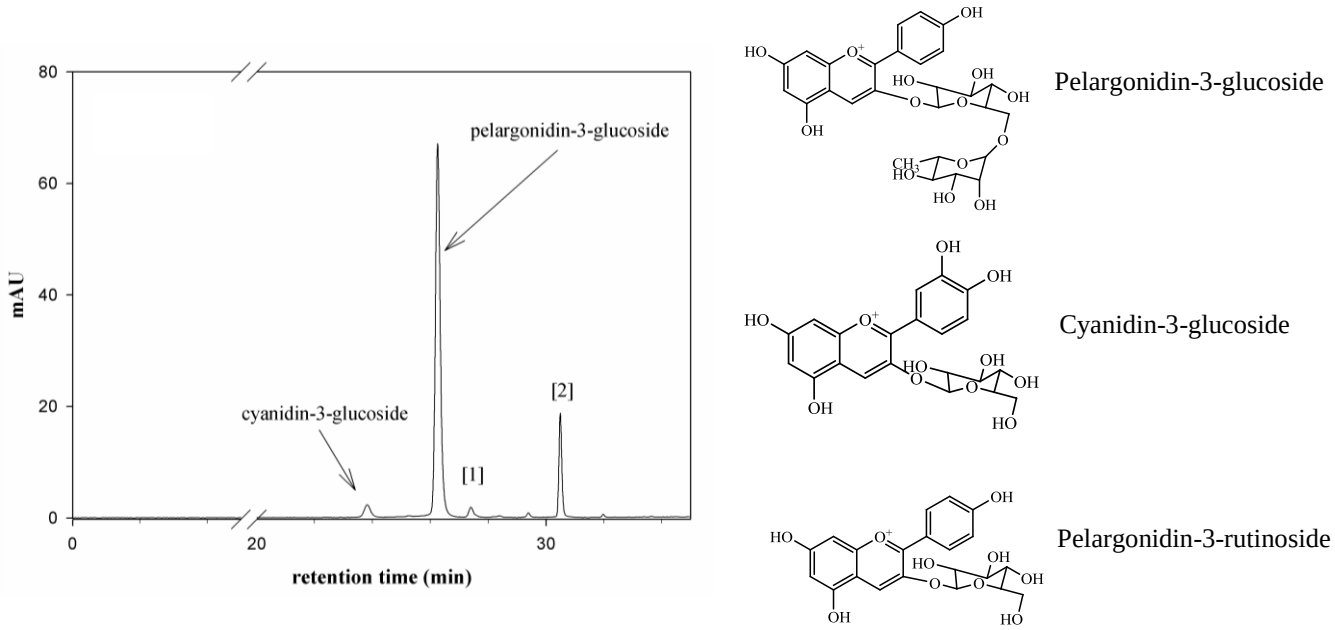


Figure E2: Anthocyanin profile of strawberry fruits (cv. Matis) and chemical structure of main anthocyanins identified in strawberry fruits

Lopes da Silva *et al.* (2007) detected through a detailed study of strawberry pigments (by means of HPLC coupled to DAD and MS detection) up to 25 different anthocyanins within the five different strawberry cultivars analysed. The authors highlighted the notable variability among anthocyanin content in samples of the same variety and harvest and therefore pointed out the strong influence of degree of maturity, edaphic-climatic conditions and postharvest storage on the concentration of these pigments. Terry *et al.* (2007) identified three major anthocyanins (*viz*. cyanidin-3-glucoside, pelargonidin-3-glucoside derivative and pelargonidin-3-glucoside at concentrations of 2.165, 33.56 and 121.54 μg g^{-1} FW respectively) in strawberry cv. Elsanta fruit grown under glasshouse and submitted to full or deficit irrigation (**Figure E3**). Indeed, in the same work the authors demonstrated that differences exist in anthocyanin content between primary and secondary fruit from the same primary truss (**Figure E3**). Similar concentrations were later reported by Crespo *et al.* (2010) when studying anthocyanin concentrations in fruits from four different cultivars and grown at different Swiss production sites.

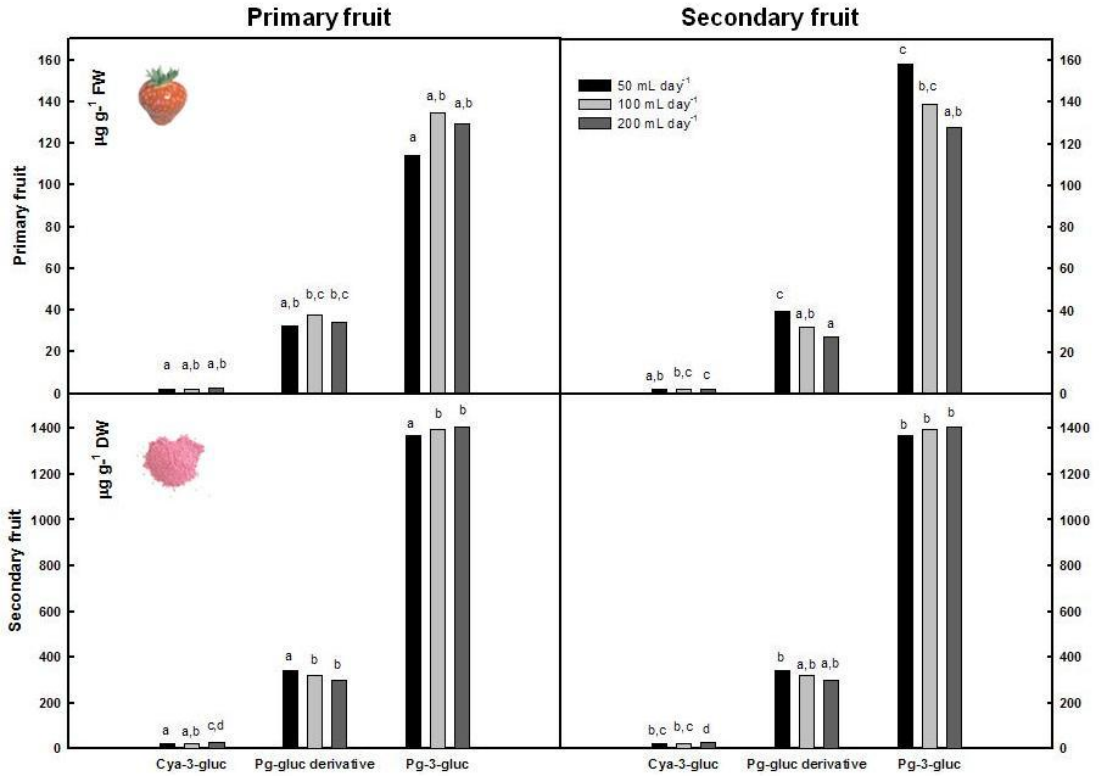


Figure E3: Effect of different water irrigation regimes (mL day^{-1}) on the concentration of main anthocyanins in strawberry (cv. Elsanta) primary and secondary fruit and expressed on a fresh weight (FW; upper panel) and dry weight (DW; lower panel) basis.

In addition to fruit position, anthocyanin profiles differ spatially with different tissues/locations within the fruit. Aaby *et al.* (2005) showed the different anthocyanin profile in receptacle tissue and achenes from two different strawberry cvs. (Totem and Puget Reliance). Both cvs. had pelargonidin-3-glucoside as the main anthocyanin in the flesh whereas similar amounts of this anthocyanin and cyanidin-3-glucoside were detected in the achenes of both cultivars. Almeida *et al.* (2007) demonstrated alter on that anthocyanin levels increased during ripening whereas flavan-3-ols decreased in both cvs. Queen Elisa and Korona. Indeed, the authors demonstrated that at white stage, flavan-3-ol content was mainly associated with vascular epidermial tissue and pith, but was mainly limited to vascular tissues at more advanced development stages. Recently, Zhang *et al.* (2008) when studying the antioxidant properties of different strawberry fractions found that anthocyanins including cyanidin-3-glucoside (7156 µM Trolox mg^{-1}), pelargonidin (4922 µM Trolox mg^{-1}) and pelargonidin-3-rutinoside (5514 µM Trolox mg^{-1}) gave greater antioxidant activities as determined by the popular trolox equivalent antioxidant capacity (TEAC) assay (Chapter 18) than other

purified fractions from the same berries. In the same study the authors highlighted that strawberry extracts and their purified compounds had antiproliferative activity in a dose-dependent manner when assessed in different lines of human cancer cells (oral, colon, and prostate).

The biological activity of strawberry fruits is in part due to their biological activity of its polyphenols, including anthocyanins. Thereby, it is crucial to determine the bioavailability of these compounds by means of human and animal studies targeted to (i) obtain information on target organ and organ-tissue distribution, (ii) assess the concentrations reached in the organs, tissues or fluids, and also (iii) to assess the potential toxic effects, if any. Although not much information exists to this regard, it is evident that the body of literature regarding bioavailability of strawberry bioactives is currently growing, and facts are available on the bioavailability of ellagic acid, ellagitannins, anthocyanins, procyanidins and flavonols (Tomás-Barberán, 2008). In the particular case of strawberry anthocyanins, it has been reported that consumption of 200 g of strawberries resulted in concentrations of pelargonidin-3-glucuronide, in blood plasma of 274 nmol L^{-1} (Mullen *et al.*, 2008). Another human study with six healthy volunteers, 3 from each gender, was that conducted by Felgines *et al.* (2003). In this particular case, results showed that after consumption of 200 g of strawberries, pelargonidin -3-glucoside, its aglycon, three monoglucuroniodes as well as a sulfo-conjugate were detected as urinary metabolites. This said, the total urinary excretion of anthocyanin metabolites represented no more than 1.80 ± 0.29 % of the total pelargonidin-3-glucoside ingested. Hollands *et al.*, (2008) concluded that strawberry anthocyanins were partially bioavailable with a linear relation between intake and urine excretion (each additional unit of dose ingested resulted in 0.0166 units excreted). Similar findings were shown by Carkeet *et al.* (2008) where the linear response-dose was reported to be in the range of 15 – 60 μmol. In all cases, pelargonidin, the main anthocyanin detected in strawberry fruits, seems to better absorbed and excreted (with recoveries in urine over 24h of 0.58%) as compared to other anthocyanins commonly present in strawberry fruits (0.084-0.087%) (Carkeet *et al.*, 2008). Even though, anthocyanins are mainly absorbed in the duodenum with very little or no absorption taking place in the small intestine (Tomás-Barberán, 2008), Andres-Lacueva *et al.* (2005) showed that these compounds were even detected in the brain. For the interested reader an excellent review on the bioavailability and

absorption of anthocyanins is that published by McGhie and Walton (2007). Regardless, of the poor bioavailability, it is accepted that the little portion that is absorbed may be very biologically active (Carkeet *et al.*, 2008)

Other types of flavonoids are flavonols, which are present in strawberries as glucosides and glucuronides of quercetin and kaempferol aglycons (Seeram *et al.*, 2006c). Epidemiological data suggests an inverse relationship between intake of flavonols and cardiovascular disease or incidence of certain cancer types (Knekt *et al.*, 2002). In this context, Häkkinen *et al.* (1999) studied the flavonol content of different berries including those from strawberry fruits cvs. Senga Sengana and Jonsok and found both quercetin (8 μg g^{-1} FW) and kaempferol (5-8 μg g^{-1} FW) in relatively low concentrations. In another study, Seeram *et al.* (2006c) identified quercetin-rutinoside, quercetin-glucoside, quercetin-glucuronide and kaempferol-glucuronide as the main flavonols in strawberry fruits. However, flavonol content in strawberries varies drastically according to the literature. These great variations may be, in part, explained by differences in the genotypes studied, growing conditions as well as the sample preparation and extraction methodology used (Häkkinen, *et al.*, 1999). In addition, as observed for anthocyanins (Terry *et al.*, 2007), it may be feasible to speculate that flavonol concentrations may also vary according to fruit position in the cymose inflorescence. The bioavailability of flavonols has been investigated too, and so far, there seems to be enough scientific evidence to suggest that monoglucosides are absorbed better than their corresponding aglycones which in part seems to be related the presence of glucose transporters in the intestinal wall (Tomás-Barberán, 2008).

There is a paucity of research data available describing the effect that different food matrices may have on the absorption and metabolism of strawberry bioactives in general, but specifically on flavonoids (Mullen *et al.*, 2008). Mullen and co-authors demonstrated that consumption of strawberries with 100 mL of double cream delayed absorption of pelargonidin-3-glucoside by more than 1h but had no effect on the C_{max} as compared to fruits ingested without cream (Mullen *et al.*, 2008).

E.2.4 Ellagic acid, ellagitannins and derivatives

Ellagic acid is a polyphenol naturally occuring in both the free form and esterified to glucose in water soluble hydrolysable tannins in certain fruits and nuts. Although concentrations of ellagic acid for strawberry differ markedly according to the literature (19.9 to 522 µg g^{-1} FW; Gil *et al.*, 1997 and Häkkinen and Törrönen, 2000, respectively), strawberry fruits are one of the richest sources of this polyphenol especially if compared to other commonly consumed fruits (Williner *et al.*, 2003). Fruit tissue distribution and variability among cultivars has been highlighted by several works (Maas *et al.*, 1991; Atkinson *et al.*, 2006). Ellagic acid content was showed to decrease during fruit development in all of the five cvs. studied by Williner *et al.* (2003). For instance, cv. Chandler had 2.07 ± 0.10 mg g^{-1} DW at white stage and decreased to 1.08 ± 0.11 and 0.46 ±0.07 mg g^{-1} DW at 50% and 100% red stage fruits (Williner *et al.*, 2003). Ellagic acid, *per se*, has been associated with numerous health-promoting properties and up to now many researches support the role of this phenolic compound as a chemopreventive agent (Hannum, 2004). Häkkinen *et al.*, (2000) reported that ellagic acid in strawberries accounted for approximately 51% of the phenolic profile from this berry, although significant differences in ellagic acid concentrations existed between cultivars (396 µg g^{-1} FW Senga Sengana as compared to 522 µg g^{-1} FW in cv. Jonsok). Genotypic differences for ellagic acid concentrations have been further confirmed by others (60-341 µg g^{-1} FW) as also has been the ratio between conjugated ellagic acid and free ellagic acid (Atkinson *et al.*, 2006). Häkkinen *et al.* (2000) detected other selected phenolic acids in strawberries at lower proportions than those described for ellagic acid (*viz*. Kaempferol (3.1 %), quercetin (6.0%), myrecitin (1.6%), p-coumaric acid (34.3%), p-hydroxybenzoic acid (4%)). Whereas for most berries ellagic acid seems to be concentrated in the seeds, Daniel *et al.* (1989) found that most of the ellagic acid in strawberries was found in the pulp (95.7%). In the same study, ellagic acid contretions (~63 µg g^{-1} FW) in strawberry fruits were below the concentrations found in other berries (raspberry and blackcberries; ~150 µg g^{-1} FW) but far superior to that found in other fruits analysed. The higher concentrations in pulp as compared to achenes was later highlighted as the possible explanation for the greater bioavailability of ellagic acid from strawberries as compared to that of other fruits (Hannum, 2004). In contrast, in a more recent study conducted by Aaby and collaborators (2005), the achenes from two different

strawberry cultivars were the main source of ellagic acid (cv. Totem 87.3±14.6 mg $100g^{-1}$ FW and cv. Puget 34.4 ±3.7 mg 100 g^{-1} FW) or ellagic acid derivatives as compared to the flesh (cv. Totem 0.3 mg 100 g^{-1} FW and cv. Puget 0.2 mg 100 g^{-1} FW) (Aaby *et al.* 2005). In this context, the discrepancies encountered between the study of Aaby *et al.* (2005) and Daniel *et al.* (1989) are most probably due to variations in the methodologies used such as hydrolysis conditions, extraction solvents used, etc.

Aaby *et al.* (2005) not only reported greater amounts of ellagic acid and anthocyanins for achenes than pulp but found up to twenty-fold greater total antioxidant activities, and 10 times larger total phenolic values in achenes than in the flesh of the different strawberry cvs. investigated. In agreement, Terry *et al.* (2002 & 2004) also shown, by thin layer chromatography (TLC), that strawberry achenes contained larger amounts of antifungal compounds, many of them being phenolic compounds, than other fruit tissues. (**Figure E4**).

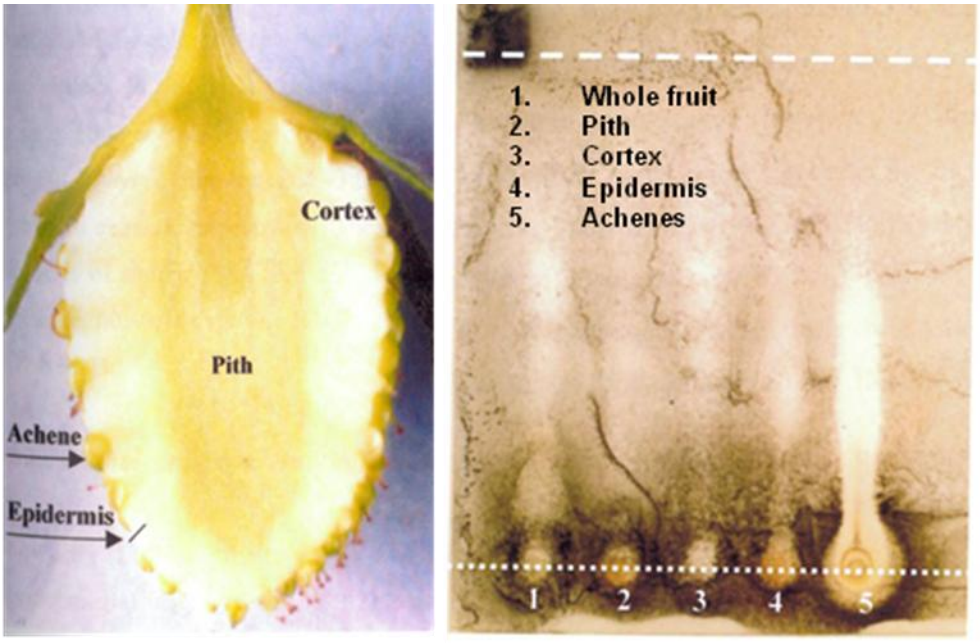


Figure E4: TLC *Cladosporium cladosporioides* bioassay of cv. Elsanta fruit crude ethanol extracts (100 µl, 0.2 ml g^{-1} FW), from different tissues of Green I strawberry fruits, run in hexane: ethyl acetate: methanol (60:40:10 v/v/v) (Terry, 2002; Terry *et al.*, 2004).

Nevertheless, most of the achenes from the fruit tend to pass intact through the alimentary canal ending up in the faeces and hence making any bioactives present in this fruit tissue most probably unavailable.

Other strawberry compounds derived from ellagic acid are ellagitannins, which are water-soluble polyphenols belonging to the hydrolysable tannins class. This type of compounds can occur in complex polymeric forms of high molecular weight. Ellagitannins can be quantified directly or indirectly by hydrolysing the polymer with an acid or base to yield ellagic acid (Häkkinen *et al.*, 2000) thus resulting in the concept of free ellagic acid or conjugated ellagic commonly found in the literature (**Figure E5**).

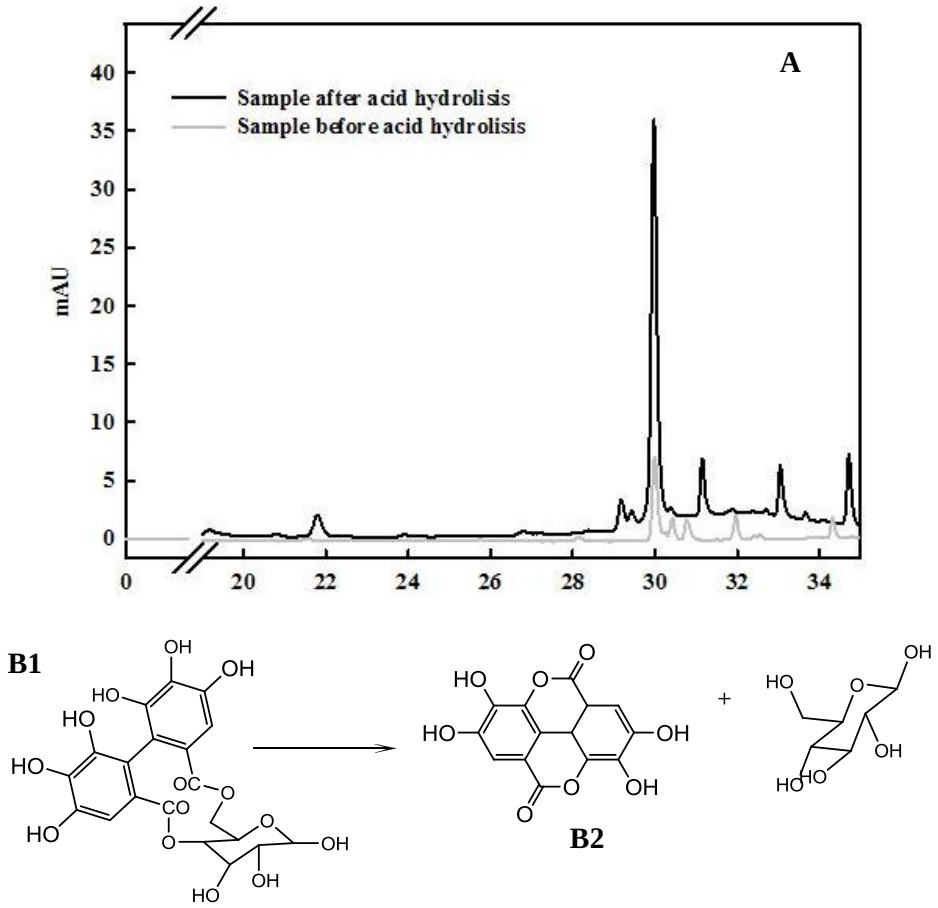


Figure E5: (**A**) Chromatographic profile of ellagic acid in strawberry fruits (cv. Camarosa) prior to and after 90 min hydrolysis. (**B**) General scheme for the hydrolysis of ellagitanins from strawberry fruits; before hydrolysis the principal components correspond to ellagic acid glycosides, simple ellagitannins (B1) and complex oligomeric ellagitannins that when hydrolysed give rise to ellagic acid (B2), methyl gallate and methyl sanguisorboate.

Seeram *et al.* (2006c) identified sanguiin H-6 and ellagic acid glycosides among the major hydrolysable tannins present in strawberry fruits whereas other type of ellagitannins were further reported by Cerdà *et al.* (2005). Cerdà and collaborators studied the metabolism of ellagitannins from strawberries and identified urolithin B, a previously identified antiangiogenic and hyaluronidase inhibitor compound, as a suitable biomarker for ellagitannin consumption in healthy humans. After strawberry (cv. not speficied) intake, Urolithin B excretion was ca. 2.8% of the ingested dose and varied considerably between individuals. Similar findings were observed by Seeram et al (2006c) when studying the pharmacokinetic parameters of ellagic acid after ingestion of pomegranate juice. Despite the limited absorption of either ellagic acid or ellagitannins (Tomás-Barberán, 2008) these compounds and metabolites have been detected in kidneys and liver of rats as well as the prostate gland of mice (Cerdà *et al.*, 2003; Seeram *et al.*, 2007).

E.2.5 Follate and other bioactives

With constant advances in analytical sciences new strawberry-derived compounds have been identified and associated with potential health-promoting properties. Resveratrol, a compound commonly found in grapes and synthesised from cinnamic acid derivatives was indentified in strawberry fruits by Wang *et al.* (2007). During the past decades several studies have shown the potential health-promoting properties of resveratrol (*viz*. antioxidant, anticarcinogenic, anti-inflammatory, cardioprotection) (for the interested reader see review by Aggarwal *et al.* (2004)) and as a result increasing interest has raised to identify new sources of this phytoalexin among fruits and vegetables. Even though there was evidence of this compound in other berries, the first detailed study on strawberries was probably that conducted by Wang *et al.* (2007). The authors not only studied resveratrol content of cvs. Kent and Earliglow of strawberries grown in glasshouse but also detailed the effect that different preharvest factors (*viz*. genotype, fruit maturity, cultural practices and environmental conditions) had on its concentration. Resveratrol concentration was greater in fully ripe berries that early ripe as well as being higher in achenes (~2 μg g^{-1} DW) as compared to pulp (~0.12 μg g^{-1} DW). Overall, values for resveratrol concentrations in strawberry fruits were far below the ~10 μg g^{-1} FW that can be found in the skin of red mature grapes cv. Napoleon (Cantos *et al.*, 2000).

Another compound found in strawberry fruits with reported anti-inflamatory, anti-proliferative and anti-angiogenic (Sung *et al.*, 2007) as well as memory-enhancing properties (Maher *et al.*, 2006) is fisetin. Fisetin is a flavonoid naturally occurring in certain fruits and vegetables with concentrations ranging from 2 to 160 µg g^{-1}(Arai *et al.*, 2000). The mechanisms by which fisetin exerts its anticarcinogenic effects still remains unclear although recent research has shown that it inhibits cyclin-dependent kinase 6 and downregulate NF-κB-regulated cell proliferation (Sung *et al.*, 2007).

In addition to all the polyphenols mentioned earlier, strawberry fruits are one of the richest natural sources of the water soluble vitamins folate and ascorbate. Inadequate folate status in humans has been associated with an increased risk for chronic diseases that may particularly affect the elderly population (Rampersaud, *et al.*, 2003; Tulipani *et al.*, 2009). Similarly, ascorbate deficiency it is known to be related with detrimental health effects (See appendix D). For the determination of folate content in strawberry fruits both a microbial or a radio-protein binding assays have been recently developed and validated in berry fruits (Strasljo *et al.*, 2003; Tulipani *et al.*, 2008) In a recent study, total folate content from nine Italian strawberry cultivars ranged from ~0.2 to ~1.0 µg g^{-1} FW (Tulipani *et al.*, 2008) and was in agreement with earlier studies conducted on Swedish-grown strawberries (0.73 - 0.99 µg g^{-1} FW; Strasljo *et al.*, 2003). In this context, it is generally accepted that 250-350 g of berries (~125 µg follate) can provide with the totality of European daily intake recommendations (200-300 µg day^{-1}) (Bailey and Gregory, 1999), making strawberries one of the most appealing sources of this vitamin. Suplementation with folate to individuals with homocysteinemia has been shown to reduce levels of homocystein in plasma and therefore reduce the risk of heart disease (Spiller and Dewell, 2003).

Ascorbic acid content in strawberry fruits varies drastically among different genotype (Figure 6) and agroclimatic conditions (Cordenunsi *et al.*, 2005; Atkinson *et al.*, 2006; Terry *et al.*, 2007; Giné Bordonaba and Terry, 2009; Crespo *et al.*, 2010) with reported concentrations ranging from 0.2 to 0.9 mg g^{-1} FW (Hakala *et al.*, 2003; Atkinson *et al.*, 2006; Terry *et al.*, 2007; Giné Bordonaba and Terry, 2009; Crespo *et al.*, 2010).

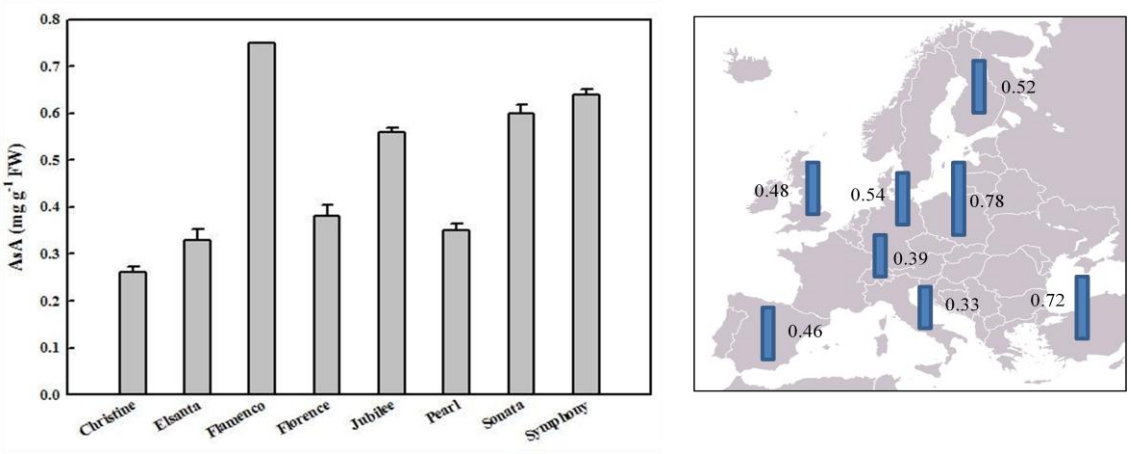


Figure E6: (A) Ascorbic acid (AsA) concentrations (mg g^{-1} FW) in a range of UK-grown strawberry cultivars (Giné Bordonaba and Terry, 2009). (**B**) Reported AsA concentrations (mg g^{-1} FW) in strawberry fruits from different locations within Europe (N.B. Values correspond to average concentrations from different cultivars and different harvest years obtained from various literature)

E.3 Chemopreventive and health-related properties

E.3.1 Introduction

The antioxidant properties of strawberries are well documented (Wang and Lin, 2000; Terry *et al.*, 2007; Wolfe *et al.*, 2008). Since oxidative stress has been suggested to play an important role in the development of certain conditions including cancers, it was initially expected that antioxidant capacity of the fruit would correlate well with its antiproliferative properties. Nevertheless, Meyers *et al.* (2003) demonstrated that antioxidant activity from eight different strawberry cultivars was not related to their antiproliferative properties. Nowadays, a plethora of research studies have shown that berries, including strawberry, or berry purified phenolic compounds, inhibit cancer cell proliferation, regulate cell cycle arrest and in some cases induce apoptosis by multi-mechanistic means of actions beyond antioxidation (reviewed by Seeram and Heber, 2007). Besides, little or no cytotoxic effect have been observed when strawberry extracts were tested on normal cells resulting in no doubts of the potential anticancer effects of strawberry fruits (Seeram, 2008). As for many other fruits and vegetables, most of the reported anticancer activity of strawberry fruits is based on *in-vitro* studies rather than *in-vivo* or intervention trials. Among the limitations of *in-vitro* tests, several authors (Roques *et al.*, 2002; Kern *et al.*, 2007), have pointed out

that the results of *in-vitro* assays may be an artefact from the generation of hydrogen peroxide in the culture media by the antioxidants tested and hence it is evident that results from this type of assays should be interpreted cautiously.

In this context, the following sections aims to describe the latest scientific evidence obtained from *in-vitro*, *in-vivo* experiments or intervention studies regarding the beneficial effects that strawberry fruits or their extracts (*viz*. freeze-dried powders, concentrates, etc.) may have on preventing or fighting against cancer as well as other illnesses (**Table E2**).

E.3.2 Cancer studies

Several studies have demonstrated that strawberry extracts inhibit the growth of human carcinoma cells when tested *in vitro* (**Table E2**). For instance, strawberries effectively inhibited by different mechanisms the growth of oral, breast and prostate (Seeram *et al.*, 2006a) or liver cancer cell lines (Ramos *et al.*, 2005) in a dose-dependent manner. Ramos *et al.* (2005) proved that whole strawberry extracts arrested the G1 phase showing therefore pro-apoptotic effects. In a more recent study, Wu and collaborators (2007) investigated whether strawberry and other berry extracts had any effect on cell viability and expression of apoptotic cell markers in human HT29 colon cancer cells. The results suggested that berry extracts inhibited cell proliferation through the cyclin kinase inhibitor pathway p21WAF1 (Wu *et al.*, 2007). Other *in vitro* studies with extracts from two different strawberry cvs. (*viz*. Sweet Charlie and Carlsbad) showed the potential of both cultivars to inhibit breast cancer and cervical cancer cell proliferation (Wedge *et al.*,2001). Li *et al.* (2008) not only demonstrated the anti-carcinogenic properties of strawberry extracts but elucidated that strawberry extracts specifically inhibited nuclear factor of activated T cells (NFAT) and tumoral necrosis factor alpha (TNF-α). Results from the same study suggested that the chemopreventive properties of black raspberry and strawberry bioactives may be targeted through different signalling pathways. In an earlier study, Wang *et al.* (2005) showed that strawberry extracts suppressed cancer cell proliferation and transformation by means of inhibiting the transcription factors, activating protein-1 (AP-1) and nuclear factor kappa B (NFkB). In the same study the authors postulated that the antioxidant properties and the ability to reduce oxidative stress were most

probably related to the ability of the same extracts to block ultraviolet B (UVB) and TPA-induced AP-1 and NFkB activation.

Besides the earlier highlighted mechanisms of action, strawberry fruits are thought to possess anticarcinogenic effects by inhibiting possible mutations. In this context, Hope *et al.* (2004) pointed out that strawberry tannin fractions were very effective in inhibiting mutations caused by both methyl methanesulfonate and benzopyrene.

Whereas animal and human studies are still limited, many research groups are currently investigating the possible role of strawberry fruits, not only in preventing cancer but in several other diseases. The dietary intake of products rich in ellagitannins such as strawberries has been shown to inhibit the initiation and promotion of oesophageal cancer (Stoner *et al.*, 1999). Stoner and collaborators included 5 or 10% of freeze-dried strawberries into the diet of rats prior to the induction of oesophagus cancer with *N*-nitrosomethylbenzylamine (NMBA) and observed an inhibition effect depending on the dose-concentration. Using a similar approach, the chemopreventive effect of strawberry liophilised extracts on NMBA-induced rat oesophagus carcenogenesis was studied by Carlton *et al.* (2001). They proved that although the berry extract had no effect on tumour incidence it significantly reduced tumour multiplicity as compared with control or non-treated cells. This said, an earlier study by Stoner *et al.* (1999) did not detect any effect of the strawberries in the reduction of lung cancer in rats when induced by other carcinogenic compounds. In a different study conducted by Chung *et al.* (2002), the effect of strawberries against the endogenous generation of carcinogens in healthy individuals consuming a diet with excessive nitrates was evaluated. Results from that study demonstrated that consumption of 300 g of strawberry resulted in a reduction of 70% in the urinary concentration of the carcinogen NMBA. Recently, Warner *et al.* (2008) demonstrated that lyophilized strawberries or selenium-enriched lyophilised strawberries inhibited tumour formation by 43% and 59%, respectively. Overall, the authors concluded that based on their hamster cheek pouch experiments, strawberries and strawberries with selenium could prevent or delay the development of oral cancer (Warner *et al.* 2008).

Table E2: Reported health-promoting properties of *Fragaria* x *ananassa* fruits or derived products.

Activity	Action	System	Dose	Extract type*	Reference
Anticarcinogenic	Inhibit the initiation and promotion of oesophageal cancer	Rat	Diet (AIN76) containing 5 and 10% of strawberries (~ ellagic acid concentration was 0.34 and 0.67 mg kg^{-1} diet, respectively)	Freeze-dried strawberry puree	Stoner *et al.*, 1999
Anticarcinogenic	Reduction of tumor multiplicity in oesophageal cancer	Rat	Diet (AIN76) containing 5 and 10% of strawberries	Freeze-dried strawberry (var. Comander)	Carlton *et al.*, 2001
Anticarcinogenic	Protective effect against endogenous generation of carcinogens	27 male and 13 female volunteers (10 healthy subjects in each group)	300g on the 4^{th} day of a 4-days trial	Fresh strawberries	Chung *et al.*, 2002
Anticarcinogenic	Inhibition of cancer cell proliferation by inhibiting transcription factors and activating protein-1 (AP1) and nuclear factor kappa B (NFkB)	*In vitro*: human lung epithelial cancer A549 cells and Mouse epidermal JB6 P^{+} cell lines	-	Filtered and diluted strawberry homogenates	Wang *et al.*, 2005
Anticarcinogenic	Growth inhibition of liver cancer cells	*In vitro*: HepG2 cell cultures	0.1–0.8 mg ml^{-1}	Lyophilized strawberry extract	Ramos *et al.*, 2005
Anticarcinogenic	Growth inhibition of oral, brest and prostate cancer cells	*In vitro*; cell cultures	25-200 µg ml^{-1}	Polyphenolic enriched strawberry extract (sugars and acids removed by C18)	Seeram *et al.*, 2006a

Anticarcinogenic	Reduced cell proliferation through the cyclin kinase inhibitor pathway p21WAF1	*In vitro*; HT29 colon cancer cells	0-60 mg ml^{-1}	Homogenised strawberry extract	Wu *et al.*, 2007
Anticarcinogenic	Inhibition of nuclear factor of activated T cells (NAFT) and tumoral necrosis factor alpha (TNF-α)	*In vitro*: Mouse epidermal JB6 Cl 41 cell lines	1-100 µg ml^{-1}	Lyophilized strawberry extract	Li *et al.*, 2008
Anticarcinogenic	Inhibition of tumour formation of oral cancer cells	Hamster	Diet (AIN76) containing 10% of strawberries or strawberries enriched with selenium (0.5ppm)	Liophilised strawberry or selenium enriched liophilised strawberries	Warner *et al.*, 2008
Anticardiovascular disease	Endothelium-dependent vasorelaxation through the activation of Pl3kinase/akt	Rabbit aorta	0.1-10 mg ml^{-1}	Freeze-dried strawberry powder	Edirisinghe *et al.*, 2008
Anticardiovascular disease	Greater reduction in oxidative damage to LDL	Human intervention study on 28 hyperlipidemic subjects	454 g/d in a randomized 1-month crossover study with a 2-week washout	Fresh berries	Jenkins *et al.*, 2008
Anticardiovascular disease	Antiplatelet activity	Mice	~11 ml kg^{-1}	Strawberry filtrate	Naemura *et al.*, 2008
Anticardiovascular disease	borderline significant, multivariate 14% lower likelihood of an elevated CRP of ≥3 mg/L	Cohort study	≥2 servings/week	-	Sesso *et al.*, 2008
Anticardiovascular disease	Hypocolesterolemic effects and reduced lipid peroxidation	Women suffering metabolic syndrome	25 g day^{-1}	Freeze-dried strawberry powder	Basu *et al.*, 2009
Antiinflammatory	Inhibition key inflammation enzymes (COX1)	Cell culture	125 µg ml^{-1}	Lyophilised crude anthocyanins from strawberries cv.	Seeram *et al.*, 2001

				Honeoye	
Anti-neurodegenerative	Reversion of the aging process by protecting against decrease in mental performance	Rat	9.5 g kg^{-1} of standard diet for 6 months	Lyophilised strawberry powder	Joseph *et al.*, 1998
Anti-neurodegenerative	Reduction of oxidative stress-induced neurotoxicity	Neuronal cells	100-2000 µg ml^{-1}	Phenolics extracted from 10 g of dried sample	Heo & Lee, 2005
Anti-diabetic	Limiting post-meal blood glucose levels by reducing α-amilase	*In vitro* assays	0-500 µg $assay^{-1}$	Phenolic rich fractions from breeding variety 932034 and cv. Elsanta from local growers.	McDougall *et al.*, 2005
Anti-obesity	Increased weight gain	Mice	Diet containing 10% freeze-dried strawberry powder	Whole fruit	Prior *et al.*, 2008
	Reduced weight gain		Purified anthocyanins from strawberry given in the water	Purified anthocyanins from strawberries	

* Most strawberry extracts used for *in vitro* studies are based on acidified methanol aqueous extraction followed by an acetone-water extraction, evaporation of the solvents and resuspension of the extracts in water prior to be deposited onto the cell cultures. Whenever data is available the cultivars used are specified. (-) concentration dose not specified

E.3.3 Cardiovascular disease.

Epidemiological data suggests that consumption of fruit and vegetables may lower the risk of cardiovascular disease. Again, antioxidant activity has been cited as the possible mechanism by which strawberries or specific polyphenols found in strawberries may exert their beneficial effects. In this context, it is postulated that the antioxidant activity of strawberry is crucial in the prevention of atherosclerosis, since the oxidation of the low-density lipoprotein (LDL) is a key phenomenon associated with the development of such conditions (Diaz *et al.*, 1997; Edirisinghe *et al.*, 2008).
In addition, specific compounds found in strawberries, for instance anthocyanins, are known vasodilators and help towards reducing the incidence of coronary diseases. Edirisinghe *et al.*, (2008) showed for the first time that not only freeze-dried strawberry powder from California strawberries caused endothelium-dependent relaxation in the rabbit aorta (EDR), but also that this was achieved through the activation of Pl3 kinase/akt (**Table E2**). The major phenolic compounds present in the strawberry extracts were pelargonidin-3-O-glucoside, coumaryl-3-O-glucoside, and trans-cinnamoyl-O-glucoside. Previous studies demonstrated that also ascorbate had EDR effects at concentrations similar to those found in strawberry fruits and therefore the authors pointed out the possible synergistic effect between ascorbate and polyphenols. The loss of proper endothelial function is frequent in people suffering from diabetes mellitus, hypertension and other chronic conditions that can therefore increase the risk of heart disease. The role that certain polyphenols, present in strawberry fruits (*viz*. kaempferol, catechin and anthocyanins), have in inhibiting the formation of atheroma plaque and therefore reducing the incidence of thrombosis has been demonstrated (Rein *et al.*, 2000). In a cohort study conducted by Sesso *et al.* (2008) higher strawberry intake (> 2 servings/week) was associated with a reduced borderline but significant likelihood of having elevated CRP levels. This said, in the same study, no association were found between the risk of incident CVD, lipids, or CRP in middle-aged and older women, and consumption of strawberry fruits. The authors pointed out that additional epidemiologic data was needed to clarify any role of strawberries in CVD prevention.
Jenkins and co-workers (2008) assessed the effect of adding strawberries as a source of antioxidants to improve the antioxidant effect of a cholesterol lowering diet on 28 hyperlipidemic subjects. Results from this study revealed that supplementation with

strawberry fruits resulted in a greater reduction in oxidative damage to LDL while preserving reductions in blood lipids and enhancing diet palatability (Jenkins *et al.*, 2008).

In another study conducted by Naemura *et al.* (2008) an *in vitro* platelet function test (haemostatometry) was used to screen different strawberry cultivars. In the same study, those cultivars showing significant antiplatelet function were further examined *in vivo* by means of a laser-induced thrombosis test in mice. Results suggested that strawberry varieties KYSt-4 (Nohime), KYSt-11 (Kurume IH-1) and KYSt-17 (Kurume 58) showed significant antiplatelet activity both *in vitro* and *in vivo*.

Recently, the effect of freeze-dried strawberry powder supplementation was evaluated on women suffering metabolic syndrome (Basu *et al.*, 2009). Short-term supplementation of a strawberry drink, consisting of 25 g of freeze dried strawberry powder (unspecified cultivar), one cup of water, artificial sweeteners and vanilla essence, resulted in hypocholesterolemic effects and decrease in lipid peroxidation (Basu *et al.*, 2009) at 4 weeks as compared to baseline values. When a similar study was undertaken with liperlipidemic subjects ingesting 453 g of fresh strawberries daily for 4 weeks, no differences were observed in lipid levels (Jenkins *et al.*, 2008). In the same study, however, strawberry consumption was associated with reduction of lipid oxidative damage.

E.3.4 Other beneficial effects

E.3.4.1 Anti-inflammatory

It is possible that certain phenolic compounds from strawberry fruits may exert positive effects on the immune system. For instance, Seeram *et al.* (2001) reported on the inhibitory effect of berry anthocyanins on cyclo-oxygenase (COX). COX is a key enzyme in inflammation and its inhibition is the target mechanism of different drugs including the common aspirin. Strawberries have been proven to be very effective inhibiting COX2, though not so effective against COX1. Given that inflammation is a process involved in the ethiology of several pathologic conditions including cancer, cardiovascular, Alzheimer's etc., the findings by Seeram *et al.* (2001) highlighted the potential of strawberries for the treatment of multiple conditions (Tomás-Barberán, 2008).

E.3.4.2 Anti-Neurodegenerative

Scientific evidence suggests that strawberries (**Table 2**) and other berries may have a role in delaying or even overturning age-related degenerative diseases. Already studies performed during the last decade showed the ability of diets supplemented with strawberries to retard and reverse the aging process in rats (Joseph *et al.*, 1998; Shukitt-Hale *et al.*, 1999; Bickford *et al.*, 2000). In the study carried out by Joseph and collaborators, 6 months age rats were fed with strawberries for eight months. Results revealed the potential of strawberry fruits on the cerebral function and protective effect against decrease in mental performance associated with aging (Joseph *et al.*, 1998). Other studies conducted on aged rats showed that strawberries improved the motor and learning skills as well as reduced the reduction in the cognitive capacity of the animals (Bickford *et al.*, 2000). More recent data, obtained generally from *in vitro* studies, reveals the potential of strawberry fruits as well as their anthocyanins or other polyphenols to reduce oxidative-stressed induced apoptosis in neuronal cells. In the study conducted by Heo and Lee (2005), strawberries significantly reduced oxidative stress-induced neurotoxicity in neuronal cells in a greater manner than that observed for banana and oranges respectively (Heo and Lee, 2005). Accordingly, Lau *et al.* (2006) suggested that combinations of antioxidants and anti-inflamatory polyphenols from berries may be key compounds to help preventing, suppressing or inhibiting age-related deficits by several mechanisms. Recently, Shukitt-Hale *et al.* (2007) studied the ability of strawberry and other berry extracts had on irradiated rats. Exposing young rats to irradiation enhances oxidative stress and disrupts the dopaminergic system in a similar way than that observed in aged rats (Shukitt-Hale *et al.* 2007). In this context, the authors demonstrated the ability of the strawberry extracts to protect against spatial deficits.

E.3.3.3 Antiobesity and anti-metabolic disorders

Following earlier studies in which anthocyanins were hypothesised to have important implications in the prevention of antiobesity and diabetes (Tsuda *et al.*, 2004), Prior and collaborators investigated whether whole blueberry and strawberries, or their purified anthocyanins extracts, had any antiobesity effect on a mouse model. The authors proved that while feeding mice with the whole fruit did not prevent and even

increase obesity, feeding purified anthocyanins from the same extract had a crucial effect on reducing obesity (Prior et al., 2008). The potential therapeuric effects of strawberry extracts (limiting post-meal blood glucose levels by reducing α-amylase were also demonstrated by McDougall *et al.* (2005). The authors found that, from a range of berry extracts, strawberry and raspberry were the most effective in inhibiting α-amylase, which was hypothethised by the authors to be due to their high concentrations of soluble tannins.

E.4 Effect of preharvest and postharvest continuum

Strawberry growth and development is characterised by changes in colour, texture and flavour (Manning, 1993) with four or five different stages commonly described in the literature and based on the development of non ovarian receptacle tissue (Culperpper *et al.*, 1935; Spay and Morris, 1981; Huber, 1984; Terry *et al.*, 2004). These stages include *small green*, *large green*, *white* and *full red.* The content of certain bioactives, which account for the potential health-promoting properties of the fruits markedly vary depending on developmental stage. Changes on anthocyanins and other bioactives in strawberry fruits have recently been described in detail (Carbone *et al.*, 2009). In addition, during fruit growth and development, exposure of the plant to certain abiotic and biotic conditions may result in enhancing oxidative stress and therefore generation of ROS. It is believed that under such conditions the plant responds by increasing bioactive-related gene expression and thus enhancing the production of ROS scavengers, mainly antioxidants which may counteract ROS at different levels. For strawberry fruits, it has been demonstrated that growing the plants under different agroclimatic conditions may result in fruits with different contents of health-promoting components (Atkinson *et al.*, 2006; Terry *et al.*, 2007).
Recent studies elucidated that exposing the plant to different stress conditions (viz. deficit irrigation, salinity, etc.) resulted in enhanced content of specific bioactives (Terry *et al.*, 2007; Keutgen and Pawelzik 2008; **Table E3**). Specifically, Terry *et al.* (2007) showed that anthocyanin content, total phenolics and antioxidant capacity were greater in 'Elsanta' plants irrigated with 50 mL day^{-1} as compared to those plants receiving greater amounts of water (100 or 200 mL day^{-1}) (**Figure E3**).

The antiproliferative effect of strawberry fruits grown conventionally or organically was recently assessed by Olsson *et al.* (2006). The organically grown

strawberries showed greater antiproliferative activity, which the authors related to their higher content of secondary metabolites found in those berries and which in turn may be associated with the exposure of the plants to greater stress by pathogens when grown under organic cultivation systems. In contrast, others (Hargreaves *et al.*, 2008) could not find differences in several bioactive constituents between organically and conventionally grown strawberries and hence further research is required to elucidate whether or not organic production may result in "healthier" berries.

Clearly, metabolism is known to continue beyond fruit harvest, but, this is often ignored or more commonly overlooked or not appreciated. The concentration of certain metabolites is expected to change during postharvest storage and through the supply chain. Given this, however, few works have studied the role that postharvest treatments have on strawberry quality and bioactives. In addition, the effectiveness of any postharvest treatment is dependent on whether the treatment is focused on preserving appearance or maintaining the health-related composition of the fruits. Earlier works have shown that postharvest treatments focused on maintaining appearance do not correlate for instance with better maintenance of certain bioactives (Pelayo *et al.*, 2003). Postharvest storage temperature (5 or 22ºC) did not have a significant effect on ellagic acid content in strawberries stored during 24h (Häkkinen *et al.*, 2000). Under longer storage conditions, Gil *et al.* (1997) reported that content of ellagic acid increased over the course of 10 days in fruits stored at 5ºC and pointed out that such a phenomenon was probably the result of degradation of ellagitannins (**Figure E5**). In another study, Cordenunsi *et al.* (2005) evaluated the chemical composition and antioxidant activity changes of different strawberry cultivars (*viz*. Dover, Campineiro and Oso Grande) stored at 6, 16 and 25ºC for 6 days. The authors concluded that low temperatures negatively affected anthocyanins and ascorbic acid whereas it had no significant effect on the content of flavonols, ellagic acid and total phenolics. Antioxidant activity, on the other hand, was similar between cultivars and in all cases decreased after harvest independent of temperature. Interestingly, the observed increased in ascorbate in all three cultivars during storage (10% greater than initial values) was only experienced at 16ºC and was therefore in disagreement with previous studies (Nunes *et al.*, 1998; Cordenunsi *et al.*, 2003) which highlighted that low temperatures and high humidity during storage may retard ascorbate degradation (Nunes, 2008). Nunes *et al.* (1998) showed that in strawberry fruits stored for 8 days

at 1 or 10ºC as well as 4 days at 20ºC, ascorbic degradation was greater at higher temperatures. In the same study, postharvest shelf-life was enhanced and ascorbic acid degradation reduced by 7.5-fold in fruits stored at 1ºC. In this context, Cordenunsi *et al.* (2005) postulated that ascorbic acid synthesis may occur during postharvest storage and it is indeed affected by lowering temperature. Hakkinen *et al.*, (2000) also demonstrated that quercetin content markedly increased in strawberries or strawberry jams stored for 9 months whereas the ellagic acid tended to decline in fresh fruit but not in jams from the same berry. Similarly, others also reported that flavonol content increases in strawberries or other fruits stored under refrigerated conditions (Gil *et al.*, 1997). Controlled atmosphere (CA) during storage may reduce the rate of accumulation of anthocyanins normally observed on strawberry fruits after harvest (Zheng *et al.*, 2007). Nevertheless, Gil *et al.* (1997) showed that enriched CO_2 atmospheres had a minimal effect on anthocyanin concentrations of cv. Selva fruit. In the particular case of anthocyanins, it is likely that changes in other components within the fruit (i.e. organic acids) may affect the pH and hence the stability, copigmentation and spectra of these pigments within the fruit (Terry *et al.*, 2009), hence accounting for the experimental changes during postharvest storage. In addition, given the variability in colour within different genotypes (Giné Bordonaba and Terry, 2009) it may be feasible to speculate that changes in anthocyanins and other pigments as a result of any postharvest treatment may be genotype-dependent, and this should be further investigated.

The effect that certain non-conventional treatments have on preharvest or postharvest strawberry bioactives have also been increasingly studied over the last decade. As an example, Zabetakis *et al.* (2000) studied the effect that treatment with high hydrostatic pressure and further storage at different temperatures had on strawberry anthocyanins. High hydrostatic pressure may be an alternative to conventional heat treatments for preservation of strawberry derived products. The authors observed that samples pressurised under 800 MPa for 15 min (the greatest of the different treatments applied) resulted in the lowest losses of anthocyanins. Furthermore, after high hydrostatic pressure treatments, storage at 4ºC also resulted in the best matinenance of both pelargonidin-3-glucoside and pelargonidin-3-rutinoside. Accordingly, treatment with 400 and 600 MPa, resulted in greater enzymatic activity involved in anthocyanin degradation as compared to 800 MPa (Zabetakis *et al.* 2000).

In a different study, Wang *et al.* (2007), showed that strawberries treated postharvest with essential oils better inhibited human HT-29 colon cancer cell proliferation than those non-treated.

The application of essential oils preharvest, for instance methyl jasmonate, resulted in marked changes in anthocyanin concentrations in different strawberry cultivars (Giné Bordonaba and Terry, unpublished)

Table E3: Effect of preharvest factors (A) and postharvest treatments (B) on the health-related composition of strawberries.

(A) Preharvest factors	Effect on bioactives	Reference
Conventional vs. organic cultivation systems	No effect on total phenolics	Häkkinen and Törrönen, 2000
Growing temperature	Strawberry grown at higher T (ºC) showed higher concentrations of bioactive compounds	Wang and Zheng, 2001
Cultural system (Hill plasticulture vs. matted row)	Hill plasticulture systems resulted in higher content of phenolics, flavonoids and ascorbate	Wang *et al.*, 2002
Ozone exposure	No significant effect on antioxidant activity or bioactives	Keutgen and Pawelzik, 2007
Salinity stress	Moderate salinity resulted in increase antioxidant activity and bioactives	Keutgen and Pawelzik, 2007
Deficit irrigation	Higher content of certain anthocyanins, total phenolics and antioxidant activity	Terry *et al.*, 2007
Inoculation with *Botrytis cinerea*[1]	No effect on strawberry (cv. Elsanta) bioactives or antioxidant activity	Terry *et al.*, 2007
Organic and conventional nutrient amendments	No significant differences between treatments on antioxidant activity	Hargreaves *et al.*, 2008
Methyl jasmonate (MeJa) applied in fully or deficit irrigated plants	Higher concentrations of anthocyanins found in MeJa-treated plants and changes in fruit and leaves antioxidant capacity	Giné Bordonaba and Terry, unpublished

1 at anthesis of primary flower

(B) Postharvest treatment	Effect on bioactives	Reference
1-MCP	Lower accumulation of phenolics anthocyanins in cv. Everest treated fruits	Jiang *et al.*, 2001
Heat-treatment before refrigerated storage	Treated fruits (cv. Selva) showed less anthocyanin accumulation than controls when held at 20ºC	Vicente *et al.*, 2002
Exogenous Absicic acid (ABA) application	Anthocyanin and phenolic contents and PAL activity increased during storage of ABA-treated strawberry fruits (cv. Everest) more rapidly than in non-treated fruits	Jiang and Joyce, 2003
Storage temperature	Higher temperatures (5º and 10ºC) resulted in greater antioxidant capacity, total phenolics and anthocyanins of the fruits (cv. Chandler) as compared to fruits stored at 0ºC	Ayala-Zavala *et al.*, 2004
UV-C (4.1 kJ m^{-2}) and heat treatment (45°C, 3 h in air) either separately or combined	All treatments reduced the accumulation of anthocyanins in strawberries cv. Seascape	Pan *et al.*, 2004
Strawberry wrapping with polyvynil chloride and stored at 1ºC	Wrapped fruits (cv. Oso Grande) suffered lower water loss and maintain better anthocyanin and other soluble phenolics as compared to unwrapped fruits.	Nunes *et al.*, 2005
Storage temperature	Lower temperatures negatively affected anthocyanins and ascorbate but had no effect on flavonols, ellagic acid and total phenolics and antioxidant capacity	Cordenunsi *et al.*, 2005

MeJA in combination with ethanol	Enhanced antioxidant capacity, volatile compounds and postharvest life of strawberries (cv.Chandler)	Ayala-Zavala *et al.*, 2005
Superatmospheric storage conditions	High oxygen atmospheres (>40 kPa) resulted in fruits (cv. Chandler) with higher antioxidant capacity, total phenolics, less decay, and longer postharvest life than those stored in air	Ayala-Zavala *et al.*, 2007

E.5 Future research needs and conclusions

Unlike many other fruits and vegetables there appears to be substantial scientific data to confirm that strawberries as one of the main source of vitamin C, folic acid, dietary fibre as well as an excellent source of dietary polyphenols in the diet. From the survey of the literature presented herein, anthocyanin pigments, together with hydrolyzable and non-hydrolyzable tannins are among the main compounds with reported health-promoting properties (*viz*. anticarcinogenic, anti-cardiovascular and anti-neurodegenerative diseases, anti-inflammatory, etc). This said, in order to fully understand the health-promoting properties of this berry more studies are still required to further elucidate not only the heterogeneity in bioactive compounds amongst strawberry genotypes as affected by preharvest/ postharvest continuum, but also to broaden investigative research on the bioavailability of specific strawberry bioactives in different food matrices.

In vitro studies must continue since they provide vital information on the mechanisms and actions of specific bioactives, but it is clear that such studies may present certain limitations and the results can not be translated *in vivo*. Consequently, further *in vivo* and intervention studies must be conducted to sustain the information obtained so far, as well as to study the long term beneficial or toxic effect, for instance allergenic, derived from strawberry consumption.

Daily consumption of dietary polyphenols can vary from a few hundred mg to almost two g per capita. As described throughout this chapter, only a very minor fraction of the polyphenols found in strawberry fruits are directly absorbed in their original form. Generally, most polyphenols pass the stomach and come across the gut microflora, resulting in a diverse range of metabolites, most of them still unknown, which undoubtebly may have potential health-related properties. Further work should aim to clarify whether the metabolites generated by the interaction between gut microflora and polyphenols exert health-promoting properties.

APPENDIX F

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

APPENDIX F

Published literature

- Chope, G.A., Terry, L.A. and **Giné Bordonaba**, J. (2007). Effect of water deficit irrigation and inoculation with Botrytis cinerea on strawberry (Fragaria x ananassa) fruit quality. *Journal of Agricultural and Food Chemistry*, 55, 10812-10819.

10812 *J. Agric. Food Chem.* **2007**, *55*, 10812–10819

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Water Deficit Irrigation and Inoculation with *Botrytis cinerea* on Strawberry (*Fragaria x ananassa*) Fruit Quality

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Deficit irrigation (DI) detrimentally affected berry size but had a profound effect on fruit physiology and biochemistry. Strawberry cv. Elsanta fruit from DI-treated plants had higher levels of abscisic acid (ABA). Dry matter content as a proportion of fresh weight was increased by a quarter in fruit from water-stressed plants as compared to fruit harvested from plants held at or near field capacity. Concomitant to this, the concentration of some taste-related (viz. monosaccharides and sugar/acid ratios) and health-related compounds/parameters (viz. antioxidant capacity and total phenolics) were generally much greater in DI-treated fruit. The effect of inoculation with *Botrytis cinerea* on fruit quality was also tested. Fruit derived from inoculated plants displayed symptoms of gray mold postharvest disease earlier than noninoculated fruit and had double the concentration of ABA. Inoculation had no significant effects on all other target analytes measured. There was no interaction between water treatment and inoculation. The possible mechanisms for increased synthesis of ABA and the different effects of pathogen-induced stress versus drought stress on fruit quality are discussed.

KEYWORDS: Abscisic acid; anthocyanins; FRAP; organic acids; sugars; total phenolics.

- **Giné Bordonaba**, J. and Terry, L.A. (2008). Biochemical profiling and chemometric analysis of seventeen UK-grown blackcurrant cultivars. *Journal of Agricultural and Food Chemistry*, 56, 7422-7430.

7422 *J. Agric. Food Chem.* **2008**, *56*, 7422–7430

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Biochemical Profiling and Chemometric Analysis of Seventeen UK-Grown Black Currant Cultivars

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Black currant fruits are recognized as being an important dietary source of health-related compounds, such as anthocyanins and ascorbic acid. In the present study, the biochemical composition (viz., nonstructural carbohydrates, individual anthocyanins, total anthocyanins, total phenolics, and organic acids, including ascorbic acid) from 17 UK-grown black currant cultivars was analyzed. Berry composition was significantly affected by genotype. Nonstructural carbohydrates ranged from 85.09 to 179.92 mg g^{-1} on a fresh weight (FW) basis, while concentration for organic acids ranged from 36.56 to 73.35 mg g^{-1} FW. Relative concentrations of cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside and delphinidin 3-rutinoside were 3.1−7.9%, 35.4−47.0%, 7.6−12.5% and 36.9−50.9%, respectively. Differences in the biochemical profile among cultivars were emphasized by principal component analysis (PCA) and hierarchical cluster analysis (HCA). PCA was able to discriminate between cultivars, especially on the basis of health-related compounds. Initial exploration revealed that individual anthocyanins, total phenolics, and ascorbic acid could be used to characterize and classify different cultivars. HCA showed that the biochemical composition of the different cultivars was related to parentage information.

KEYWORDS: Nonstructural carbohydrates; anthocyanins; organic acids; total phenolics; principal component analysis; hierarchical cluster analysis

- **Giné Bordonaba**, J. and Terry, L.A. (2009). Development of a glucose biosensor for rapid assessment of strawberry fruit quality: relationship between biosensor response and fruit composition. *Journal of Agricultural and Food Chemistry*, 57, 8220-8226.

8220 *J. Agric. Food Chem.* **2009,** *57,* 8220–8226
DOI:10.1021/jf901596w

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Development of a Glucose Biosensor for Rapid Assessment of Strawberry Quality: Relationship between Biosensor Response and Fruit Composition

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Sugars are intimately related to the taste of strawberry fruit and therefore to quality. A disposable prototype glucose biosensor was constructed using glucose oxidase (GOx) immobilized onto Meldolas Blue mediated screen printed electrodes, to determine glucose content in diluted strawberry juices. Several experimental variables that affect biosensor performance, such as applied potential, GOx loading, and pH of the buffer/electrolyte solution were optimized by means of a 2^3 central composite design. Optimum applied potential, pH, and enzyme loading were +300 mV (versus Ag/AgCl), 7.2, and 20 U, respectively. Although the linear range (0−10 mM) was not significantly affected by the optimization process, the signal given by the biosensor was increased by as much as 3-fold as compared to preliminary experiments, and the reproducibility of the measurements was improved. Unlike total soluble solids (TSS), and as hypothesized, the constructed GOx biosensor was able to discriminate and rank eight different strawberry cultivars on the basis of their glucose content when compared to known concentrations measured by standard HPLC. A detailed study of the possible interferences (viz. total phenolics, antioxidant capacity, and organic acids) found in strawberry samples that could affect biosensor performance was also performed to further understand the relationship between biosensor response and sample composition. Under the imposed experimental conditions the constructed biosensor acted interference-free.

KEYWORDS: Antioxidant capacity; organic acids; sugars; total phenolics; TSS

- **Giné Bordonaba**, J. and Terry, L.A. (2010). Manipulating the taste-related composition of strawberry fruits (Fragaria x ananassa) from different cultivars using deficit irrigation. *Food Chemistry* (in press).

Food Chemistry xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Manipulating the taste-related composition of strawberry fruits (*Fragaria* × *ananassa*) from different cultivars using deficit irrigation

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ARTICLE INFO

Article history:
Received 30 June 2009
Received in revised form 8 February 2010
Accepted 15 March 2010
Available online xxxx

Keywords:
Berry size
Dry matter
Organic acids
Sugars
Sweetness

ABSTRACT

Demand and, consequently, production of strawberry fruits has increased over the past few years and, as a result, the water abstractions for cultivation of this fruit have risen considerably. To limit the amounts of water used for several horticultural crops, water deficit irrigation (DI) has been seen as a potential alternative for new cultivation systems. DI in strawberry fruits is generally associated with reduction in berry size and yield; however, a recent study demonstrated that DI on strawberry can increase the concentration of some taste- and health-related compounds in fruits from cv. Elsanta. Hence, the aim of the present study was to further corroborate such findings and to assess the response (and variability) among different strawberry cultivars (namely Christine, Elsanta, Florence, Sonata and Symphony) to imposed water-DI conditions. Water-DI affected both fruit physiology and biochemistry. Nevertheless, the response to drought stress was different for each of the cultivars tested. Plants from cvs. Elsanta, Sonata and Symphony showed a greater reduction in berry size, accompanied by a significant increase in dry matter content for fruit harvested from DI-treated plants. Concomitant to this, and where dry matter was increased, the concentrations of sugars and some acids were generally higher in DI-derived fruit. In contrast, cvs. Florence and Christine did not show significant variations in berry weight or any of the target analytes measured when grown under the conditions imposed in this study. The results presented herein suggest that reducing water irrigation between flower initiation and fruit harvest may be a viable technique for increasing the concentrations of taste-related compounds in cvs. Elsanta, Sonata and Symphony and it may not have a negative impact on overall fruit size of cvs. Christine and Florence.

- Crespo, P., **Giné Bordonaba, J**., Carlen, C. & Terry, L.A. (2010) Characterisation of major taste and health-related compounds of four strawberry genotypes grown at different Swiss production sites. *Food Chemistry* 122, 16-24.

Food Chemistry 122 (2010) 16–24

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Characterisation of major taste and health-related compounds of four strawberry genotypes grown at different Swiss production sites

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ARTICLE INFO

Article history:
Received 13 October 2009
Received in revised form 17 December 2009
Accepted 3 February 2010

Keywords:
Altitude
Anthocyanins
Antioxidant capacity
Fragaria × *ananassa*
Organic acids
Sugars

ABSTRACT

Individual sugars, organic acids, anthocyanins and vitamin C were quantified in strawberry fruits of four newly-bred cultivars grown at two production sites in Switzerland with different soil, climatic conditions and altitudes (1060 and 480 m above sea level). All the measured compounds were significantly influenced by genotype. Pelargonidin-3-glucoside was the main anthocyanin present in all cultivars, while the presence of other pelargonidin derivatives was genotype-dependent. Differences of about 2-fold were observed among the studied cultivars for their vitamin C content. In the mountain region, where plants produced a higher fruit yield over a shorter period, the concentration of both health and taste-related compounds was detrimentally affected. In particular, the vitamin C content in the fruits was negatively related to the average yield per day. However, the compositional variations of strawberry fruits in response to different production sites were genotype specific. Within the four cultivars studied, cv. Antea was most affected by the production site, showing generally lower contents of all analysed compounds when cultivated at higher altitudes, whereas cv. Clery seemed to have the more consistent chemical composition, regardless of production site. The results presented in this work corroborate the dominant role of strawberry genotype over environmental factors.

Other publications in press or under reviewing process:

- **Giné Bordonaba, J**., Chope, G.A. & Terry, L.A. (2010). Maximising blackcurrant anthocyanins: temporal changes during ripening and storage in different genotypes. *Journal of Berry Research* (in press)
- Chope, G.A., **Giné Bordonaba, J**. & Terry, L.A. Maturity at harvest determines fruit quality and nutritional value of blackcurrant berries (cv. Ben Hope) under different storage regimes using a bespoke temperature block. (submitted)
- **Giné Bordonaba, J**., Crespo, P. & Terry, L.A. A new acetonitrile-free mobile phase for HPLC-DAD determination of individual anthocyanins in blackcurrant and strawberry fruits: a comparison and validation study (submitted)

List of conference presentations:

- Oral presentation at the ***2nd International Symposium on Human Health Effects of Fruits and Vegetables (FAV 2007)*** held in Houston (USA) in October 2007. Title: *Characterisation of seventeen blackcurrant (Ribes nigrum) cultivars based on chemometric profiling of health related compounds.* Giné Bordonaba, J. and Terry, L. A.
- Poster presentation at the ***XXIV International Conference on Polyphenols (ICP)*** (July 2008 – Salamanca, Spain). Title: *Fast separation and identification of anthocyanins from blackcurrant by HPLC coupled to photodiode-array detection.* Giné Bordonaba, J. and Terry, L.A.
- Poster presentation at ***Cranfield Health Postgraduate Conference*** (September 2008). Title: *Biosensor array for improved soft fruit quality control*. Giné Bordonaba, J. and Terry, L. A. **Awarded** with the prize for the best poster.
- Oral presentation at the Workshop (COST863) on ***Berry production in changing climate conditions and cultivation systems*** held in Geisenheim (October 2008). Title*: Differential effect of deficit irrigation on fruit quality of five June-bearing strawberry cultivars.* Giné Bordonaba, J. and Terry, L. A.
- Poster presentation at the ***III Postharvest Unlimited symposium*** held in Berlin, November 2008. Title: *Routine quality control for blackcurrant fruit is poorly correlated with the real composition of the samples.* Giné Bordonaba, J. and Terry, L. A.

- Oral presentations at the Joint Meeting (COST863; W1+W4) on ***Bioactive compounds in berry fruits: genetic control, breeding, cultivar, analytical aspects and human health*** held in Zurich, December 2008. Titles*:*
    - *"The role of genotype and maturity at harvest on blackcurrant bioactives";* Giné Bordonaba, J. and Terry, L. A.
    - *"Effect of preharvest factors on strawberry (Fragaria x ananassa) bioactives"* Terry, L. A. and J. Giné Bordonaba
- Oral and poster presentations at the ***6th International Postharvest Symposium*** held in Antalya (Turkey), April 2009. Titles:
    - "*Development and optimisation of amperometric biosensors for improved postharvest quality control*". Terry, L. A., Giné Bordonaba, J. and Abayomi, L.A.
    - "*Use of a bespoke temperature block to study the detailed effect of postharvest storage temperature on blackcurrant berries*". Chope, G. A., Giné Bordonaba, J. and Terry, L. A.
- Oral presentations at the ***3rd International Symposium on Human Health Effects of Fruits and Vegetables (FAV 2009)*** held in Avignon (France) on October 2009. Titles:
    - *"Electrochemical behaviour of soft fruit juices on screen printed carbon electrodes: Towards a rapid index of antioxidant activity"*. Giné Bordonaba, J. and Terry, L. A.
    - *"Effect of genotype and growing site on individual anthocyanins and ascorbic acid content of strawberry fruits".* Crespo, P., Giné Bordonaba, J., Terry, L.A. and Carlen, C.
- *Poster presentations at the 4th* ***International Conference on Polyphenols and Health*** *joint with Euroberry (COST 863 WG4) meeting held in Harrogate (UK) on December 2009.* Titles:
    - "*Maximising blackcurrant anthocyanins: temporal changes during ripening and storage in different genotypes*" Giné Bordonaba, J. and Terry, L.A.
    - *"Development of a disposable electrochemical sensor for the amperometric determination of ascorbic acid in selected berries".* Giné Bordonaba, J. and Terry, L.A.
- *Accepted for poster presentation at the* ***20th World Biosensor congress*** *to be held in Glasgow (UK) on 24-27 May, 2010. Title:*
    - *"Increasing consumption of berries through biosensor-based evaluation of fruit composition and antioxidant capacity"* Giné Bordonaba, J. and Terry, L.A.

- *Accepted for short oral poster presentation at the **28th International Horticultural Conference** to be held in Lisbon (Portugal) on 22-27 August, 2010. Titles:*
    - *"Preharvest manipulation of strawberry fruit quality: unravelling the impacts of deficit irrigation and related strategies"* Giné Bordonaba, J. and Terry, L.A.
    - *"The Application of biosensors for rapid screening of taste- and health-related compounds in berries"* Giné Bordonaba, J. and Terry, L.A.